Cobalamin-dependent methionine synthase

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ABSTRACT

Cobalamin-dependent methionine synthase catalyzes the transfer of a methyl group from N^5-methyltetrahydrofolate to homocysteine, producing tetrahydrofolate and methionine. Insufficient availability of cobalamin, or inhibition of methionine synthase by exposure to nitrous oxide, leads to diminished activity of this enzyme. In humans, severe inhibition of methionine synthase results in the development of megaloblastic anemia, and eventually in subacute combined degeneration of the spinal cord. It also results in diminished intracellular folate levels and a redistribution of folate derivatives. In this review, we summarize recent progress in understanding the catalysis and regulation of this important enzyme from both bacterial and mammalian sources. Because inhibition of mammalian methionine synthase can restrict the incorporation of methyltetrahydrofolate from the blood into cellular folate pools that can be used for nucleotide biosynthesis, it is a potential chemotherapeutic target. The review emphasizes the mechanistic information that will be needed in order to design rational inhibitors of the enzyme. — BANERJEE, R. V.; MATTHEWS, R. G. Cobalamin-dependent methionine synthase. *FASEB J.* 4: 1450–1459; 1990.

Key Words: methionine synthase · cobalamin · vitamin B_{12} · nitrous oxide · methyltetrahydrofolate

METHIONINE SYNTHASE (5-methyltetrahydrofolate-homocysteine methyltransferase) (EC 2.1.1.13) catalyzes the transfer of a methyl group from methyltetrahydrofolate (CH_3-H_4folate)[1] to homocysteine, generating H_4folate and methionine as shown in *Eq. 1.*

$$CH_3-H_4\text{folate} + \text{Homocysteine} \rightarrow H_4\text{folate} + \text{Methionine} \quad (1)$$

In prokaryotes that synthesize methionine de novo, this reaction represents the terminal step in methionine biosynthesis. Methionine synthase is thus poised at the point of convergence of two major biosynthetic pathways: the tetrahydrofolate-dependent pathway for biosynthesis of methyl groups and the homocysteine biosynthetic pathway. Mammals, on the other hand, are unable to synthesize homocysteine de novo; rather they use methionine synthase to regenerate methionine from homocysteine to provide one-carbon units for S-adenosylmethionine (AdoMet)-dependent methylation reactions.

Escherichia coli synthesize two distinct proteins with methionine synthase activity. The *metH* gene product is a cobalamin-dependent enzyme (EC 2.1.1.13) that uses monoglutamate as well as polyglutamate forms of CH_3-H_4 folate as substrates, and must be activated for catalysis by a reductive methylation involving AdoMet and an electron donor (1, 2). The *metE* gene product is a cobalamin-independent form of the enzyme (5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase, EC 2.1.1.14) that has a strict requirement for the polyglutamate form of the substrate CH_3-H_4folate (3). In vitro assays can easily distinguish between the two activities; MetE requires magnesium and phosphate ions for activity (3), whereas MetH has an absolute requirement for AdoMet and a reducing system (2). In addition, the standard assay for cobalamin-dependent methionine synthase includes the monoglutamate form of CH_3-H_4folate and thereby excludes reaction of MetE.

Although the prokaryotic cobalamin-dependent methionine synthase has been studied extensively (reviewed in refs 4 and 5), the mammalian enzyme is much less well characterized. The *metH* gene from *E. coli* has been cloned and its gene product overexpressed (6). The mammalian enzyme appears very similar in its catalytic properties to the *metH* gene product from *E. coli.* Thus, the mammalian enzyme also contains cobalamin (7) and is competent to react with the monoglutamate form of CH_3-H_4folate. It, too, is isolated in an inactive form and requires AdoMet-dependent reductive methylation for activation (7). The apparent similarity of the catalytic mechanisms of the cobalamin-dependent enzymes from bacterial and mammalian sources, and the ready availability of the bacterial MetH protein, make this enzyme an attractive choice for mechanistic studies.

In this review, we begin by presenting a brief overview of methionine synthase and its role in folate

[1]Abbreviations: AdoMet, S-adenosyl-L-methionine; CH_3-H_4folate, N^5-methyltetrahydrofolate; CH_2-H_4folate, N^5,N^{10}-methylenetetrahydrofolate; AdoHCy, S-adenosyl-L-homocysteine; kDa, kilodalton; HCy, homocysteine; $CH=H_4$folate, N^5,N^{10}-methenyltetrahydrofolate; $CHO-H_4$folate; N^{10}-formyltetrahydrofolate; H_2folate, dihydrofolate; bp, base pairs.

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metabolism and then consider the mammalian enzyme as a potential target for chemotherapeutic intervention. The inactivation of enzyme from both mammalian and bacterial sources by nitrous oxide (N_2O) is discussed. Studies of the nucleotide sequence analysis of the *metH* gene and structure-function studies of the encoded protein are summarized. Finally, salient chemical problems associated with catalysis of this methyl transfer reaction are discussed within the framework of a possible mechanism of catalysis.

OVERVIEW OF METHIONINE SYNTHASE AND ITS ROLE IN FOLATE METABOLISM

The pioneering studies by du Vigneaud and co-workers (8) and by Bennett (9) demonstrated that dietary requirements for methionine in the rat could be replaced by supplying homocysteine and cyanocobalamin. Studies from the laboratories of Buchanan, Kisliuk, and Woods (reviewed in refs 4 and 5) demonstrated the presence of a cobalamin cofactor in methionine synthase purified from both *E. coli* and pig liver.

The structure of the cobalamin cofactor of methionine synthase is shown in **Fig. 1.** The cobalt ion in cobalamin is coordinated by four coplanar nitrogen ligands in a reduced tetrapyrrole structure, the corrin ring. Two additional coordination sites exist: the upper and lower axial positions that lie above and below the plane of the ring, respectively. In methylcobalamin, a methyl group is bonded to cobalt in the upper axial position, and the

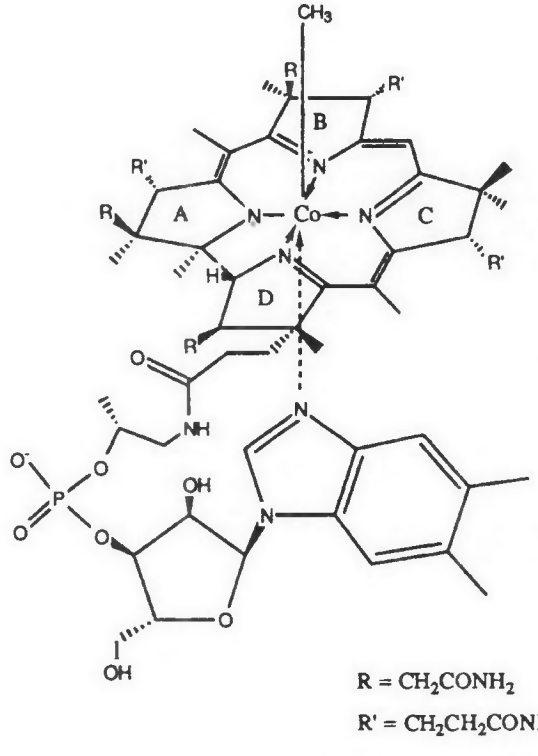

Figure 1. Structure of the cobalamin cofactor of methionine synthase. The structure drawn is that of methylcobalamin, with the methyl group occupying the upper axial coordination site of cobalt.

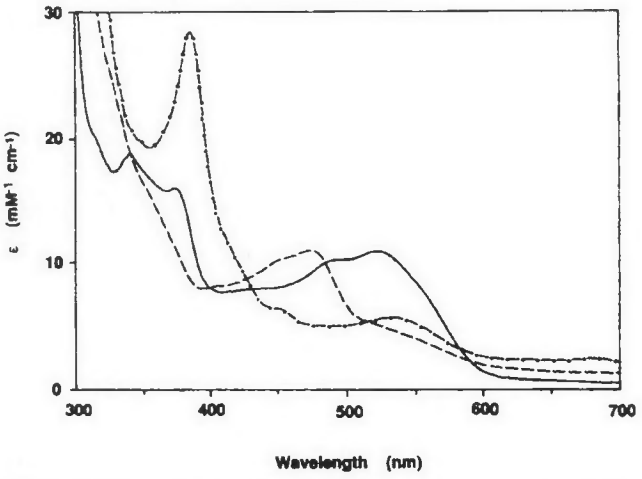

Figure 2. Electronic absorbance spectra of enzyme-bound cobalamin at different states of reduction and of methylated enzyme. —, methylcobalamin; ----, cob(II)alamin; -·-·-·-, cob(I)alamin.

lower axial position is occupied by a nitrogen of the dimethylbenzimidazole nucleotide substituent of the corrin ring. During turnover, the cobalamin cofactor of methionine synthase shuttles between methylcobalamin and cob(I)alamin (which contains a pair of electrons in the dz^2 orbital oriented perpendicularly to the plane of the corrin ring). The enzyme-bound cob(I)alamin can be oxidized to cob(II)alamin, with a single electron in the dz^2 orbital, or to cob(III)alamin. The different forms of cobalamin can readily be distinguished by their ultraviolet and visible electronic absorbance spectra (**Fig. 2**).

The cobalamin-dependent enzyme from *E. coli* B was extensively purified by Taylor and Weissbach (10) and was purified to homogeneity from *E. coli* K-12 by Fujii and Huennekens (11). Studies from these two laboratories, and from those of Kisliuk and Woods (reviewed in refs 4, 5), suggested the catalytic mechanism shown in **Fig. 3,** in which the enzyme cycles during turnover between methylcobalamin and cob(I)alamin. The participation of methyl cobalamin as a catalytic intermediate was demonstrated by radiolabel tracer studies (12). Cob(I)alamin was formed by demethylation of methylated enzyme under anaerobic conditions, as deduced by characteristic changes in the visible electronic absorbance spectrum when homocysteine was added to the enzyme-bound methylcobalamin (13, 14).

Concomitant studies of the enzymes involved in the conversion of the β-carbon of serine into the methyl group of AdoMet (**Fig. 4**) elucidated the regulation of AdoMet biosynthesis. Serine hydroxymethyltransferase catalyzes the transfer of the β-carbon of serine to H_4folate to generate CH_2-H_4folate and glycine. Methylenetetrahydrofolate reductase (NADPH) (EC 1.5.1.20) catalyzes the reduction of CH_2-H_4folate to CH_3-H_4folate. The overall NADPH-linked reduction of CH_2-H_4folate to CH_3-H_4folate is irreversible (15) and commits folate-bound one-carbon units to use for the methylation of homocysteine. Methionine synthase catalyzes the transfer of the methyl group of CH_3-H_4folate to homocysteine

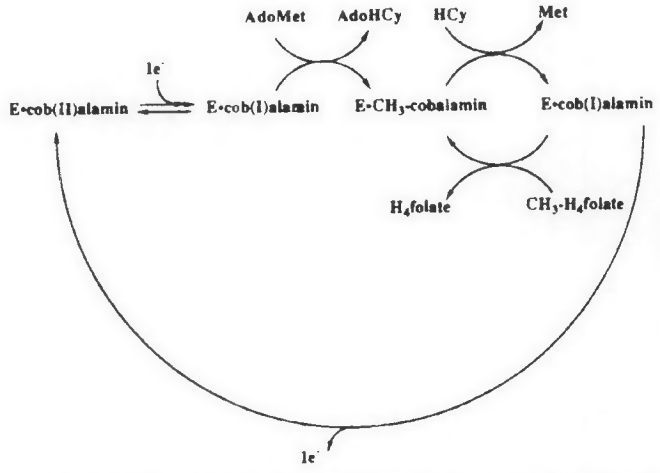

Figure 3. Schematic mechanism for activation and catalysis of methionine synthase. The cob(II)alamin form of the enzyme is inactive. In vitro activation involves a reductive methylation with AdoMet as the methyl donor. The methylated enzyme, once formed, is catalytically active and cycles between cob(I)alamin and methylcobalamin in catalysis, as shown. Occasional oxidation of the cob(I)alamin intermediate to the inactive cob(II)alamin mandates the continuing requirement for the activation system during catalysis.

to form methionine and H_4folate, and methionine can then be used for protein synthesis or converted to AdoMet. In mammals, this pathway is regulated by the AdoMet/AdoHCy ratio, and AdoMet has been shown to be an allosteric inhibitor of methylenetetrahydrofolate reductase from mammalian sources (16, 17). AdoHCy, the product of AdoMet-dependent methylation reactions, blocks the binding of AdoMet to methylenetetrahydrofolate reductase, but does not itself inhibit or activate the enzyme. In *E. coli,* AdoMet, rather than methionine, is also the major regulator of methionine biosynthesis and combines with an aporepressor protein, the *metJ* gene product, to inhibit transcription of enzymes involved in methionine biosynthesis (reviewed in ref 18).

Inhibition of methionine synthase interferes with AdoMet-dependent regulation of the cellular levels of CH_3-H_4folate. Under these circumstances, inadequate intake of dietary methionine leads to a fall in AdoMet concentrations that can no longer be prevented by remethylation of homocysteine. Methylenetetrahydrofolate reductase remains fully active, and cellular folates accumulate as CH_3-H_4folate. These changes result in a cellular methyl trap as first proposed by Noronha and Silverman (19) and by Herbert and Zaluskey (20). Cobalamin deficiency also leads to an accumulation of the CH_3-H_4folate pool at the expense of the other cellular folate pools owing to the decreased activity of methionine synthase. The depletion of CH_2-H_4folate required for thymidylate biosynthesis appears to be the primary cause of megaloblastic anemia associated with either folate or cobalamin deficiency in humans. Megaloblastic anemia is not associated with folate or cobalamin deficiency in other mammals.

Inhibition of methionine synthase leads not only to redistribution of cellular folate derivatives, but also to a decline in the total intracellular folate level. The major circulating form of folate is CH_3-H_4folate, and methionine synthase activity is required to convert this derivative to forms that can be used for nucleotide biosynthesis (15). Moreover, CH_3-H_4folate is a poor substrate for folylpolyglutamate synthase (21), the enzyme that catalyzes sequential poly-γ-glutamylation of intracellular folate derivatives to generate folylpolyglutamates, which are the predominant intracellular forms of folic acid. The polyglutamate forms of folic acid are essential for intracellular retention of the vitamin. Hence a shift in the cellular folate pool to methyl derivatives results in a shift toward shorter-chain forms that can traverse the cell membrane more readily and lead to an intracellular folate deficiency.

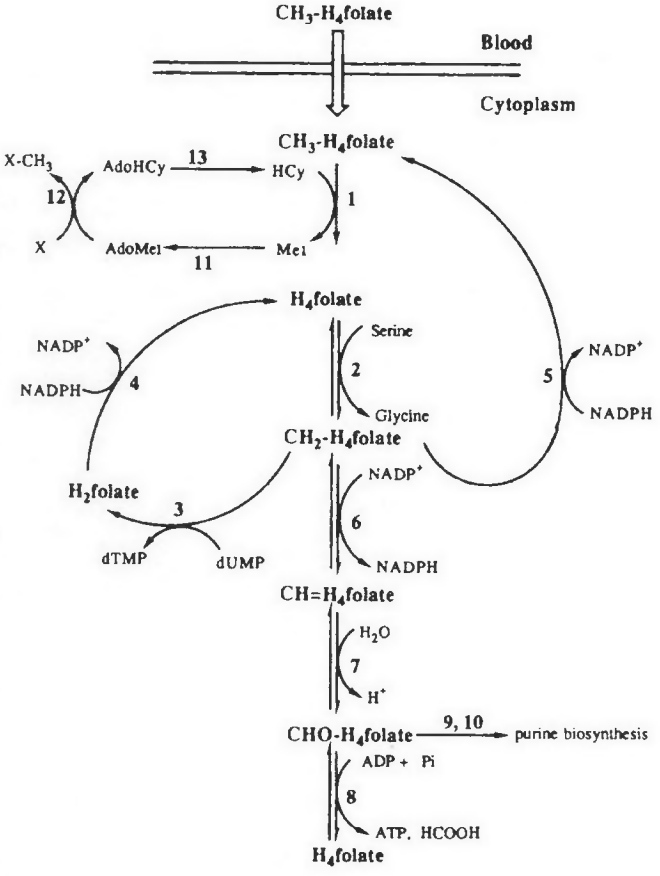

Figure 4. Outline of the major tetrahydrofolate-dependent biosynthetic pathways. The enzymes involved are: 1, methyltetrahydrofolate-homocysteine methyltransferase (methionine synthase); 2, serine hydroxymethyltransferase; 3, thymidylate synthase; 4, dihydrofolate reductase; 5, methylenetetrahydrofolate reductase; 6, methylenetetrahydrofolate dehydrogenase; 7, methenyltetrahydrofolate cyclohydrolase; 8, formyltetrahydrofolate synthetase; 9, glycineamide ribonucleotide transformylase; 10, aminoimidazolecarboxamide ribonucleotide transformylase; 11, adenosylmethionine synthetase; 12, adenosylmethionine-dependent methyltransferases; and 13, adenosylhomocysteine hydrolase.

Methionine synthase thus may be viewed as a crucial player in folate metabolism, poised at the point of entry of folates into the cell and responsible for converting the vitamin from CH_3-H_4folate into a more widely useful form that can participate in nucleotide biosynthesis. Methionine, the other product of the methionine synthase-catalyzed reaction, is an essential amino acid in mammals, and serves in protein synthesis and as the precursor for AdoMet. The latter is a crucial methyl group donor involved in the biosynthesis of a variety of compounds including creatine, phosphatidylcholine, and epinephrine. AdoMet shortage in cells lacking active methionine synthase may be alleviated by the provision of exogenous methionine, whereas conversion of CH_3-H_4folate to H_4folate would remain compromised. Methylenetetrahydrofolate reductase activity should remain inhibited under this regime, preventing the accumulation of cellular folylpolyglutamates as CH_3-H_4folate.

The relationship between impaired methionine synthase activity and diminution of cellular folate pools should make it an attractive chemotherapeutic target. Rapidly dividing cells should have an elevated requirement for uptake of folate from the blood, and may be particularly hard hit by inhibition of methionine synthase. Several enzymes involved in folate metabolism, including thymidylate synthase and dihydrofolate reductase, have already been realized as chemotherapeutic targets. Inhibition of methionine synthase, with its potential for depleting the folate pool with consequent inhibition of nucleotide biosynthesis, holds a similar promise. As yet no antineoplastic drug directed against methionine synthase is available.

INACTIVATION OF METHIONINE SYNTHASE BY NITROUS OXIDE

Exposure of humans to nitrous oxide (N_2O), a commonly used anesthetic agent, leads to inactivation of methionine synthase (22). Prolonged exposure leads to the development of megaloblastic anemia in humans but not in other mammals (23), and in cases of repeated exposure may even result in the neurological symptoms characteristic of pernicious anemia or cobalamin deficiency, such as subacute combined degeneration of the spinal cord (24). Treatment of rodents with N_2O has been shown to lead to a rapid decrease in the levels of methionine synthase activity in liver homogenates (25–27). Recovery of activity required several days after discontinuation of N_2O treatment and presumably was linked to synthesis of new enzyme. Long-term administration of N_2O to monkeys resulted in subacute combined degeneration of the spinal cord, and these symptoms could be alleviated by methionine supplementation of the diet (28). Since the effect of nitrous oxide appears to be specific for methionine synthase, an understanding of the events associated with this inactivation might point the way to the design of other inhibitors of methionine synthase.

Model studies of the interaction of the cob(I)alamin in aqueous solution with N_2O have established that cob(I)alamin is oxidized and N_2 is liberated (29, 30), presumably according to *Eq. 2* and *3*.

$$\text{cob(I)alamin} + N_2O + 2H^+ \rightarrow \text{cob(III)alamin} + N_2 + H_2O \quad (2)$$

$$\text{cob(III)alamin} + \text{cob(I)alamin} \rightarrow 2\,\text{cob(II)alamin} \quad (3)$$

As these experiments were conducted in the presence of an excess of cob(I)alamin, the formation of cob(III)alamin was not directly demonstrated but was implied from the observed formation of cob(II)alamin.

The similarity of cobalamin-dependent methionine synthase enzymes from prokaryotic and eukaryotic sources extends to their susceptibility to inhibition by N_2O. Purified methionine synthase preparations from *E. coli* B and from pig liver have been used to demonstrate the in vitro inactivation of the enzyme by N_2O (31). Inactivation occurred only in the presence of all components required for turnover. The ratio of moles of methionine formed to moles of enzyme inactivated was approximately 3900 for the bacterial enzyme. Presumably because of the requirement for multiple turnovers per inactivation event, inactivation occurred only in very dilute enzyme solutions ($\sim$6 nM enzyme), so that absorbance changes associated with enzyme inactivation could not be monitored directly. When the inactivated enzyme was reconcentrated from the assay solution so that its absorbance properties could be examined, significant loss of the cobalamin was observed in the N_2O-treated enzyme as compared to control N_2-treated enzyme, but both samples retained the spectrum of cob(II)alamin. Based on the model studies, it was postulated that N_2O intercepts enzyme-bound cob(I)alamin that is generated transiently during turnover, with formation of cob(II)alamin and hydroxyl radical as shown in *Eq. 4*. Generation of a rogue hydroxyl radical at the active site could explain the observed irreversible loss of enzyme activity.

$$\text{cob(I)alamin} + N_2O + H^+ \rightarrow \text{cob(II)alamin} + N_2 + OH\cdot \quad (4)$$

Studies aimed at directly monitoring the reaction of enzyme bound cob(I)alamin with N_2O are in progress in our laboratory.

CLONING AND SEQUENCE ANALYSIS OF THE METH GENE

In *E. coli*, methionine synthase catalyzes the terminal step in the de novo biosynthesis of methionine. Under conditions of aerobic growth the bacteria are unable to synthesize cobalamin, although the MetH apoenzyme is synthesized. In the absence of methionine or cobalamin in the medium, aerobic cell growth requires the MetE protein. When coablamin is added to the medium, the MetH holoenzyme is formed and MetE synthesis is repressed. A methionine-requiring nutritional auxotroph of *E. coli* (RK4536: *metH*$^-$, *metE*$^-$) was used to isolate a recombinant clone that was prototrophic for methionine in the presence of cobalamin (32, 33). The identity

of the cloned *metH* gene was confirmed by nucleotide sequence analysis, maxicell expression, and enzyme activity assays. The sequence of both strands was completely determined, including 220 bp upstream and 180 bp downstream of the protein coding sequence (6). The open reading frame encodes 1,124 amino acids with a predicted molecular mass of 123,640 daltons, within 9% of the value given by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and by gel filtration (34). More recently, the nucleotide sequence of the *metH* gene from *E. coli* K-12 has been determined (35) and a 3600-bp open reading frame that encodes a peptide with a predicted molecular weight of 132,628 was found. The amino acid sequence deduced by the two groups diverges at position 3337. The difference appears to result from omission of a G from the sequence TGGGG starting at position 3334, which results in a stop codon at position 3374 of the shorter open reading frame. The validity of the COOH-terminal extension was established by the expression of β-galactosidase activity by a *metH-lacZ* fusion located 190 bp downstream of the putative stop codon at position 3374 (35).

Comparison of the deduced amino acid sequences of the *E. coli metH* gene and the proximal third of the *Salmonella metH* gene (36) revealed a remarkable similarity, with only 21 of the 371 amino acids being different. In contrast, comparison with all other sequences in the NBRF and GenBank databases failed to identify any significant homologies. Both *metH* and *metE* genes have been shown to be subject to positive regulation by the *metR* gene product, a *trans*-acting transcriptional activator (37, 38). Although no extensive regions of homology are apparent in the sequences of the *metE* and *metH* control regions of *Salmonella typhimurium,* a short region of interrupted dyad symmetry was found (36). Mutations in this region of the *metE* gene disrupt the normal regulation of the *metE* gene by the *metR* gene product (36). The *metR* gene product has been shown to bind to an upstream region in the *E. coli metE* gene (39). Upstream of the *E. coli metH* gene a similar sequence has been found (35, 40). The four sequences are shown in **Fig. 5.** The finding of only partial homology between the control regions of the two genes is not surprising, since they are regulated to vastly different extents by the *metR* gene product (36). Hence, although the range of activation of the *metE* gene is nearly 100-fold, that of the *metH* gene is only five- to sixfold. The observation that the dyadic symmetry is more extensive in the two *metE* sequences than in the analogous *metH* sequences is consistent with the differential effect of the *metR* gene on transcriptional regulation of these two genes.

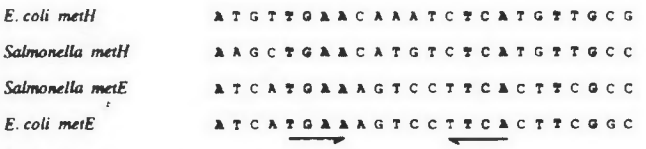

Figure 5. Comparison of the upstream sequences of the *metE* and *metH* genes that are presumably involved in regulation by *metR*. The regions compared show the interrupted dyad symmetry sequence; mutations in this region disrupt the normal regulation of the *metE* gene by the *metR* gene product in *Salmonella* (36). Residues conserved in all four genes are shown in bold type. Each of the sequences is upstream of the corresponding -35 region of its gene. The direction of transcription of all three genes is to the right. The papers from which these sequences are drawn are noted in the text.

THE COBALAMIN-BINDING DOMAIN: AN α/β STRUCTURE?

Since primary sequence comparison of MetH with other folate- and cobalamin-binding proteins failed to reveal any significant relationship, we sought to use limited proteolysis to identify a fragment of the enzyme containing the cobalamin-binding site (6). Tryptic digestion of the native recombinant enzyme resulted in the formation of two daughter fragments of 95 and 35 kDa, and was accompanied by loss of activity. NH_2-terminal sequence analysis of the 35-kDa fragment established its position at the COOH-terminal end of the protein. Although the 35-kDa fragment was fairly stable over the time course of the digestion, the 95-kDa peptide was cleaved to form 68- and 28-kDa peptides. Separation of the peptides on a native nondenaturing gel resulted in the identification of a pink fragment that retained the cobalamin-binding site and had a molecular mass of 28,000. NH_2-terminal analysis of the 28-kDa fragment established the limits of the cobalamin-binding domain. The hypersensitive site at the COOH-terminus of the cobalamin-binding domain, between residues 900 and 901, lies in one of the most hydrophilic internal regions of the protein as predicted by the method of Kyte and Doolittle (41). The 28-kDa fragment is immediately upstream of the 35-kDa fragment, and is characterized by the prevalence of hydrophobic residues.

Comparison of the deduced sequence of the 28-kDa fragment with those of other soluble cobalamin-binding proteins such as BtuR (a cobalamin-binding protein from *E. coli* implicated in the metabolism of adenosylmethionine) (42), MutA and MutB (the two subunits of methylmalonyl CoA mutase from *Propionibacterium shermanii*) (43), and EalA and EalB (the two subunits of ethanolamine ammonia lyase from *Salmonella typhimurium*) (L. P. Faust, J. A. Connor, D. M. Roof, J. A. Hoch, and B. M. Babior, unpublished results), failed to detect any significant homologies. However, predicted secondary structure comparisons of the four proteins revealed a striking similarity in the pattern and spacing of alternating α-helices and β-sheets spanning a length of about 100 amino acids in the deduced sequences of each of the four proteins, as shown in **Fig. 6.** In the absence of a crystal structure for any member of the cobalamin-binding protein family, it was postulated that the cobalamin pocket may contain an α/β type of structure (6). The alternation of α-helices and β-sheets is suggestive of the Rossman fold motif that is involved in binding nucleotides (44). The structure of cobalamin contains dimethylbenzimidazole in an unusual nucleotide. A comparison of the

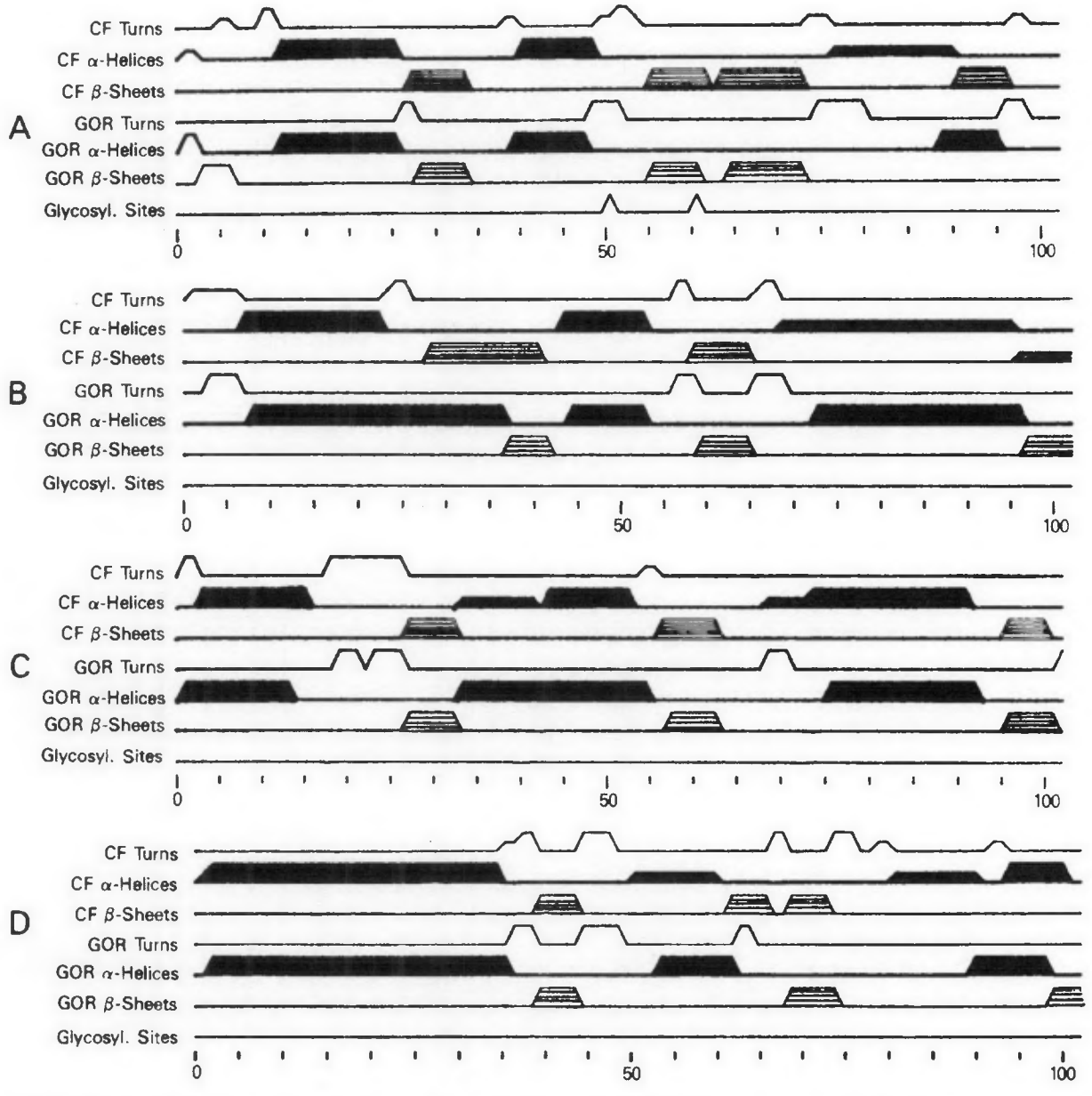

Figure 6. Predicted secondary structure comparisons of segments of four cobalamin-binding proteins. A, Residues 800-900 of MetH (6); B, residues 96-196 of BtuR (42); C, residues 505-605 of MutB (43); D, residues 110-210 of EalB (B. M. Babior, personal communication). CF, Chou-Fasman secondary structure prediction; GOR, Garnier-Osguthorpe-Robson secondary structure prediction. The thickness of the black bars is proportional to the strength of the prediction.

benzimidazole-ribofuranosyl portion of cobalamin with the adenine ribose of NAD bound to crystalline malate dehydrogenase revealed a good alignment of the two molecules with a calculated root mean square distance between corresponding atoms of 1.05 Å (45). The region of identified secondary structural homology in the cobalamin-binding proteins may be involved in docking the dimethylbenzimidazole nucleotide.

Conservation of secondary and tertiary structure in binding domains in the absence of primary sequence conservation is not without precedent. Hence, in the globins and cytochromes, peptides with poor primary sequence homology fold into proteins of striking architectural similarity. In the globins, for instance, the pocket in which the heme cofactor binds is created by the assembly of eight α-helices. Only 5 of the 116 positions that are involved in conserved interactions with the cofactor are identical in all globins, and even they are not contiguous in the primary sequence (46). This illustrates the difficulty in predicting the relatedness of the globin family members from primary sequence comparisons alone.

ACTIVATION OF METHIONINE SYNTHASE AND ITS CATALYTIC MECHANISM

The properties of the *E. coli* B enzyme that has been purified to homogeneity (34) are similar to those de-

scribed for the partially purified enzyme from the same source (10) and for the homogeneous wild-type (11) and recombinant (6) enzymes from *E. coli* K-12. The enzyme is monomeric, and contains 1 mol of cobalamin per mol of enzyme. Electron spin resonance (EPR) spectroscopy of the enzyme as isolated in its inactive form reveals the presence of cob(II)alamin (34), which is consistent with the electronic absorption spectrum of the enzyme. The EPR signal disappears upon reductive methylation, as expected for the conversion of paramagnetic cob(II)alamin to diamagnetic methylcobalamin (Fig. 3). In addition to cobalt, the *E. coli* B enzyme was found to contain 1 mol of copper per mol of enzyme-bound cobalamin (34). The copper was tightly bound and EPR silent in the native enzyme, but became detectable when the enzyme was denatured. The copper bound to the enzyme is in the Cu^{+1} oxidation state as judged by X-ray absorption edge spectroscopy (Clark, K., Penner-Hahn, J., Banerjee, R. V., and Matthews, R. G., unpublished results). The recombinant enzyme from *E. coli* K-12 lacks stoichiometric copper (it contains about 0.1 mol copper per mol cobalamin) although its turnover number under V_{max} conditions is comparable to that of the wild-type *E. coli* B enzyme. These results lead us to conclude that copper does not play a crucial catalytic role.

Methionine synthase has also been purified to homogeneity from human placental tissue (47) and found to contain two equivalents of iron per mol of enzyme-bound cobalamin. The role of iron in this enzyme is as yet unknown. The homogeneous enzyme from human placenta had a specific activity of 0.014 μmol min^{-1} mg $protein^{-1}$, measured at 37°C, which would correspond to a turnover number of 2.2 min^{-1}. In contrast, a turnover number of 670 min^{-1} was estimated for partially purified enzyme from pig liver (7), and a specific activity of 0.9 μmol min^{-1} mg $protein^{-1}$ at 37°C was measured for the partially purified pig kidney enzyme (48). Thus there is concern that the enzyme isolated from human placenta (47) was grossly inactive. The turnover number of 670 min^{-1} estimated on the basis of cobalamin determinations in partially purified enzyme fractions (7) is quite comparable to the turnover number of the enzyme from *E. coli* ($\sim$1300 min^{-1} at 37°C) (34).

The overall reaction catalyzed by methionine synthase is a transmethylation from CH_3-H_4folate to homocysteine, as shown in *Eq. 1,* and has an equilibrium constant of 10^5 in favor of products (49). Under in vitro assay conditions, the reaction displays an absolute requirement for catalytic amounts of AdoMet and a reducing system (1, 2). Inactive cob(II)alamin is primed for turnover by a reductive methylation reaction, as shown in Fig. 3. The methylated enzyme so generated is catalytically competent and mediates the transfer of a methyl group from CH_3-H_4folate to homocysteine during turnover. However, about once in every 100 turnovers, the enzyme is disabled by an adventitious oxidation of cob(I)alamin (50), and needs to be reactivated for turnover by the reducing system and AdoMet.

A variety of different activation systems have been employed with the enzyme from *E. coli.* The standard assay includes dithiothreitol and aquocobalamin in phosphate buffer as the source of electrons. The chemical reductants can be substituted by NADPH and two flavoproteins from *E. coli,* designated the R and F proteins (11), that have properties analogous to ferredoxin: NADPH oxidoreductase and flavodoxin, respectively. With the physiological reducing system, the ultimate source of electrons is NADPH, an obligate two-electron donor. Although the R flavoprotein undergoes a facile two-electron reduction by NADPH, the second protein in the electron transfer chain, the F protein, stabilizes the one-electron reduced semiquinone state (50). Hence the role of the two flavoproteins in this electron wire between NADPH and methionine synthase is presumably to mediate conversion from two to one electron transfer. With the chemical reducing system, aquocobalamin may assume a similar role in bridging the gap between dithiothreitol, a two-electron donor, and methionine synthase, a one-electron acceptor. In addition, or alternatively, aquocobalamin plays the role of an oxygen scrubber, providing semianaerobic conditions for assays conducted with initially aerobic solutions. Apoenzyme from bacterial sources is inactive in assays containing aquocobalamin and requires methylcobalamin for holoenzyme formation (51).

Conversion of cob(II)alamin to cob(I)alamin is a particularly challenging reduction in the cellular milieu because the midpoint potential for this redox couple is one of the lowest found in biological systems (**Fig. 7**). The cob(II)alamin/cob(I)alamin couple of the aqueous cofactor at pH 7 was determined to be -610 mV vs. the standard hydrogen electrode (52). The reduction potential for the methionine synthase-bound cob(II)alamin/cob(I)alamin couple was found to be -526 mV (53). Hence, although the binding of the cofactor to the protein does render the reduction somewhat easier, reduction remains a thermodynamically unfavorable reaction with the reducing partners of methionine synthase in in vitro assays, viz. flavodoxin or dithiothreitol, as shown in Fig. 7.

An endergonic reduction could in principle be driven by a large free energy decrease accompanying methylation of the reduced cobalamin, if the two reactions were coupled. Evidence for such a mechanism for reductive methylation of methionine synthase has recently been obtained (53). The highly exergonic methyl transfer from AdoMet to cob(I)alamin could be used to drive a highly endergonic reduction of the cob(II)alamin redox center of the enzyme at potentials as high as -82 mV. From the shift in the apparent standard potential for the cob(II)alamin/cob(I)alamin couple in the presence of AdoMet, $\Delta G^{o'}$ for the methyl transfer was estimated to be greater than -9 kcal per mol. In contrast, $\Delta G^{o'}$ for the CH_3-H_4folate-dependent methylation was calculated to be -0.09 kcal per mol, and the apparent standard potential for the cob(II)alamin/cob(I)alamin couple in the presence of CH_3-H_4folate is only shifted to -450 mV. These results elucidate the enigmatic requirement for AdoMet during in vitro activation,

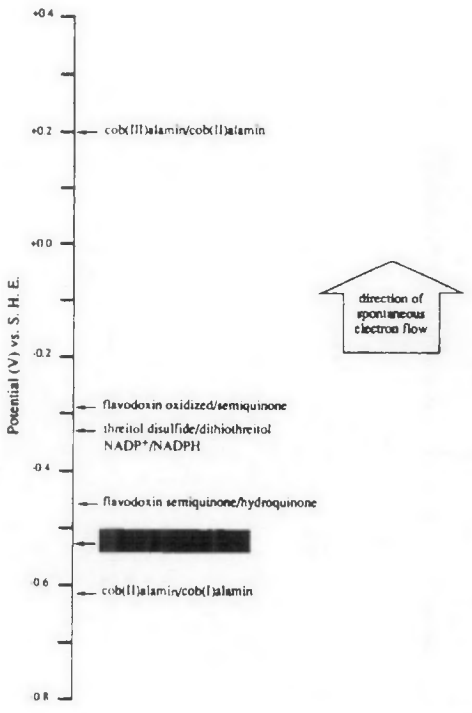

Figure 7. Redox potential ladder demonstrating the thermodynamic difficulty of reducing cob(II)alamin in the cellular milieu, and the rationale for employing AdoMet vs. CH_3-H_4folate to couple methylation of cob(I)alamin to reduction of cob(II)alamin to cob(I)alamin. For a two electron transfer at 25°C, a 30 mV potential difference corresponds to an ~10-fold difference in concentrations of the two species at equilibrium, and for a one electron transfer, a 60 mV potential difference corresponds to an ~10-fold difference in concentrations of the two species at equilibrium. All potentials shown are vs. the standard hydrogen electrode and are standard potentials at pH 7.

despite the ready reaction of CH_3-H_4folate with cob(I)alamin during catalytic turnover. The equilibrium constants for the two transmethylations differ by more than 10^9; hence AdoMet, but not CH_3-H_4folate, is able to drive reduction of cob(II)alamin at ambient potentials during aerobic growth and under in vitro assay conditions.

There is no evidence for the presence of a pyridine nucleotide-dependent activation system analogous to the R and F protein in pig liver cells. However, several thiols, such as homocysteine, cysteine, and dithiothreitol, are capable of supporting methionine synthase activity in the absence of added cobalamin (54). Partially purified enzyme is associated with thiol oxidase activity (54), as shown in *Eq. 5.*

$$\text{Dithiothreitol} + O_2 \rightarrow \text{Threitol disulfide} + H_2O_2 \qquad (5)$$

It has been postulated that the thiol oxidase activity is an intrinsic property of the mammalian enzyme, and that thiols may serve as the physiological reductants for the mammalian enzyme (54). A group of patients with severe megaloblastic anemia and homocystinuria (but no methylmalonic aciduria) have been described (55), and their symptoms appear to be associated with a genetic defect in cobalamin metabolism (cblE). Cells from these patients had normal methionine synthase activity under standard assay conditions, but the enzyme activity was greatly reduced when measured with suboptimal concentrations of thiol reducing agents. Significantly, the patient's fibroblasts were insensitive to exposure to nitrous oxide, suggesting that methionine synthase was inactive in these cells. Although the identity of the loci affected by the cblE mutation is unknown, it is speculated that it affects a reducing system analogous to that in *E. coli.* Characterization of the mutation should provide a powerful tool for understanding the mechanism of activation of the human methionine synthase.

Once methylcobalamin enzyme is formed, catalysis proceeds with alternation of demethylation and methylation as shown in Fig. 3. The stereochemistry of the overall methyl transfer reaction from CH_3-H_4folate to homocysteine was investigated using CH_3-H_4folate that was chiral at the 5-methyl position by virtue of containing deuterium and tritium (56). The reaction was found to proceed with retention of configuration at the transferred carbon, as expected for two consecutive methyl transfers from CH_3-H_4folate to cobalamin and then from methylcobalamin to homocysteine.

We have used stopped-flow kinetic studies to determine the kinetic competence of the cob(I)alamin and methylcobalamin species at 25°C, under rigorously anaerobic conditions (Banerjee, R. V., Frasca, V., Ballou, D. P., and Matthews, R. G., unpublished data). Under such strictly anaerobic conditions, the need for an activation system is obviated because of the reduced susceptibility of cob(I)alamin to oxidation. Hence, the turnover reaction can be studied starting with the methylcobalamin form of methionine synthase in the absence of added AdoMet and aquocobalamin. These experiments were performed with methylated methionine synthase that had been generated by electrochemical reduction in the presence of AdoMet (53). The enzyme was rapidly mixed with either homocysteine or a mixture of homocysteine and CH_3-H_4folate. The formation of cob(I)alamin from methylcobalamin was monitored either by observing the increase in absorption at 390 nm or the decrease in absorption at 520 nm (see Fig. 2). Conversely, the formation of methylcobalamin from cob(I)alamin was followed by monitoring the increase in absorbance at 520 nm. In these experiments, evidence has been obtained that both cob(I)alamin and methylcobalamin are kinetically competent catalytic intermediates.

Although there is good evidence for the intermediacy of methylcobalamin in the methyl group transfer from CH_3-H_4folate to homocysteine, and for the role of cob(I)alamin as a nucleophile, the mechanism of activation of CH_3-H_4folate prior to nucleophilic displacement of the methyl group remains obscure. A simple S_N2 displacement at the α-carbon of a tertiary amine would constitute a chemical reaction without precedent, albeit cob(I)alamin is an excellent nucleophile. As shown in **Fig. 8,** the tertiary amine could be activated by two-electron oxidation giving rise to a quaternized sp^2 nitrogen (**structures** 1 and 2), by one electron oxi-

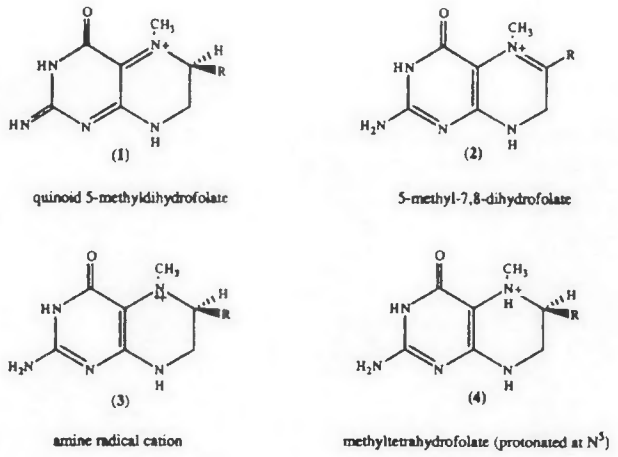

Figure 8. Possible activated forms of CH_3-H_4folate.

dation to generate the amine radical cation (structure 3), or by quaternization via protonation at N^5 (structure 4). Formation of **structures** 1, 2, or 3 would require an as-yet-unidentified high potential electron acceptor on the enzyme. Whatever the mode of activation, an understanding of the structure of the activated species (i.e., whether N^5 is trigonal or tetrahedral; charged or neutral) would have an important bearing on the rational design of CH_3-H_4folate analogs as inhibitors of the enzyme.

In summary, although the availability of large amounts of the bacterial methionine synthase from *E. coli* K-12 has led to advances in our understanding of the activation and turnover mechanisms, studies with the mammalian enzyme have lagged behind. Although the enzymes from the two sources share many common properties, the activation systems appear to be different. In particular, it is not known what factors are required for reductive activation of the mammalian enzyme in vivo. Nor is the mode of activation of CH_3-H_4folate preceding displacement of the methyl group understood. An understanding of the reaction mechanism and solution of the crystal structure of methionine synthase holds promise for the rational design of inhibitors with potential antineoplastic activity. FJ

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ANNALS OF WESTERN MEDICINE AND SURGERY

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The Biochemical Basis of Betaine-Glycocyamine Therapy

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THE CLINICAL investigations described in this series of articles had, as point of departure, the general idea that, for the recovery of a cell with impaired function, more of certain substances may be required than for the maintenance of the cell when it is healthy. The example in mind was vitamin therapy, where at least several times the maintenance dose is required to cure a clinical deficiency.

Rationale

Phosphocreatine is the main reservoir of immediately available energy in muscle and nerve.† Perhaps then, the presentation to a damaged muscle or nerve of more creatine than is normally synthesized in the body might facilitate its recovery? The inference was that by providing extra creatine there would be more retention of creatine in the tissue and, as a consequence, more extra phosphocreatine in muscle or nerve.

There is some experimental evidence in support of this possibility in the findings of Benedict and Osterberg[4], who fed small amounts of creatine to three normal, adult dogs and found, besides an increased excretion of creatinine, a storage of nitrogen and a small but persistent weight gain in two of the animals. Chanutin[5], using larger doses of creatine, confirmed these observations in men.

Further support is seen in the close correlation between characteristic changes in muscle phosphocreatine, urinary creatinine and creatine on the one hand, and certain experimental and clinical pathological conditions on the other. This correlation has not been pointed out in the literature. The interpretation of much of the clinical data needed the finding that the immediate precursor of most of the urinary creatinine is the tissue phosphocreatine[6]. In progressive muscular dystrophy muscle phosphocreatine is low [7-8], urinary creatinine also is low [9-17] and parallels closely the severity of the disease[17]. The lesion in progressive dystrophy is in the muscle. In myasthenia gravis, on the other hand, where the lesion is in the myoneural junction, both the muscle phosphocreatine and urinary creatinine are within the normal range[7, 18, 18-22].

Bearing on Treatment of Cardiac Failure

The findings in thyroid disorders bear more directly on the treatment of cardiac failure. In thyroxinized animals the heart is hypertrophied and its concentrations of creatine and phosphocreatine are very low; on the other hand the concentrations are within normal limits or only slightly lowered in the skeletal muscles[23-28]—except in severely myasthenic animals where they were definitely low[23]. As the heart is only a small fraction of the total muscle mass, the reduction in cardiac phosphocreatine alone does not lower the urinary creatinine[28-30]. In Graves' disease the trend of creatinine excretion is from normal in the milder forms of the disease[31], to somewhat subnormal when the condition is severe and there is

ANNALS OF WESTERN MEDICINE AND SURGERY devotes much of this issue to the publication of four papers on the use in combination of betaine and glycocyamine, two materials related to amino acids, which promise potential therapeutic usefulness. Use of the combination is new to medicine. These papers no doubt will stimulate further investigation.

The first paper outlines the basic biochemical background which encouraged the preliminary clinical investigation of the betaine and glycocyamine combination. The other three report the preliminary clinical results obtained when the combination was used in certain heart ailments.

Additional studies by these and other workers are currently in progress, not only in the cardiac field but in poliomyelitis, arthritis and other pathological conditions. For the present, the combination of betaine and glycocyamine is available for investigational use only.

This work was supported in part by a grant to the California Institute of Technology from the International Minerals and Chemical Corporation, Chicago, and by a contract with the Office of Naval Research. The major portion of the glycocyamine and all of the betaine used was provided by the International Minerals and Chemical Corporation.

The Doctors Borsook prepared the first paper of this series with complete equality of authorship.

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†Reviews of the development of knowledge of the normal and abnormal metabolism of creatine and creatinine have been published by Hunter[1], Schoenheimer[2], and Du Vigneaud[3].

muscle wasting[32]. The muscular weakness in Graves' disease has been likened to that in the myopathies by several authors[22, 34,35].

Space does not permit review of the extensive literature on the urinary creatine and creatinine in thyroid disorders. All the data are in accord with the view that most of the urinary creatinine is derived from the total tissue phosphocreatine, that neither increased creatine production nor feeding of creatine increases the urinary creatinine unless the creatine is "fixed" first as phosphocreatine.

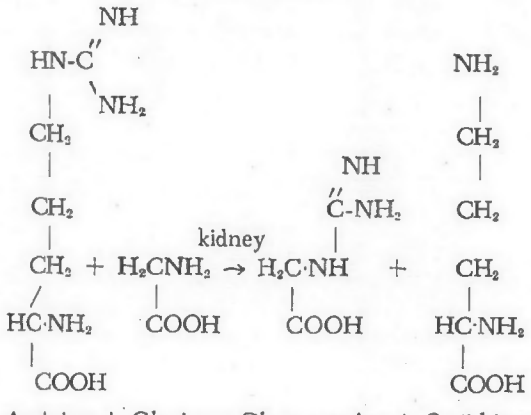
All of these observations are consistent with the hypothesis that larger than normal amounts of creatine may be beneficial in conditions of muscular weakness or degeneration, including cardiac failure, and in some neurological conditions, because phosphocreatine is the reservoir of immediately available energy also in nerve. The feeding of massive doses of creatine is unphysiological. It seemed preferable to follow the physiological process more closely by feeding the immediate precursors of creatine.

Precursors of Creatine

The immediate precursor of creatine in animals is glycocyamine (guanidoacetic acid). It is synthesized from arginine and glycine in the kidney[35-37]. Glycocyamine is then transported to the liver where it is methylated by methionine to form creatine[37, 38]. But there is experimental evidence that betaine can supply the necessary methyl group. This is accomplished via homocysteine and betaine[37].

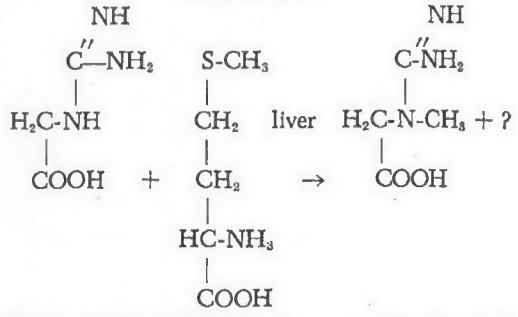
The reactions by which creatine is formed in animals may be summarized as follows:

Reaction I



Arginine + Glycine→ Glycocyamine + Ornithine

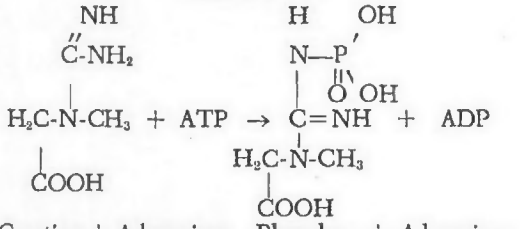
Reaction II



Glycocyamine + Methionine → Creatine + ?

Creatine is converted in muscle, nerve, et cetera to phosphocreatine by reaction III.

Reaction III

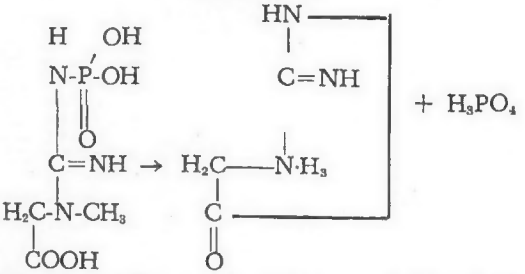


Creatine + Adenosine-triphosphate → Phospho-creatine + Adenosine-diphosphate

In the N-P bond of phosphocreatine resides energy for physiological and metabolic work. This energy becomes available for metabolic work by rapid back transfer of the phosphate of phosphocreatine to adenosinediphosphate, thereby converting the latter to adenosinetriphosphate. This is the reverse of reaction III. In muscle there is more phosphocreatine than adenosinetriphosphate; and hence phosphocreatine is a reservoir of available energy. The ultimate source of the energy is, of course, food stuff combustion.

The reaction by which phosphocreatine is converted to creatinine is

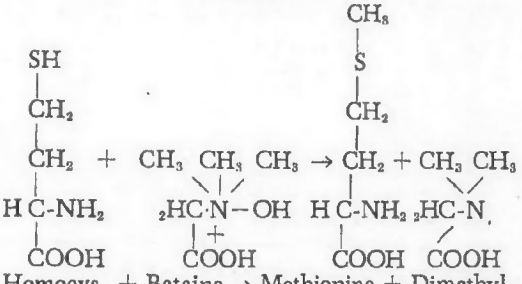
Reaction IV



Phosphocreatine → Creatinine + Phosphate

The product which arises after methionine has lost its methyl group in the synthesis of creatine (equation II) is not known. One of the possible, even probable, products is homocysteine; and it has been established that homocysteine is converted in the liver to methionine by interaction with betaine[38-39]. The reaction is as follows:

Reaction V



The methionine replenished by reaction V can methylate glycocyamine to form creatine by reaction II. The biochemical basis of betaine-glycocyamine therapy is in reactions II and V.

Determination of Dose

In the course of the last six to twelve months, over 200 patients have taken glycocyamine together with betaine. The daily dose, by mouth, is glycocyamine, 30 mg. per pound of bodyweight, and betaine hydrate four to five times the dose of glycocyamine. This amount of glycocyamine, after methylation to creatine, is approximately three times the normal production of creatine. The above quantity of betaine was given because of the inefficiency of the methylation process. The two substances were taken together, the glycocyamine in tablets, the betaine dissolved in water, and the total daily dose divided into four or five portions through the day.

Possible Toxicity

The daily ingestion of betaine and glycocyamine at approximately the above levels may be considered as nontoxic in view of the following experimental and clinical data. Many times the above level of betaine (on a per kilo basis) could be given to rats and dogs orally or parenterally, for weeks, without any evidence of toxicity[40]. The question of the possible toxicity of the glycocyamine needs to be considered in somewhat more detail. Glycocyamine fed to rats on a diet suboptimal in methionine, devoid of choline, and low in folic acid and vitamin B_{12}, brought about cessation of growth or fatty liver[38-42]. The level of glycocyamine in these experiments (on a per kilo basis) was seven times or more the therapeutic level prescribed above; the excessive glycocyamine fed drained the depleted stores of labile methyl groups (see equation II above), and the signs of methyl group deficiency appeared. Betaine fed with the glycocyamine together six to twelve months. These formation of fatty liver[38], (see equations V and II above).

Even when the diet is devoid of methionine or choline but adequate in folic acid and vitamin B_{12} glycine and serine can provide the quantity of labile methyl groups (in choline and methionine) necessary for normal growth[43-46].

Clinical Study

Aproximately two hundred patients have taken daily the above amounts of betaine and glycocyamine together six to twelve months. These patients evidently had an adequate intake of folic acid and vitamin B_{12}, as they showed no evidence of folic acid or vitamin B_{12} deficiency. The glycine and serine alone from the protein in their diets, without taking into account the methionine and choline it contained, could have provided enough methyl groups to methylate the glycocyamine administered. In addition they received three to five times the betaine required to methylate the glycocyamine.

Accordingly, as was to be expected, neither the symptoms, nor extensive blood and urine analyses indicated any toxicity of the betaine and glycocyamine ingested[40].

Betaine may prove useful in the treatment of liver damage where the therapeutic indications are for large doses of labile methyl groups: DL-methionine is toxic[47]; betaine is not. Furthermore, as betaine is much more soluble than methionine, it is much easier to administer.

The papers that follow report the effects of betaine with glycocyamine in patients with heart disease. The effects of betaine with glycocyamine have been studied in addition in cases of muscular weakness following poliomyelitis one and one-half years or more previously, in certain myopathies of peripheral and central origin, and in cases of arthritis; the findings in these groups will be reported later.

Sthenic Effect

One effect has been outstanding. Within one or a few weeks after the beginning of therapy many patients reported an improved sense of well being, less fatigue, and an increased desire for and performance of physical and mental work, according to the occupation of the patient. This sthenic effect has appeared and persisted whatever the degree of specific objective improvement and is referable, presumably, to the nervous system.

Further Studies in Process

The clinical results tend to support the hypothesis stated above. It needs to be tested further by

studying the effects of creatine, betaine and glycocyamine alone. Such studies are planned. Also studies on the metabolism of betaine and glycocyamine are now under way. Until the results of clinical and metabolic studies such as these become available the hypothesis from which this work began can be held only tentatively.

Acknowledgement: We are indebted to and wish to thank Capt. C. C. Shaw (MC, USN), Director of Research, Bureau of Medicine and Surgery, for making possible certain of the clinical tests of the hypothesis proposed in this paper.

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UNITED STATES AIR FORCE REQUESTS APPLICATIONS FROM DOCTORS

Men and women practitioners in the medical profession and specialized vocations allied to medicine and surgery now have an opportunity to step directly from civil life into the ranks of commissioned officers in the United States Air Force, according to an announcement released by Lt. Colonel Charles D. Morat, Jr., Director of Personnel Procurement at headquarters of the First Air Force, Mitchel Air Force Base, N. Y.

The classifications from which officers are sought by the Air Force are: doctors of medicine, medical research and allied science specialists, physical and occupational therapists, medical supply and administration specialists, environmental and industrial hygiene engineering specialists, dietitians and nurses.

In each category, a definite set of educational and qualifying experience standards are required, with increasing professional attainment minimal for progressively higher ranks. In all cases, degrees must have been granted by colleges and institutions acceptable to the surgeon general of the U. S. Air Force. For women, an age limit of 45 years has been established. While the commissions offered under this program are in the U. S. Air Reserve, applicants must be ready to be called to active duty at the discretion of the Air Force.

Because each classification has its own set of requirements for each grade from second lieutenant to the higher ranks, the First Air Force has set up clerical facilities for prompt servicing of all inquiries regarding these commissions. Men and women who are interested in any of the specified professions are invited to write to the Air Surgeon, Headquarters First Air Force, Mitchel Air Force Base, New York.

Treatment of Cardiac Decompensation With Betaine and Glycocyamine

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THIS REPORT describes our experience in the use of betaine and glycocyamine in the treatment of patients with heart disease. The theoretical considerations underlying this form of therapy have been presented in the foregoing paper.[1] The following represents a logical extension of these implications into the field of applied therapeutics.

Specific Considerations

At autopsy, hearts with abnormally large muscle mass are sometimes found, and yet the cause of death is cardiac failure. Again, hearts which have suffered extensive infarction resulting in reduced muscle mass maintain an adequate circulation. To do so, the remaining myocardial cells must do more work. Hence, cardiac failure cannot be explained on anatomical grounds alone. Perhaps, then, a large reservoir of immediately available energy to an inadequately functioning heart would be helpful. The reservoir of immediately available energy in heart as in skeletal muscle is phosphocreatine, which constitutes the cardiac reserve. The creatine content has been found to be subnormal in the hearts of patients who died of cardiac failure.[2–5] These findings are in accord with those obtained in animal experiments.[7–9]

Experimental Design

Suitable data were obtained from 28 patients; of these, eight had rheumatic and 20 had arteriosclerotic heart disease. The same general procedure was followed in each case. After the initial evaluation, all medication was withdrawn and betaine and glycocyamine therapy was begun. In nearly every instance the daily amount given was 30 mg. of glycocyamine and 150 mg. of betaine per pound of body weight, administered in four equal doses. All patients were initially office or home cases. The two who died were hospitalized shortly before death. All patients were seen at intervals of five to seven days, or oftener, when necessary. The evaluation was based on subjective and objective findings. Laboratory studies included the examination of the blood and urine, radiography, electrocardiography, and the determination of urinary creatine and creatinine. In some instances, additional tests were made. The patients were observed for periods varying from six to ten months.

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Results

The patients studied can be divided, according to the clinical course, into the following groups:

Group I: Fourteen patients experienced no untoward symptoms after the substitution of betaine and glycocyamine for other therapy. They improved rapidly and retained the improvement, except that one patient suffered coronary occlusion while under treatment.

Group II: Six patients improved but not until after a few stormy and uncomfortable days following the substitution of betaine and glycocyamine for other forms of therapy. Except for this, they are not distinguishable clinically from the patients in Group I.

Group III: Four patients, at least for a time, required other forms of therapy in addition to betaine and glycocyamine.

Group IV: In three instances the treatment failed and two of the patients died.

Presentation of Group I

This group includes ten patients with arteriosclerotic, three with rheumatic, and one with arteriosclerotic and thyrotoxic heart disease. Five patients had one or more coronary occlusions and one of these (D. L.), in addition, had heart block. Six of the group had hypertension; one had hypotension (70 mm. Hg. systolic and 50 diastolic pressure); none were fibrillating; one was a diabetic. Their break in cardiac tolerance was of seven months' to six years' duration. Ten patients were women and four men, with an age range of 45 to 76 years.

A brief outline of the clinical course of ten representative cases of the group is given here. They depict the clinical course of the entire group and, in addition, certain special features.

Case 1

A 46-year-old white man was first seen May 10, 1950. Until the age of 45 he was well and strong. Then he suffered an attack of coronary occlusion and myocardial infarction. Four months later, he had a similar injury. Prior to these events, he had never been afflicted with any illness ordinarily considered to affect the heart. His family history was good. Following recovery from the second infarction, the electrocardiogram had the typical

pattern of healed posterior wall infarction. He slowly improved but was greatly limited in his activities by dyspnea, anginal pain and fatigue. He was unable to return to work, because of the distressing nature of his symptoms. His presenting complaints were severe chest pain and shortness of breath on slight effort. He was weak and had little endurance. A slow walk of 50 feet caused angina. He slept poorly on three pillows. Therapy consisted of extra rest and nitroglycerin for pain; digitalis was not given.

Physical Examination: This obese white man, 5 ft., 8 in. tall, weighed 204 pounds. The heart was not enlarged, the sounds were of fair quality, and no murmurs were heard. The blood pressure was 110 mm. Hg. systolic and 80 diastolic, and the pulse rate 84. The chest was clear, the liver was not palpable, and no edema of the extremities was present.

In the teleroentgenograms the heart was normal in size, measuring 13.2 cm. in its transvere diameter, the predicted normal value being 14.4.

The red blood cell count was 5,300,000 and the leukocyte count was 6,800, with a normal differential distribution. The fasting blood sugar was 82, and the N.P.N. 31 mg. per cent. The blood electrolytes were within normal limits. The sedimentation rate was normal. Urinalysis revealed no abnormality.

Glycocyamine and betaine therapy was initiated and nitroglycerin discontinued on June 9, 1950. No significant change occurred during the next ten days. During the succeeding two weeks he reported less pain and very little dyspnea; he felt stronger and had greater endurance. He was able to walk 500 feet without symptoms. This was a topic of comment by his wife and friends. His weight remained unchanged. He continued to improve, with increasing exercise tolerance. Five weeks after the initiation of betaine and glycocyamine therapy, he was so much improved that he experienced neither dyspnea nor angina on walking over a mile at a moderate pace. He tired at the end of the day but slept soundly and awakened refreshed.

Three weeks later, he returned to his work as a truck dispatcher. While not experiencing dyspnea nor angina, he became faint and dizzy in the early afternoon. During the following week, this recurred and was accompanied by hunger and a cold perspiration. The electrocardiogram did not indicate any further heart injury. It was discovered that in an effort to reduce weight he ate a scanty breakfast and no lunch. He presented, clinically, the classical picture of hypoglycemia. His blood sugar was 48 mg. per cent at 3:00 p.m. He was advised not to try to reduce but to eat a good breakfast and lunch. Should these symptoms recur, he was to drink a glass of sweetened orange juice. Following this, slight faintness still occurred in the early afternoon but was quickly abolished by the ingestion of orange juice. Otherwise, he remained strong and well and placed no limitation on his activities.

His exercise tolerance continued to increase during the next four months. At no time did he develop symptoms referable to the heart. He did a full day's work. His weight remained unchanged. His blood pressure slowly rose to 118 mm. Hg. systolic and 80 diastolic, at which level it has remained, with minor variations. On November 11, 1950, he made a ten-day automobile trip, driving approximately 400 miles a day without experiencing distressing symptoms or undue fatigue. On December 15, 1950, he left the city permanently to take up farming. He reported by mail that he continued to feel well and that he experienced no physical difficulty in his new occupation.

Comment: Within ten days after taking betaine and glycocyamine, this patient reported that he felt much better, was capable of increased activity to a significant degree, and he experienced less fatigue. Shortly thereafter, he was able to return to work. Noteworthy was the development of moderately severe hypoglycemia. No significant electrocardiographic changes or alterations in heart size were observed. These events coincided with the administration of glycocyamine and betaine.

Case 2

A 73-year-old white woman first came for examination on July 4, 1950. From the age of 22, she had rheumatism which affected her hands and knees. In 1945, she underwent a cholecystectomy for empyema of the gallbladder, from which she had made a good recovery. She was in her usual state of health until the autumn of 1948, when she had experienced shortness of breath and slight constricting chest pain on effort. These attacks had increased in frequency. They lasted from a few seconds to about three minutes and subsided with rest. Excitement frequently provoked an attack. During the past two years, her feet and ankles had become swollen. She had also been afflicted with cramps in her calves at night, which were relieved by vigorous rubbing or walking. Nocturia had been troublesome. The family history was good.

The chief presenting complaints were shortness of breath on effort, swelling of the feet and nocturnal leg cramps. She could walk one block at a moderate pace, when angina and dyspnea caused her to rest. She did light housework with frequent

rests. She found it necessary to sleep on three pillows; her hands and knees were always stiff in the morning and frequently would be painful and swollen.

Physical Examination: The patient was a large white woman, with lips and finger-tips slightly cyanotic, who became dyspneic on walking 36 feet to the x-ray room. She weighed 166 pounds; her height was 5 ft., 8 in. The heart was moderately enlarged, the apex beat being in the sixth interspace, at the anterior axillary line. The blood pressure was 178 mm. Hg. systolic and 90 diastolic, and the pulse rate 98. Fine râles were heard over both pulmonary bases. The liver was palpable one finger-breadth below the costal margin. Edema of the extremities extended to the tibial tuberosities. Her hands were stiff. She could not close her fingers. Her knees were slightly swollen, tender, and had a 15-degree flexion deformity.

The teleroentgenogram showed the heart to be moderately enlarged, chiefly to the left; the transverse diameter was 15.6 cm., and the predicted normal value 13.3 cm. The electrocardiogram showed normal rhythm at a rate of 90. There was moderate left axis deviation and the T waves were inverted in Leads I and CF_5. The record was consistent with the diagnosis of left ventricular strain and hypertrophy.

The red blood cell count was 5,900,000; the hemoglobin 15.6, and the leukocyte count 11,300, with a normal differential distribution. The fasting blood sugar was 88, and the N.P.N. 34.2 mg. per cent. The urine was of 1.012 specific gravity, contained a trace of albumin and occasional hyaline and granular casts, but was not otherwise abnormal. The sedimentation rate was slightly rapid, being 28 mm. in one hour (Wintrobe).

Clinical and laboratory findings were consistent with the diagnosis of hypertensive and arteriosclerotic heart disease, moderate cardiac enlargement and congestive failure; additional diagnoses were nocturnal claudication, and rheumatoid arthritis.

Beginning July 15, 1950, she received 5 gm. of glycocyamine and 25 gm. of betaine in five equally divided doses daily. During the following week, she felt much worse. Her hands and knees were more painful, and her cardiac symptoms just as severe. The signs of cardiac decompensation, however, had lessened. No basal râles were heard, the liver was not palpable, and the edema of the extremities less. The blood pressure had dropped to 150 mm. Hg. systolic and 80 diastolic, and the pulse rate to 92.

Two weeks after the start of treatment with betaine and glycocyamine, she reported that during the previous four days, she had not noticed any chest pain, and her dyspnea was much less. She felt stronger and had greater endurance. The flare-up of her arthritis had subsided and her hands and knees were no longer painful. She had experienced no leg cramps during the past five days. Examination revealed no signs of cardiac decompensation. Slight edema of the right ankle, which she had injured, was present.

The patient continued to improve during the next two weeks. By August 12, 1950, she no longer had any cardiac complaints and was able to do five hours' continuous housework without distress. She slept well and was not troubled by nocturnal leg cramps. There was no sign of cardiac decompensation. The blood pressure was 134 mm. Hg. systolic and 90 diastolic and the pulse rate 86. She noted that her hands and knees were much more mobile and the morning stiffness was absent. The 15-degree deformity which was formerly present in her knees was now absent. She could close her hands completely without difficulty.

During the next six months, she increased her activity and developed a remarkable sense of well-being. When last seen March 10, 1951, she was discharging all her household duties and was able to walk two miles without distress, the pulse rate and blood pressure returning to their former level in 70 seconds. During the six previous months, her blood pressure varied between 144 mm. Hg. systolic and 90 diastolic and 200 mm. Hg. systolic and 100 diastolic. There were no signs of cardiac decompensation. No significant changes appeared in the serial electrocardiograms and teleroentgenograms.

Comment: In this patient, clinical improvement coincided with the administration of glycocyamine and betaine. She felt much better. Exercise tolerance was significantly increased. The disappearance of nocturnal claudication, improvement of the rheumatoid arthritis of the hands and knees, and a transient fall in blood pressure occurred.

Case 3

A 62-year-old housewife, on June 6, 1950, presented complaints of severe chest pain, shortness of breath on effort and occasionally even at rest. She tired easily and could walk slowly three blocks, when she was compelled to rest. She slept poorly on three pillows. Her family history was good. She had been well and strong until October 1948, when she suffered an attack of coronary occlusion and myocardial infarction. Previous to this she had been perfectly well and had not suffered from any illness which ordinarily is considered to affect the heart. She slowly recovered following her hospital stay but was greatly limited in her activity by shortness of breath and chest

pain. Early in February, 1950, her symptoms had become more severe and swelling of the feet and ankles had developed. From March 17, 1950, when she was digitalized, she had received mercupurin® every five to seven days, with salt and fluid intake moderately restricted and activities limited. However, she continued to go downhill.

Physical Examination: The white woman, 5 ft., 2 in. tall, weighed 139 pounds. The heart was not enlarged. The heart sounds were of fair quality and no murmurs were heard. The blood pressure was 100 mm. Hg. systolic and 80 diastolic. The pulse was 102 and regular. Fine râles were heard over both pulmonary bases. The liver was palpable two fingerbreadths below the costal margin and edema of the lower extremities extended to the mid-calf.

In the teleroentgenogram, the size of the heart was within normal limits, measuring 11 cm., in its transverse diameter, the predicted normal value being 11.8 cm. The electrocardiogram showed normal rhythm at a rate of 96. There was left axis deviation and the QRS complexes were prolonged (0.12). In the precordial leads, V_4 through V_6, the ST segments were depressed but were otherwise normal. The fasting blood sugar was 86 and the N.P.N. 29 mg. per cent. The sedimentation rate was normal. No abnormality was found in the examination of the blood and urine.

The clinical and laboratory findings were consistent with a diagnosis of coronary heart disease and slight congestive failure.

On June 6, 1950, all previous therapy was discontinued and replaced by 6 gm. of glycocyamine and 24 gm. of betaine in four equally divided doses. No significant changes occurred during the next six days following which her symptoms increased slightly during the next two days.

Five days later she reported that the pain and dyspnea were much less. She felt stronger, tired less easily and could do more housework. She slept better and awakened refreshed. She had lost three pounds. No basal râles were heard, the liver was not palpable and only slight ankle edema was present.

On June 24, 1950, she reported that she had experienced no angina nor dyspnea for the past three days. She felt fit, stronger, had greater endurance and was doing all her housework. This was confirmed by her husband. They both stated that she had walked one and a half miles at a moderate pace. She had gained three pounds. She was able to lie flat on the examining table without distress. No signs of cardiac decompensation were present. On July 1, 1950, she was without symptoms ordinarily referable to the heart and felt as well as she did before her coronary occlusion. She was doing anything she wished to do, without distress, and was without signs of cardiac decompensation.

When last seen March 15, 1951, she had been without signs or symptoms of cardiac decompensation for a period of approximately nine months. She felt fit and strong. Her weight had slowly risen to 146 pounds. Early in September 1950, she reported that her breasts were slightly larger and more tender than before and that there was a considerable increase of libido. These changes have persisted. No significant electrocardiographic changes have occurred.

Comments: In this patient, within two weeks after the discontinuance of her previous therapy and the substitution of betaine and glycocyamine, improvement was noted. One week later she was without the signs and symptoms of cardiac decompensation. She developed a remarkable sense of well-being, increased strength and endurance, and she appreciably increased her activities. Her breasts enlarged and libido increased. These changes coincided with the administration of glycocyamine and betaine.

Case 4

A 63-year-old white woman was first seen May 26, 1950. Her presenting symptoms were extreme shortness of breath at rest and more on effort, palpitation, weakness, and nervousness. At the age of 12, she had mild thyrotoxicosis and was treated with bed rest at first and later, in middle life, with iodine. During the past three and one-half years, she had become increasingly short of breath, had experienced some vague chest pain, and developed swelling of the lower extremities. She gradually became worse, with increasing dyspnea and dependent edema. For the past two years, she had slept in a stuffed chair. She had been unable to wear shoes but had worn slippers which were split down the front. With assistance, she could walk to the bathroom — a matter of a few feet. She slept poorly. She had been continually tired and extremely weak. No medical attention was received during the past few years.

Physical Examination: The orthopneic white woman, 5 ft., 1½ in. tall, weighed 128 pounds on bathroom scales. She was examined in a sitting position. Her heart was moderately enlarged, the apex being in the fifth interspace, close to the anterior axillary line. The heart sounds were of poor quality. The blood pressure was 144 mm. Hg. systolic and 60 diastolic and the pulse rate was 114. Fine râles could be heard over both pulmonary bases. The liver was palpable three finger-breadths below the costal margin. Slight ascites was detected and edema of the extremities

extended to above the knees. A fine tremor was present. When the patient swallowed, a small nodular goiter was felt.

The teleroentgenogram showed the heart to be moderately enlarged, the transverse diameter measuring 14 cm., the predicted normal being 13 cm. The electrocardiogram showed normal rhythm at a rate of 108. In the limb leads the QRS complexes showed left axis deviation and the T waves were flat in Leads 1 and 2 and diphasic in Lead 3. In Lead CF_5 there were prominent QS waves, and the T waves were inverted.

The red blood cell count was 4,050,000, hemoglobin 76 per cent, and the white blood cell count 9,800, with a moderate lymphocytosis. Fasting blood sugar was 88, and the N.P.N. 36.4 mg. per cent. The total cholesterol and cholesterol esters were within the low limits of normal, being 130, and 78 mg. per cent, respectively. The blood iodine was 6.2 micrograms per cent.

Clinical and laboratory findings were consistent with a diagnosis of arteriosclerotic and thyrotoxic heart disease, moderately enlarged heart, sinus tachycardia, and congestive failure.

Therapy consisted of 6 gm. of glycocyamine and 30 gm. of betaine daily, in five divided doses. The patient was seen for the first ten days at home and made a surprising and dramatic improvement. Three days after the initiation of betaine and glycocyamine therapy, she stated she felt better, stronger, and was less tired. On the fifth day, the liver was barely palpable and only an occasional râle could be heard over the pulmonary bases. Ascites could not be detected and the edema of her extremities was considerably less. The pulse rate had dropped to 100.

One week later, she was able to come to the office, when she stated that she was experiencing no chest pain, that her dyspnea had greatly diminished, and that she was now sleeping in bed on two pillows. She had washed some dishes. These statements were confirmed by her husband and daughter. She was wearing shoes for the first time in two years. She was able to lie flat on the examining table without distress. No basal râles were heard. The liver was not palpable, and only moderate ankle edema was present. The blood pressure was 140 mm. Hg. systolic and 80 diastolic, and the pulse rate 82. She had lost seven pounds.

Ten days later, the patient reported no chest pain, dyspnea, nor palpitation, except when she hurried or became tired. She felt much stronger, was tired only at night, but slept well on two pillows and awakened refreshed. She was doing light housework, such as washing dishes and cooking, and had walked as far as a third of a mile without distress. She had gained five pounds, without clinical evidence of edema.

During the next ten days, she continued to improve. She then stated that she was doing all her housework, had even worked in the garden, and had walked slowly one mile, without dyspnea or undue fatigue. There were no signs of cardiac decompensation present. The blood pressure was 142 mm. Hg. systolic and 78 diastolic, and the pulse rate 80.

She continued to improve. One month later, she felt extremely well, strong, and had greater endurance. She was able to do all her household chores, some gardening, and had even painted the kitchen. She had gained three more pounds without clinical evidence of edema. The blood pressure at this time was 190 mm. Hg. systolic and 90 diastolic, and the pulse rate 84. Because her blood pressure had risen so high, it was felt advisable to reduce the dose of glycocyamine to 4 gm. and betaine to 20 gm.

During the next six months, the patient continued to feel extremely fit, with appreciably increased activities, and at no time, until March 10, 1951, when she was seen last, did she experience symptoms referable to the heart, nor did signs of cardiac decompensation occur. She slowly gained ten pounds since the initiation of betaine and glycocyamine therapy, without clinical evidence of edema. During a three-day period of extremely hot weather, September 2 — 5, 1950, she drank a great deal of water and had a urinary output of 12,350 cc. No significant changes were observed in serial teleroentgenograms or electrocardiograms, with the exception of reduction of heart rate. The blood pressure during the past six months varied between 148 mm. Hg. systolic and 90 diastolic and 164 mm. Hg. systolic and 92 diastolic, and the pulse rate varied between 72 and 88.

Comment: A remarkable and dramatic clinical improvement occurred in this patient, which coincided with the administration of glycocyamine and betaine. The patient felt much better, became compensated, greatly increased her activities, and gained ten pounds in weight, the latter without clinical evidence of edema.

Case 5

A 57-year-old white woman was seen for the first time June 2, 1950, complaining of severe chest pain and shortness of breath on effort. She felt poorly and weak and had little endurance. She had been perfectly well and strong until February 1949, when she suffered a coronary occlusion with myocardial infarction. She slowly recovered but was greatly limited in her activities by dyspnea and anginal pain. She was unable to do any housework, could walk slowly one block on the

level, but not on the upgrade. She had received digitalis, aminophylline and phenobarbital; salt had been restricted; she was advised to limit activity.

Physical Examination: The well-developed and well-nourished white woman weighed 131 pounds and was 5 ft., 2 in. tall. The heart was not enlarged, the apex beat being in the fifth interspace close to the midclavicular line. The heart sounds were of good quality and no murmurs were heard. The blood pressure was 160 mm. Hg. systolic and 92 diastolic, and the pulse rate 80. A few râles were heard over the pulmonary bases. The liver was not palpable, and edema of the extremities was not present. An anginal attack occurred during the course of the examination.

In the teleroentgenogram, the heart was well within the normal limits of size, measuring 11.8 cm. in its transverse diameter, the predicted normal value being 12.3 cm. The electrocardiogram showed normal sinus rhythm, at a rate of 90. In the limb leads the QRS complexes showed left axis deviation, and the T waves were low and diphasic in Lead 1. No other significant changes were present. In the precordial leads the T waves were inverted in CF_5 and CF_6. The examination of the blood gave evidence of no abnormalities of the erythrocytes or leukocytes. The fasting blood sugar was 97 and the N.P.N. 31.2 mg. per cent. The blood electrolytes were normal. No abnormality was found in the urinalysis.

All previous medication was discontinued and replaced by 4 gm. of glycocyamine and 20 gm. of betaine in four equally divided doses daily. One week later, she reported that her angina and dyspnea were much diminished. She was stronger had greater endurance, and slept better. The blood pressure was 110 mm. Hg. systolic and 80 diastolic, and the pulse rate was 74.

During the following week, she experienced neither angina nor dyspnea on effort. There were no signs of cardiac decompensation. The blood pressure was 124 mm. Hg. systolic and 80 diastolic, and the pulse rate 76. She had gained one and one-half pounds in weight. She felt remarkably better, stronger, had greater endurance and was doing some of her housework.

During the ensuing month she appreciably increased her activities, felt remarkably fit, and was without symptoms referable to the heart. She stated (and her family confirmed the statement) that she was doing all her housework, including the washing and ironing, and had walked over a mile including a steep upgrade without experiencing any distress. The blood pressure remained within normal limits.

During the next six months, her clinical course was characterized by a remarkable sense of well-being, increased strength and endurance, and appreciably increased activity, all with no symptoms referable to the heart nor signs of cardiac decompensation nor hypertension. She slowly gained 12 pounds. Serial electrocardiograms, taken at intervals of three weeks, at no time indicated any significant change.

Comment: Following the removal of all previous therapy, the patient reported no untoward symptoms, and in a few days, observed the diminution and finally the disappearance of angina and dyspnea on effort. She developed a remarkable sense of well-being and appreciably increased her activities, without suffering undue fatigue. A significant and persisting drop in blood pressure was observed, and a substantial gain in weight occurred. These changes coincided with the administration of betaine and glycocyamine.

Case 6

A 63-year-old white man was first seen July 31, 1950. He had an easily provoked chest pain and dyspnea on effort, fatigue, weakness and insomnia. His family history was good. He had been exceptionally strong and well and had never been afflicted with any disease which ordinarily is considered to affect the heart until July 1947, when he experienced severe constricting substernal pain which lasted several hours. Following this event, a short walk of one block had provoked pain which lasted about one minute and was relieved by rest. The frequency and severity of these attacks of pain had caused him to discontinue going to his place of business. In the fall of 1947, he had consulted a physician who had restricted his activities, prescribed digitalis, papaverine, and nitroglycerin.

Physical Examination: The patient was a nervous person, 5 ft., 6½ in. tall, weighing 129 pounds. The blood pressure was 114 mm. Hg. systolic and 80 diastolic, and the pulse rate 56. The heart was not enlarged and the heart sounds were of good quality. No murmurs were heard. There were no congestive phenomena.

A teleroentgenogram showed the heart to be well within normal limits as to size, the transverse diameter being 10.6 cm. and the predicted normal value 11.8 cm. An electrocardiogram recorded partial A-V block, with a ventricular rate of 60 and an auricular rate of 92. In the limb leads, the QRS complexes were normal in axis and amplitude, and slightly prolonged in duration, being 0.11 second. The S-T segments were isoelectric in Lead 1 and slightly depressed in Leads 2 and 3, with upright T waves. R waves were absent in the

precordial leads V_1 through V_4, which were otherwise normal.

The blood and urine were normal, the fasting blood sugar being 102 and the N.P.N. 33.6 mg. per cent. Blood electrolyte values were within normal limits. The clinical and laboratory findings were thought to be consistent with the diagnosis of coronary heart disease, old healed myocardial infarction, partial A-V block and angina pectoris.

On July 31, 1950, all previous therapy was discontinued and replaced with 4 gm. of glycocyamine and 20 gm. of betaine daily, in four equally divided doses. No significant change occurred until August 8, 1950, at which time exercise which formerly provoked pain no longer did so, but he was still short of breath. He slept better. The blood pressure and pulse rates were unchanged. He had gained four pounds in weight.

During the next three weeks, he occasionally experienced angina and slight dypsnea on effort but generally felt better, with less fatigue.

He slowly improved during the next two months with decreasing frequency and severity of his cardiac symptoms. His strength and endurance continued to increase and two months after the initiation of betaine and glycocyamine therapy he had walked a mile without distress. His blood pressure and pulse rate were unchanged. He had gained 13 pounds in weight. In order to ascertain whether the rapid gain in weight was due to retention of fluid or not, he was given, as a clinical test, a mercurial diuretic intravenously. No substantial diuresis nor weight loss occurred during the ensuing three days.

On December 26, 1950, he had been without chest pain or shortness of breath for two months. He was able to do 50 bends without distress. His clinical condition remained the same during the following three months. The only noteworthy event was the slow rise in his weight to 145 pounds. No significant change appeared in the serial electrocardiograms. The blood pressure varied between 110 mm. Hg. systolic and 80 diastolic and 126 mm. Hg. systolic and 84 diastolic, the pulse rate between 60 and 64.

Comment: In this patient the substitution of betaine and glycocyamine for other therapy coincided with clinical improvement. He felt much better, angina and dyspnea disappeared, and his exercise tolerance was significantly increased.

Case 7

A 62-year-old white man, on July 17, 1950, complained of severe angina and dyspnea on slight effort or excitement. He had been well and strong until the age of 56, when he suffered an attack of coronary occlusion and myocardial infarction. His family history was good. Prior to this injury he had never been afflicted with any illness which ordinarily is considered to affect the heart. Following the infarction, he slowly improved but was greatly limited in his activity by dyspnea and anginal pain. He had not been able to work since his heart attack. A walk of half a block or any slight exertion or excitement caused severe chest pain and dyspnea. The pain radiated up to his left shoulder and both upper arms would become numb. He slept poorly on two pillows. He was tired and weak. His treatment consisted of morphine and nitroglycerin, with a salt-poor diet and restricted activity.

Physical Examination: The well-nourished white man was 5 ft., 4 in. tall, weighing 150 pounds. The heart sounds were of good quality. No murmurs were heard. The chest was clear, the liver palpable three finger-breadths below the costal margin and there was no edema of the extremities. The apex beat was in the fifth interspace just to the left of the midclavicular line. The blood pressure was 160 mm. Hg. systolic and 80 diastolic and the pulse rate 72.

In the teleroentgenogram the heart was at the upper limits of normal size, measuring 13.4 cm. in its transverse diameter, the predicted normal value being 13.0 cm. The electrocardiogram showed normal rhythm at a rate of 80. In the limb leads the RS-T segments were depressed in Leads 1 and 2, and the T waves were low in Lead 1. In the precordial leads, the R waves were absent in CF_3, and the ST segment slightly elevated. In Lead CF_5, the T wave was inverted.

The red blood cell count was 5,450,000 and the hemoglobin 15.5 gm. The leukocyte count was 7,400 with a normal differential distribution. The fasting blood sugar was 88, and the N.P.N. 31.4 mg. per cent. The blood electrolytes were normal. No abnormality was found in the urinalysis.

The clinical and laboratory findings were thought to be consistent with the diagnosis of coronary heart disease, slightly enlarged heart, angina pectoris, and mild congestive failure.

On July 15, 1950, all therapy was discontinued. Six grams of glycocyamine and 30 grams of betaine, in four equally divided doses, were given daily. Three days later, he reported that a "weight has been lifted from my chest." Nine days after the initiation of betaine and glycocyamine therapy, he stated he had no angina, very slight dyspnea, and he felt remarkably fit. He felt stronger, tired less easily, and had walked six blocks without distress. He was sleeping well. He was of a happier disposition and was more active. He had gained five pounds in weight, the chest was clear and the liver barely palpable. Edema of the extremities was no longer present.

One week later, he reported that for the past four days he had experienced neither dyspnea nor angina, felt better and stronger, and did not tire so easily as formerly. He had walked up 15 steps, without distress, and he slept well without sedation. He was examined lying flat on the examining table, without complaining of either dyspnea or angina. There were no signs of cardiac decompensation. The blood pressure was 144 mm. Hg. systolic and 70 diastolic, and the pulse rate 74.

Ten days later, under a low spinal anesthesia (pontocaine®) his coccyx, which had been fractured for many years and was causing pain, was excised. It was noted, during surgery, the blood pressure did not fall nor the pulse rate increase but remained remarkably stable. His convalescence was uneventful. He continued to improve, without signs or symptoms of cardiac decompensation, with increasing strength and endurance, and an excellent sense of well-being.

During the ensuing seven months his blood pressure varied from 130 mm. Hg. systolic and 82 diastolic to 144 mm. Hg. systolic and 80 diastolic and the pulse rate between 72 and 78. He gained ten pounds in weight, without clinical evidence of edema. At that time, he was able to walk approximately one mile and was able to work a little in his tailor shop without distress. Serial teleroentgenograms and electrocardiograms at no time showed any significant change.

On March 30, 1951, he experienced constricting midline pain and shortness of breath. The blood pressure dropped to 100 mm. Hg. systolic and 80 diastolic, and the pulse rate rose to 116. Serial electrocardiograms during the next three days indicated that he had suffered another coronary occlusion with posterolateral myocardial infarction.

The patient is at present hospitalized and is making a slow recovery. Betaine and glycocyamine therapy is being continued.

Comment: Following the discontinuation of the previous therapy and substitution of betaine and glycocyamine the patient rapidly improved. Symptoms referable to the heart and the signs of cardiac decomposition disappeared. He felt remarkably fit, developed increased strength and endurance and appreciably increased his activities. A persisting drop in blood pressure to within normal limits and a weight gain of ten pounds was observed. These changes persisted for eight months, at which time the patient suffered a second coronary occlusion and myocardial infarction.

Case 8

A 51-year-old white woman was first seen on June 16, 1950, when her chief complaints were severe numbness over the precordium and arms and dyspnea which was always present and most severe at night. Since adolescence she had been afflicted with asthma. In 1947 she suffered a coronary occlusion with anterior myocardial infarction. Afterwards, she had progressively become worse. Her asthma had increased in severity and frequently at night necessitated the administration of oxygen and the use of benadryl® and aminophylline. She could do light housework with frequent rests. She slept poorly in a hospital bed with three pillows. She took 1½ grains of digitalis daily and 50 mg. of benadryl® every six hours She found it difficult to breathe, even with the aid of benadryl®.

Physical Examination: The patient was a white woman who appeared much older than her stated age. She was orthopneic and cyanotic. She weighed 130 pounds and was 5 ft., 3 in. tall. The blood pressure was 90 mm. Hg. systolic and 70 diastolic, and the pulse rate 98. The heart was not enlarged. Heart sounds were poor and no murmurs were heard. Coarse and moist râles were heard over the entire chest. The liver was not palpable and no edema was present. In the teleroentgenograms, the heart was well within normal limits, measuring 11.6 cm. in its transverse diameter, the predicted normal value being 12.2 cm. The electrocardiogram showed normal rhythm at a rate of 90. In the limb leads, the T waves were inverted in Lead 1. In the precordial leads, the T wave was diphasic in CF_3, and in CF_5 the R wave was absent and the T wave inverted. This record was typical of those obtained during the previous year and a half and was thought to be consistent with an old lateral infarction.

The leukocyte count was 12,400 with 1,290 eosinophils, the red blood cell count 5,200,000, the hemoglobin 15 gm. The fasting glood sugar was 84 and the N.P.N. 34 mg. per cent. Urinalysis disclosed nothing abnormal.

On June 16, 1950, all previous therapy was discontinued and replaced with 6 gm. of glycocyamine and 30 gm. of betaine in four equally divided doses. Five days later, she reported the numbness to be unchanged, but her dyspnea was much less. She felt stronger, tired less easily, and could do more housework. She slept better and awakened refreshed. The chest was almost clear, the blood pressure was 100 mm. Hg. systolic and 70 diastolic and the pulse rate 90. The eosinophil count was 433.

Five days later, the numbness also had decreased, and she no longer complained of dyspnea. In the company of her daughter she walked at a moderate pace, half a mile. The chest was clear, the blood pressure 106 mm. Hg. systolic and 80 diastolic, and the pulse 86. The eosinophil count had

dropped to 340 and the white blood cell count to 9,200. There were no significant changes in the electrocardiogram.

She continued to improve, and two weeks later reported that she had been without dyspnea or numbness or any other symptom ordinarily referable to the heart or lungs during the past week. She was doing all her housework and had walked a mile without distress. She slept soundly, felt very well, and had gained three pounds. Leukocyte count was 8,200, with 88 eosinophils per cubic millimeter.

She continued to improve during the next seven months, and when last seen on March 15, 1951, she had been without numbness of the chest or shortness of breath, and she felt fit and strong during the entire period. Her weight had slowly risen to 143 pounds. The leukocyte and eosinophil counts had remained within normal limits. Significant electrocardiographic changes were not observed.

Comment: Within two weeks after the discontinuance of her previous therapy and substitution of betaine and glycocyamine, this patient felt better and was capable of increased activity. Coinciding with this improvement were the disappearance of chest signs, a slowing of the pulse rate, and a moderate rise in blood pressure. Increased strength, disappearance of fatigue, and the return of the leukocyte and eosinophil counts to within normal levels also occurred.

Case 9

A 53-year-old white housewife was first seen on July 1, 1950. Her presenting complaints were chest pain and moderate shortness of breath on exertion and occasionally at rest. During childhood and through early womanhood, she suffered several attacks of rheumatic fever. She had also been afflicted with recurring attacks of pain and stiffness involving the hands and knees during the past ten years. In 1944 she underwent a hysterectomy for menorrhagia. She remained in her usual state of health until August 1948, when she developed swelling of the ankles and increasing shortness of breath. A salt-poor diet and digitalis therapy were instituted and extra rest advised. Six months later, the administration of a mercurial diuretic weekly was necessary.

She felt ill, tired easily, and did her housework with difficulty. She could walk slowly five to six blocks, when she was compelled, because of dyspnea, to rest. She had received no treatment for the past six months.

Physical Examination: The well-developed and well-nourished woman weighed 150 pounds; she 5 ft., 4 in. tall. The heart was not enlarged. A thrill could be felt at the apex and a rough mitral presystolic murmur was heard. The blood pressure was 130 mm. Hg. systolic and 60 diastolic and the pulse rate 96. An occasional fine râle was heard over both pulmonary bases. The liver was palpable two finger-breadths below the costal margin. Moderate edema was present to the level of the mid-calf.

The electrocardiogram showed normal rhythm, at a rate of 80 per minute. The P waves were prominent, and the P-R interval was 0.18 second. There was right axis deviation and the T waves were upright in Lead 1, diphasic in Lead 2, and inverted in Lead 3. The S-T segments were isoelectric in Lead 1, and slightly depressed in Leads 2 and 3. In the precordial leads, QRS was upright in CF_1 and CF_2, and inverted in CF_4 and CF_6, with inverted T waves in CF_1 and CF_2, and upright T waves in CF_4 and CF_5. The S-T segments were slightly elevated in CF_1 and CF_2, and the chest leads were not otherwise abnormal. The teleroentgenogram showed the heart to be within the upper limits of the normal size, measuring 12.8 cm. in its transverse diameter, the predicted value being 13 cm. No abnormality was found in the routine blood examination and urinalysis. The fasting blood sugar was 102, and the N.P.N. 31 mg. per cent. The sedimentation rate was normal, being 18 mm. in one hour (Wintrobe).

The clinical and laboratory findings were consistent with a diagnosis of rheumatic heart disease with mitral stenosis, right ventricular enlargement, and congestive failure.

When the patient was first seen, betaine and glycocyamine therapy was started. During the first week there were no significant changes. However, she frequently experienced nausea and gaseous eructations after she took her medication. Five days later, she had so improved that she experienced neither chest pain nor dyspnea on effort and she ran from the back of her yard to the telephone, a distance of approximately 100 feet. She felt much better, had greater endurance, and slept better. The chest was clear, the blood pressure 160 mm. Hg. systolic and 60 diastolic, the pulse 80, the liver not palpable, and no edema of the extremities was present. She had gained five pounds in weight.

She continued to improve during the next three months and when seen on October 12, 1950, was without signs or symptoms of cardiac decompensation. She had gained eight pounds. At that time, she stated that she was going to stop treatment because she was concerned about her weight gain and she did not see the necessity for further treatment.

Eleven weeks later, November 30, 1950, the patient returned. She stated that during the past

month she was again experiencing chest pain and shortness of breath, and no longer felt well, but was weak and had none of the endurance that had developed while she was under treatment. She felt about the same as at the time of initiation of therapy on July 1, 1950. She weighed 162 pounds, the liver was palpable three finger-breadths below the costal margin and edema was present to the top of the calf. No râles were heard over the pulmonary bases. The heart sounds and murmur were the same, and the blood pressure was 100 mm. Hg. systolic and 80 diastolic, the pulse rate 96. X-ray and electrocardiographic findings were unchanged from those originally present.

She again started betaine and glycocyamine therapy. One week later, she was much improved with only slight chest pain and dyspnea on effort. She had greater endurance, felt strong and was able to do most of her housework. She slept better, weighed 161 and was without signs of cardiac decompensation. The blood pressure was 110 mg. systolic and 70 diastolic, the pulse rate 72. Two weeks later, her exercise tolerance had again increased, and this improvement continued during the next three months. Her weight then was 168 pounds. No significant electrocardiographic or x-ray changes were observed.

Comment: The disappearance of the signs and symptoms of decompensation, the development of a remarkable sense of well-being and performance of increased activity coincided with the administration of betaine and glycocyamine therapy. Approximately seven weeks after the cessation of betaine and glycocyamine therapy, she reverted to her former state. She again became compensated and capable of increased activity shortly after therapy was resumed. A causal relationship, in this patient, between her improvement and betaine and glycocyamine therapy, seems likely to exist.

Case 10

A 76-year-old woman of Spanish descent was first seen June 9, 1950. Her presenting symptoms were severe constricting substernal pain which radiated down the left arm and shortness of breath on effort. She also experienced vertigo, blurred and poor vision, and, frequently, severe headaches. She was in her usual state of health until 15 years ago, when she developed symptoms associated with diabetes and was treated with insulin and diet. At the age of 64, she complained of symptoms presumably associated with high blood pressure. She had little limitation of activity, however, until six years ago, when she experienced severe chest pain and shortness of breath on effort. These symptoms had increased in severity and had caused her to curtail sharply her activities. She was treated with digitalis, salt restriction, and nitroglycerin. She had also received supplemental vitamin therapy for a concomitant peripheral neuritis. Two years ago, because her ankles began to swell, mercurial diuretics were a necessary addition to her therapy. She also had experienced vertigo, blurred vision, and frequent headaches. She had been unable to help with the housework or the care of her grandchildren, for the past five years. The family history was significant in that there was a high incidence of diabetes and deaths due to disease of the heart and arteries.

Physical Examination: The patient was a short, slightly obese, elderly woman, weighing 112 pounds and measuring 4 ft., 11 in. in height. The heart was enlarged, the apex beat being in the fifth interspace, 2.5 cm. to the left of the midclavicular line. The heart sounds were of exceptionally good quality, A_2 being greater than P_2. Fine râles were heard over both pulmonary bases, the liver being palpable at the umbilical plane, and edema was present to the mid-calf. The blood pressure was 190 mm. Hg. systolic and 110 diastolic and the pulse rate 116.

The teleroentgenogram showed the heart was enlarged, chiefly to the left, the transverse diameter measuring 13.5 cm., the predicted normal value being 11.6 cm. The electrocardiogram showed normal rhythm at a rate of 120. In the limb leads there was slight left axis deviation of QRS, the RS-T segments were moderately depressed in Leads 1 and 2 and the T waves were low in Lead 1. In the precordial leads, R was absent in CF_3, and in CF_5 there were depressed ST segments.

The white blood cell and red cell counts, hemoglobin, and hematocrit values were within normal limits. The fasting blood sugar and sugar tolerance tests had a typical diabetic curve (see fig. 1). The N.P.N. was 34.4, total cholesterol was 290, and the esters 230 mg. per cent, respectively. Urinalysis specified two plus sugar and one plus albumin with one granular and two hyaline casts, per high power field, and was not otherwise abnormal.

The clinical and laboratory findings were thought to be consistent with hypertensive and arteriosclerotic heart disease, moderately enlarged heart and congestive failure. Diabetes mellitus was an additional diagnosis.

On June 9, 1950, all previous therapy, including insulin, was discontinued, and replaced with 4 gm. of glycocyamine and 20 gm. of betaine in four equally divided doses, daily. Three days later, she reported that she felt much better. Her dyspnea

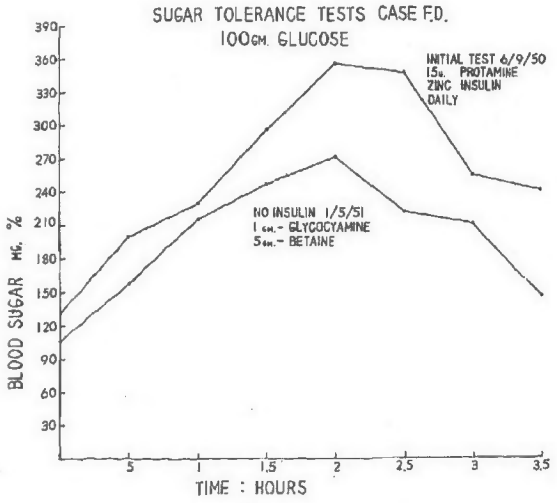


Fig. 1

and angina were considerably less. She was sleeping better and tired less easily.

Ten days after the initiation of betaine and glycocyamine therapy, she reported that she had experienced neither angina nor dyspnea during the past two days. Her vision was better and the headaches were gone. She felt stronger and was helping a little at home. The chest was clear, the liver could not be palpated, but slight ankle edema was present. She had lost four pounds in weight. Her blood pressure had dropped to 150 mm. Hg. systolic and 86 diastolic, and the pulse rate was 72. There was no sugar in a three-day specimen of urine.

One week later, she was without heart symptoms on moderate effort, felt much stronger, had greater endurance and was able to help with the cooking and the washing of dishes. For the first time in many years, she had made her own bed. She had walked two miles and did extensive shopping without distress. These activities were confirmed by her daughter. There were no signs of cardiac decompensation. The blood pressure was 180 mm. Hg. systolic and 80 diastolic and the pulse rate 74. No sugar was present in a three-day specimen of urine.

The patient continued to improve during the next month, becoming more active, feeling stronger, with greater endurance, and tiring only at the end of the day. No signs of cardiac decompensation were present. Her vision had improved considerably, but her neuritis had returned and supplemental vitamin therapy was again instituted. The urine at this time contained a moderate amount of sugar. She was advised to use 10 units of protamine zinc insulin daily, but her diet was not restricted.

She continued to feel well and increased her activities during the next three months without signs or symptoms of cardiac decompensation. Her weight had slowly risen to 112 pounds. At this time certain changes occurred in the electrocardiographic tracings. The RS-T segments were now normal and the T waves in Leads 1 and CF_5 were taller. These changes were considered to be due to the omission of digitalis.

The following week, she experienced slight faintness and vertigo associated with hunger. The fasting blood sugar was normal. Insulin was again discontinued. She continued to feel extremely fit, stronger, with appreciably increased activities, and was without signs and symptoms of cardiac decompensation, nor did glycosuria occur during the next three months. She gained two more pounds.

As a clinical test, on January 7, 1951, she was given two cubic centimeters of a mercurial diuretic. No weight loss nor substantial diuresis occurred. During the following week she gained one pound. The remainder of her clinical course to March 28, 1951, has been characterized by the absence of signs and symptoms of cardiac decompensation and by increasing activity and a remarkable sense of well-being. Her weight has slowly risen to 117 pounds, without clinical evidence of edema. The blood pressure has fluctuated between 180 mm. Hg. systolic and 100 diastolic and 140 mm. Hg. systolic and 84 diastolic. The heart size has remained unchanged. The electrocardiographic changes, as stated above, have persisted. No glycosuria has occurred and the values are given in figure 1.

Comment: Coinciding with the administration of betaine and glycocyamine, and discontinuance of digitalis, insulin and dietary restrictions, the patient became compensated, increased her activities, had improved vision, and developed a remarkable sense of well-being. Her diabetes has been controlled without the use of insulin or diet, for a period of six months.

Discussion of Cases in Group I

These patients in Group I, after betaine and glycocyamine therapy was begun, received no other therapy, their diets were not restricted in any respect, nor was extra rest emphasized or specifically ordered. They began to improve shortly after the beginning of betaine and glycocyamine therapy and, within two weeks to two months, became free of the signs and symptoms of cardiac decompensation. They experienced no distress following the removal of other therapy.

They increased their activities to a significant degree and, with one exception, have remained well since.

Before much objective clinical improvement was apparent, all reported an improved sense of well-being and stated that they slept better. Later their strength and endurance improved. Twelve patients gained 3 to 20 pounds in weight; in one, the weight remained unchanged; one lost two pounds. The weight gain was not due to edema. One patient, Case 7, suffered a coronary occlusion after eight months of betaine and glycocyamine therapy. The six patients with hypertension had a transient drop in blood pressure; in three, it fell to within normal limits and, to date, has remained so. In the one patient with hypotension, the blood pressure has slowly risen to 110 mm. Hg. systolic and 70 diastolic.

In all cases of this group, except the patient with heart block, the pulse rate has dropped to within normal limits, where it has remained. No significant change in rate was noticed in the patient with heart block.

One patient, Case 9, because of her weight gain and because she felt so well, did not see the necessity of further treatment and discontinued therapy after three months. She returned 11 weeks later, presenting her original signs and symptoms. Therapy was resumed. Three weeks later, she was again free of signs and symptoms of cardiac decompensation and has remained so.

No untoward symptoms were observed, except mild nausea and diarrhea in a few patients, which cleared up spontaneously.

Early in the course of this experiment, one patient, Case 1, while taking glycocyamine and betaine, in an effort to control weight, omitted lunch. Repeatedly, in the early afternoon, he exhibited the clinical symptoms of hypoglycemia. A blood sugar determination indicated that the blood sugar had dropped to 48 mg. per cent, at 3:00 p.m. In order to check this observation more critically, the fasting blood sugar of seven patients was determined. They were then given 1 gm. of glycocyamine and 5 gm. of betaine hydrate. In every patient, the blood sugar dropped from 15 to 20 mg. per cent. Two of these patients appeared clinically to suffer from acute hyperinsulinism, with rapid pulse, nervousness, anxiety, weakness, hunger, profuse sweating and vomiting. We do not consider the transient hypoglycemia, usually, important enough to require extra carbohydrate with betaine and glycocyamine therapy.

Presentation of Group II

This group includes five patients with arteriosclerotic and one with rheumatic heart disease; three males and three females, with an age range of 47 to 72 years. Their break in cardiac tolerance was of three to four years' duration. Three had one or more coronary occlusions; one had an aneurysm of the aorta, (arteriosclerotic); one was a diabetic with asthma; one was fibrillating; two had enlarged hearts; five had hypertension. All were decompensated in varying degree, although they had received the usual forms of therapy for cardiac decompensation.

A brief outline of the clinical course of three representative cases of the group is given below. They depict the clinical course of the entire group, and in addition certain special features.

Case 1

A 72-year-old white woman was first seen on August 11, 1950. Her presenting symptoms were constricting chest pain and shortness of breath on effort. She was in her usual state of health until 1946, when she suffered a coronary occlusion and myocardial infarction. Previous to this injury, she was quite well and strong and able to discharge her household duties without difficulty. After the infarction, she slowly improved but with activity greatly limited by chest pain and dyspnea. Treatment consisted of digitalis, nitroglycerin, and extra rest. Two years ago, following a severe attack of chest pain which lasted approximately 36 hours, she began to fibrillate. A second coronary occlusion with infarction was diagnosed. Since that time, her condition slowly deteriorated and she developed signs and symptoms of increasing cardiac decompensation. During the past eight months, she had been unable to do any housework and had chiefly sat in a chair. With assistance, she could go to the bathroom. She felt weak and ill and could do nothing without help, at the time of the first examination.

Physical Examination: The well-developed and well-nourished white woman weighed 158 pounds and was 5 ft., 4 in. tall. The heart did not appear enlarged. Her heart sounds were chaotic and no murmurs were heard. The blood pressure was 160 mm. Hg. systolic and 92 diastolic. Fine râles were heard over both pulmonary bases, the liver being barely palpable, and no edema of the extremities was present.

The electrocardiogram indicated auricular fibrillation with an approximate ventricular rate of 100. The QRS complex was of normal duration and axis, but the Q waves were abnormally prominent in Leads 2 and 3. The T waves were low but upright in Lead 1 with shallow inversion in Lead 2 and deeper inversion in Lead 3. The RST segments were slightly depressed. In all the precordial leads, the Q waves were prominent with inversion of the T waves in CF_4 and CF_5. Teleroentgenograms showed the heart to be slightly

enlarged, measuring 13.8 cm. in its transverse diameter, the normal being 13.3 cm. The red blood cell count was 4,850,00 and the leukocyte count 10,800. The fasting blood sugar was 82, and the N.P.N. 36.4 mg. per cent. Blood electrolytes were well within normal limits. One plus albumin, an occasional red blood cell, a few pus cells, and one granular cast per high power field were found in the urinalysis.

The clinical and laboratory findings were consistent with the diagnosis of coronary heart disease, slight cardiac enlargement, auricular fibrillation, and congestive failure.

All previous therapy was discontinued and replaced by 6 gm. of glycocyamine and 30 gm. of betaine, in four equally divided doses daily. Five days after the initiation of betaine and glycocyamine therapy, the patient was definitely worse, with signs and symptoms of increasing cardiac decompensation. She felt quite ill, was weaker and very tired. Her weight had risen four pounds. A slight increase in the number of râles was heard over the pulmonary bases. The liver was palpable two finger-breadths below the costal margin, and slight ankle edema was present. The blood pressure was 168 mm. Hg. systolic and 92 diastolic.

The patient was seen twice daily, during the next five days, and thereafter slowly improved, so that ten days later, she stated that she felt considerably better and that the angina and dyspnea were much less than when betaine and glycocyamine were started. She still felt weak and was extremely tired. The chest was clear, the liver not palpable, and edema of the extremities was not present. The blood pressure was 160 mm. Hg. systolic and 90 diastolic. She weighed two pounds less. No significant change was observed in the electrocardiogram or teleroentgenogram.

She continued to improve and five days later reported only slight angina and dyspnea on effort. She felt generally better, stronger, and had greater endurance. She slept better. No signs of cardiac decompensation were present. Her weight remained unchanged. The blood pressure was 134 mm. Hg. systolic and 84 diastolic.

She continued to improve during the next ten days, experiencing angina and dyspnea only when hurrying. She stated that this was the best she had felt for the past five years. She ate at the table and walked around the house without assistance. She slept soundly and awakened refreshed. She gained two pounds without clinical evidence of edema. The blood pressure was 124 mm. Hg. systolic and 84 diastolic.

Two weeks later she reported she was able to help a little with the housework, even went to a movie, and had walked slowly three blocks without distress. This was confirmed by her daughter. Two weeks later, she reported that she had walked approximately half a mile, in the company of her daughter, and experienced no untoward effects, beyond slight fatigue and dyspnea on the return trip.

During the next two months she continued to improve, with appreciably increased activity and an excellent sense of well-being. She had assisted in the care of her grandchildren and only occasionally experienced angina and dyspnea. She slept soundly on two pillows. Usually she tired by 4:00 p.m., but a short nap before dinner refreshed her.

During the ensuing two and a half months, her clinical condition remained essentially the same. She felt reasonably fit, experiencing angina and dyspnea only when hurrying. She walked as far as two miles without distress. Her activities appreciably increased, without causing undue fatigue. She gained ten pounds during the seven months without clinical evidence of edema. Her blood pressure during the last four and a half months varied between 160 mm. Hg. systolic and 92 diastolic and 124 mm. Hg. systolic and 86 diastolic. No significant changes were found in the serial teleroentgenograms and electrocardiograms, other than a reduction of the ventricular rate to approximately 90.

Comment: In this patient, after the withdrawal of her previous therapy, signs and symptoms of increasing cardiac decompensation developed for a short time, after which she improved and continued to do so during the ensuing six months. She developed increased tolerance, a remarkable sense of well-being, and gained ten pounds of weight without clinical evidence of edema. These changes coincided with the administration of betaine and glycocyamine.

Case 2

A 63-year-old white man was first seen on August 15, 1950. His presenting complaints were moderate chest pain and shortness of breath which were most severe at night, experienced during the past two weeks. He had been exceptionally well and strong and had had no disease which ordinarily is considered to affect the heart, until three years ago, when he became afflicted with asthma. The attacks usually occurred at night and had increased in severity. Frequently, it had been necessary to administer oxygen.

About the first of January, 1950, he was moved by airplane ambulance to Chicago, where his son, a physician, lived. There he was admitted to a hospital, where a diagnosis of arteriosclerotic heart

disease, healed infarction and cardiac asthma was established. He was also found to be suffering from diabetes mellitus, the fasting blood sugar being 260 mg. per cent. He was digitalized and the diabetes brought under control. Therapy thereafter consisted of one digilanid® tablet daily, aminophylline and phenobarbital three times a day, and insulin and dietary control of his diabetes. Salt and fluid intake and activities were moderately restricted. He felt weak, ill and very tired, at the time of his first visit. He could walk at most two and a half blocks slowly without angina and had not been able to work for the past two and a half years. His sleep was frequently interrupted by asthma.

Physical Examination: The patient was a nervous, well-developed white man, 5 ft., 6 in. tall, weighing 153 pounds. He was orthopneic and slightly cyanotic. The heart was not enlarged. The heart sounds were somewhat obscured by many musical and fine râles, which were heard over both pulmonary fields. Slight ankle edema was present. The blood pressure was 160 mm. Hg. systolic and 92 diastolic, and the pulse rate 98.

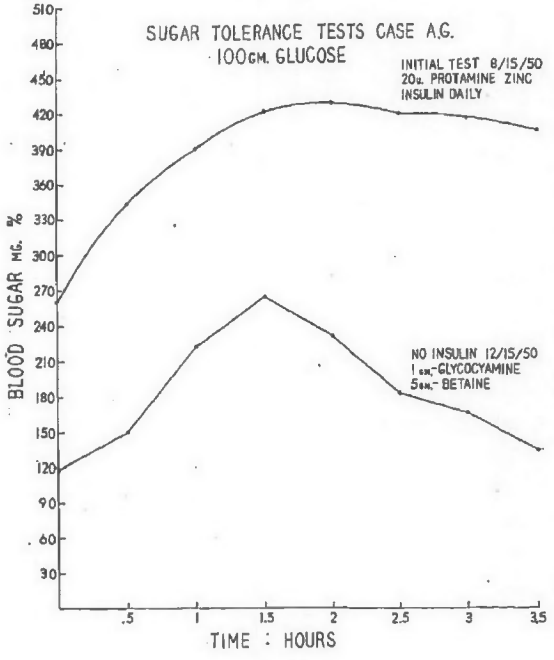


Fig. 2

In the teleroentgenogram, the heart was within normal limits, measuring 12.2 cm. in its transverse diameter, the predicted normal value being 12.8 cm. The significant electrocardiographic findings were as follows: There was slight depression of the RS-T segments in Leads 2 and 3. In the precordial leads R was absent and T inverted in CF_2 and CF_5. The erythrocyte count was 5,200,000, the hemoglobin 15.5 cm.; the white blood cell count was 13,200, with 650 eosinophils per cubic millimeter. The urine was normal with the exception of a two plus glycosuria. The blood electrolytes and N.P.N. were both within normal limits. The fasting blood sugar and sugar tolerance tests were typical of diabetes mellitus (fig. 2).

On August 15, 1950, all therapy including insulin and dietary restrictions was discontinued and replaced by 1.5 gm. of glycocyamine and 6 gm. of betaine four times daily. His condition remained unchanged during the next four days, but during the following three days became much worse, with signs and symptoms of increasing cardiac decompensation, so that he was forced to curtail his activities. He gained three pounds. The blood pressure was 170 mm. Hg. systolic and 94 diastolic and the pulse rate 106. The ankle edema had increased considerably but no glycosuria was present. A nocturnal asthmatic attack necessitated the intravenous administration of benadryl.® Betaine and glycocyamine therapy was continued. Thereafter, he began to improve so that on August 30, 1950, he experienced only slight angina and less dyspnea on effort than formerly. He felt much better and stronger and had slept soundly the previous two nights.

One week later, he touched the floor 62 times without appreciable distress, a fact confirmed by his wife. Both lung fields were then clear and no ankle edema was present. He had lost four pounds and the pulse was 90, the blood pressure 148 mm. Hg. systolic and 84 diastolic.

He continued to improve so that by October 7, 1950, he had been without signs or symptoms of cardiac decompensation, with increased activity, for ten days. He stated he had mowed the lawn without undue fatigue or symptoms ordinarily referable to the heart. A three-day specimen of urine showed no glycosuria. In the electrocardiographic tracing the RS-T segments in Leads 2 and 3 were isoelectric and the T wave, in CF_3 was upright (fig. 3), indications of considerable improvement which were considered to be due to the omission of digitalis. The leukocyte and eosinophil counts were within normal limits.

From September 17, 1950, to January 30, 1951, when the patient was seen before his return to Chicago, he had been remarkably well and strong, without signs or symptoms of cardiac decompensation or asthma and with appreciably increased activity. His weight slowly rose to 162 pounds. The blood pressure varied between 136 mm. Hg. systolic and 86 diastolic and 160 mm. Hg. systolic

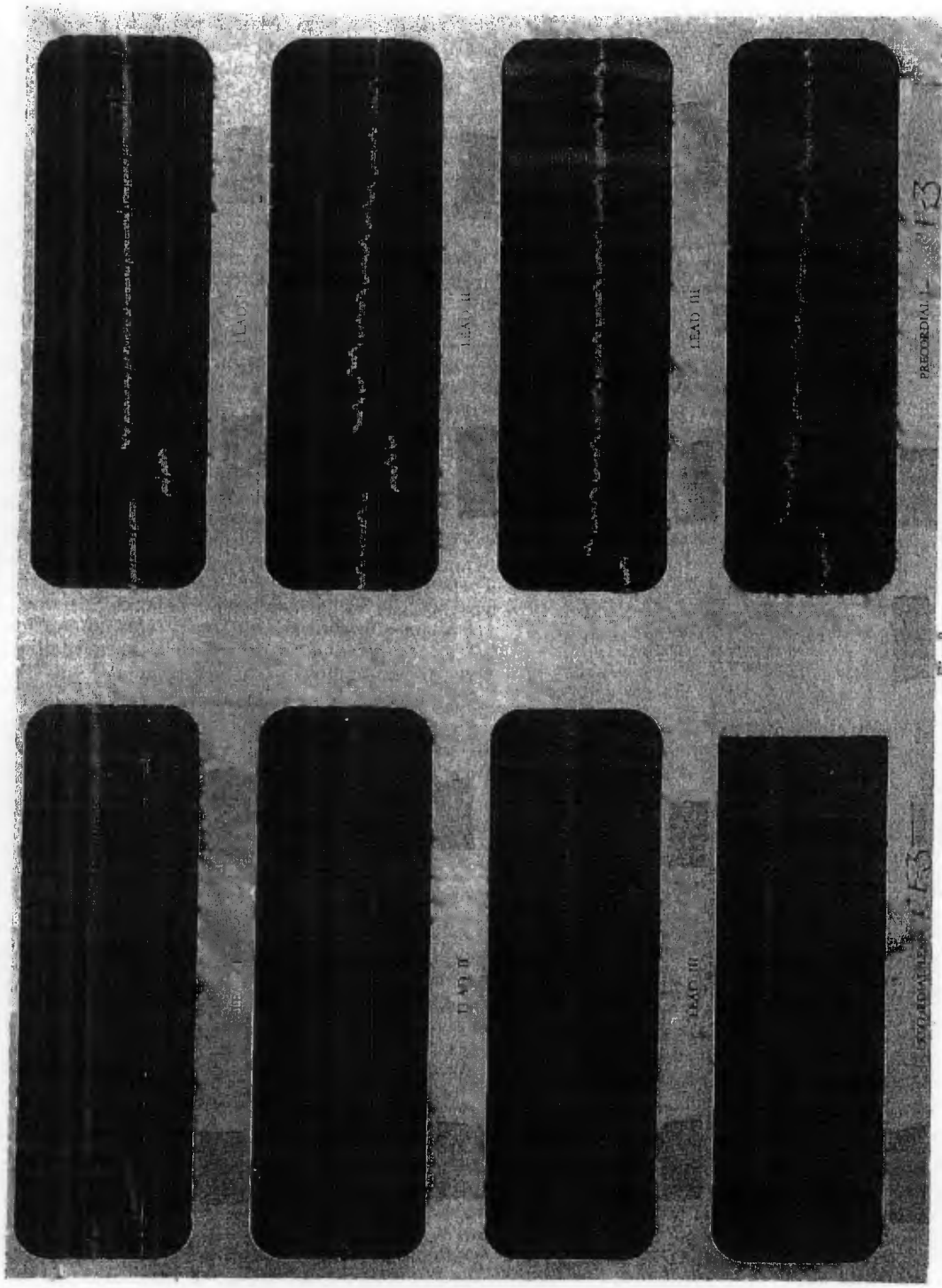


Fig. 3

A. G. 6/1/50. Electrocardiographic tracing showing in standard Lead I low-voltage QRS and flat T waves, with depressed S-T segments in Leads II and III, and with a positive R, deep S, and diphasic T waves in CF_2.

A. G. 10/3/50. Approximately four months later, the S-T segments in Leads II and III are isoelectric. T is positive in CF_2.

and 90 diastolic, and the pulse rate between 70 and 84 per minute.

Teleroentgenograms recorded no change in heart size. Electrocardiograms taken at two- to three-week intervals, during the course of his treatment, showed the changes considered to be due to the removal of digitalis (fig. 3).

Comment: The patient was a diabetic with severe cardiac asthma, the latter a consequence, apparently, of myocardial infarction. Both conditions improved to a remarkable degree. The improvement, the development of a sense of well-being, and increase in activity coincided with the administration of betaine and glycocyamine.

Case 3

A 64-year-old white man, a night watchman, was first seen on June 20, 1950. He had enjoyed good health until the age of 51, when he was discharged from the Army because of symptoms presumably associated with high blood pressure. He continued, with little limitation, however, until Christmas day, 1949, when he experienced severe chest pain, shortness of breath, and perspired profusely all that day. Thereafter, these symptoms were easily produced by effort and occasionally would occur at rest. On February 9, 1950, a diagnosis was made by his physician of hypertensive and arteriosclerotic heart disease, greatly enlarged heart, aneurysm of the aorta, and congestive failure. He was digitalized, and potassium iodide therapy was instituted, with salt-poor diet and fluid restriction. The administration of a mercurial diuretic every fourth day was necessary, because of the swelling of the legs. During the ensuing two months, he improved considerably and lost 22 pounds in weight. Thereafter, he failed to improve and became slightly worse, with signs and symptoms of increasing cardiac decompensation.

His presenting symptoms were easily provoked, severe constricting chest pain, which radiated down both arms and to the epigastrium, extreme dyspnea and profuse perspiration of effort. He felt weak and he tired easily. Angina limited his activities to a few paces. He had to sit in a chair at the place of his employment. He slept poorly, propped up on three pillows.

Physical Examination: The patient was an orthopneic, large, obese man who weighed 240 pounds and was 6 ft., 3 in. tall. The face was livid, the lips and fingertips cyanotic, and he was perspiring freely. An attempt to examine him in the recumbent position failed, because of his obvious distress, his face having become ashen and his respirations labored. He was assisted to a sitting position and allowed to rest a few minutes before the examination was continued. Tortuosity and nicking of the retinal vessels were marked. The heart was greatly enlarged, the apex beat being in the sixth interspace at the anterior axillary line. Heart sounds were of good quality, A_2 being greater than P_2, and no murmurs were heard. The blood pressure was 180 mm. Hg. systolic and 110 diastolic, and the pulse rate 112. Coarse and moist râles were heard over both pulmonary bases. The liver was palpable four finger-breadths below the costal margin, and edema of the lower extremities was present to the mid-calf.

In the teleroentgenogram, a large aneurysm of the aortic arch was seen, which measured 9 cm. in diameter. The heart was greatly enlarged, measuring 17 cm. in its transverse diameter, the predicted normal value being 14.5 cm. (fig. 4). The electrocardiogram showed sinus tachycardia, with a ventricular rate of 180, a P-R interval of 0.20 second. In the limb leads the QRS complexes showed left axis deviation and the T waves were low in Lead 1. In the precordial leads, the significant findings were deep Q and inverted T waves in CF_1 and minimal R waves, with deep S and upright T waves in CF_3. The erythrocyte count was 5,900,000, the white blood cell count 10,200, with a normal differential distribution. The blood and spinal fluid Wassermann tests were negative. Examination of the spinal fluid gave evidence that the cell count, sugar, and protein determinations were within normal limits. The colloidal gold curve was normal. Determination of the fasting blood sugar, the N.P.N., and the blood electrolytes were within normal limits. Examination of the urine denoted a specific gravity of 1.008, a trace of albumin, and an occasional red blood cell, and three granular and one hyaline cast per high power field.

Clinical and laboratory findings were consistent with the diagnosis of arteriosclerotic and hypertensive heart disease, greatly enlarged heart, aneurysm of the aorta and congestive failure.

All previous therapy was discontinued and replaced with 8 gm. of glycocyamine and 40 gm. of betaine, daily, in divided doses. The signs and symptoms remained unchanged during the first week but increased in severity during the ensuing four days, with evidence of increasing cardiac decompensation. He was unable to sleep, experienced nausea, diarrhea, and severe headache, and he gained three pounds in weight. Thereafter, he slowly improved, with less angina and dyspnea, and decreasing signs of cardiac decompensation.

One month after the initiation of betaine and glycocyamine therapy, he stated that he felt much better and stronger and had greater endurance. He slept better. He was able to walk about the factory without distress and no longer perspired

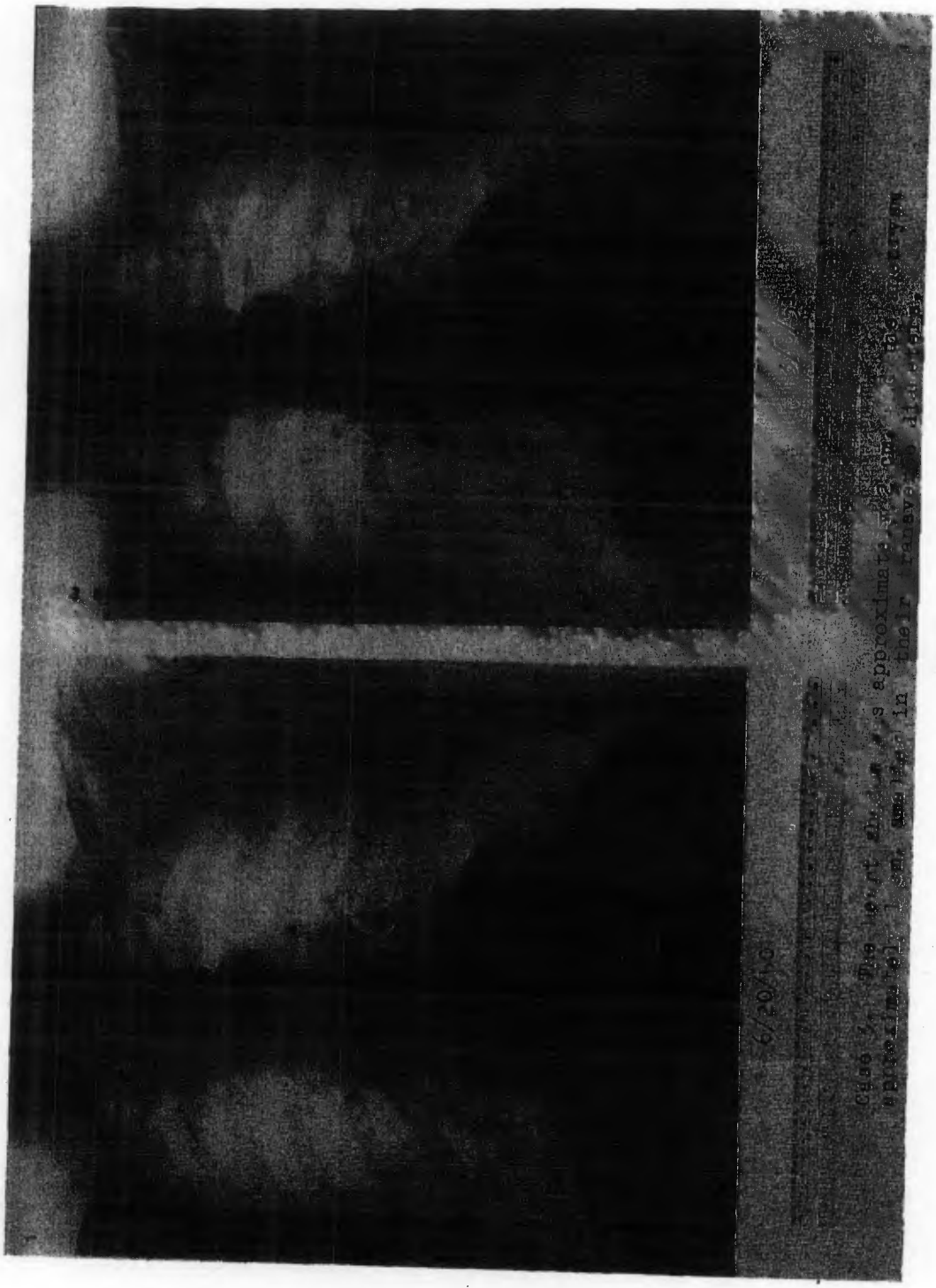


Fig. 4

excessively. The chest was clear, the liver not palpable, and ankle edema was not present. The blood pressure was 180 mm. Hg. systolic and 110 diastolic and the pulse rate 90. The teleroentgenogram and electrocardiogram showed no significant change. In an effort to evaluate his exercise tolerance, he bent and touched the floor 25 times at a moderate rate, without distress. His blood pressure and pulse returned to their previous level in 90 seconds.

He continued to improve thereafter and was without symptoms referable to the heart. He felt much stronger, had greater endurance, and felt extremely fit. He was tired at the end of the night's work but he slept well and awakened refreshed.

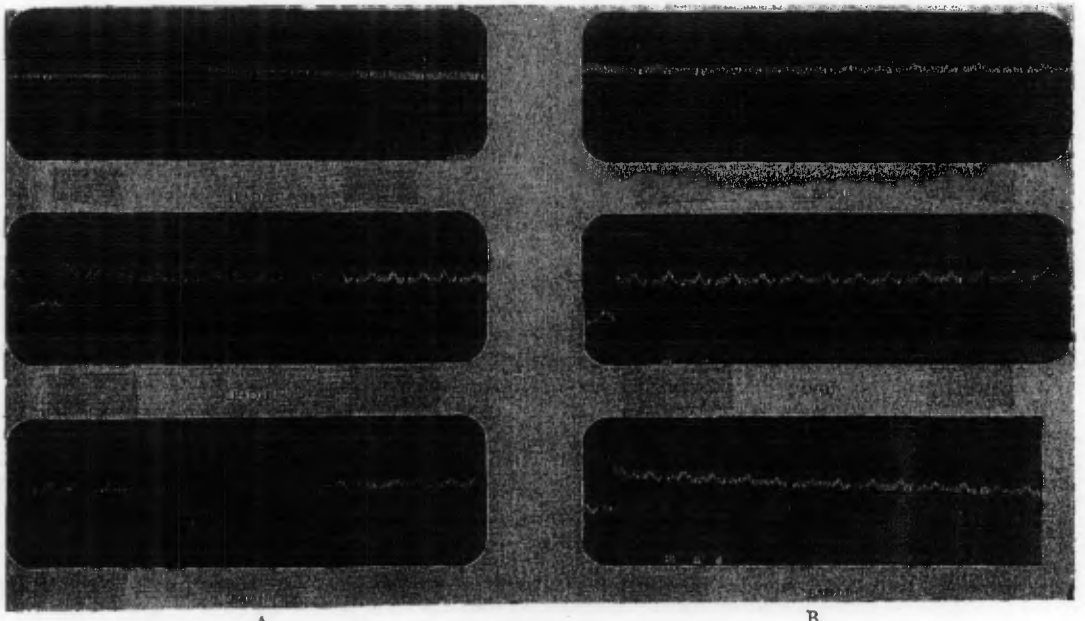
Seven months after the initiation of betaine and glycocyamine therapy, he stated that he had walked approximately two miles without difficulty, had worked in his garden, and painted his home. These statements were confirmed by his wife. He had gained five pounds in weight without clinical evidence of edema. In this interval the blood pressure had varied between 230 mm. Hg. systolic and 130 diastolic and 170 mm. Hg. systolic and 110 diastolic.

On December 5, 1950, he experienced the symptoms and was found to have signs and x-ray evidence of cholecystolithiasis. He was seen in consultation with his family physician, on March 8, 1951, to ascertain whether surgery was advisable. The remarkable circulatory improvement was confirmed and commented on. At this time the heart shadow was two centimeters and the aneurysm one centimeter less in their transverse diameters (fig. 4). There was no significant change in the serial electrocardiograms other than a greater voltage in T_1, which was probably due to the omission of digitalis (fig. 5).

Comment: The patient, while improving somewhat on his previous therapy for a short time, was severely decompensated, when first seen. Coincident with the discontinuation of his previous therapy and the substitution of betaine and glycocyamine, he became for a few days more decompensated but thereafter slowly improved. At the end of approximately one month, he became free of the signs and symptoms of cardiac decompensation. He developed a remarkable sense of well-being, appreciably increased his activities, and gained weight, without clinical evidence of edema. During the same period, the heart and aneurysm became smaller.

Summary of Group II

With the initiation of betaine and glycocyamine therapy and the discontinuation of all other therapy (including dietary restriction), the condition of these six patients became worse but not to an



A. B.

Fig. 5

(A.) R. B. 6. 6/20/50. T waves in Lead I are flat.

(B.) R. B. 3/8/51. T waves in Lead I have greater voltage and are almost normal. This was the only significant change observed in the electrocardiogram. The heart sizeand the aneurysm are smaller.

alarming degree, for ten days to three weeks, after which they began to improve. Within one to three months, they became free of the signs and symptoms of cardiac decompensation and they have remained so since. (Typical case histories for three patients in this group are reported.)

These patients gained six to nine pounds in weight, without clinical evidence of edema. Five of the six had elevated blood pressure. A transient fall in blood pressure occurred after betaine and glycocyamine was begun and was maintained in only one case. In all patients, the pulse rates are now normal. In Case 3, the size of the heart and aneurysm has become smaller.

Case 2, a diabetic, without insulin or dietary restriction, has been without symptoms of diabetes or evidence of glycosuria for four months. He has gained weight. His sugar tolerance curves are shown in figure 2. One patient had a recurrence of rheumatic fever while receiving betaine and glycocyamine therapy.

The characteristic of this group is a worsening of their condition on the removal of their previous therapy and the substitution of betaine and glycocyamine. Although they eventually became free of the signs and symptoms of cardiac decompensation, they attained this state more slowly than did the patients in Group I. At the present time, they cannot be distinguished clinically from the patients in Group I.

Presentation of Group III

This group includes two patients with arteriosclerotic and two of rheumatic heart disease. All were women, with an age range of 42 to 69 years. Their break in cardiac tolerance was of six to ten years' duration.

All of this group had elevated blood pressures, enlarged hearts, and were fibrillating. All had been treated with the usual forms of therapy and were decompensated at the time betaine and glycocyamine therapy was started.

The clinical course of two representative cases is given here:

Case 1

A 54-year-old white woman was first seen June 27, 1950. Her presenting complaints were extreme shortness of breath and palpitation on effort. She first had rheumatic fever at the age of 13 and had many recurrences since, the last at the age of 42. For five years following the last attack, she was in her usual state of health, received no medication, and was capable of moderate activity. Soon, thereafter, she became increasingly short of breath. A few months later, swelling of the feet and ankles occurred. At first, the edema would disappear at night but later persisted continuously. The edema and dyspnea gradually increased. In the latter part of 1948, she consulted a physician who made a diagnosis of rheumatic heart disease, enlarged heart, mitral stenosis and insufficiency, aortic insufficiency, and auricular fibrillation. Therapy included digitalization, moderate salt and fluid restriction, and limitation of activity. In the fall of 1949, the administration of mercurial diuretics became necessary, first at weekly intervals, later every fourth day.

Physical Examination: The patient was a well-nourished woman, orthopneic, with slight cyanosis of the lips and finger tips. She weighed 198 pounds, with a height of 5 ft., 4 in. The heart was obviously enlarged, the apex beat being at the anterior axillary line in the sixth interspace. A rough mitral diastolic, a blowing mitral systolic, and an aortic diastolic murmur were heard. The rhythm was chaotic. A few fine râles were heard over the pulmonary bases. The blood pressure was 168 mm. Hg. systolic and 70 diastolic. The liver was palpable three fingerbreadths below the costal margin. Edema of the lower extremities extended to the tibial tuberosities.

In the teleroentgenogram, the transverse diameter of the heart was 16.2 cm., the predicted value being 15 cm. In the electrocardiogram, auricular fibrillation was seen, with an approximate ventricular rate of 90. The QRS voltage in all limb leads was moderately low, and there was right axis deviation. The ST segments were isoelectric in Lead 1, and slightly depressed in Leads 2 and 3. In Lead 1, the T waves were low and inverted in Leads 2 and 3. In the precordial leads, the T waves were diphasic in CF_3, and inverted in CF_5. The ST segments were depressed in CF_5.

Examination of the blood showed no abnormality of the erythrocytes. The leukocyte count was 11,200, with a normal differential distribution, and the hemoglobin was 82 per cent. The fasting blood sugar and N.P.N. were within normal limits. Urinalysis, with one plus albumin, and low specific gravity — 1.008, was not otherwise abnormal.

The clinical and laboratory findings were consistent with a diagnosis of rheumatic heart disease, moderately enlarged heart, mitral stenosis and insufficiency, aortic insufficiency, auricular fibrillation, and congestive failure.

On June 27, 1950, all previous medication was discontinued and replaced with 6 gm. of glycocyamine and 30 gm. of betaine daily in five equally divided doses. The patient did not return until ten days later and then reported that her symptoms had increased and that she was forced to curtail her activities. She had gained seven pounds. The liver was palpable four fingerbreadths below the costal margin, and the edema of her lower ex-

tremities had increased. The electrocardiogram showed the ventricular rate to be approximately 120. She was ordered to bed, digitalized during the next three days, salt was forbidden, and fluids were restricted to 1,500 cc. daily. A mercurial diuretic was administered every fourth day. Betaine and glycocyamine therapy was continued. She rapidly improved and one week later was permitted out of bed. Two weeks later, there was only slight dyspnea on moderate activity. She had lost 17 pounds since June 27, 1950. The liver was not palpable, the chest was clear, and only slight ankle edema was present. The blood pressure was 174 mm. Hg. systolic and 90 diastolic. On August 5, 1950, she stated she was much better and stronger; she had greater endurance and slept soundly. No signs of cardiac decompensation were present. Mercurial diuretics were discontinued. She continued to feel well, with increasing activity during the next month. On September 4, 1950, digitalis was reduced to ¾ grain daily. Eight days later, she noticed a little more shortness of breath, and, in addition, experienced considerable nausea. She had gained two pounds and was otherwise unchanged. The dyspnea and nausea spontaneously disappeared in a few days. She became stronger, had increased endurance and could walk ten blocks without distress. She was sleeping soundly. Her weight remained stationary and there were no signs of cardiac decompensation.

On October 2, 1950, she reported she had walked approximately one and a half miles and was doing all her housework without experiencing any dyspnea or undue fatigue. Five days later digitalis was discontinued. No untoward effects were reported or observed following the discontinuance of digitalis. She remained compensated. She continued to increase her activities during the next month and only occasionally experienced dyspnea on hurrying. The electrocardiogram showed an approximate ventricular rate of 80. On November 20, 1950, salt and water restrictions were lessened.

During the next two weeks she gained three pounds, experiencing slight dyspnea and curtailed activities. Slight ankle edema was present. Two weeks later, she stated she was not so well as when salt and water were restricted and voluntarily reduced the quantities consumed. She continued to feel fit and strong with increasing activities so that on March 15, 1951, she could walk two miles without distress. Her weight was 201 pounds, and she was without signs of cardiac decompensation. Serial electrocardiograms and teleroentgenogram indicated no significant changes other than reduction of the approximate ventricular rate to 80.

Comment: It was found possible in this patient gradually to remove digitalis over a period of three months. As her clinical condition warranted, mercurial diuretics were discontinued and salt and fluid restrictions were lessened, without sacrificing the substantially increased activity, cardiac compensation and a general sense of well-being which had developed. These changes coincided with the administration of betaine and glycocyamine.

Case 2

A 67-year-old white woman was seen for the first time August 1, 1950, presenting symptoms of constricting chest pain and moderate shortness of breath on effort. She enjoyed good health until the age of 55, when she complained of symptoms presumably associated with high blood pressure. She continued with little limitation until nine years ago, when she suffered a heart attack, for which she was confined to her bed for three months. Thereafter, she had experienced mild chest pain of variable duration, provoked on effort, and relieved by rest. Her physician instituted digitalis therapy and curtailed her activities. She continued with moderate limitation for approximately six months, when she developed swelling of her feet and ankles and increasing shortness of breath. She was advised to curtail her salt and fluid intake. Shortly thereafter, intravenous medication was administered; first at ten-day intervals and later every fourth day. She had not been under the care of a physician for the past two years and had taken no medication except digitalis and that only intermittently. She had slightly restricted her salt intake.

At the time of examination, she felt poorly and tired and was unable to help with the housework. She could walk with rests, about one and one-half blocks. She slept propped up in bed, on three pillows.

Physical Examination: The well-developed orthopneic woman weighed 169 pounds, being 5 ft., 5 in. tall. She could not be examined in the recumbent position because of dyspnea. The heart was enlarged, the apex beat being in the sixth interspace at the anterior axillary line. Heart sounds were chaotic. No murmurs were heard. The blood pressure was 182 mm. Hg. systolic and 90 diastolic. A few râles were heard over the pulmonary bases. The liver was palpable for six finger-breadths below the costal margin, and edema of the extremities was present almost to the knees.

In the teleroentgenogram, the transverse diameter of the heart was 15.2 cm., the predicted normal value being 13.7 cm. The electrocardiogram taken at this time showed auricular fibrillation, with a ventricular rate of approximately 98. Premature beats arose from different foci in the ventricles. In the limb leads QRS was of very

low voltage, there was left axis deviation and the RS-T segments were slightly depressed. The T waves were inverted in Lead 1 and flattened in Lead 2. The precordial leads from V_1 through V_6 had deep S waves. Examination of the blood indicated no abnormality of the white and red blood cells. The sugar tolerance test was normal. Nonprotein nitrogen was at the high limit of normal, being 38.4 mg. per cent. The blood electrolytes were within normal limits. The urine was of 1.012 specific gravity and contained four granular and an occasional hyaline cast per high power field, being otherwise normal.

On August 1, 1950, all medication was discontinued and replaced by 5 gm. of glycocyamine and 25 gm. of betaine in five equally divided doses daily. She failed to improve and during the next nine days developed signs and symptoms of increasing cardiac decompensation. The electrocardiogram showed an approximate ventricular rate of 116. She was ordered to bed, digitalized during the next three days; salt was forbidden, and fluids restricted to 1,800 cc. per day. A mercurial diuretic was administered every fourth day thereafter and digitalis 1½ grains daily. Betaine and glycocyamine therapy was continued She looked and felt very ill and was incapable of any ordinary physical activity. She slept more poorly. She had gained ten pounds. The liver was about the same size, the basal râles had increased and edema was present well above the knees. The blood pressure was 190 mm. Hg. systolic and 100 diastolic.

She slowly improved during the next 12 days and was able to come, at that time, to the office for examination. Her shortness of breath was much less and she felt generally better. She had lost 14 pounds since August 10, 1950. The liver was not palpable, the chest was clear, and only moderate ankle edema was present. The blood pressure was 190 mm. Hg. systolic and 90 diastolic. The approximate ventricular rate was 104. At this time, the fluid allowance was increased and moderate activity was permitted.

Ten days later she reported only slight dyspnea and no pain on effort. She felt better, was stronger, and she had greater endurance. Her weight was unchanged. No signs of cardiac decompensation were present. The mercurial diuretic was discontinued, but digitalis, betaine and glycocyamine, and salt restriction were continued. She was advised to limit her activities to those which she could do without distress.

Two weeks later, she was without symptoms referable to the heart, felt fit, and had considerably increased her activities. She was doing the household shopping for her daughter. She slept soundly on three pillows and awakened refreshed. Her weight had increased four pounds without clinical evidence of edema. Her blood pressure was 176 mm. Hg. systolic and 90 diastolic, and the apical rate 90. At this time the moderate use of salt was permitted.

During the next two weeks, she experienced only slight dyspnea on effort. There were no signs of decompensation present. During extremely hot weather, the patient drank copiously of water and excreted 11,700 cc. in 72 hours. During the next three weeks, her exercise tolerance increased.

She continued to feel extremely well. When she was seen November 1, 1950, she reported no angina nor shortness of breath for the previous 12 days. Her weight had slowly increased to 173 pounds. The liver was barely palpable and slight ankle edema was present. The blood pressure was 190 mm. Hg. systolic and 94 diastolic, and the approximate ventricular rate 96. A mercurial diuretic was administered and she was advised to curtail somewhat her fluid intake. She continued to improve during the next two weeks with increasing physical activity and with a disappearance of the signs of cardiac decompensation. The mercurial diuretic was not repeated.

From that time on, she remained well compensated, without symptoms referable to the heart and with no significant change in her physical status until December 15, 1950, when she again developed slight ankle edema and again was given a mercurial diuretic. The ankle edema disappeared in four days and the mercurial diuretic was not repeated. During the next three weeks, she continued to feel extremely well, did some Christmas shopping but tired by 3:00 p.m. She then would rest until dinner time. On December 19, 1950, digitalis was reduced to a dose of ¾ grain a day. Since that time, until March 7, 1951, she was without symptoms referable to the heart or signs of cardiac decompensation.

Comment: Following the attainment of cardiac compensation, it was possible to reduce the number of injections of diuretics, permit moderate use of salt, and reduce the dose of digitalis without sacrificing the increased exercise tolerance and the remarkable sense of well-being which occurred. A substantial increase in weight was observed, which was not due to edema. These changes coincided with the administration of glycocyamine and betaine.

Summary of Group III

Without exception, all of the patients in this group, on the withdrawal of their usual therapy and its replacement with betaine and glycocyamine, promptly became much worse and required

the assistance of their previous therapy. Only in one patient has it been possible to remove all previous therapy, and in this patient, only in the last three months. All these patients are now compensated and are feeling much better than before they started betaine and glycocyamine therapy. Their strength, endurance, and activities have appreciably increased. They all have developed an improved sense of well-being. They all exhibited a transient fall in blood pressure. They have gained weight, 3 to 16 pounds, without clinical evidence of edema.

In this group, betaine and glycocyamine appears to be a useful addition to their previous therapy, as the improvement coincided with its use. The clinical course of these patients suggests a mode of management which could safely be followed in all cases: that betaine and glycocyamine be added to the therapy the patient is receiving and slowly, as the clinical condition permits, restrictions of diet and activity and other forms of therapy be reduced or withdrawn.

Presentation of Group IV

This group includes two cases of arteriosclerotic and one case of rheumatic heart disease. Two were males and one female, with an age range of 47 to 88 years. All were severely decompensated, with enlarged hearts. One patient, who was fibrillating, had suffered a recent coronary occlusion and myocardial infarction. Two have died and in the third betaine and glycocyamine therapy is considered to have failed.

Case 1

A 60-year-old Negress was first seen June 13, 1950. Her presenting complaints were severe dyspnea, weakness, fatigue and swelling of the lower extremities. She was in her usual state of health until two years previously when she noticed increasing shortness of breath which was most severe at night. One year later, her lower extremities began to swell; at first, this disappeared with rest at night but, during the past six months, it persisted. Recently she had become progressively weaker, so that she could walk only a few steps with assistance. She slept on four pillows. The patient had received no cardiac therapy.

Physical Examination: The large obese woman, 5 ft., 9 in. tall, weighed 204 pounds. She was orthopneic and could be examined only in the sitting position. The heart was greatly enlarged, the apex beat being in the sixth interspace at the anterior axillary line. The heart sounds were of poor quality, A_2 being greater than P_2. No murmurs were heard. The blood pressure was 210 mm. Hg. systolic and 120 diastolic and the pulse rate at least 152. Coarse râles were heard over both pulmonary fields. The breath sounds could not be heard over the pulmonary bases. The liver was palpable six fingerbreadths below the costal margin and edema of the extremities was present to a hand-breath above the knees.

The red blood cell count was 6,050,000 and the white blood cell count 15,800, with moderate polymorphonucleosis. The fasting blood sugar was 108 and the N.P.N. 54 mg per cent. Only 585 cc. of urine were excreted in 24 hours and according to the urinalysis there were a three plus albumin and many hyaline and granular casts. In a teleroentgenogram the presence of fluid was noted in both pleural spaces and the heart was greatly enlarged, measuring 18.2 cm. in its transverse diameter, the predicted value being 14.5 cm. The electrocardiogram showed sinus tachycardia at a rate of 160. In the limb leads the QRS complexes were slurred and showed moderate left axis deviation. The RS-T segments were depressed in Leads 1 and 2 and the T waves were diphasic in Lead 1. In the precordial leads the RS-T segments were depressed and the T waves inverted in CF_5.

The clinical and laboratory findings were consistent with the diagnosis of hypertensive and arteriosclerotic heart disease, great cardiac enlargement, left ventricular strain and severe congestive failure. Chronic Bright's disease was an additional diagnosis.

Bed rest was advised and she was given 6 gm. of glycocyamine and 30 gm. of betaine, in five equally divided doses, daily. During the next week, she felt miserable, experienced nausea and general malaise. She was capable of increased activity, however, feeling slightly stronger, less tired and was less short of breath.

Seven days after the initiation of betaine and glycocyamine therapy, orthopnea was relieved and examination of the patient in a prone position was possible. The chest was clear, the liver was not palpable, and edema of the lower extremities was present to a hand-breadth above the ankles. The blood pressure was 190 mm. Hg. systolic and 120 diastolic and the pulse rate 92.

Two weeks after the initiation of betaine and glycocyamine therapy, her cardiac status was further improved. She was less short of breath, with appreciably increased activity. The chest was clear, the liver not palpable, and the edema of the extremities the same. The blood pressure was 142 mm. Hg. systolic and 80 diastolic. However, she had vomited a great deal during the preceding week.

Three days later she reported that she had walked two blocks without distress. She complained that the betaine nauseated her. Edema extending to the mid-calf was absent in the morn-

ing, appeared in the late afternoon, and disappeared at night with rest. The chest was clear, the liver not palpable, the blood pressure was 180 mm. Hg. systolic and 120 diastolic.

She slowly improved during the next two weeks. Her weight gradually increased to 210 pounds without evidence of increasing fluid retention. She was stronger and had walked three blocks at a good pace.

On August 1, 1950, she suffered an extensive painful sunburn of her left leg, which developed into a large painful necrotic ulcer, which failed to heal. It was felt that the slight dependent edema present was interfering with the healing of the ulcer. A mercurial diuretic was administered. The ulcer was treated with local therapy and antibiotics. The blood pressure at this time was 130 mm. Hg. systolic and 84 diastolic and the pulse rate 84. The only sign of cardiac decompensation was slight edema of the ankles.

Thereafter, her condition continuously worsened. She refused to eat, lost 33 pounds in weight, and became increasingly drowsy. The N.P.N rose in three weeks to 126 mg. per cent, and the urinary output rarely exceeded 300 cc. daily. The blood pressure remained at 132 mm. Hg. systolic and 80 diastolic. At this point, in order to avoid any addition to the nonprotein nitrogen, betaine and glycocyamine were discontinued. She became progressively more toxic and vomited considerably. The urinary output dropped to approximately 200 cc. per day in spite of the fact she was encouraged to drink more fluids. She was hospitalized, and there received parenteral fluids. This failed to check her downhill progress. The N.P.N. finally rose to 426, and she expired on October 10, 1950.

At no time did the blood pressure, during the preceding two months, exceed 136 mm. Hg. systolic and 84 diastolic. There was no evidence, at the time of death, of dependent edema. No significant x-ray or electrocardiographic changes occurred except the disappearance of fluid from the pleural spaces and the reduction in heart rate.

Comment: It is difficult to evaluate the several factors contributing to this patient's death. The chronic Bright's disease, the effect of betaine and glycocyamine on the blood pressure and nitrogen retention, the sunburn and ulcer, are considered to be contributing factors. She did not die a cardiac death. The significant drop in blood pressure may well have been a large contributing factor.

Case 2

A 47-year-old Negro, on June 6, 1950, presented the complaints of extreme shortness of breath, abdominal pain and swollen lower extremities. At the age of ten, he developed rheumatic fever and had suffered many recurrences since. In 1943, at the age of 39, he became decompensated and had been treated intermittently since that time with digitalis, ammonium chloride and mercurial diuretics.

Physical Examination: The thin Negro, 6 ft., 1½ in. tall, weighed 169 pounds. He was very orthopneic. The heart was greatly enlarged, the apex beat being in the sixth interspace at the anterior axillary line. A thrill could be felt at the apex. A mitral systolic and presystolic murmur and an aortic diastolic murmur were heard. The pulse rate was 130 and the blood pressure 126 mm. Hg. systolic and 90 diastolic. The patient sat, gasping, in a chair, complaining of constricting pains in his abdomen and legs; he was unable to move without assistance. Coarse and moist râles were heard over both pulmonary fields. The liver was palpable. Moderate ascites was demonstrated and edema of the extremities was present to the knees.

The electrocardiogram showed sinus tachycardia at a rate of 140. In the limb leads QRS showed right axis deviation, the RS-T segments were depressed in Leads 2 and 3 and the T waves were low in Lead 1. The T waves were inverted and the S-T segments depressed in CF_5. In the teleroentgenogram, the heart measured 17 cm. in its transverse diameter, the predicted value being 13 cm. The red blood cell count was 4,900,000, with 14.5 gm. of hemoglobin. The leukocyte count was 13,400, with 78 per cent polymorphonuclear cells. The fasting blood sugar and N.P.N. were within normal limits. The urinalysis with two plus albumin and a specific gravity of 1.012 was not otherwise abnormal.

Clinical and laboratory findings were consistent with the diagnosis of rheumatic heart disease, great cardiac enlargement, mitral stenosis, aortic insufficiency, right ventricular strain and congestive failure.

On June 6, 1950, all previous therapy was discontinued and replaced with 6 gm. of glycocyamine and 30 gm. of betaine in five equally divided doses daily. When the patient was seen one week later, he was definitely worse and reported that he had not taken his medication. He felt very ill; his dyspnea and abdominal pain were much greater. He was completely helpless. Moist râles were heard over both pulmonary fields. The liver was palpable four finger-breadths below the costal margin and marked ascites was present. Edema extended to the groin. The blood pressure was 118 mm. Hg. systolic and 80 diastolic, and the pulse rate at least 150. He had gained 11 pounds in weight. He was persuaded to take his

medication and was seen twice daily during the next three days.

Three days later, he was definitely better. The shortness of breath was much less; the leg and abdominal pains were gone. He walked into the office. The chest was clear, the liver palpable for one finger-breadth below the costal margin, and his ascites less. Edema was present to the mid-calf. The blood pressure was 118 mm. Hg. systolic and 80 diastolic, and the pulse rate 100. He had lost six pounds of weight.

During the next two weeks, he stated he felt stronger and had increased his activities. He still was experiencing considerable dyspnea. On examination, signs of increasing cardiac decompensation were present, in that coarse râles were again heard over the pulmonary fields. The liver was palpable five finger-breadths below the costal margin, increased ascites was present, and the edema of the extremities was the same. He had gained four pounds in weight.

During the next two weeks, his course was characterized by signs and symptoms of increasing cardiac decompensation, so that it was necessary to confine the patient to bed and administer a mercurial diuretic, restrict fluids to 1,500 cc., and institute digitalization. At this time, many more râles were heard over the pulmonary fields, the liver was palpable to the level of the iliac crest, the ascites had increased and the edema of the extremities had extended. He was incapable of any movement without assistance. On this date, the patient discontinued betaine and glycocyamine therapy.

Comment: In this patient, over a short period of time, following the removal of other forms of therapy and its replacement with glycocyamine, slight improvement was noticed for a few days. Then he developed the signs and symptoms of increasing cardiac decompensation. Betaine and glycocyamine therapy was discontinued after three weeks and, in this instance, is considered to have failed.

Case 3

An 88-year-old man was first seen June 20, 1950. His presenting complaints were severe dyspnea, angina on effort, swelling of the lower extremities, and abdominal pain. He had been in his usual state of health, until one month previously, when, while working in the cellar, he had experienced severe constricting chest pains, which lasted approximately two hours. Thereafter, he had become dyspneic on slight effort and was compelled to sleep on three pillows. He had been able to walk a few steps at a time, when chest pain and shortness of breath compelled him to stop. He had received no therapy.

Physical Examination: The elderly, somewhat senile man, 5 ft., 2 in. tall, weighed 122 pounds. He was orthopneic. Cyanosis of the lips, ears and fingertips was observed. The heart was enlarged, the apex beat being at the anterior axillary line, in the sixth interspace. The rhythm was chaotic and no murmurs were heard. The blood pressure was 160 mm. Hg. systolic and 110 diastolic, and the pulse rate, at the apex, 92. Coarse, moist râles were heard over both pulmonary fields. The liver was palpable two finger-breadths below the costal margin. The abdomen was distended with ascites. Edema of the lower extremities extended up to the iliac crest.

In the teleroentgenogram, the heart was moderately enlarged, measuring 14.2 cm. in its transverse diameter, the predicted value being 11.9, and fluid was seen in both pleural spaces. The electrocardiogram showed auricular fibrillation with a ventricular rate of 100. In the limb leads the QRS interval was 0.12 second and right axis deviation was present. The RS-T segments were slightly elevated in Lead 1 and slightly depressed in Leads 2 and 3. The T waves in Lead 1 were low but upright. There was a small Q and an inverted T wave in CF_3. In CF_5 the ST segment was slightly depressed and the T wave inverted. The red blood cell count was 4,800,000, hemoglobin 13.4 gm. and the leukocyte count 15,200 with 82 per cent polymorphonuclear leukocytes. The sedimentation rate was rapid, being 28 mm. in one hour. The fasting blood sugar was 94, and the N.P.N. 46 mg. per cent. Urinalysis showed a specific gravity of 1.002, three plus albumin, and five to eight granular casts per high power field.

The clinical and laboratory findings were considered to be consistent with a diagnosis of hypertensive and arteriosclerotic heart disease, moderately enlarged heart, recent coronary thrombosis and infarction, intraventricular conduction defect, and auricular fibrillation and severe congestive failure. Nephrosclerosis was an additional diagnosis.

The patient was advised to enter the hospital immediately but he refused. He was taken home and attempts to confine him to bed were unsuccessful. He was given 4 gm. of glycocyamine and 25 gm. of betaine in four equally divided doses daily. Fluid and salt restriction were advised but not followed.

Serial electrocardiograms during the next three days confirmed the impression of a recent myocardial infarction. In Lead 1, the voltage of QRS became lower, the RS-T segments became more elevated and the T waves flat. In CF_1 there was a higher take-off of the ST segment and in CF_3

and CF_5, a greater depression of the ST segments with deeper inversion of the T waves.

The patient was seen twice daily at home during the ensuing week. He was very short of breath and cyanotic. He felt poorly, experienced severe abdominal pain, and could do nothing without assistance. Coarse and moist râles were heard over the chest. The edema of the extremities was unchanged, the ascites was less, and the liver barely palpable. The blood pressure was 180 mm. Hg. systolic and 110 diastolic, the apical heart rate, 96.

The following day he suffered more pain and presumably an extension of the myocardial infarction. Following this the signs and symptoms of increasing cardiac decompensation appeared. A pericardial friction rub could be heard. He was again advised hospitalization, which he refused. He was put to bed at home, his fluids restricted to 1,500 cc. daily, a mercurial diuretic administered, intravenously, and morphine prescribed for pain. Anticoagulant therapy was not administered because of the impossibility of controlling it at home. Betaine and glycocyamine therapy was continued.

The râles heard over both pulmonary fields and the edema of his extremities considerably diminished. He lost two pounds in weight. He slowly improved with decreasing signs and symptoms of cardiac decompensation, so that he appeared somewhat better during the next six days.

Two weeks later, he suffered another coronary occlusion and developed bronchopneumonia for which he received antibiotics. Folic acid and B_{12} were administered also. Betaine and glycocyamine therapy was continued. His temperature subsided during the next three days, following which he was moderately dyspneic. Only a few râles could be heard over the pulmonary fields; the liver was barely palpable and no ascites could be detected.

Three days later he suffered still another coronary occlusion and six hours later developed a left hemiplegia. Arterial pulsations were also absent in both lower extremities. Twelve hours later he died. At the time of death, there was no clinical evidence of fluid in the chest or dependent edema.

The important finding at autopsy was the evidence of recent myocardial infarctions, mural thrombus formation, embolism of the right middle cerebral artery and its tributaries, saddle-back thrombus at the bifurcation of the aorta, resolving bronchopneumonia, marked nephrosclerosis, slight chronic passive congestion of the liver, spleen and kidneys. No evidence of fatty degeneration of the liver was present.

Comment: In this instance, multiple coronary occlusions occurred during the course of treatment. While the signs of cardiac decompensation lessened, the initiating cause of death was cardiac and resulted in fatal embolism. Betaine and glycocyamine therapy, in this patient, is considered to have failed.

Summary of Group IV Cases

Two died and in the third, betaine and glycocyamine therapy is considered to have failed. One patient, Case 2, for a few days exhibited signs and symptoms of clinical improvement. Thereafter, he rapidly became severely decompensated and it became necessary to institute digitalis, bed rest and dehydrating measures. The patient discontinued betaine and glycocyamine therapy. Betaine and glycocyamine therapy in this instance is considered to have failed. Of the two patients who died, one did not die a cardiac death and was without signs of cardiac decompensation for the previous two months. The immediate cause of death in Case 3 was embolism, a consequence of coronary infarction and mural thrombus formation. He also was without signs of excessive fluid retention at the time of death. Postmortem examination confirmed the clinical impression in this respect. No evidence of toxicity due to betaine and glycocyamine therapy was found. Here also, betaine and glycocyamine therapy is considered to have failed.

Effect of Betaine and Glycocyamine Therapy on Blood Sugar

Early in this experiment, one patient (E.B.W.), while taking glycocyamine and betaine, in an effort to control his weight, omitted lunch. Repeatedly, in the early afternoon, he exhibited the symptoms of hypoglycemia. A blood sugar determination indicated it had dropped to 45 mg. per cent, at 3:00 p.m. In order to check this observation more critically, the fasting blood sugar of seven patients was determined. They were then given 1 gm. of glycocyamine and 5 gm. of betaine hydrate. In every case, the blood sugar dropped from 15 to 20 mg. per cent. Two of these patients appeared clinically to suffer from acute hyperinsulinism, with rapid pulse, nervousness, anxiety, weakness, hunger, profuse sweating and vomiting. We do not consider the transient hypoglycemia, usually, important enough to require extra carbohydrate with betaine and glycocyamine therapy.

The sugar tolerance curves of two diabetics who are patients in this heart series, whose diabetes is controlled without insulin, are shown in figures 1 and 2. They are without signs and symptoms of diabetes and are gaining weight. No glycosuria has occurred in either of these patients for the last five months. At the present time the value, if any, of betaine and glycocyamine therapy in the control of diabetes is under study. Most of the

diabetic patients in this series continue to require insulin.

Summary

1. Twenty-eight patients with cardiac decompensation have been treated with betaine and glycocyamine therapy over a period of six to ten months. In 20 of these patients, betaine and glycocyamine medication was the only form of therapy used, as all other forms of treatment were discontinued. These patients have become and have remained free of the signs and symptoms of cardiac decompensation. In four patients, it is felt betaine and glycocyamine were useful additions to other cardiac therapy; in three patients, results of betaine and glycocyamine therapy were inconclusive and are considered a failure. Two patients suffered a coronary occlusion, and one patient had a recurrence of rheumatic fever while under therapy.

2. Important features of betaine and glycocyamine therapy, as it has been used in these patients, are its ease of management and safety.

3. Outstanding effects have been, in nearly all patients, the early appearance of a markedly improved sense of well-being, less fatigue, greater strength and endurance. These effects have persisted but their relation to improved cardiac function was not always evident.

4. Most of the patients gained weight. The weight gain was not due to edema and probably was, in part, due to a positive nitrogen balance[10] and the effects of improved rest and better appetite.

5. In 18 patients with hypertension, there was a transient fall of the blood pressure shortly after beginning betaine and glycocyamine therapy. The decrease in blood pressure has persisted in four of these.

6. In only one patient was a change in the heart size observed (Group 2, Case 2). No significant changes were observed in the blood electrolytes. There were blood sugar changes. A large increment in the total creatinine excretion (creatine plus creatinine) was observed in all patients.[11]

7. An endocrine effect was suggested in that one third of the patients, male and female, reported increased libido and potentia. Some of the female patients reported enlargement of the breasts. A lessening of the signs and symptoms of arthritis and of asthma occurred.

8. The only untoward effects observed were transient nausea and diarrhea, which cleared up spontaneously.

9. The dextrose tolerance of both diabetic and nondiabetic patients was increased.

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THE FORMATION OF CREATINE FROM GLYCOCYAMINE IN THE LIVER

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The study of the precursors of creatine in animals has been beset by two difficulties principally. One has been the lack of really adequate biological material; the other, the lack of a specific, and at the same time sensitive analytical method. Experiments hitherto have consisted in attempts to change the urinary excretion of creatine and creatinine, or the creatine content of the tissues of intact animals or of isolated perfused organs. The normal, *i.e.* uncontrolled, fluctuations in tissue composition and urinary excretion are relatively large compared with the changes induced experimentally; it is often impossible to distinguish when experimental effects are observed, whether these have arisen from changes in the processes of excretion or synthesis; there may be variations in the water content of the tissues, thereby affecting their percentile composition; all of these have stood in the way of firm conclusions being drawn.

Some of these difficulties have been avoided in perfusion experiments on isolated organs (1). These experiments are extremely laborious, time-consuming, and costly. The experimentally induced change in creatine content is at the most—about 30 per cent—a small deviation from the normal. The normal base-line is not constant but varies with the age and weight of the animal. Since the same specimen cannot be used for the experiment and control, a large number of animals must be used first to establish the normal base-line, encompassing the variations of the normal, and then an equally large number for each single experiment in order to obtain a body of data sufficiently large for a statistical conclusion which may be significant.

In 1935 we published some observations of a slight increase in in "apparent" creatine when rat liver slices were incubated with a protein hydrolysate (2). Granted that such liver slices could synthesize creatine, most of the difficulties in working with whole animals or whole organs are avoided. With tissue slices the one specimen of tissue provides material for controls and for testing a variety of experimental variants simultaneously. This was the reason that the observation of even a slight synthesis of creatine by tissue slices seemed promising.

We were faced here, however, with the second difficulty mentioned above, uncertainty whether the material which is augmented by the liver slices is really creatine. A number of substances give a positive test with the Jaffe reagent. The amount of the material in question formed by the liver slices was too small to be identified by isolation.

Accordingly further study of this problem was postponed until a more specific analytical method which could be adapted to submicro scale was available. Such a method was provided by Dubos and Miller (3). They discovered and succeeded in culturing a soil bacterium which specifically destroys creatine and creatinine.

We again took up the problem, employing tissue slices and this new adjuvant to our former submicromethod for creatine (4). We have found that liver slices of the cat, rabbit, and rat are able to convert glycocyamine to creatine. In the experimental conditions we have observed, the increase is 5 to 20 times the amount originally present in the slices. The difficulties and uncertainties which exist in conclusions resting on statistical analysis of small differences have therefore been overcome. The liver is well suited to this study, because its normal creatine content and therefore the control or blank value is very low.

We have found further that when methionine is present in the Ringer's solution, the amount of creatine formed from the added glycocyamine was on the average about 50 per cent greater than when methionine was not added. We have tested thirty-two other substances including amino acids, methylated amines and a purine, and betaine. All of these were negative in this respect. It seems reasonable to conclude therefore that rat liver slices transfer the methyl group of methionine to glycocyamine, thus converting the latter substance to creatine.

Technique

The tissue slice technique used is only slightly modified from that described by Warburg (5). The details of the reaction vessels and the manner in which they are mounted are described in a previous communication (6).

The blank controls and each experimental variant were carried through in triplicate. We have had twenty to thirty reaction vessels, each containing three slices from the same liver, running simultaneously. It was necessary to make the sampling of the slices as uniform as possible, because the capacity to synthesize creatine may vary significantly between the lobes. The procedure we finally adopted was to arrange the reaction vessels in three rows (when each experimental variant is carried through in triplicate), one vessel in each row for each variant. Consecutive slices were transferred to a Petri dish containing Ringer's solution, one slice for each vessel in the row. After 3 minutes soaking in this vessel, with gentle agitation, the slices were transferred to the reaction vessels. The same was done for the other two rows. The whole procedure was then repeated according to the number of slices wanted in each vessel. The reason for the 3 minutes soaking in the Ringer's solution before transfer to the reaction vessels is that more creatine was formed than when the slices were transferred directly with only momentary rinsing in Ringer's solution.

At the end of the reaction period the contents of the vessels, including the slices, were transferred to test-tubes, and the bottles and transfer pipette washed with two 1 ml. portions of 0.02 N HCl. The test-tubes were placed in a boiling water bath for 10 minutes with occasional stirring or shaking. After this time the test-tubes were cooled and the volumes made up to 6 ml. with 0.02 N HCl. These test-tubes are marked at 6 ml. Usually only 0.1 to 0.2 ml. was needed to bring the volumes to the mark. The suspensions were then filtered. The coagulated slices with the coagulated protein in each vessel were transferred to small glass dishes and the weight determined after drying at 100°.

The analytical procedure was essentially that described previously (4). Some minor details were varied from time to time in addition to employment of the bacterial digestion procedure of Dubos and Miller. Table I is a protocol of an experiment in

which the complete analytical procedure was used. Instead of triplicate reaction vessels, six were used for each experimental variant. The boiled extracts in pairs of vessels were joined and filtered. To 8 ml. of this filtrate were added 2 ml. of 0.25 M phosphate buffer at pH 7.0. This solution was divided into two portions. In one-half the creatine was determined directly. The creatine in the other half was destroyed by a suspension of NC soil organisms prepared according to the prescription of Dubos and Miller. The difference between the color developed by the Jaffe reagent with and without this digestion gave the true creatine (and creatinine) in the original solution.

The bacterial digestion was carried out as follows: 5 ml. of the solution neutralized with phosphate were transferred to a 250 ml. Erlenmeyer flask. To this was added 1 ml. of a suspension of the NC soil organisms. The necks of the flasks were covered with squares of Parafilm and set away in an air bath at 38° for ½ to ¾ of an hour. At the end of this time 1 ml. of 0.5 N HCl was added to each flask. The contents were then centrifuged. 6 ml. of the clear supernatant solution were taken for analysis. The length of time the bacteria were allowed to react on the experimental solutions was based on a prior determination of the potency of the bacterial suspension used. It was the time required by 1 ml. of the bacterial suspension to destroy completely the creatine in 5 ml. of a 2 mg. per cent solution. This was a larger amount of creatine than in any of the tissue extracts submitted to digestion.

To the 5 ml. of tissue extract containing the phosphate buffer, but which had not been digested by the bacteria, were added 1 ml. of water and 1 ml. of 0.5 N HCl. 6 ml. were then taken for analysis. From this point on the analytical procedure was identical for the solutions which had been digested with bacteria and those which had not. Both sets were carried through to the completion of the analysis simultaneously.

The 6 ml. aliquots were transferred to thick walled Pyrex test-tubes with internal dimensions of 125 × 12 mm. The tubes were covered with parchment paper caps and autoclaved for 20 minutes at 125°. After they were cooled, a small amount of Lloyd's reagent was added to each. We have found that the amount of the Lloyd's reagent may vary from 10 to 60 mg. without affecting the final result. The test-tubes were now shaken for 7 minutes

on a shaker of the type devised by Fisher and Wilhelmi (7). The tubes were then centrifuged, the supernatant solution discarded with the last drop at the rim of the test-tube taken up with filter paper, and the Lloyd's reagent resuspended in 2 ml. of 0.01 N HCl. The tubes were centrifuged again, the supernatant solution again discarded, and the adherent moisture on the walls of the test-tube carefully taken up with filter paper. 3 ml. of a sodium picrate solution were added to each tube. This solution consists of 10 parts of saturated picric acid (purified) and 1 part of 10 per cent NaOH, these being mixed immediately before use. The test-tubes were again shaken for 7 minutes and centrifuged. The color was measured on a spectrophotometer with light of approximately 0.525 μ wave-length. With concentrations ranging from 0 to 2 mg. per cent there is a strictly linear relation between concentration of creatine and the intensity of color measured in this manner.

The following controls were taken through the above identical procedure including digestion by the bacteria: Ringer's solution alone, Ringer's solution containing the same concentration of glycocyamine used in the experiment, and Ringer's solution containing glycocyamine and methionine. Each of these was carried out in triplicate. In addition 1 ml. of the bacterial suspension alone and bacterial suspension plus 5 ml. of 2 mg. per cent creatine were incubated with the experimental solutions and carried through the same analytical procedure, performed in duplicate. Finally a set of five standard creatine solutions with concentrations ranging from 0.1 to 2 mg. per cent was treated in exactly the same manner as the experimental solutions except that they were not submitted to bacterial digestion. The amounts of creatine in the experimental solutions and controls were determined by interpolation from the straight line given by the readings of these standard solutions. The above controls and the standards were carried through afresh in every experiment with the experimental solutions.

It is a testimony to the convenience of this analytical method that we have frequently carried through more than 60 individual analyses from the bacterial digestion to the final spectrophotometer reading in less than 5 hours.

We found in experiments with rat and rabbit liver that essen-

TABLE I

Formation of Creatine by Rat Liver Slices in 6 Hours at 37.5° from Glycocyamine

Treatment	Composition of medium			Dry weight of tissue	Apparent creatine				Residual chromogenic material after bacterial digestion	True creatine			
	Ringer's solution	Glycocyamine	*dl*-Methionine		Total	Total amount formed	Amount formed from glycocyamine	Q(creatine) × 100 from glycocyamine		Amount present	Total amount formed	Amount formed from glycocyamine	Q(creatine) × 100 from glycocyamine
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)
	ml.	*mg.*	*mg.*	*mg.*	*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	
Analyzed immediately	4			40.8	0.12				0.07	0.05			
" "	4			41.5	0.14				0.07	0.07			
" "	4			59.8	0.17				0.10	0.07			
Incubated 6 hrs.	4			34.1	0.21	0.12			0.14	0.07	0.03		(0.15)
" 6 "	4			40.2	0.26	0.14			0.18	0.08	0.03		(0.13)
" 6 "	4			38.0	0.22	0.12			0.14	0.08	0.03		(0.15)
" 6 "	4	0.25		35.4	1.20	0.60	0.38	1.9	0.70	0.50	0.45	0.42	2.1
" 6 "	4	0.25		45.0	1.54	0.94	0.66	2.5	0.72	0.82	0.77	0.74	2.8
" 6 "	4	0.25		35.5	1.31	0.71	0.49	2.4	0.72	0.59	0.54	0.51	2.5
" 6 "	4	0.25	1.45	32.5	1.54	0.90	0.70	3.7	0.72	0.82	0.78	0.75	3.9
" 6 "	4	0.25	1.45	37.8	1.54	0.90	0.64	2.9	0.70	0.84	0.79	0.76	3.4
" 6 "	4	0.25	1.45	35.5	1.53	0.89	0.67	3.2	0.71	0.82	0.77	0.74	3.6
" 6 "	4	0.25			0.61				0.61				
" 6 "	4	0.25			0.60				0.60				

Incubated 6 hrs.	4	0.25		0.60			0.61			
" 6 "	4	0.25	1.45	0.65			0.64			
" 6 "	4	0.25	1.45	0.66			0.63			
" 6 "	4	0.25	1.45	0.61			0.64			
1 ml. bacterial suspension incubated 45 min. at 37°							0.01			
0.1 mg. creatine incubated with 1 ml. bacterial suspension 45 min. at 37°							0.007			
							0.006			
							0.006			

The factor converting mg. per cent to Q(creatine) is $4/100 \times 1.5 \times 171 \times 1/6 \times 1/$(dry weight of tissue): 4/100, since there were only 4 ml. in each reaction vessel for the amount of tissue indicated; 1.5 because these 4 ml. were diluted to 6 ml. in the transfer from the reaction vessels to the test-tubes in which they were boiled; 171, because 1 mg. of creatine would occupy 171 c.mm. at S.T.P. if it were a gas; 1/6, because the experiment was run for 6 hours. Q(creatine) is therefore the amount of creatine formed, expressed as if it were a gas in c.mm. at S.T.P., per mg. of tissue (dry weight) per hour.

tially the same result was obtained whether bacteria were used or not. Accordingly we later dispensed with the use of the bacteria, except as indicated.

Results

Table I is a condensed protocol of a typical experiment. The figures in Column 6 are the spectrophotometer readings converted to mg. per cent by interpolation from the standard curve. The figures in Column 7 are obtained by subtracting from those in Column 6 the values of the glycocyamine or glycocyamine plus the methionine blank, and the amount present in the tissue at zero time. The glycocyamine blank value is quite large. It arises from the conversion of glycocyamine to glycocyamidine during the autoclaving. It was essential that the glycocyamine blanks be treated exactly the same as the experimental solutions through all the operations from immersion in the water bath at 37.5° for the same length of time to the final development of the color. The figures in Column 8 are obtained by subtracting from those in Column 7 the amount of chromogenic material formed by the tissue in the Ringer's solution without glycocyamine. Q(creatine) (Columns 9 and 14) is the amount of creatine formed, expressed as if it were a gas in c.mm. at S.T.P., per mg. of tissue (dry weight) per hour. The figures in Column 11 are the differences between those in Columns 6 and 10. From the figures for the blanks in Columns 6 and 10, it is seen that the bacteria digested none of the glycocyamine. The figures in Columns 9 and 14 are not significantly different. They are a little higher in Column 14 than in Column 9 because in Column 7 a correction should have been applied to the glycocyamine blank value for the glycocyamine converted to creatine, from 10 to 16 per cent. If this had been done, the figures in Column 8 would have been increased by 0.06, which would have made them the same, within experimental error, as those in Column 13.

In a previous determination we have found that there was practically no creatinine in the tissue extracts.

Table I shows that the liver slices convert glycocyamine to creatine. This, as far as we know, is the first time the biological conversion of glycocyamine to creatine has been demonstrated by an unequivocally specific analytical method for creatine, and in which

the tissue used both in the controls and in the experiment came from the identical organ specimen. The increases in the experimental vessels were from 10 to 15 times the amount present in the tissue at the beginning of the experiment. The figures in Columns 9 and 14 show that with added methionine there was 40 per cent more creatine formed than when methionine was not added.

Most of the chromogenic material in the liver slices at the beginning and end of the experiment was not true creatine. This is in accord with the findings of Baker and Miller (8). There is, however, a slight increase in true creatine in the liver slices suspended in Ringer's solution containing no glycocyamine. We have found this repeatedly. Most of this non-creatine chromogenic material found in the tissue blanks is also not glycocyamine. This was ascertained by a direct determination for glycocyamine.

The values for Q(creatine) are much smaller than those found with liver slices for the formation of urea, amino acids, or hippuric acid (6). Nevertheless, this rate, small though it is, is more than sufficient to account for the total creatine plus creatinine excretion in the rat. Thus, an adult rat with a liver weighing 12 gm. might excrete 9 mg. of creatine plus creatinine in 24 hours (9). A Q(creatine) of 0.02 would correspond in such an animal to the formation of 7 mg. of creatine in 24 hours.

The following compounds and combinations of compounds were tried instead of glycocyamine to determine whether they could serve as precursors of creatine: arginine, arginine plus glycine, arginine plus glycolic acid, choline, glycine, glycine plus urea, glycolic acid, and methionine. Each of these has been tested on both rat and rabbit liver slices several times. The results have been consistently negative.

Table II is a summary of most of our experiments with glycocyamine and methionine. In the course of these experiments we have used glycocyamine from two different commercial sources, and two specimens of *dl*-methionine, one prepared in this laboratory and one obtained commercially. A different animal was used for each pair of figures.

The data in Table II show the increase in creatine formation invariably obtained when methionine was added to the glycocyamine. Approximately the same relative increase occurred regardless of age, sex, and nutritional condition of the animal, and

TABLE II

Formation of Creatine by Rat Liver from Glycocyamine with and without Added Methionine at 37.5°

From glycocyamine alone	From glycocyamine and methionine	Duration of incubation	Age and sex	Nutritional condition
Q(creatine) × 100	*Q*(creatine) × 100	hrs.		
3.2	6.2	1	Adult. ♂	Normal nutrition
6.1	9.0	2	" ♂	" "
4.0	5.5	3	" ♂	" "
4.0	6.5	3	" ♂	" "
5.7	8.1	3	" ♂	" "
1.9	3.4	3	3 mos. ♂	" "
4.2	7.4	3	3 " ♂	" "
6.6	7.9	3	3 " ♀	" "
6.5	9.0	3	3 " ♀	" "
4.6	6.6	4	Adult. ♂	" "
8.5	11.3	4	" ♂	" "
6.1	8.5	4	" ♂	" "
7.3	10.6	4	" ♂	" "
7.7	11.1	4	" ♂	" "
3.1	5.8	4	1 mo. ♂	" "
2.3	4.5	4	1 " ♂	" "
1.5	2.3	4	Adult. ♂	Fasted 66 hrs.
1.5	2.4	4	" ♂	" 66 "
4.1	6.4	4	" ♀	Normal nutrition
4.4	6.9	4	" ♀	" "
2.7*		6	" ♂	" "
2.8*	4.8*	6	" ♂	" "
2.5*	3.6*	6	" ♂	" "
2.4	3.3	6	" ♂	" "
3.1		6	" ♂	" "
2.5		6	2 mos. ♂	" "
2.2		6	Adult. ♀	" "
2.7		6	" ♀	" "

Each of the above figures is the average of a triplicate determination. Each reaction vessel contained 20 to 40 mg. (dry weight) of liver, 4 ml. of Ringer's solution containing 0.25 mg. of glycocyamine, and in the methionine series in addition 1.49 mg. of the amino acid.

* Values obtained with the employment of bacteria in the analysis.

regardless also of the duration of the experiment. The over-all average value for *Q*(creatine) × 100 from glycocyamine alone is 4.1, and from glycocyamine plus methionine 6.6.

We have no explanation as yet for the large variations in the rate of creatine formation in different experiments.

It will be noted that most of the results in Table II were obtained without the employment of bacteria in the analysis. Three experiments with rat liver and three with rabbit liver were carried out in which bacteria were used. The values for Q(creatine) were, as in Table I, essentially the same with and without the use of bacteria. We feel therefore that the results obtained without the use of bacteria with this tissue and under these experimental

TABLE III

Rate for Formation of Creatine from Glycocyamine with and without Added Methionine

Time	Without methionine		With methionine	
	Amount of creatine formed per 100 mg. (dry weight) of tissue	Q(creatine) × 100	Amount of creatine formed per 100 mg. (dry weight) of tissue	Q(creatine) × 100
hrs.	*mg.*		*mg.*	
1	0.018	2.8	0.036	6.2
2	0.070	6.0	0.101	8.6
4	0.095	4.6	0.150	6.4
6	0.144	4.5	0.203	5.8

Each of these figures is the average of a triplicate determination. The composition of the Ringer's solution and amount of tissue were the same as in the experiments of Tables I and II. Two lobes of liver were used. Consecutive slices were placed alternately in the vessels with and without methionine. The comparison at each hour, therefore, is of the activity of immediately adjacent sections of liver. Bacteria were not employed in the analyses here.

conditions are as reliable indices of true creatine as those obtained with bacteria.

Table III contains the results in more detail of an experiment in which the rate of creatine formation from glycocyamine, with and without methionine, was studied. The figures show that the methylation of glycocyamine proceeds unchecked for at least 6 hours. The maximum at 2 hours in each series is accidental. It did not occur in other similar experiments.

The absolute amount of glycocyamine methylated was not increased by a 10-fold increase in the initial concentration of gly-

cocyamine. We have not yet explored thoroughly the effect of changing the concentration of methionine. In one experiment 0.0025 M methionine was as effective as 0.01 M. The fact that addition of so much methionine increases the rate of methylation only 40 or 50 per cent leads us to suspect that the methylating agent in the liver itself may not be methionine. Another piece of evidence which points in this direction is that the ratio of the rates of creatine formation with and without methionine is nearly the same throughout the whole period of from 1 to 6 hours (Table III). In other words, the effects of the methionine and of the methylating agent in the tissues were additive. It is possible that the methylating agent in the tissues is derived from methionine.

The following compounds were tested with rat liver as possible methylating agents of glycocyamine. All gave negative results: acetylcholine, *d*-alanine, *d*-arginine, *l*-asparagine, *l*-aspartic acid, betaine, caffeine, choline, *l*-cysteine, *l*-cystine, ethanolamine, *d*-glutamic acid, *d*-glutamine, glycine, glycolic acid, guanidine, *l*-histidine, *dl*-isoleucine, *l*-leucine, *d*-lysine, mono-, di-, tri-, and tetramethylamine, *d*-ornithine, *l*-hydroxproline, *dl*-phenylalanine, *l*-proline, *dl*-serine, *d*-threonine, *l*-tryptophane, and *l*-tyrosine. The final concentration of glycocyamine in the Ringer's solution in these experiments was always approximately 0.0005 M, and 0.0025 M of the compound whose methylating possibilities were being tested. The significance of the positive effect invariably obtained with methionine is heightened obviously by the fact that all of the above compounds were negative.

Some experiments have been made with the kidneys of the cat, rabbit, and rat. Slices of the cortex with and without methionine either failed to methylate glycocyamine or the slight positive results were within the experimental error. These experiments are part of a survey not yet completed of the organs of a number of animals. The details of these experiments will be published later.

Minced liver of the rat or rabbit failed to give any measurable increase in creatine in 6 hours at 37.5° on incubation with glycocyamine, with or without methionine.

Similarly negative results were obtained with slices of heart

and sartorius. But the cell structure is not preserved in slices of these muscular structures as it is in slices of liver.

Until conditions are discovered in which positive results are obtained with minced liver, we feel that no significance can be attached to negative results with sections whose cell structure has been broken or to extracts of other organs.

DISCUSSION

Beard, Boggess, and Pizzolato (10) proposed that glycine and urea condense to form glycocyamine, which is then methylated by more glycine or glutamic acid. We have observed neither this condensation nor the proposed methylating reaction with rat or rabbit liver slices. The conclusions of Beard *et al.* are based largely on experiments on the rat. The negative results in our experiments with glycine and urea we believe are significant in view of the positive results with glycocyamine and methionine. Experiments on the synthesis of urea, amino acids, and hippuric acid have shown that results obtained with tissue slices afford reliable qualitative information, at least, regarding the potentialities of the intact tissue *in situ*.

Bodansky (11) fed glycocyamine to normal rats and at 3, 6, 12, and 24 hours after feeding measured the glycocyamine and creatine concentrations in the liver, muscle, heart, and kidney. Bodansky's interpretation of the data obtained in these experiments was that significant increases in creatine content occurred only in the kidney, and that the increases in the liver were not sufficiently clear cut to be significant. Bodansky concluded that his findings suggest "that methylation of the guanidoacetic acid may have occurred in the kidneys. . . In view of the occurrence of guanidoacetic acid in large amounts in the liver and the failure to show an increase in creatine, it is surmised that the liver plays an insignificant role, if any, in creatine production."

This surmise is in direct conflict with our observations. Bodansky's experimental observations, however, and ours are not in conflict. Thus the creatine content of the liver in two controls in Bodansky's experiments was 16.0 and 20.4 mg. per 100 gm. of tissue, and in three experiments with glycine, 18.6, 16.8, and 19.0 mg. After glycocyamine feeding the figures are 21.7 mg. in 3 hours

and 23.3 in 6 hours, and in a second experiment 30.5 in 3 hours and 22.3 in 6 hours. The relative increases over the control values in the liver with glycocyamine were of the same order of magnitude as those found in kidney. The increases in true creatine in the liver were probably relatively much larger, since most of the chromogenic material in the liver with the Jaffe reagent is not creatine (Table I, and also Baker and Miller).

There is another physiological factor which must be taken into account in interpreting the data obtained by Bodansky. This factor is that the kidney is better able to store creatine than the liver. The analyses of Baker and Miller and our own show that the true creatine content of the kidney is 4 or 5 times that of the liver. Bodansky found that when creatine was fed the creatine content of the liver was twice the control value at the 3rd hour but had declined to the control value by the 6th hour, whereas in the kidney the concentration was 70 per cent above the control value at the end of the 6th hour.

Our observations show conclusively that glycocyamine can be methylated by rat liver. The rate of methylation by kidney slices is much slower than in liver, if it is not absolutely negative.

All these observations are brought into accord by the hypothesis that in the experiments of Bodansky the creatine synthesized from glycocyamine in liver was quickly removed by the blood and stored for a relatively long period in the kidney. We have no reliable data of our own at present on the possible conversion of glycocyamine to creatine in other organs or in the muscles.

Fisher and Wilhelmi found that when isolated male rabbit heart was perfused there was an increase in creatine when arginine was added to the perfusate. No increase in creatine was observed under these conditions in the hearts of prepubertal animals. Davenport, Fisher, and Wilhelmi (12), extending these observations, found that glycolic acid was essential for the methylation of glycocyamine. They suggested the following mechanism of creatine formation in the rabbit heart. Arginine is broken down to glycocyamine and glycolic acid; the glycolic acid then methylates the glycocyamine to form creatine.

In rat and rabbit liver slices the results with arginine, with and without glycolic acid or glycine, and with the two acids alone did

not yield detectable amounts of either glycocyamine or creatine. The only substance we have yet found which is capable of methylating glycocyamine is methionine.

We have attempted to repeat the observations of Fisher *et al.* with slices of rabbit heart. These experiments were unsatisfactory because of the difficulties of obtaining uniform sampling and because the experimental effects were small compared with the amount of creatine initially present. For the reason stated above we do not attach any significance to these experiments.

The difference between the observations on the perfused heart and ours on the liver stand, for the time being at least, either as an unresolved discrepancy or as indicating important differences in the mechanism of creatine formation in the heart and in the liver.

SUMMARY

1. Liver slices of cat, rabbit, and rat convert glycocyamine to creatine.

2. This methylation is accelerated in rat liver by methionine, (other animals are now being studied).

3. Methionine is the only substance we have yet found among a large number of amino acids, methylated amines, and other compounds which is able to effect this methylation in rat liver.

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THE FORMATION OF GLYCOCYAMINE IN ANIMAL TISSUES*

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It was shown in preceding communications that glycocyamine is converted into creatine by surviving liver slices (1). Our findings indicated that the methylating agent is methionine or a derivative of methionine. Liver slices can methylate glycocyamine rapidly enough to permit assignment to the liver alone, if necessary, of the task of making good the loss of creatine and creatinine in the urine. This holds for the livers of all mammals studied. We found no evidence of this methylating mechanism in any other tissues, except possibly slight activity in the kidney. In the pigeon the kidney is as effective in this respect as the liver.

These experimental facts were corroborated (as far as the rat is concerned) by experiments on living animals with tracers. Bloch and Schoenheimer, using N^{15}, found that glycocyamine is readily converted to creatine (2). Du Vigneaud and his collaborators fed rats methionine with deuterium in the methyl group; after only 3 days a relatively large quantity of deuterium was found in the muscle creatine (3).

Glycocyamine has had a favored position among the possible precursors of creatine. It is nearer to creatine structurally than any other precursor which has been proposed; and its convertibility to creatine in the living organism has been proved. The case against glycocyamine rested on two arguments: (1) that only a small fraction, 5 to 15 per cent, of administered glycocyamine is methylated, and (2) that glycocyamine had not been found as a normal constituent of animal tissues. It has, however, been isolated by Weber from human and dog urine (4).

* A summary of this work has appeared (*Science*, **91**, 551 (1940)).

The findings with surviving liver slices and the tracer studies reinstated glycocyamine, more firmly than before, as a possible normal precursor of creatine. The quantity of creatine synthesized daily is so large that, if glycocyamine is its normal precursor, an active mechanism for the formation of glycocyamine must exist. We undertook a search for this mechanism.

The first necessity was an adequate analytical method. Such a method is described in the preceding communication (5).

With it we have found that kidney slices rapidly form glycocyamine from arginine and glycine. All other tissues tested are negative in this respect.

This interaction of arginine and glycine is also catalyzed by thoroughly macerated cell-free kidney tissue suspended in a phosphate buffer solution.

The formation of glycocyamine from arginine and glycine is a new biochemical reaction which may be called "transamidination." We propose that the enzyme be designated "glycine-transamidinase." The discovery of this reaction provides direct proof that arginine and glycine are precursors of creatine.

Bloch and Schoenheimer fed ammonia containing N^{15} to rats and later found the isotope in the amidine nitrogen of creatine. After glycine containing N^{15} was fed, the isotope was found in creatine in the sarcosine nitrogen. In a later communication the same authors presented more direct evidence (again obtained by the use of N^{15}) which confirmed the findings we had reported that glycocyamine is formed by the transfer of the amidine group from arginine to glycine. They fed $l(+)$-arginine with N^{15} in the amidine group to rats (6); afterwards the creatine in the muscles had a far higher isotopic content than after the administration of isotopic ammonia, urea, or any other amino acid except glycine. It was so high that they considered that the amidine group of creatine must have originated from arginine.

The present communication contains the details of our experimental procedure, our findings on the effects of a fairly large number of amino acids and derivatives of arginine and of glycine, surveys of the capacity for glycine transamidination of the organs of a number of common experimental animals, and the results of some studies of the effect of concentration of reactants, pH, and time on the rate of transamidination.

Technique and Results

The tissue slice technique used and the details of the reaction vessels are described in a previous communication (7).

At the end of an experiment the contents of the reaction vessels were transferred with the slices to test-tubes graduated at 20 ml.; the vessels were washed with three 2 ml. portions of 0.02 M phosphate buffer solution at pH 6.0 and the washings added to the main solution. The pH was adjusted finally to 6 with a drop of 0.5 N hydrochloric acid. The test-tubes containing the slices, the main solutions, and washings were kept in a boiling water bath for 10 minutes, after which they were cooled to room temperature, and made up to the 20 ml. mark with water and mixed by shaking. These solutions were then filtered. 5 ml. of the clear protein-free filtrate were analyzed for glycocyamine by the procedure described in the preceding communication (5).

The coagulated slices and protein in each test-tube were transferred to small glass weighing dishes, heated overnight at 105°, and, after cooling in a desiccator, were weighed.

Table I is the detailed protocol of a typical experiment. A significant amount of glycocyamine is formed when arginine alone is added to the Ringer's solution. Glycine without arginine also leads to a slight increase in glycocyamine. When both amino acids are added together, the increase in glycocyamine is more than 10 times the increase with arginine alone. The increases obtained with arginine alone and with glycine alone indicate either the presence of these amino acids in the free state (more glycine than arginine) or their formation in small amounts by autolysis within the slices.

In the experiment whose results are recorded in Table I, and in a number of others, creatine analyses were carried out with the specific bacterial (NC) enzyme of Dubos and Miller (8, 9). No evidence of creatine formation was found.

The steps in the proof that the substance we were measuring was glycocyamine were as follows: Autoclaving in acid solution produced a substance which was adsorbed by Lloyd's reagent and gave a positive test with the Jaffe reagent. This chromogenic material was not digested (before autoclaving) by the NC bacteria of Dubos and Miller under conditions in which creatine and creatinine were completely digested. A strongly positive Saka-

guchi test was obtained in the unautoclaved solution after all the arginine was removed by exhaustive adsorption on permutit. The depth of color which the unknown solution gave with the Jaffe reagent (after autoclaving) corresponded, assuming it to be glycocyamine, to the intensity of color it gave in the Sakaguchi reaction after removal of the arginine. A liter of solution was

TABLE I

Protocol of Typical Experiment Showing Formation of Glycocyamine from Arginine and Glycine by Rat Kidney Slices

Ringer's solution, 38°, 4 hours.

Dry weight of slices (1)	Ringer's solution (2)	0.02 M arginine in Ringer's solution (3)	0.04 M glycine in Ringer's solution (4)	Concentration of glycocyamine in aliquot taken for analysis (5)	Glycocyamine found per 100 gm. fresh tissue (6)
mg.	*ml.*	*ml.*	*ml.*	*mg. per cent*	*mg.*
26.4	4			0.02	6
30.4	4			0.04	11
22.5	3	1		0.10	36
21.0	3	1		0.11	42
20.0	3		1	0.04	16
22.0	3		1	0.05	18
23.6	2	1	1	1.03	349
21.4	2	1	1	0.97	363

The figures in Column 6 are obtained by multiplying those in Column 5 by 8000 and dividing them by the dry weight of the tissue in mg. (Column 1). The figure 8000 is obtained as follows: the solution is diluted 5-fold before analysis; in the course of analysis it undergoes a further 1:1 dilution; the results in Column 5 expressed as mg. per cent must be divided by 25, since there were only 4 ml. of the original reaction solution; to express the results on the basis of 100 gm. of fresh tissue, the factor $100,000/5 \times W$ is used, W being the dry weight in mg. given in Column 1. The factor therefore is $5 \times 2 \times 1/25 \times 100,000/(5 \times W) = 8000/W$.

now collected in which kidney slices had acted upon arginine and glycine, and which contained, according to analysis, about 50 mg. of glycocyamine. The glycocyamine was isolated by adsorption on Lloyd's reagent in acid solution, elution with baryta, removal of the arginine by repeated adsorption with permutit, and crystallization from glacial acetic acid as glycocyamine acetate. These crystals were the characteristic needles and thin prisms (10).

The free glycocyamine was regenerated from the acetate by boiling in dilute aqueous solution and crystallized by evaporation of the water. 25 mg. of crude glycocyamine were thus obtained. It was thrice recrystallized from water, with a final yield of 11 mg. of the pure dry material which was analyzed. It gave the following figures.[1]

Observed.	C 30.8, H 5.95, N 35.8
Theoretical for glycocyamine.	" 30.8, " 6.0, " 35.9

TABLE II

Formation of Glycocyamine by Rat Kidney Slices from Arginine and Glycine or Glycine Derivatives

Glucose-Ringer's solution, 3 hours, 38°. Concentration of arginine 0.005 M; glycine or derivatives 0.01 M.

Arginine	Glycine or glycine derivative	Glycocyamine found per 100 gm. fresh tissue
		mg.
−		8
+		35
−	Glycine	19
+	Betaine	44
+	Glutathione	296
+	Glycine	382
+	" anhydride	8
+	Glycylglycine	436
+	Glycolic acid + ammonia	6
+	Hippuric acid	35
+	Leucylglycine	254
+	Sarcosine	109
+	" anhydride	6

Table II summarizes the relative effectiveness of glycine and some glycine derivatives as precursors of glycocyamine. The effect of the glycine peptides is accounted for on the hypothesis that these are first hydrolyzed and that it is the free glycine which reacts with arginine to form glycocyamine. The argument is as follows: The rate of glycocyamine formation is proportional to the concentration of free glycine (Table VIII). When glycine

[1] We are indebted to and wish to thank Dr. A. J. Haagen-Smit for these analyses.

peptides were the source of the glycine, the concentration of free glycine was initially 0 and only in the course of the 3 hour experimental period did it approximate 0.01 M, whereas when glycine itself was added the initial concentration was 0.01 M. Hence smaller amounts of glycocyamine were formed from glutathione, leucylglycine, and hippuric acid than from the same initial concentration of glycine. Glycylglycine gave a higher value than glycine, because on hydrolysis it yields 2 molecules of glycine and as a result the concentration of glycine rose well above 0.01 M before the end of the 3 hour period.

It follows, if the above is the correct explanation of the effect of glycine peptides, that rat kidney contains a dipeptidase for leucylglycine, an enzyme which liberates glycine from glutathione, and no enzymes capable of forming free glycine at a significant rate from betaine, glycine anhydride, or hippuric acid.

The results with sarcosine indicate that this substance is demethylated without deamination in rat kidney. Separate analyses showed that no creatine was formed; this proved that demethylation of the sarcosine had occurred prior to the transamidination. The kidney contains, therefore, an enzyme which demethylates sarcosine.

These findings with sarcosine are complemented by those of Bloch and Schoenheimer (2, 11) who, using N^{15} as a tracer, found that sarcosine is converted to glycine *in vivo* and that in the course of the demethylation the glycine nitrogen originally attached to the carbon chain is not replaced. Their experiments therefore excluded intermediate deamination of sarcosine in the course of its conversion to glycine.

Analogous to its inability to hydrolyze glycine anhydride the kidney is unable to hydrolyze sarcosine anhydride.

The negative result shown in Table II with glycolic acid and ammonia indicates that rat kidney is unable to form glycine from these two substances at a significant rate.

The experiments summarized in Table III revealed that rat kidney evidently is able to synthesize arginine from citrulline. The other possible amidine donators which were tested, guanidine, ornithine, and urea, were negative.

The positive result with citrulline was retested in a number of more adequately controlled experiments. A group of typical

TABLE III

Formation of Glycocyamine by Rat Kidney Slices from Glycine and Arginine or Other Possible Donators of Amidine Group

Glucose-Ringer's solution, 3 hours, 38°. Concentration of glycine 0.01 M; of arginine or other amidine donators 0.005 M.

Glycine	Amidine donator	Glycocyamine formed per 100 gm. fresh tissue
		mg.
−		35
−	*l*(+)-Arginine	68
+	"	498
+	*l*(+)-Citrulline + ammonia	330
+	Guanidine	16
+	*d*(−)-Ornithine + ammonia	12
+	Urea	33

TABLE IV

Formation of Glycocyamine from Glycine and Citrulline by Rat Kidney Slices and by Cell-Free Macerate of Rat Kidney

Slices in glucose-Ringer's solution, macerate in 0.1 M phosphate buffer, pH 7.0, 3 hours, 38°. Glycine 0.01 M; amidine donator (ammonia, arginine, citrulline, or ornithine) 0.005 M.

	Glycine	Amidine donator	Glycocyamine formed per 100 gm. fresh tissue
			mg.
Kidney slices	−		22
	+		31
	−	Ammonia	18
	−	Arginine	68
	−	Citrulline	58
	+	Arginine	357
	+	Citrulline	200
	+	" + ammonia	227
	+	Ornithine + "	8
Cell-free macerate	−		30
	+	Arginine	120
	+	Citrulline + ammonia	59

results is shown in Table IV. Citrulline was only slightly less[8] effective than citrulline plus ammonia.

Included in Table IV are some typical results obtained with a

cell-free macerate of kidney. This suspension was less active than an equivalent amount of kidney tissue in the form of slices but qualitatively the results were in every respect the same.

The suspension was made by the homogenizing procedure of Potter and Elvehjem (12). The kidney was stripped of its capsule, split down the longitudinal axis, the pelvic fat and the medulla cut away, the remainder homogenized with 4 times its weight of 0.01 M phosphate buffer at pH 7.0, and the resulting suspension passed through gauze. Such a suspension retains all its transamidinase activity for at least 2 months if kept in an ice box.

The optimum pH of glycine-transamidinase is in the neighborhood of pH 7.0 (Table V).

TABLE V

Effect of pH on Activity of Glycine-Transamidinase

38°, 4 hours. Glycine 0.01 M; arginine 0.005 M.

pH	Glycocyamine formed per 100 gm. fresh tissue
	mg.
6.0	214
6.5	259
7.0	320
7.5	278
8.0	246

The activity of glycine-transamidinase in cell-free solution is unaffected by 0.001 M potassium cyanide or by carrying out the reaction *in vacuo*. For example, one extract under the same conditions as those described above formed 67 mg. of glycocyamine per 100 gm. of fresh tissue; in the presence of 0.001 M potassium cyanide it formed 80 mg. and anaerobically, 72 mg.

The following amino acids and amides were tested with rat kidney slices and arginine as possible precursors of the glycine radical in glycocyamine. The initial concentration in every case was 0.005 M. They were all negative: *d*-alanine, *dl*-alanine, *l*-asparagine, *l*-aspartic acid, *l*-cysteine, *l*-cystine, *d*-glutamic acid, *d*-glutamine, *l*-hydroxyproline, *l*-histidine, *dl*-isoleucine, *l*-leucine, *d*-lysine, *dl*-methionine, *dl*-norleucine, *l*-proline, *dl*-phenylalanine, *dl*-serine, *d*-threonine, *l*-tryptophane, *l*-tyrosine,

and *d*-valine. These negative results indicate that under the conditions of these experiments none of these amino acids is a precursor of glycine.

Glycine-transamidinase activity was found in the kidney of every animal tested except the frog (Table VI). Whenever activity was found in kidney slices, it was also found in the cell-free extract. The beef and sheep kidneys were used at least 24 hours after the animals were slaughtered; they were obtained in a butcher shop. In the cases of all the other animals the kidneys were removed immediately after the animals were killed and the extracts made soon afterwards. The negative results with extracts of frog kidney call for further study.

TABLE VI

Formation of Glycocyamine from Glycine and Arginine by Kidney Slices and by Cell-Free Macerate of Kidney of Various Animals

Slices in glucose-Ringer's solution, macerate in 0.1 M phosphate buffer, pH 7.0. Glycine 0.01 M; arginine 0.005 M. 38°, 3 hours.

Animal	Glycocyamine formed per 100 gm. fresh tissue by	
	Kidney slices	Cell-free macerate
	mg.	*mg.*
Beef		190
Cat	93	32
Dog	281	480
Frog		0
Guinea pig	38	14
Pigeon	27	16
Rabbit	187	160
Rat	357	120
Sheep		160

Liver slices and cell-free extracts of heart and of muscle of all the animals listed in Table VI were tested for glycine-transamidinase activity. Except in the case of the pigeon (see below) they were all negative. The blood, brain, intestine, and spleen of the rat were also examined; they were negative. Rat liver slices also gave negative results with glycine plus arginine, urea, or guanidine.

Before the advent of Weber's method no glycocyamine could be demonstrated in animal tissues. With this method glyco-

cyamine was detected in urine (4), in intestine, testes, and kidney (13).

Using our more sensitive method, we found glycocyamine to be widely distributed in the tissues of the rat. The concentrations (mg. per 100 gm. of fresh tissue) were blood 0.5 to 1; brain, heart, liver, skeletal muscle, and spleen 3 to 6; small intestine 10; kidney (cortex) 15 to 30.

The question arose whether transamidination in the kidney can be sufficiently rapid under physiological conditions to account for the total production of creatine in the body as indicated by the

TABLE VII

Rate of Glycocyamine Formation by Rat Kidney Slices at 38° from Arginine Initially 0.005 M and Glycine 0.01 M

Time	Glycocyamine per 100 gm. fresh tissue		
	Found	Formed	Average rate of formation per hr.
hrs.	*mg.*	*mg.*	*mg.*
0	32		
0.5	95	63	126
1.0	164	132	132
2.0	315	283	142
4.0	505	473	118
6.0	741	709	118

daily excretion in the urine of creatine and creatinine. A number of experiments were carried out to obtain what information we could on this point.

Table VII shows that the glycine-transamidinase in rat kidney slices remained practically unimpaired for 6 hours. This is in accord with the stability of the enzyme in cell-free extracts.

In Table VIII are some figures on the effect of the concentration of the reactants, arginine and glycine, on the rate of transamidination. With equal arginine and glycine concentrations from 0.001 to 0.0001 M the rate was approximately linear with respect to concentration.

We can estimate what the rate of glycocyamine formation in the kidney must be to make good the loss of tissue creatine which appears in the urine as creatinine. Two human kidneys

weigh approximately 300 gm. An average figure for the creatinine excreted in the urine in 24 hours is 1.7 gm. To make good this loss the average hourly production of glycocyamine which is necessary must be approximately 25 mg. per 100 gm. of tissue per hour.

This rate of glycocyamine formation would have been obtained in the experiments of Table VIII with concentrations of glycine and arginine between 0.0005 and 0.001 M. This is probably the concentration range of these amino acids in kidney. The basis of this estimate is as follows: The arginine concentration in dog

TABLE VIII

Variation in Rate of Glycocyamine Formation by Rat Kidney Slices with Different Concentrations of Arginine and Glycine

Ringer's solution, 4 hours, 38°.

Initial concentration		Glycocyamine formed per 100 gm. fresh tissue	Initial concentration		Glycocyamine formed per 100 gm. fresh tissue	Initial concentration		Glycocyamine formed per 100 gm. fresh tissue
Arginine	Glycine		Arginine	Glycine		Arginine	Glycine	
mole per l.	*mole per l.*	*mg.*	*mole per l.*	*mole per l.*	*mg.*	*mole per l.*	*mole per l.*	*mg.*
0.02	0.01	190	0.005	0.02	617	0.005	0.005	360
0.01	0.01	590	0.005	0.01	533	0.0025	0.0025	310
0.005	0.01	533	0.005	0.005	361	0.001	0.001	134
0.0025	0.01	535	0.005	0.0025	252	0.0005	0.0005	78
0.00125	0.01	303	0.005	0.00125	201	0.0001	0.0001	20
0.000625	0.01	201	0.005	0.000625	103			

blood is between 2.6 and 3.9 mg. per cent (14); we have found 2.0 to 5.0 mg. per cent in human blood. This range corresponds to 0.0001 to 0.00025 M. The concentrations of these amino acids in the kidney are certainly much higher than in blood, since the total free amino nitrogen in kidney and other tissues is 10 times or more that in blood (15, 16). The rates of glycocyamine formation recorded in Table VIII are sufficient therefore to make good the total loss of creatine from the tissues. This estimate is, of course, based on the assumption that the rate of glycocyamine formation in human kidney *in vivo* is of the same order as in rat kidney slices *in vitro*, which seems not unreasonable.

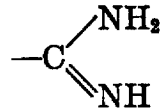
DISCUSSION

The experimental results given above, confirmed and complemented as they now are by the findings in studies with tracers, make a strong case for the following mechanism of creatine formation in animals: (*a*) arginine and glycine in the kidney form glycocyamine; (*b*) in the liver glycocyamine and methionine form creatine. The mechanisms for these reactions are widely distributed in mammals. The quantitative aspects of the data show that the transamidination mechanism for the formation of glycocyamine is fast enough to replace all the creatine lost from the body. Other mechanisms of creatine formation are not excluded; but the tracer studies indicate that the arginine-glycine-methionine mechanism is quantitatively the most important one *in vivo*.

The argument *contra* glycocyamine, that it had never been found as a constituent of animal tissues, antedated the development of suitable analytical methods. This argument is now completely disposed of by the detection of glycocyamine in practically all tissues.

The controversy whether arginine is a precursor of creatine (17, 18) may be considered as settled in favor of the affirmative. The path of its conversion to creatine, however, is different from what was generally believed. It was proposed that arginine was converted to glycocyamine by way of deamination and β oxidation; thus all of the nitrogen was derived directly from arginine. This made it necessary to explain away the evidence which suggested that glycine played some part in creatine formation, unless it be in the methylation of glycocyamine.

Bergmann and Zervas were the exception. They observed that "triacetyl anhydro arginine" and glycine ethyl ester reacted in the absence of water to give a fairly good yield of diacetyl glycocyamine ethyl ester (19); and the same arginine derivative with sarcosine ethyl ester gave diacetyl creatine ethyl ester (20). Their view was that, "der wesentliche Punkt der biologischen Kreatinbildung in einer direkten Umsetzung eines reaktionslustigen Argininabkömmlings mit einem Aminoäthanderivat beruht." This clear statement that the guanidine group of creatine arises *in vivo* by transamidination needs now to be modified in two respects: that an enzyme, glycine-transamidinase, and not a

reactive derivative or split-product renders the  group in arginine labile as it is in "triacetyl anhydro arginine." The enzyme arginase exerts a similar influence. The other modification is that glycine itself and not a derivative is the acceptor of the amidine group in the biological transamidination reaction.

We may now infer that one of the reasons for the essential character of arginine and methionine in the rat is that they participate in the formation of creatine. These amino acids, of course, also serve other functions, for example the rôle of arginine in urea formation and of methionine in other methylation reactions (3), in addition to their participation in the constitution of tissue protein.

The recent findings on essential amino acids for the chick indicate that arginine and glycine are required for creatine formation in this animal. Arnold *et al.* (21) reported that arginine is essential for rapid growth. This was confirmed by Klose *et al.* (22) who showed further that arginine is necessary for maintenance as well as growth. Recently Klose and Almquist reported that citrulline is as effective as arginine, whereas ornithine alone or with urea is ineffective (23). We have found (Tables III and IV) that glycocyamine is formed from citrulline and glycine, the citrulline presumably being first converted to arginine, while ornithine, with or without added ammonia, is completely negative.

The parallel between the amino acid requirements for creatine formation and for growth was extended further when Almquist *et al.* found that glycine is essential for the growth of the chick (24) and that creatine as a substitute for glycine is even more effective than glycine itself. Glycolic acid and betaine could not replace creatine (25).

We have examined the organs of the pigeon for transamidinase activity. Activity was found not only in the kidney but also in heart, liver, and skeletal muscle. The limiting amino acid appeared to be glycine; *i.e.*, nearly as much glycocyamine was formed when glycine alone was added as from glycine and arginine together. The differences between experimental and control were, however, small in absolute terms compared with those found in the kidneys of other animals. Although these

differences were greater than could be ascribed to analytical or sampling variations, we cannot consider our findings in the pigeon as established until more determinations have been made. For this reason the detailed figures are not presented here. We hope that other workers with facilities for a study such as this on birds may undertake the investigation. Our laboratory does not at present possess such facilities.

SUMMARY

1. Beef, cat, dog, guinea pig, pigeon, rabbit, rat, and sheep kidney form glycocyamine from arginine and glycine. This reaction is catalyzed by cell-free extracts of kidney as well as by surviving kidney slices.

2. It is proposed that this reaction be designated "transamidination," and the enzyme "glycine-transamidinase." The optimum pH of this enzyme is about 7.0. It is not affected by potassium cyanide nor by anaerobiosis.

3. Transamidination does not occur in the liver, heart, or skeletal muscle of the animals mentioned above; the blood, brain, and spleen of the rat were tested also and found inactive. It is possible that in the pigeon a low glycine-transamidinase activity resides in liver, heart, and skeletal muscle as well as in kidney.

4. Glycocyamine is also formed in the kidney from glycine and citrulline. Glycine plus ornithine (with or without ammonia), urea, or guanidine is negative in this respect.

5. A large number of amino acids, several amides, and anhydrides were tested as possible precursors of the glycine radical of glycocyamine. They were all negative, as was also glycolic acid plus ammonia.

6. Glycocyamine is formed from arginine and sarcosine. Evidence is presented that the sarcosine is first demethylated, thus being converted to glycine, indicating the presence of a demethylating enzyme in kidney. Sarcosine anhydride is negative.

7. The above findings, complemented by the tracer studies in the laboratories of Schoenheimer and of du Vigneaud, and in conjunction with our previous findings, prove the existence of the following mechanism of creatine formation in animals: arginine and glycine form glycocyamine in the kidney; the glycocyamine is methylated in the liver by methionine (or a derivative of methionine) to form creatine.

8. Quantitative aspects of the data indicate that all of the creatine formed in animals may normally be formed by this mechanism.

9. Evidence of the generality of transamidination is seen in the close parallel between the above findings and those on amino acids essential for the growth of the chick.

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Betaine and Glycocyamine in the Treatment of Disability Resulting from Acute Anterior Poliomyelitis

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THIS report describes our experience with betaine and glycocyamine in the treatment of patients with paralysis and paresis resulting from acute anterior poliomyelitis. Discussion of the physiologic and biochemical properties of betaine and glycocyamine in part and their application to the treatment of heart diseases, including cardiac decompensation, was presented in earlier papers.[1-4] The following represents a similar logical extension into the field of therapeutics. This clinical investigation is based on two general ideas: (1) the recovery of a cell with impaired function may require more of certain substances than is necessary for cell maintenance alone, analagous to general experience with vitamin therapy, and (2) a larger reservoir of immediately available energy should be helpful to cells under conditions of stress or those burdened with extra function.

DL-Methionine—Glycocyamine Combination Disappointing

The first experiment was with glycocyamine in combination with DL-methionine. One of us (M.E.B.) found that large doses of DL-methionine were tolerated poorly—anorexia, nausea, vomiting, oliguria and hyperproteinemia frequently accompanied its use—and results obtained were discouraging. The use of DL-methionine was abandoned when betaine hydrate was made available.§

Betaine and Glycocyamine Promising

Our first experience with betaine and glycocyamine therapy occurred in December 1949 and included patients suffering from the general debility of old age, asthenia, parcsis and cardiac failure of varying degrees and varied etiology. Betaine and glycocyamine were given in addition to the usual forms of treatment. Results obtained were so encouraging as to warrant investigation of the effects of these two substances alone.

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Dosage of Betaine and Glycocyamine

As little as three milligrams of glycocyamine per pound of body weight per day, in divided doses, was given at first. As experience and confidence increased, larger doses of glycocyamine covered with betaine were administered. Optimum dosage is probably 30 mg. per pound of body weight per day.

When glycocyamine and betaine were fed in stoichiometric proportions, waste resulted in that the amount of extra creatine formed was relatively small, and most of the glycocyamine was excreted unchanged. However, the percentage of glycocyamine converted to creatine rose progressively, as the ratio of betaine to glycocyamine was increased. When the betaine-glycocyamine ratio administered was 3:1, approximately 30 per cent of the glycocyamine was converted to creatine. When a 5:1 ratio was used, 55 per cent of the glycocyamine was methylated, and with a 7:1 proportion optimum methylation occurred. However, nausea and regurgitation happened too frequently with this last proportion to permit its use. Clinically, we have found that a ratio of five or six parts of betaine to one part of glycocyamine is well tolerated and moderately efficient.

Role of Phosphocreatine

Phosphocreatine is the main reservoir of immediately available energy. Perhaps, then, the presentation to a damaged muscle or nerve of more creatine than is normally synthesized would facilitate its recovery? The thought was that if extra creatine were provided, some of the increment might be retained in the tissues and more phosphocreatine be made available to muscles and nerve.

Muscular involvement subsequent to disease of the nervous system results in excessive creatine excretion and a lowered preformed creatinine coefficient proportional to the degree of muscle wasting and the loss of functional capacity.[5-8]

The preformed creatine coefficient is low during the acute phases and the early convalescence of poliomyelitis and slowly rises with clinical improvement.[9] This finding is clinically significant, as the urinary creatine bears a direct relationship

to the body creatine—the 24 hours' excretion represents two per cent of the body stores.[10,11] and points to the value of creatine in clinical recovery.

These observations are consistent with the hypothesis that larger amounts of creatine might be beneficial in conditions of muscular weakness or degeneration, *providing the creatine could be "fixed" in the tissues as phosphocreatine.* Experimental evidence in support of this possibility is present in the findings of Benedict and Osterberg,[12] and Chanutin.[13]

Creatine feeding is unphysiologic. It interferes with the transmethylation of endogenous glycocyamine[14] and has been disappointing clinically. Amino acid therapy, which results in the formation of additional creatine, has been more encouraging. An excerpt from a paper by Tripoli and Beard[15] is pertinent:

> The results of our own work and that of many investigators show that the patient who is suffering from certain types of myopathies cannot "fix" or retain creatine in muscles. This valuable substance is continually lost in the urine and is accompanied by a gradual loss of muscle tissue and function. If creatine is given as such, most of it passes through the body and is excreted in the urine. Hence feeding creatine alone does not increase the creatine content of the muscles and no clinical improvement in the patient's condition takes place.
>
> On the other hand, amino acids form creatine which is first lost in fairly large amounts. After a few weeks it is retained in the muscles and at this time the patient begins to show clinical improvement.
>
> It would seem therefore that the amino acids supply a deficiency in muscle metabolism. This creatine, retained as phosphocreatine, serves over and over again to supply the energy for muscular contraction, and muscular efficiency improves at a remarkable rate. That the amino acids play a definite part in muscle metabolism is supported by the fact that as long as they are fed to the patient, he improves, yet the improvement may cease as soon as the therapy is discontinued, and the patient may revert to his original condition.

The implications of these statements are clear. Certain amino acids must first be incorporated in tissue proteins before creatine can be "fixed."

Betaine and glycocyamine therapy differs materially from creatine feedings; it not only supplies biological precursors of creatine but also makes available for incorporation into tissue proteins the amino acid dimethylglycine,[16] thus providing a "building stone" for additional protein for the storage of phosphocreatine and for repair. Herein lies the crux of this form of therapy.

Dimethylglycine is derived from betaine after it has donated one of its methyl groups to homocysteine for the conversion of the latter to methionine.[17–20] Methionine is the immediate methyl donor for the methylation of glycocyamine to creatine.[21] Betaine also replenishes the body stores of labile methyl groups[22–25] and is nontoxic.

Motor Units and Motion

Motion in the animal body is produced by the activities of a combination of motor units, with two or more muscles usually sharing in its production. A motor unit is a functional unit of the nervous system and is composed of one anterior horn cell, one axone and its dependent group of 100 to 150 muscle fibers.[26]

As a consequence of poliomyelitis and allied neuropathies, a variable part or all of the motor nerve cells supplying a given muscle or muscle group may be injured or destroyed. The muscle fibers with functionally intact innervation remain the only active components and are responsible for the residual function.

The performance of increasing amounts of work by a paretic muscle requires each surviving motor unit to do more work. Hence a large reservoir of immediately available energy should result in extra power and delay onset of fatigue. Likewise, the presence of an amino acid which can be incorporated in tissue proteins should be of value in processes of regeneration and repair.

Experimental Design

Suitable data were obtained on 31 patients who had had poliomyelitis 18 or more months previously and whose recovery was followed by a constant degree of paresis. After initial evaluation was made, all other therapy was withdrawn. No therapy other than the administration of betaine and glycocyamine was permitted during the experimental period in order that all improvement noted could be attributed unequivocally to these compounds.

In every instance the patients received approximately 30 milligrams of glycocyamine and 150 milligrams of betaine per pound of body weight daily, in four or more equal doses. All were office patients and were seen at weekly intervals.

Evaluation was based on objective and subjective findings. Laboratory studies at regular intervals included examinations of blood and urine and determinations of urinary creatine and creatinine. Electromyography was done in some instances. The Lovett system of rating muscle power was used, and rating was always done by the same person. A careful evaluation of the functional capacity to execute movements, and the efficiency with which useful movement was accomplished was noted in each case. The patients have been observed for three to eleven months.

Results

In 31 patients with a constant degree of paresis and paralysis resulting from acute anterior poliomyelitis 403 muscles were studied. At least 18 months had elapsed since infection. Two hundred

Table I. Clinical Muscle Evaluation Effect of Betaine-Glycocyamine Therapy on Muscles with Some Residual Function

After Initiation of Betaine-Glycocyamine Therapy	Number of Muscles Exhibiting Increased Functional Ability (Number)	(Per Cent)
Two weeks	79	36
One month	203	74
Two months	260	94.6
Four months	262	98
Eight months	268	98 plus

and seventy-five muscles, or 68.3 per cent, were found to have some residual function. In 128, or 31.7 per cent, no residual function was discernible at the time this study was begun. At no time did the latter group exhibit any evidence of improvement during the course of study.

That the effect of betaine-glycocyamine administration on muscles with some residual function at the start of treatment was quite different is shown in Tables I and II.

Urinary Creatine and Creatinine

Urinary creatine and creatinine were determined from 72-hour samples obtained weekly at the beginning of the period studied, and at fortnightly intervals thereafter. The average daily value in grams at monthly intervals is given for patients who failed to demonstrate clinical improvement, patients who showed clinical improvement, and patients who gained a significant increase in muscle mass during this study. See Tables III, IV, and V.

A large increment in total urinary creatine occurred in every case. Although no significant rise in creatinine excretion occurred in any group in the first month of treatment, an appreciable rise of approximately 20 per cent occurred in the group showing clinical improvement during the first eight months of therapy. Patients with a significant increase in muscle mass showed a striking increment of approximately 40 per cent during the same period.

Electromyography

Repeated electromyographic examinations were done on a group of 125 paralyzed and paretic "polio" muscles over a period of nine months; the monopolar needle electrode technic was used with cathode-ray oscillograph recording. Ten areas of several regions (proximal, middle and distal) were tested in each muscle during each examination, and electromyograms were obtained, in order to measure the wave form, repetition frequency, and magnitude of the motor unit and denervation fibrillation voltages. In addition, an integrating meter was employed, which gave a relative power reading expressed in terms of effective microvolts. However, the relative power reading for each region represents the average value for the ten areas sampled in that particular region, and an increase of 50 microvolts or more is regarded as a significant microvolt increase. Although the patients were examined frequently enough to enable the examiner to place the needle electrode in the same region of the muscle each time, exactly the same areas in each region may not have been sampled at each examination.

Of 114 regions in 38 paralyzed muscles having nothing but denervation fibrillation prior to the start of betaine and glycocyamine therapy, not one showed any motor unit activity—even after nine months of treatment. The relative power reading, expressed in effective microvolts, remained exactly the same after betaine and glycocyamine therapy as it did prior to instituting the therapy. It is of some interest that no significant decrease occurred in the number of fibrillating areas during the study.

Of 138 regions in 46 paretic muscles having both denervation fibrillation and motor unit activity prior to the start of betaine and glycocyamine therapy, 90, or 65 per cent, showed 50 microvolt increase or greater after two weeks of

Table II

Degree and Rate of Improvement of 275 Paretic Muscles

Duration of Therapy	Number of Muscles: No Apparent Improvement		Less Than One Lovett Grade		Improvement More Than One, But Less Than 1½ Lovett Grades		Improvement Greater Than 1½ Lovett Grades	
	Number	Per Cent	Number	Per Cent	Number	Per Cent	Number	Per Cent
One month	72	26.0	151	55.0	46	16.5	6	2.5
Two months	15	5.4	95	34.5	137	49.9	28	10.2
Four months	8	2.9	49	18.1	143	52.0	75	27.6
Eight months	7	2.8	36	13.1	131	47.6	101	44.0

Table III
Five Patients* Who Failed to Improve Clinically
(Twenty-Four Hour Average of a Seventy-Two Hour Sample)

Duration of Therapy	Grams of Creatine	Grams of Creatinine
Initial	0.189	0.879
One month	3.953	0.866
Two months	2.912	0.760
Four months	2.371	0.837
Six months	3.235	0.792
Eight months	3.381	0.901

*Four adults and one child.

treatment. During the next several weeks a significant microvolt increase was demonstrated by an even larger percentage of muscle regions and, by the end of the third month, 138, or 100 per cent, of the regions showed a significant microvolt increase. At this time, average increase for the 138 regions was 104 microvolts. During the next three months, this average value steadily increased. By the end of the sixth month, average increase for the 138 regions was 126 microvolts. Again, no significant decrease in the number of areas exhibiting denervation fibrillation was noted during this time interval.

Motor unit activity but no denervation fibrillation had been noted in 123 regions of 41 paretic muscles prior to the institution of betaine and glycocyamine therapy; 60 regions, or 48.7 per cent, showed at least a 50 microvolt increase at the end of the second week. In the next several weeks a steady increase was noted in the percentage of muscle regions showing a significant microvolt increase. However, at the end of the third month only 100, or 81.3 per cent of the regions showed a significant increase. The average gain for all regions was 82 microvolts at this time. Not until the end of the sixth month did all regions show a gain of at least 50 microvolts, an average increase for all regions of 97 microvolts. It is noteworthy that no significant number of

Table IV
Twenty-Six Patients* Who Improved Clinically
(Twenty-Four Hour Average of a Seventy-Two Hour Sample)

Duration of Therapy	Grams of Creatine	Grams of Creatinine
Initial	0.249	0.935
One month	1.823	1.010
Two months	1.476	1.323
Four months	1.762	1.201
Six months	1.581	1.251
Eight months	1.629	1.262

*Seven adults and 19 children.

Table V
Eleven Patients With a Significant Increase in Muscle Mass
(Twenty-Four Hour Average of a Seventy-Two Hour Sample)

Duration of Therapy	Grams of Creatine	Grams of Creatinine
Initial	0.416	0.910
One month	1.315	1.008
Two months	1.834	1.139
Four months	1.402	1.241
Six months	1.175	1.338
Eight months	1.916	1.395

regions in the muscles showed denervation fibrillation during this course of treatment.

Most of the data described here will be discussed in more detail in a later paper, but we should like to discuss several observations at this time. As mentioned previously, the number of regions having denervation fibrillation in one group of paralyzed and in one group of paretic "polio" muscles did not diminish during the course of the experiment. Theoretically, it is possible that results would have been the same without betaine-glycocyamine therapy, but these data suggest that such therapy encourages denervation fibrillation in denervated muscles and consequently assists in maintaining denervated muscle as contractile tissue during the denervation period.

The relative electromyogram reading expressed in microvolts is a direct function of not only the repetition frequency and magnitudes of individual motor units, but also the number of motor units being activated by voluntary effort. In this experiment initial significant microvolt increases were generally caused by the development of a more rapid and regular repetition frequency in individual motor units. In other words, individual motor units were promptly brought under better voluntary control. The continued microvolt increases in a given region appeared to result from the development of greater magnitudes by these same motor units, rather than from a significant increase in the number of motor units in a given region.

Summary

1. Thirty-one patients with paralysis and a constant degree of paresis resulting from acute anterior poliomyelitis were treated with glycocyamine and betaine. Twenty-six patients showed objective evidence of improvement, which was frequently of a useful degree; five patients showed no evidence of muscle improvement.

2. All patients demonstrated an improved sense of well-being; less fatigue and greater general strength and endurance were apparent early in

the course of treatment. These effects have persisted and were apparently independent of any objective improvement in the paretic muscles. Most patients gained weight.

3. Whenever motor unit activity or residual power remained in a muscle or muscle group, a substantial increment of motor unit activity and muscle power occurred with betaine-glycocyamine therapy.

4. Conversely, in muscles exhibiting nothing but denervation fibrillation or where no residual function or motor unit activity existed, at no time during the course of therapy did any increase in activity or muscle power occur.

5. Of the 275 muscles with residual function, 268 clinically and all electromyographically improved.

6. The majority of muscles responding to betaine-glycocyamine therapy did not present clinical evidence of improvement during the first three weeks of therapy, although most of these showed electromyographic evidence of improvement during this period. Following this initial period a significant increment in power occurred during the next two months. This clinical improvement could be predicted by the earlier substantial increase in motor unit activity as demonstrated electromyographically. During the subsequent period the rate of improvemente was much less, but continuous, and closely paralleled the increasing bulk of the paretic muscles.

7. A significant rise in preformed creatinine coefficient occurred whenever the muscle bulk increase was substantial.

8. The rate of improvement can be described by an exponential curve.

9. Increase in functional ability of normal and paretic muscles in the region of completely paralyzed muscles or parts of muscles frequently resulted in increased functional capacity of the part.

10. No evidence of toxicity was observed.

Conclusion

A means of increasing muscle power in paretic "polio" muscles of patients is presented, which is of value in the rehabilitation of the patient.

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Effects of folic acid and combinations of folic acid and vitamin B-12 on plasma homocysteine concentrations in healthy, young women[1,2]

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ABSTRACT

Background: Elevated plasma homocysteine concentrations are considered to be a risk factor for vascular disease and fetal malformations such as neural tube defects. Recent studies have shown that plasma homocysteine can be lowered by folic acid in amounts corresponding to 1–2 times the recommended dietary allowance. Preliminary evidence indicates that vitamin B-12 may be beneficial when included in supplements or in a food-fortification regimen together with folic acid.

Objective: We aimed to compare the homocysteine-lowering potential of a folic acid supplement with that of 2 supplements containing different doses of vitamin B-12 in addition to folic acid.

Design: Female volunteers of childbearing age (n = 150) received a placebo for 4 wk followed by a 4-wk treatment with either 400 μg folic acid, 400 μg folic acid + 6 μg vitamin B-12, or 400 μg folic acid + 400 μg vitamin B-12.

Results: Significant reductions ($P < 0.001$) in plasma homocysteine were observed in all groups receiving vitamin treatment. The effect observed with the combination of folic acid + 400 μg vitamin B-12 (total homocysteine, −18%) was significantly larger than that with a supplement containing folic acid alone (total homocysteine, −11%) ($P < 0.05$). Folic acid in combination with a low vitamin B-12 dose (6 μg) affected homocysteine as well (−15%).

Conclusions: These results suggest that the addition of vitamin B-12 to folic acid supplements or enriched foods maximizes the reduction of homocysteine and may thus increase the benefits of the proposed measures in the prevention of vascular disease and neural tube defects. *Am J Clin Nutr* 1998;68:1104–10.

KEY WORDS Folic acid, vitamin B-12, supplementation, homocysteine, neural tube defect, cardiovascular disease, women

INTRODUCTION

Homocysteine is being scrutinized as independent risk factor for coronary, cerebral, and peripheral vascular diseases. Most case-control studies and several, though not all, prospective studies have confirmed such an association over a wide range of plasma total homocysteine (tHcy) concentrations (1–4).

In the absence of vitamin B-6 or vitamin B-12 deficiency or genetic defects in non-folate-dependent enzymes, folic acid intervention lowers plasma tHcy concentrations. This has been observed even when presupplementation plasma folate concentrations were well within the range of values currently accepted as reflecting adequate status (5, 6). In several studies, daily folic acid administration in high (pharmacologic) doses of 0.5 (7) to 10 mg (8) resulted in significant reductions in plasma tHcy. However, for both sexes, additional folic acid intakes of 200–400 μg/d, corresponding to 1–2 times the recommended dietary allowance of 400 μg dietary folate equivalents (9), seem to be sufficient to lower plasma tHcy concentrations (5, 6, 10). Indirect evidence for the protective effect of low plasma tHcy concentrations comes from a recent prospective study linking high intakes of folate to a considerably diminished risk for coronary artery disease in 80082 US nurses (11). Besides an involvement in the pathogenesis of vascular disease, maternal tHcy concentrations may further play a role in the etiology of fetal malformations such as neural tube defects (NTDs) (12–14).

As of January 1, 1998, the US Food and Drug Administration ruled that the fortification of grain and grain products with folic acid be mandatory to increase folic acid intakes and contribute to the prevention of NTDs (15). However, it has been suggested that vitamin B-12 be added to foods as well or that supplements be offered containing both folic acid and vitamin B-12 (12, 16, 17). The rationale for this proposition is that the sole addition of folic acid may mask pernicious anemia resulting from vitamin B-12 deficiency, which may slowly lead to irreversible nerve damage. Further support for this proposition is that both folic acid and vitamin B-12 are cofactors of methionine synthase, the enzyme catalyzing the formation of methionine from homocysteine. A defect in this enzyme, also resulting in elevated tHcy concentrations, was proposed to be the cause for some (although not all) NTDs.

The present study aimed to determine whether the addition of vitamin B-12 to a folic acid supplementation regimen recommended for women capable of becoming pregnant (9) potentiated the tHcy-lowering capacity of this regimen. Two different

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doses of vitamin B-12 were chosen to explore whether there was a dose-response relation with increasing vitamin B-12 dose. The low dose was approximately double the recommended dietary allowance of 2.4 μg/d (9) and reflects an amount frequently contained in vitamin supplements for adults (18). The high pharmacologic dose of vitamin B-12 takes into account the greatly reduced absorption rate of vitamin B-12 from high doses after saturation of the active absorption pathway (19). Women of childbearing age were chosen as the target population group because they may especially benefit from tHcy reduction.

SUBJECTS AND METHODS

The study was approved by the Ethical Committee of the University Hospital of Bonn. After a washout phase of 4 wk, 156 female participants aged 20–34 y received a placebo daily during the first 4 wk of the study. For the next 4 wk, the volunteers were randomly assigned to one of the following treatment groups: group A, 400 μg folic acid/d; group B, 400 μg folic acid + 6 μg vitamin B-12/d; and group C, 400 μg folic acid + 400 μg vitamin B-12/d. The vitamin capsules were specially prepared by Allpack (Schorndorf, Germany) with synthetic folic acid (pteroylmonoglutamic acid) from Takeda (Osaka, Japan) and vitamin B-12 (cyanocobalamin) from Merck (Darmstadt, Germany). Placebo and vitamin capsules were identical in appearance as the result of a colored gelatin cover so that the participants were not aware of the identity of the capsules. Capsules were provided in excess and participants were asked to return the remaining capsules after each 4-wk period to enable pill counting as a measure of compliance.

At the beginning of the washout phase, all subjects were instructed to continue their usual dietary habits for the duration of the study but to refrain from intake of other vitamin supplements or foods enriched with vitamins. Six participants opted to withdraw during the study; the 150 participants completing the study were included in the statistical analysis.

After subjects had fasted overnight, blood was drawn from each participant at the start of the study (week 0), after the placebo period (week 4), and after the treatment period (week 8). Blood was immediately cooled on ice and centrifuged within 15 min at 2000 × *g* and 4°C for 10 min. Plasma was stored at −20°C until analyzed.

Laboratory investigations

All samples for each participant were analyzed within one run to minimize measurement errors. EDTA-treated plasma was analyzed for tHcy by HPLC with fluorescence detection according to the method described by Araki and Sako (20) and Vester and Rasmussen (21) with minor modifications. The CVs for this assay were as follows: within-assay variation <5.6% and between-assay variation <5.7%. Folate and vitamin B-12 were measured in heparin-treated plasma with commercially available chemiluminescence kits (Chiron Diagnostics, Fernwald, Germany; within-assay CV <4.6% and <6.5%, respectively, and between-assay CV <13.0% and <9.1%, respectively). For the measurement of red blood cell (RBC) folate, the same chemiluminescence kit as for plasma folate was used (within-assay CV <10.7%, between-assay CV <13.1%). Hemolysis for this assay was achieved by incubating whole blood with 0.2% ascorbic acid at room temperature for 90 min before freezing the mixture according to the directions of the manufacturer of the kit. The assays for tHcy, folate, and vitamin B-12 were validated externally through participation in national and international interlaboratory comparisons (for tHcy: European External Quality Assurance Scheme for homocysteine in serum; for the vitamins: ringtest of the German Society for Clinical Chemistry). Vitamin B-6 was measured as pyridoxal-*P* (PLP) by HPLC (within-assay CV <2.3%, between-assay CV <5.9%) (22). All samples were analyzed in duplicate.

Statistical analysis

Because of positively skewed distributions, the natural logarithms of tHcy, folate, RBC folate, vitamin B-12, and PLP were used in all analyses as continuous variables. Therefore, besides presentation of arithmetic means, geometric means for these variables are given. The treatment groups were compared with respect to body mass index (BMI), plasma tHcy, folate, RBC folate, vitamin B-12, and PLP by means of parametric models [paired *t* test for within-subject comparisons and analysis of variance (ANOVA) for between-subject comparisons]. The primary analysis variable was the change in a plasma index after 4 wk of vitamin treatment. This change was expressed as the ratio of the concentration at week 8 to that at week 4, and a one-way ANOVA model was fitted to the ln-transformed ratio including a treatment effect. To account for the influence of tHcy and vitamin concentrations before treatment on the change in tHcy, these parameters were also included in the ANOVA as covariates. Post hoc tests used the Scheffe test. The age of the groups was compared by using a Kruskal-Wallis one-way ANOVA because a skewed distribution of the data remained after logarithmic transformation. Differences in proportions between the groups were tested by using a paired chi-square test. Correlation analysis used logarithmically transformed variables for calculation of Pearson's correlation coefficients. Differences were considered significant at $P < 0.05$; all P values are two-tailed. Data analyses were performed with the statistical program SPSS (version 6.1.3; SPSS Inc, Chicago).

RESULTS

The demographic characteristics of the study participants are summarized in **Table 1**. No significant differences were observed among groups with respect to age, BMI, use of oral contraceptives, or prevalence of smoking. Previous use of B vitamin supplements before the washout phase, which included regular intake of supplements containing vitamin B-6, vitamin B-12, or folic acid, was also not significantly different among groups. An estimation of compliance with intake of the capsules was possible for 146 of 150 participants (97.3%). Good compliance, defined as intake of ≥6 capsules/wk, was noted for all groups, ranging from 98.0% in group A to 100% in groups B and C.

Total homocysteine

At week 0 participants were normohomocysteinemic, with concentrations ranging from 3.5 to 14.3 μmol/L; the geometric mean value of all groups combined was 7.6 μmol/L. tHcy concentrations in plasma correlated inversely with concentrations of folate and vitamin B-12 in plasma ($r = -0.2828$, $P < 0.001$, and $r = -0.3774$, $P < 0.001$, respectively), but not with vitamin B-6 (PLP) ($r = -0.0771$, $P = 0.3$). The association with RBC folate (computed from measurements at week 4) was weaker ($r = -0.1752$, $P = 0.03$) than that observed with plasma folate at week 0.

TABLE 1
Demographic characteristics of the study participants[1]

Group	Group A ($n = 51$)	Group B ($n = 49$)	Group C ($n = 50$)
Age (y)	24.9 ± 3.5	24.0 ± 1.9	23.5 ± 2.1
BMI (kg/m^2)	20.9 ± 1.8	21.4 ± 2.2	21.3 ± 2.5
Use of OC (%)	58.8	55.1	54.0
Smoking (%)	9.8	14.3	22.0
Previous B vitamin supplementation (%)	25.5	22.4	18.0

[1] $\bar{x}$ ± SD or proportions. Group A received 400 μg folic acid/d, group B received 400 μg folic acid + 6 μg vitamin B-12/d, and group C received 400 μg folic acid + 400 μg vitamin B-12/d. The 3 groups did not differ significantly with respect to age ($P > 0.05$, Kruskal-Wallis ANOVA), BMI ($P > 0.05$, ANOVA), or prevalence of use of oral contraceptives (OC), smoking, and previous B vitamin supplementation ($P > 0.05$, chi-square test).

After 4 wk of placebo treatment, the geometric mean tHcy concentration of all study subjects increased slightly but significantly by a mean value of 0.28 μmol/L ($P < 0.01$). A subgroup analysis showed that this was true for groups A and B, although no significant change in the mean tHcy concentration occurred in group C during placebo treatment (**Table 2**). The change in tHcy was not significantly different in subjects reporting regular intake of B vitamins from that in those not regularly taking supplements before the washout phase. Despite these fluctuations, tHcy concentrations at the start of the vitamin treatment period did not differ significantly among groups.

After vitamin treatment for 4 wk, the mean tHcy concentration was reduced significantly in all groups ($P < 0.001$). The decrease in tHcy varied according to the treatment regimen: the most pronounced tHcy reduction was observed in group C (−18% compared with the tHcy concentration at week 4, corresponding to a geometric mean ratio of 0.82; Table 2). In group B, the tHcy concentration at week 8 was 15% lower than that at week 4. When folic acid was given alone (group A), the reduction in the plasma tHcy concentration was 11%. The difference in tHcy-lowering effect between group A and group C was significant ($P < 0.05$, ANOVA with Scheffe post hoc test).

The individual changes in tHcy concentration after 4 wk of vitamin treatment are shown in **Figure 1**. The change in tHcy was dependent on the tHcy concentration before vitamin supplementation (week 4); subjects with a high initial tHcy concentration responded to treatment with larger reductions in tHcy than those in subjects with initially low tHcy concentrations. Above a tHcy concentration of 8 μmol/L, each subject responded to vitamin supplementation with a decrease in tHcy after 4 wk. The extent of tHcy reduction was more pronounced when only the subjects with a tHcy concentration >8 μmol/L before treatment were considered (group A:−16%; group B:−20%; group C:−22%), even though the additional effect of vitamin B-12 on tHcy reduction was smaller ($P = 0.08$, ANOVA). Below a tHcy concentration of 8 μmol/L, the extent of tHcy reduction in group C (−14%) was significantly larger than in group A (−5%) ($P < 0.05$, ANOVA with Scheffe post hoc test), whereas it was intermediate in group B (−10%).

The change in tHcy was also dependent, although to a lesser extent, on the plasma folate concentration before vitamin supplementation. The largest reductions in tHcy were observed in women with the lowest initial plasma folate concentrations (**Figure 2**). In every subject with a plasma folate concentration <20 nmol/L, tHcy concentrations decreased after vitamin treatment, whereas this was not always the case for women with higher plasma folate concentrations. However, when plasma folate at the onset of vitamin treatment was >20 nmol/L, subjects seemed to benefit from the addition of vitamin B-12 (tHcy reduction in group C:−17%, group B:−12%) compared with the administration of folic acid alone (tHcy reduction in group A:−10%) (A compared with C: $P < 0.05$, ANOVA with Scheffe post hoc test). In contrast, for women with a plasma folate concentration ≤20 nmol/L, the change in tHcy was slightly more pronounced but not significantly different across treatment groups.

When tHcy and plasma folate concentrations before vitamin treatment (week 4) were included in the ANOVA as covariates, in addition to the significant differences between groups C and A, the tHcy reduction observed in group B was significantly larger than that observed in group A ($P < 0.03$). Change in tHcy was not related to RBC folate or plasma vitamin B-12 concentrations before vitamin supplementation; inclusion of these variables as covariates in the ANOVA did not improve the model.

Folate

At week 0, participants had plasma folate concentrations in the normal range (all but one >9.9 nmol/L; all >6.8 nmol/L) (23, 24). As expected, during the placebo phase, no changes in plasma folate concentrations were observed (**Table 3**), indicating also that any effects due to vitamin supplementation before the study began could be neglected.

Because of the laborious procedure for measurement of RBC folate, this index was measured only at weeks 4 and 8 to investigate the relation with plasma folate and tHcy as well as the response to folic acid supplementation. A strong positive associ-

TABLE 2
Response of plasma total homocysteine (tHcy) concentrations to placebo (week 4) and supplementation with folic acid or folic acid plus vitamin B-12 (week 8)[1]

	Plasma tHcy			
	Week 0 (baseline)	Week 4	Week 8	Mean ratio, week 8/week 4[2]
Group A ($n = 51$)	7.88 ± 2.22 (7.58)	8.13 ± 2.14 (7.84)[3]	7.18 ± 1.62 (6.99)[4]	0.89
Group B ($n = 49$)	7.52 ± 1.78 (7.31)	8.18 ± 2.41 (7.87)[3]	6.81 ± 1.46 (6.65)[4]	0.85
Group C ($n = 50$)	8.18 ± 1.74 (8.01)	8.12 ± 1.92 (7.91)	6.59 ± 1.12 (6.50)[4]	0.82

[1] $\bar{x}$ ± SD; geometric mean in parentheses. Group A received 400 μg folic acid/d, group B received 400 μg folic acid + 6 μg vitamin B-12/d, and group C received 400 μg folic acid + 400 μg vitamin B-12/d.

[2] Geometric mean ratio of tHcy at week 8 divided by tHcy at week 4; values <1 indicate a decrease in tHcy after vitamin treatment.

[3] Geometric mean significantly different from week 0 (baseline), $P < 0.05$ (paired t test).

[4] Geometric mean significantly different from week 4, $P < 0.001$ (paired t test).

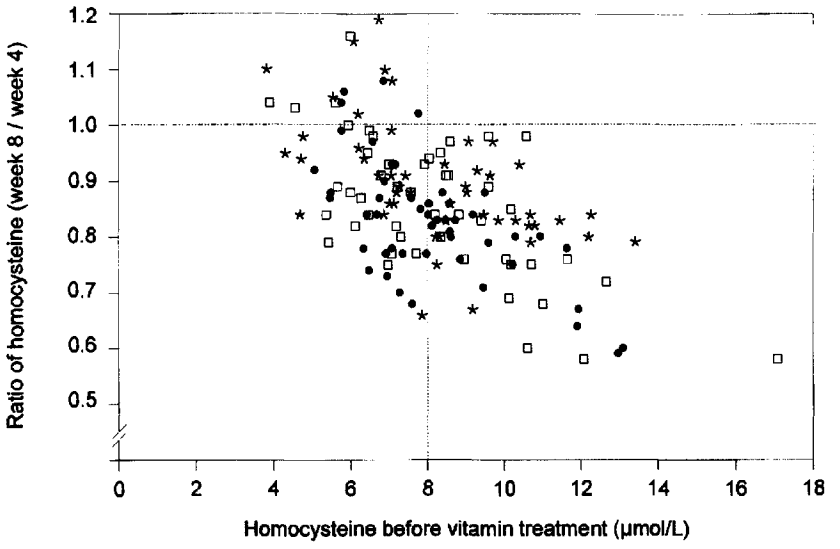

FIGURE 1. Change in the total homocysteine (tHcy) concentration in relation to the tHcy concentration before vitamin supplementation (week 4). The ratio was derived by dividing the concentration of tHcy at week 8 by the concentration at week 4. Values <1 indicate a decrease in tHcy after vitamin supplementation. *, supplementation with 400 μg folic acid/d; □ supplementation with 400 μg folic acid + 6 μg vitamin B-12/d; ● supplementation with 400 μg folic acid + 400 μg vitamin B-12/d.

ation was observed between plasma folate and RBC folate at week 4 ($r = 0.5436$, $P < 0.001$). Even though 3 of 150 subjects had low RBC folate concentrations at this time point (<317 nmol/L, or 140 ng/mL), their folate status seemed to be normal as indicated by their corresponding plasma folate and tHcy concentrations. Mean plasma folate and RBC folate concentrations at the start of the vitamin treatment did not differ among the 3 groups ($P > 0.05$, ANOVA). After 4 wk of vitamin supplementation, all treatment groups showed significant increases in mean plasma and RBC folate concentrations ($P < 0.001$ for both). The extent of the mean increase varied between 52% and 55% (plasma folate) and 69% and 78% (RBC folate) when compared with the values at week 4, and were not significantly different between the groups ($P > 0.05$, ANOVA) (Table 3).

Vitamin B-12

At week 0, the geometric mean plasma vitamin B-12 concentration for all groups combined was 268 pmol/L (Table 3). Five

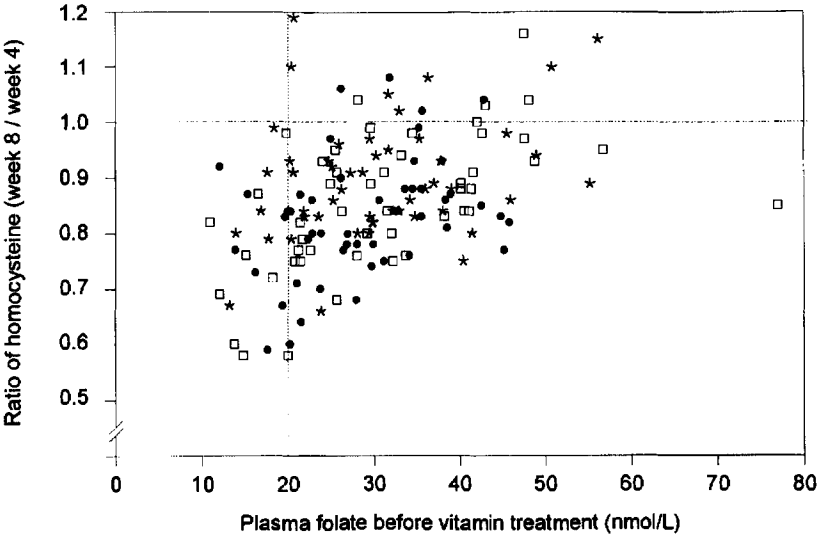

FIGURE 2. Change in the total homocysteine (tHcy) concentration in relation to the plasma folate concentration before vitamin supplementation (week 4). The ratio was derived by dividing the concentration of tHcy at week 8 by the concentration at week 4. Values <1 indicate a decrease in tHcy after vitamin supplementation. *, supplementation with 400 μg folic acid/d; □ supplementation with 400 μg folic acid + 6 μg vitamin B-12/d; ● supplementation with 400 μg folic acid + 400 μg vitamin B-12/d.

TABLE 3
Response of plasma vitamin indexes to placebo (week 4) and supplementation with folic acid or folic acid plus vitamin B-12 (week 8)[1]

	Week 0 (baseline)	Week 4	Week 8
Total group (n = 150)			
Folate (nmol/L)	29.8 ± 11.4 (27.6)	30.2 ± 10.7 (28.3)	45.6 ± 14.3 (43.5)[2]
RBC folate (nmol/L)	—	847 ± 381 (782)	1485 ± 615 (1359)[2]
Vitamin B-12 (pmol/L)	292 ± 119 (268)	276 ± 110 (253)[3]	345 ± 144 (317)[4]
PLP (nmol/L)	51.5 ± 25.0 (46.2)	—	—
Group A (n = 51)			
Folate (nmol/L)	30.1 ± 10.4 (28.2)	30.5 ± 10.2 (28.8)	46.6 ± 17.4 (43.8)[2]
RBC folate (nmol/L)	—	810 ± 295 (759)	1438 ± 643 (1296)[2]
Vitamin B-12 (pmol/L)	268 ± 100 (251)	251 ± 102 (233)[3]	259 ± 104 (240)
PLP (nmol/L)	53.9 ± 27.9 (47.8)	—	—
Group B (n = 49)			
Folate (nmol/L)	31.7 ± 13.4 (28.9)	31.3 ± 12.9 (28.8)	46.1 ± 13.4 (44.1)[2]
RBC folate (nmol/L)	—	896 ± 428 (812)	1551 ± 593 (1443)[2]
Vitamin B-12 (pmol/L)	329 ± 123 (307)	313 ± 111 (292)	371 ± 125 (353)[2]
PLP (nmol/L)	53.5 ± 26.0 (47.7)	—	—
Group C (n = 50)			
Folate (nmol/L)	27.6 ± 10.0 (25.9)	28.8 ± 8.6 (27.4)	44.0 ± 11.4 (42.5)[2]
RBC folate (nmol/L)	—	836 ± 412 (779)	1468 ± 616 (1344)[2]
Vitamin B-12 (pmol/L)	279 ± 125 (250)	265 ± 109 (239)	407 ± 158 (377)[2]
PLP (nmol/L)	47.0 ± 20.4 (43.4)	—	—

[1] $\bar{x}$ ± SD; geometric mean in parentheses. Group A received 400 μg folic acid/d, group B received 400 μg folic acid + 6 μg vitamin B-12/d, and group C received 400 μg folic acid + 400 μg vitamin B-12/d. RBC, red blood cell; PLP, pyridoxal-*P*.

[2] Geometric mean significantly different from week 4, $P < 0.001$ (paired t test).

[3] Geometric mean significantly different from week 0 (baseline), $P < 0.01$ (paired t test).

[4] No statistical test performed because of the difference in vitamin B-12 treatment among the 3 groups.

participants had plasma concentrations indicative of a suboptimal vitamin B-12 status (<111 pmol/L, or <150 pg/mL) (25). Their corresponding plasma tHcy concentrations ranged from 7.3 to 12.0 μmol/L.

After 4 wk of placebo treatment, the geometric mean vitamin B-12 concentration of the whole group was slightly but significantly lower than at week 0 ($P < 0.01$). This was mainly attributable to changes in group A ($P < 0.01$). The observed change did not correlate with the change in tHcy concentration during the placebo period ($P = 0.2$). At week 4, the plasma vitamin B-12 concentration of group A was significantly lower than that of group B ($P < 0.05$, ANOVA with Scheffe post hoc test).

From week 4 to week 8, no further changes in vitamin B-12 concentrations occurred in the group receiving folic acid only. In group B, the vitamin B-12 concentration increased significantly by a mean value of 58 pmol/L ($P < 0.001$). In group C, the plasma vitamin B-12 concentration increased by a mean value of 142 pmol/L ($P < 0.001$). This increase was significantly higher than that in group B or group A ($P < 0.05$, ANOVA with Scheffe post hoc test).

Vitamin B-6

Because vitamin B-6 was not a target index in this study, plasma PLP concentrations were measured only at the beginning of the study. PLP concentrations ranged between 16.4 and 168.9 nmol/L, with a geometric mean across all groups of 46.2 nmol/L.

DISCUSSION

The role of folic acid in the prevention of NTDs and vascular diseases and the potential additional effect of vitamin B-12 is a matter of debate. In this study, we investigated whether a combination of these vitamins had a more pronounced tHcy-lowering effect than supplementation with folic acid alone. Of the young women participating in this study, the vast majority had an adequate status of the vitamins involved in homocysteine metabolism, according to currently accepted guidelines, and they were normohomocysteinemic as defined by Kang et al (26). Despite this, folic acid supplementation (alone or in combination with vitamin B-12) resulted in significant reductions in plasma tHcy concentrations.

Ward et al (6) administered folic acid in amounts that could be reached through optimal food selection or use of fortified foods (100, 200, and 400 μg folic acid/d) to lower tHcy concentrations in middle-aged, healthy men. Because in their study the supplementation regimen was increased over the course of the study in 6-wk intervals, no information on the tHcy-lowering effect attributable solely to supplementation with 400 μg folic acid can be obtained. However, as in our study, the tHcy-reducing effect was clearly dependent on the tHcy concentration at baseline. Before supplementation, men in the 2 lowest tertiles together had a mean plasma tHcy concentration of 8.09 μmol/L, which is almost identical to that observed in group A in our study. After folic acid administration, the extent of tHcy reduction in group A was comparable with the reduction observed by Ward et al (6) in the 2 lowest tertiles over a much longer supplementation period.

Boushey et al (4) and Tucker et al (27) estimated that an increase in folic acid intake of ≈200 μg/d results on average in a reduction in tHcy concentration of 4 μmol/L, or 12%. However, this rather high effect of folic acid supplementation may be restricted to subjects with moderate or intermediate hyperhomocysteinemia, as observed previously (10, 28). Our findings and results from others (6) indicate that in normohomocysteinemic subjects, twice the amount of additional folic acid, ie, 400 μg/d is required for a mean reduction of the plasma tHcy concentration of 11%. Even though we are still awaiting results from interven-

tion studies of the protective effect of vitamin supplements in lowering plasma tHcy and concurrently risk for vascular disease, evidence for this association is increasing. A recent prospective study in 80 082 US nurses showed that in women in the highest quintile of folate intake (median intake: 696 μg/d), the relative risk for coronary artery disease, controlling for other cardiovascular risk factors, was 0.69 (95% CI: 0.55, 0.87) compared with women in the lowest quintile (median intake: 158 μg/d) (11). Calculations showed that with each 200-μg increase in folate, the relative risk was 0.89 (95% CI: 0.82, 0.96); this held true (relative risk = 0.91; 95% CI: 0.84, 0.99) even for intakes > 180 μg/d, the former recommended dietary allowance for women (29).

In this study, vitamin B-12 supplementation increased the tHcy-lowering potential of folic acid; this was especially obvious when vitamin B-12 was given in pharmacologic amounts (400 μg). In subgroup analyses, the extent of the tHcy reduction was significantly higher with the addition of increasing doses of vitamin B-12 in women with a plasma folate concentration >20 nmol/L. Because folate and vitamin B-12 have a synergistic function as cofactors of methionine synthase, sufficiency of both seems to be important to increase enzyme activity, whereas a higher availability of only one cofactor, especially in subjects with an already good supply of this cofactor, might lead to only a limited increase in enzyme activity.

After 4 wk of supplementation with vitamin B-12, the mean plasma concentration of vitamin B-12 clearly increased. This was observed with the dose of 6 μg vitamin B-12/d as well. Thus, on average, for this group of women, the low dose of vitamin B-12 was quite bioavailable. The increase in plasma vitamin B-12 concentration was even more distinct after supplementation with the high (pharmacologic) dose of 400 μg vitamin B-12. For this dose, the extent of the plasma vitamin B-12 increase observed was comparable with what Ubbink et al (28) found after supplementing young, healthy men with the same dose for 6 wk. However, it seems evident that this intake of vitamin B-12 can be reached neither by use of commonly available multivitamin supplements nor through diet, unless vitamin B-12 is included in the food-fortification scheme along with folic acid.

The results of this study suggest that the addition of vitamin B-12 to supplements containing 400 μg folic acid or to enriched foods maximizes the reduction of tHcy through the synergistic potential of both vitamins. This was most evident with the dose of 400 μg vitamin B-12/d together with folic acid. Others have suggested that, in addition to an effect on tHcy, combined supplementation with 400 μg folic acid + 400 μg vitamin B-12/d could counter the higher prevalence of vitamin B-12 deficiency in the elderly and the possibility of masked pernicious anemia with supplementation of folic acid alone (16, 28, 30). Doses of 200–400 μg vitamin B-12/d are generally regarded as safe and effective in case of loss of intrinsic factor (28, 31), but amounts as low as 25 μg vitamin B-12/d have been proposed as well (16).

A combined additional uptake of vitamin B-12 and folic acid may also be beneficial for at least a portion of women with an NTD child. There is evidence that, at comparable plasma concentrations of vitamin B-12 and folate, some of these women are less efficient at metabolizing homocysteine than are control women, thus probably pointing to a higher demand for vitamin B-12 (12).

In summary, the present study showed that additional folic acid intake, as recommended for the prevention of NTDs, results in significant reductions in the tHcy concentration of normohomocysteinemic women without vitamin deficiency. Above a plasma tHcy concentration as low as 8 μmol/L, each subject responded to vitamin supplementation with a reduction in tHcy. Beyond the presumed prevention of nerve damage in persons with pernicious anemia, the dose of 400 μg vitamin B-12/d along with the folic acid supplementation regimen showed the largest additional effect in lowering tHcy. With current public health measures, however, the additional uptake of both 400 μg folic acid/d and 400 μg vitamin B-12/d may not to be realized. Thus, further efforts should aim to optimize public health strategies to effectively reduce the risk of vascular disease and prevent a considerable number of birth defects.

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ENZYMATIC MECHANISM OF CREATINE SYNTHESIS*

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It is well established that the last step in the biosynthesis of creatine involves the methylation of guanidinoacetic acid.[1] This conclusion is based upon experimental evidence derived from two independent lines of investigation. By application of the isotopic tracer technique, du Vigneaud *et al.* (2) have demonstrated that the methyl group in creatine is derived from L-methionine; furthermore, these authors obtained conclusive evidence that, *in vivo*, the methyl group of L-methionine is transferred to the methyl acceptor as a unit. In an independent study of this transmethylation reaction *in vitro*, Borsook and Dubnoff (3) reached similar conclusions using guinea pig liver slices. Subsequently (4), these authors have shown that cell-free liver homogenates fortified with adenylic acid and an oxidizable substrate such as α-ketoglutaric acid are able to form creatine under aerobic conditions. It was assumed by these authors and by others (5, 6) that these requirements were a reflection of the endergonic nature of this transmethylation reaction and an indication of the ability of ATP to serve as an energy source in this system. These conclusions appeared to have been borne out by the findings of Cohen (5) that the methylation of guanidinoacetic acid proceeds anaerobically in the presence of ATP and Mg^{++}.

The biosyntheses of creatine and N^1-methylnicotinamide are similar. In both cases the methyl group is derived from L-methionine and, furthermore, ATP and Mg^{++} are required. Recent investigations (6, 7) have

* Fifth paper of a series on enzymatic mechanisms in transmethylation. This investigation was supported in part by grants-in-aid from the Williams-Waterman Fund for the Combat of Dietary Diseases of the Research Corporation of New York and from the American Cancer Society. Presented in part at the Forty-third annual meeting of the American Society of Biological Chemists, New York, April, 1952 (1).

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[1] The following abbreviations are used: GA, guanidinoacetic acid or guanidinoacetate; AMe, *S*-adenosylmethionine, *i.e.* active methionine; ASR, adenosylhomocysteine; NMeN, N^1-methylnicotinamide; ATP, adenosinetriphosphate; ADP, adenosinediphosphate; GSH, reduced glutathione; IP, orthophosphate; Tris, tris-(hydroxymethyl)aminomethane.

clarified the enzymatic mechanisms involved in the biosynthesis of NMeN and have indicated that this transmethylation reaction proceeds in a stepwise fashion according to Reactions 1 and 2 which, respectively, are catalyzed by the methionine-activating enzyme and by nicotinamide methylpherase.

$$\text{(1)}\quad \text{L-Methionine} + \text{ATP} \xrightarrow{Mg^{++},\ GSH} S\text{-adenosylmethionine} + 3\text{IP}$$

$$\text{(2)}\quad \text{AMe} + \text{nicotinamide} \longrightarrow \text{NMeN} + S\text{-adenosylhomocysteine}$$

$$\text{(1)} + \text{(2)},\ \text{L-methionine} + \text{ATP} + \text{nicotinamide} \longrightarrow \text{NMeN} + 3\text{IP} + \text{ASR}$$

It has been estimated (8) that the methylsulfonium bond in AMe and other sulfonium compounds is energy-rich and, tentatively, it has been assumed that the onium bond energy might account for the biological lability of the methyl group in AMe.

It has been suggested (8) that the activation of methionine might be a prerequisite to the transfer of its methyl group to any one of a variety of methyl acceptors. According to this hypothesis the biosynthesis of creatine from L-methionine, ATP, and GA should involve the coupling of Reaction 1 with Reaction 3.

$$\text{(3)}\quad \text{AMe} + \text{GA} \longrightarrow \text{creatine} + \text{ASR} + H^+$$

$$\text{(1)} + \text{(3)},\ \text{L-methionine} + \text{ATP} + \text{GA} \xrightarrow[Mg^{++}]{\text{GSH}} \text{creatine} + \text{ASR} + H^+ + 3\text{IP}$$

Preliminary experiments gave indications that, indeed, creatine synthesis followed this pattern, and it was considered of interest to study the synthesis of creatine from GA and AMe in more detail. A soluble enzyme[2] which catalyzes Reaction 3 has been found in cell-free extracts of guinea pig, rabbit, beef, and pig liver. The enzyme from pig liver has been purified approximately 20-fold by means of ammonium sulfate fractionation followed by treatment with alumina C_γ. The partially purified enzyme is free of methionine-activating enzyme and of nicotinamide methylpherase. Glutathione or other reducing substances are required for the optimal activity of the enzyme. No evidence has yet been obtained to indicate the participation of metal ions or other cofactors in the reaction.

Creatine was conclusively identified as one of the products of the enzymatic reaction by (*a*) the close agreement in its chemical determination by two different methods, namely, the α-naphthol-diacetyl reaction and the Jaffe alkaline picrate test, (*b*) the ability of the reaction product to

[2] By analogy with the nomenclature adopted in earlier studies of this series, the enzyme catalyzing Reaction 3 will be referred to as guanidinoacetate methylpherase (GA methylpherase).

function as a substrate for creatine kinase, an enzyme which catalyzes Reaction 4

(4) Creatine + ATP $\rightleftharpoons$ creatine phosphate + ADP + H^+

and (*c*) the isolation of creatinine as the potassium picrate double salt from the protein-free filtrate obtained in a large scale enzymatic run.

EXPERIMENTAL

Enzyme Preparations

GA Methylpherase—GA methylpherase was found in cell-free extracts of rabbit, guinea pig, beef, and pig livers. Pig liver extracts were selected for purification. For preparation of the enzyme fresh pig liver was obtained at the slaughter-house, packed in ice, and brought to the laboratory in a vacuum container. All the manipulations were carried out in a cold room maintained at 2°. The purification may be interrupted after each ammonium sulfate fractionation and the preparation can be stored at −20° as an ammonium sulfate paste. The liver was diced, rinsed free of excess blood with a buffer solution (sodium acetate 0.075 M, pH 5.0), weighed, and homogenized in a Waring blendor with 2.5 volumes of the same buffer solution. Next the homogenate was centrifuged at 9000 r.p.m. for 30 minutes. The supernatant material, which was slightly opalescent, was packed in ice and solid ammonium sulfate was added slowly with mechanical stirring (19.5 gm. per 100 ml.). The precipitate was removed by centrifugation in a Servall high speed centrifuge and discarded, and ammonium sulfate (10.5 gm. per 100 ml.) was added to the supernatant solution. The precipitate collected as above contained essentially all of the activity. For further purification the ammonium sulfate paste was dissolved in a small volume of 0.10 M sodium acetate and dialyzed for 3 hours against running 0.05 M acetate buffer, pH 5.6. At the end of the dialysis an inactive precipitate was removed by centrifugation. The protein content of the supernatant material was then determined, and the protein concentration was adjusted, by dilution with the same acetate buffer, to 20 mg. per ml.; 0.33 volume of alumina Cγ (dry weight, 35 mg. per ml.) was added, with good mechanical stirring, the suspension was centrifuged at 3000 r.p.m., and the supernatant fluid discarded. The residue was eluted four times with phosphate buffer (0.0125 M, pH 6.35), a volume of buffer equal to that of the alumina Cγ suspension being used each time. The eluates having the highest specific activity, usually the first two, were pooled, and the pH of the solution was adjusted to 7.2 with dilute NaOH, and then buffered at this pH by addition of 0.05 volume of 2 M phosphate buffer, pH 7.2. Next, saturated ammonium sulfate, pH 7.2, was added to 47.5 per cent saturation and the inert precipitate removed at high speed

centrifugation as above. Solid ammonium sulfate was added slowly with stirring to the supernatant solution (1 gm. for each 10 ml.) and, after 30 minutes, the precipitate was collected by centrifugation. The precipitate was dissolved in dilute phosphate buffer (pH 7.4) and, if convenient, dialyzed against 0.05 M KCl or 0.025 M phosphate buffer (pH 7.4) for 3 hours. The results from a representative run are presented in Table I.

Other Enzyme Preparations—Creatine kinase was prepared from a water (2.5 volumes) extract of rabbit muscle. The muscle extract was dialyzed against running distilled water for 12 hours in the cold. A heavy flocculent precipitate formed and was discarded and the supernatant material was fractionated by ammonium sulfate. The fraction which precipitated

TABLE I

Preparation of Guanidinoacetate Methylpherase from Pig Liver

	Units per ml.*	Specific activity	Yield
		units per mg. protein	*per cent*
Acetate buffer extract	1.1	0.034	100
Ammonium Sulfate Ppt. I (25–40% saturated)	3.2	0.13	90
Treatment with alumina C_γ			
Supernatant	0.56	0.14	19.5
Eluate 1	2.76	1.6	28.7
" 2	2.38	1.7	25.6
" 3	0.83	1.66	9
Ammonium Sulfate Ppt. II (48–65% saturated)	15.5	2.6	34.5

* 1 unit = 1 μM of creatine formed in 120 minutes at 37°.

between 60 and 70 per cent saturation[3] was collected and dissolved in cold 0.85 M NaCl. The ATPase activity of this fraction was very slight and could be reduced to insignificant values by dilution.

The preparations of nicotinamide methylpherase and of methionine-activating enzyme were as described earlier (7, 9).

Chemical Preparations—*S*-Adenosylmethionine was prepared enzymatically and purified as described by Cantoni (9). Unless indicated the preparation of AMe contained L-methionine, but was free of organic and inorganic phosphate compounds and of Mg^{++}. Preparations of AMe, free of methionine and approximately 80 per cent pure (AMe 80), obtained by paper chromatography were used in some of the experiments. Guanidinoacetic acid obtained commercially was recrystallized from water before use. Reduced glutathione and ATP were commercial preparations.

[3] 60 per cent saturation = 42.3 gm. per 100 ml.

p-Chloromercuribenzoic acid and methionine methylsulfonium iodide were generously supplied by Dr. T. Singer and Dr. R. McRorie. Alumina Cγ was prepared as described by Bauer (10).

Measurement of Enzyme Activity—The reaction was carried out in small test-tubes. The cold enzyme solution was added to the reaction mixture at room temperature and the reaction run for 60 to 120 minutes in a water bath at 37°. Under the conditions of the assay the activity of the enzyme was linear with time and proportional to enzyme concentration (Fig. 1).

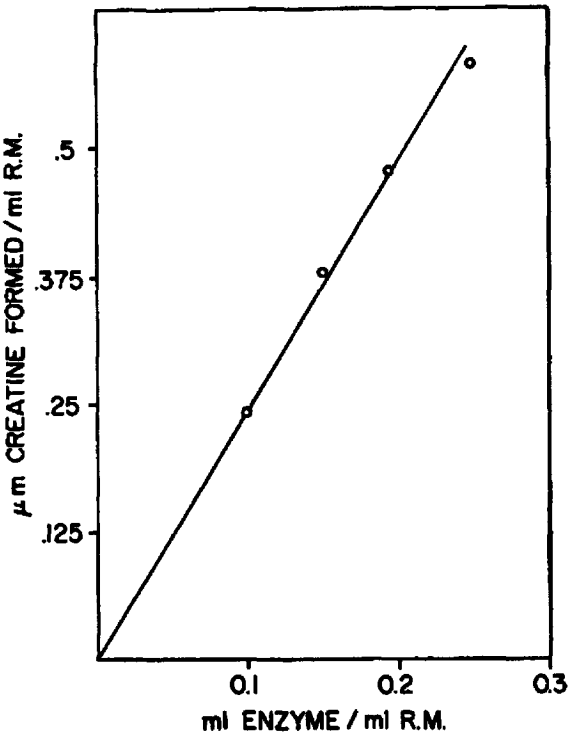

FIG. 1. Relationship of enzyme concentration to activity

After stopping the reaction by the addition of trichloroacetic acid, an aliquot of the protein-free filtrate was autoclaved in 0.5 N HCl for 30 minutes at 15 pounds pressure and the resulting creatine determined by Borsook's modification (11) of the alkaline picrate method of Folin (12).

The amount of creatine formed was determined by the difference between the color developed in the complete system and that developed in a duplicate sample in which AMe had been added after the completion of the incubation. This procedure was aimed at correcting for any preformed creatine, as well as for any chromogenic material derived from guanidinoacetic acid. In addition, a variety of control experiments were performed to make sure that the increase in creatinine was in reality due to creatine synthesis and not to the formation of chromogenic "creatine"-

like substances derived from AMe, GA, or the enzyme preparation itself. Under the experimental condition used there was no detectable increase in chromogenic material unless all of the components of the reaction mixture were added (Table II).

Specificity and Properties of GA Methylpherase—It was found that the activity of GA methylpherase is a function of the concentration of the substrate both with regard to GA and to AMe. In the presence of an excess of the acceptor, GA, the transfer of the methyl group of *S*-adenosylmethionine appears to go to completion as indicated by the stoichiometric relationship between the amount of substrate furnished and the amount of creatine formed. Likewise, in the presence of an excess of the methyl

TABLE II

Enzymatic Methylation of Guanidinoacetate

Components of system, 0.15 ml. of guanidinoacetate methylpherase (C_γ eluate pool containing 1.75 mg. of protein) in a final volume of 0.8 ml. The complete system contained GA, 0.0033 M; AMe, 0.0021 M; BAL, 0.00016 M; and Tris buffer, pH 7.4, 0.1 M. Incubation time, 60 minutes at 37°. The results are expressed as micromoles of creatine formed per ml. of enzyme preparation per hour.

	Creatine formed	Per cent of complete system
Complete system	1.10	100
No GA	0	0
" AMe	0	0
" enzyme	0	0
" BAL	0.605	55

donor, all of the guanidinoacetate supplied can be methylated to creatine (Table III).

A number of *S*-methyl compounds related chemically to AMe, such as adeninethiomethylpentose, and methionine methylsulfonium iodide were tested for their ability to function as methyl donors in this system; only AMe was active as a methyl donor. L-Methionine was not active as a methyl donor, either in the presence or in the absence of ATP and Mg^{++}. However, when the system was supplemented with a preparation of methionine-activating enzyme from rabbit liver, creatine synthesis was readily achieved, thus providing excellent support for the reaction mechanism described in Reactions 1 and 3 (Table IV).

The specificity of the enzyme for the methyl acceptor has been tested only with respect to nicotinamide; this compound was not methylated in this system.

The pH optimum for the reaction was found to be around 7.5; phosphate, bicarbonate-CO_2, and tris(hydroxymethyl)aminomethane can be used to buffer the reaction mixture.

TABLE III

Equivalence of Guanidinoacetic Acid and S-Adenosylmethionine Utilization to Amount of Creatine Formed

0.3 ml. of enzyme in a final volume of 1.0 ml. Tris buffer, 0.1 M, pH 7.5; BAL, 0.0002 M; AMe 80 and GA added as micromoles per ml. of reaction mixture as indicated. Incubation time, 180 minutes at 37°. The results are expressed as micromoles of creatine formed per ml. of reaction mixture.

Experiment A*			Experiment B†			
Additions		Creatine formed	Additions		Creatine formed	
GA	AMe 80		AMe 80	GA		
						per cent AMe 80
	1.7			2.5		
0.085	1.7	0.084	0.2	2.5	0.17	84.0
0.17	1.7	0.169	0.36	2.5	0.31	87.2
0.26	1.7	0.284	0.48	2.5	0.44	91.2
0.34	1.7	0.351	0.6	2.5	0.53	89.0
0.42	1.7	0.423	0.9	2.5	0.73	82.0
			1.2	2.5	0.96	80.0

* Enzyme (Ammonium Sulfate II) containing 9.1 mg. of protein per ml.

† Enzyme (Ammonium Sulfate I) containing 60.4 mg. of protein per ml.

TABLE IV

Synthesis of Creatine by Coupling of Methionine-Activating Enzyme and GA Methylpherase

The reaction mixture contains ATP, 0.013 M; L-methionine, 0.02 M; GSH, 0.001 M; MgCl, 0.166 M; Tris buffer, pH 7.4, 0.075 M; Enzyme 1 and Enzyme 2 as indicated. Enzyme 1 (methionine-activating enzyme) contains 22 mg. of protein per ml.; Enzyme 2 (guanidinoacetate methylpherase) contains 6.0 mg. of protein per ml. Final volume, 1 ml. Incubation time, 120 minutes at 37°. The results are expressed as micromoles of creatine formed per ml. of reaction mixture per 120 minutes.

Enzyme 1	Enzyme 2	Creatine formed
ml.	*ml.*	
	0.15	0.0
0.025	0.15	0.8
0.05	0.15	0.975
0.1	0.15	0.975
0.1		0.0
0.1	0.025	0.32
0.1	0.05	0.56
0.1	0.1	0.85

Borsook and Dubnoff (4) observed that addition of cyanide was inhibitory to creatine synthesis in liver homogenates. Inasmuch as these authors also found that oxygen was an absolute requirement for creatine synthesis, it was not clear whether cyanide inhibition was due to disruption of the aerobic generation of energy-rich phosphate compounds or to interference with the transmethylation reaction itself. On reinvestigation it was found that cyanide not only is not inhibitory but, in fact, stimulated the activity of GA methylpherase. In addition, other reducing compounds such as glutathione, cysteine, and BAL increased the activity of the enzyme. The activation by —SH and other reducing compounds became more pronounced as the degree of purification of the enzyme

TABLE V

Reversible Inactivation of Guanidinoacetic Methylpherase by p-Chloromercuribenzoic Acid

0.1 ml. of guanidinoacetate methylpherase enzyme, Ammonium Sulfate I containing 11.7 mg. of protein; Tris buffer, pH 7.4, 0.01 M; guanidinoacetic acid, 0.003 M; *S*-adenosylmethionine, 0.004 M; glutathione, 0.005 M. Incubation time, 60 minutes at 37°. The results are expressed as micromoles of creatine formed per ml. of enzyme.

Preliminary treatment of enzyme	GSH	Creatine formed
None	−	3.04
"	+	4.83
p-Chloromercuribenzoic acid*	−	0
" " (followed by GSH)	+	4.93

* 0.001 M; 45 minutes at room temperature.

increased. Thus the activity of crude liver homogenates was not increased by the addition of —SH reagents; there was a moderate activation of the initial ammonium sulfate fraction and a very marked activation of the alumina Cγ eluates, or the ammonium sulfate fractions obtained from them. Further evidence for the dependence of GA methylpherase on the presence of free —SH groups for activity was obtained by use of *p*-chloromercuribenzoic acid. This reagent at a concentration of 1×10^{-3} M caused complete inhibition of creatine synthesis. This inhibition could be reversed quantitatively by subsequent addition of glutathione in sufficient excess (Table V).

Ca^{++} or Mg^{++} is not required in the reaction catalyzed by GA methylpherase; sodium fluoride, folic acid, and *Leuconostoc citrovorum* factor have no effect on the activity of the enzyme.

Evidence for Formation of Creatine—Studies of creatine synthesis have

been severely handicapped by the lack of a specific micromethod for creatine determination. Two methods are available for the determination of creatine: one is based on the α-naphthol-diacetyl reaction for creatine (13); the other depends on the conversion of creatine to creatinine and determination of the latter by the Jaffe alkaline picrate reaction. GA interferes in both determinations; the degree of interference, however, is different. Different also are the specificities of the two determinations. It is well known that the determination of creatine, after conversion to creatinine, by the Jaffe alkaline picrate reaction, is fraught with pitfalls. In earlier studies (4, 5, 14, 15) one of the principal sources of difficulty was the frequent occurrence in crude homogenates of α-keto acids in gen-

TABLE VI

Creatine Synthesis As Determined by Two Methods

0.3 ml. of GA methylpherase; Ammonium Sulfate I containing 25.3 mg. of protein in a final volume of 1.0 ml.; AMe, 0.0018 M; GA, 0.0026 M; Tris buffer (pH 7.4), 0.075 M; GSH, 0.001 M. Incubation time, 60 minutes at 37°. The results are expressed as *micrograms* of creatine formed per ml. of reaction mixture per hour.

Incubation time	Creatine determination	
	Method I*	Method II†
min.		
0	115	31.4
60	195	121
Creatine formed	+80	+89.6

* Alkaline picrate method of Borsook (11).

† α-Naphthol-diacetyl method of Ennor and Stocken (16).

eral and of α-keto-γ-methiolbutyric acid in particular; when treated with alkaline picrate these α-keto acids give rise to chromogenic products which are indistinguishable from those produced by creatinine. Treatment with Lloyd's reagent, however, appears to reduce greatly errors from these sources (5).

Direct determination of creatine by the α-naphthol-diacetyl reaction is not particularly suitable for routine use with this system because the presence of —SH groups interferes with the color development. Such interference can be overcome, however, by appropriate treatment with *p*-chloromercuribenzoic acid, as suggested by Ennor and Stocken (16). In view of the relative lack of specificity of the two methods it appeared desirable to measure simultaneously the formation of creatine by the two methods. Table VI shows the result of such an experiment. The values obtained for creatine synthesis as determined by the two methods agree

within 10 per cent. These results present strong support for identification of creatine as one of the products of the GA methylpherase reaction.

However, it was believed important to identify creatine more directly. It is known that in the presence of creatinekinase, ATP will phosphorylate creatine to form creatine phosphate. The substrate specificity in this case is very high, guanidinoacetate and other guanidino compounds being inactive in the system. As is well known, phosphocreatine is very labile at acid pH and is completely hydrolyzed to orthophosphate and creatine during the course of the procedure employed for phosphorus determination

TABLE VII

Formation of Creatine As Demonstrated Enzymatically by Means of Creatine Kinase

0.1 ml. of creatine kinase containing 60 γ of protein; ATP, 0.004 M; borate buffer, pH 9.1, 0.068 M; $MgCl_2$, 0.01 M. Additions as indicated. Incubation time, 15 minutes at 37°. The results are expressed as micrograms of "orthophosphate" formed per ml. of reaction mixture.

Additions	"Orthophosphate" formed
No additions	0.55
Creatine, 1 μM	11.00
" 2 "	18.90
Product of guanidinoacetate methylpherase, Reaction Mixture 2 (0.25 ml.)*	6.40
Reaction Mixture 1, control†	0.60

* Reaction Mixture 2 deproteinized at end of incubation and concentrated. 1 ml. contained 3.5 μM of creatine, as determined by the alkaline picrate method.

† Reaction Mixture 1 deproteinized at zero time and concentrated to same volume as Reaction Mixture 2. Reaction Mixtures 1 and 2 are the same as the complete system of Table II.

(17). Thus, if creatine were formed by the GA methylpherase reaction, addition of a suitable aliquot of the incubated reaction mixture to creatine kinase in the presence of ATP and Mg^{++} at pH 9.0 should result in the formation of phosphocreatine which can be determined as "apparent orthophosphate." Table VII describes the result of such an experiment from which it is concluded that creatine is the product of the enzymatic methylation of GA.

Isolation of Reaction Product and Identification As Creatinine Potassium Picrate—The purified enzyme (3 ml., 66 mg. of protein) was incubated with AMe (190 μM), GA (380 μM), BAL (0.01 ml.), and glycylglycine buffer in a final volume of 25 ml. for 4 hours at 37°. The reaction was terminated by addition of 0.1 volume of 100 per cent trichloroacetic acid. After cen-

trifugation, the supernatant solution was autoclaved in 0.05 N HCl for 30 minutes at 15 pounds pressure for conversion of the creatine present to creatinine. Next the creatinine was adsorbed on 1 gm. of Lloyd's reagent and eluted therefrom with 20 ml. of saturated $BA(OH)_2$ as described by Bloch and Schoenheimer (18). In addition to creatinine the Lloyd eluate contained an unidentified contaminant showing an ultraviolet absorption with a maximum of 250 mμ. Prior to the crystallization of creatine as the potassium picrate double salt, it was deemed desirable to remove this contaminant, since it also gave an insoluble derivative when treated with picric acid. For this purpose the eluates were freed of barium, adjusted to pH 7.8 with dilute phosphate buffer, and passed through a Dowex 50 (H^+) column (15 × 45 mm.) which then was washed with 20 ml. of water. For elution 0.1 N HCl in 0.1 N NaCl was used and the eluates were collected in 10 ml. lots. The first three fractions were discarded and the next nine were pooled. The combined eluates were adjusted to 0.1 per cent with respect to both potassium picrate and picric acid and crystallization was allowed to proceed in the ice box. After repeated recrystallizations, approximately 15 mg. of the double salt were obtained. An aliquot was ground in Nujol and its infra-red absorption spectrum determined in a Perkin-Elmer spectrophotometer. The material exhibited a spectrum practically identical to that given by an authentic sample of creatine potassium picrate and clearly different from the corresponding salt of guanidinoacetic acid anhydride. This provides further evidence for the formation of creatine as the product of the enzymatic methylation of guanidinoacetic acid.

DISCUSSION

On the basis of the evidence presented it appears justified to conclude that the enzymatic mechanisms involved in the biosynthesis of creatine conform to the pattern revealed earlier in studies of the methylation of nicotinamide. The two systems differ, of course, in the specificity and properties of the transmethylating enzymes, but the mechanisms for activation of methionine and for the transfer of the methyl group are presumably the same in both cases. du Vigneaud *et al.* (2) have produced conclusive evidence that *in vivo* the methyl group of methionine is transferred *as such* to creatine. The exact mechanism underlying the migration of the methyl group is not yet known with certainty; in view of recent findings on the structure of AMe, it would appear reasonable to postulate that the methyl group might migrate as a positively charged methylcarbonium ion which could be transferred from the methyl donor either directly to the substrate or first to the enzyme catalyst and then to the substrate.

A comparison of Reactions 2 and 3 indicated that H^+ formation[4] accompanies the methylation of GA, but not that of nicotinamide. This is related to the fact that in the methylation of GA a tertiary amine is formed, whereas the methylation of nicotinamide results in the formation of a new onium compound containing a methylpyridinium bond.

Liener and Schultze (19), Stekol *et al.* (20), and others (21, 22) have reported that vitamin B_{12}- or folic acid-deficient rats show decreased ability to methylate, as indicated by lowered creatine and N^1-methylnicotinamide synthesis. Since the rôle of vitamin B_{12} and of folic acid, or its derivative, *L. citrovorum* factor, in the biosynthesis of methyl groups and in the synthesis of purines is well established, it would appear that these results might be related to decreased synthesis of methyl groups or of adenine nucleotides in the deficient animal. This conclusion is supported by the observation reported above that *L. citrovorum* factor, folic acid, or vitamin B_{12} had no effect on the enzymatic synthesis of creatine. However, the possibility that the enzyme guanidinoacetate methylpherase contains tightly bound *L. citrovorum* factor as its prosthetic group has not been ruled out.

It would be anticipated that the product of demethylation of AMe should be adenosylhomocysteine. In fact, preliminary results[5] indicate that a compound having chemical properties expected for ASR can be detected by means of chromatographic techniques following the transmethylation reaction. Further work aiming at the isolation and characterization of this demethylation product is in progress in this laboratory.

SUMMARY

1. The methylation of guanidinoacetic acid by *S*-adenosylmethionine to form creatine has been studied in partially purified preparations of pork liver.

2. The enzyme, which is referred to as guanidinoacetate methylpherase, has been partially purified and some of its properties have been investigated. The partially purified enzyme requires reduced thiol groups for optimal activity.

3. Creatine has been identified as a reaction product (*a*) by simultaneous determinations by two different methods, (*b*) enzymatically by means of creatine kinase, and (*c*) by conversion to creatinine and the isolation of creatinine potassium picrate.

[4] Hydrogen ion formation was measured experimentally by running the reaction in a Warburg vessel and using bicarbonate-CO_2 buffers. It was clearly evident that H^+ formation accompanied creatine synthesis, but the exact stoichiometric relationships could not be worked out because of the limited sensitivity of the method and the large amount of protein required.

[5] E. Scarano and G. L. Cantoni, to be published.

4. The mechanism underlying creatine biosynthesis has been discussed.

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Regulation of homocysteine metabolism and methylation in human and mouse tissues

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ABSTRACT **Hyperhomocysteinemia is an independent risk factor for cardiovascular disease. Homocysteine (Hcy) metabolism involves multiple enzymes; however, tissue Hcy metabolism and its relevance to methylation remain unknown. Here, we established gene expression profiles of 8 Hcy metabolic and 12 methylation enzymes in 20 human and 19 mouse tissues through bioinformatic analysis using expression sequence tag clone counts in tissue cDNA libraries. We analyzed correlations between gene expression, Hcy, *S*-adenosylhomocysteine (SAH), and *S*-adenosylmethionine (SAM) levels, and SAM/SAH ratios in mouse tissues. Hcy metabolic and methylation enzymes were classified into two types. The expression of Type 1 enzymes positively correlated with tissue Hcy and SAH levels. These include cystathionine β-synthase, cystathionine-γ-lyase, paraxonase 1, 5,10-methylenetetrahydrofolate reductase, betaine:homocysteine methyltransferase, methionine adenosyltransferase, phosphatidylethanolamine *N*-methyltransferases and glycine *N*-methyltransferase. Type 2 enzyme expressions correlate with neither tissue Hcy nor SAH levels. These include SAH hydrolase, methionyl-tRNA synthase, 5-methyltetrahydrofolate:Hcy methyltransferase, *S*-adenosylmethionine decarboxylase, DNA methyltransferase 1/3a, isoprenylcysteine carboxyl methyltransferases, and histone-lysine *N*-methyltransferase. SAH is the only Hcy metabolite significantly correlated with Hcy levels and methylation enzyme expression. We established equations expressing combined effects of methylation enzymes on tissue SAH, SAM, and SAM/SAH ratios. Our study is the first to provide panoramic tissue gene expression profiles and mathematical models of tissue methylation regulation.—Chen, N. C., Yang, F., Capecci, L. M., Gu, Z., Schafer, A. I., Durante, W., Yang, X.-F., Wang, H. Regulation of homocysteine metabolism and methylation in human and mouse tissues. *FASEB J.* 24, 2804–2817 (2010). www.fasebj.org**

Key Words: gene expression · homocysteine metabolism · methylation

HYPERHOMOCYSTEINEMIA (HHCY) HAS been identified as an independent risk factor for cardiovascular disease (CVD), diabetes, and Alzheimer's disease. Numerous studies have established that homocysteine (Hcy) has profound biological effects, including accelerating atherosclerosis, impairing postinjury endothelial repair and endothelial function, dysregulating lipid metabolism, and inducing thrombosis. However, the biochemical basis by which HHcy contributes to CVD remains largely unknown. Tissue-specific distribution of Hcy metabolic enzymes, as well as the contribution of each enzyme to tissue Hcy metabolism and methylation has not been previously studied.

Hcy is a non-protein-forming, sulfur-containing amino acid that functions as a key intermediate in methionine (Met) metabolism. Met is first demethylated to form Hcy, which is then metabolized through two pathways: transsulfuration to cysteine (Cys) and remethylation to Met (**Fig. 1*A***). Deficiency in Hcy metabolic enzymes could lead to abnormal Hcy levels in both humans and mice. It is known that cystathionine-β-synthase (CBS) and methylenetetrahydrofolate reductase (MTHFR) are key enzymes for Hcy metabolism (1, 2). Patients with CBS deficiency have plasma Hcy levels of 200–300 μM compared to ~10 μM in healthy individuals (1). Similarly, CBS-deficient mice develop HHcy (plasma Hcy~200 μM). In addition, polymorphisms of MTHFR are associated with HHcy in humans (2), and MTHFR-deficient mice develop moderate HHcy (plasma Hcy 33 μM) (3). Although the link between systemic deficiency of certain Hcy metabolic enzymes and global HHcy has been described, the contribution of tissue enzyme expression to tissue-specific Hcy metabolism remains to be explored.

We have proposed hypomethylation as a specific biochemical mechanism by which Hcy induces vascular injury (4, 5). Hcy can form *S*-adenosyl-homocysteine (SAH), a potent inhibitor of cellular methylation. It has been reported that abnormal DNA methylation of genes such as PPARα (6), Pdx1 (7), and type 1B adrenal angiotensin receptor (8) contribute to the development of cardiovascular and metabolic diseases (9). We have demonstrated that Hcy arrests endothelial

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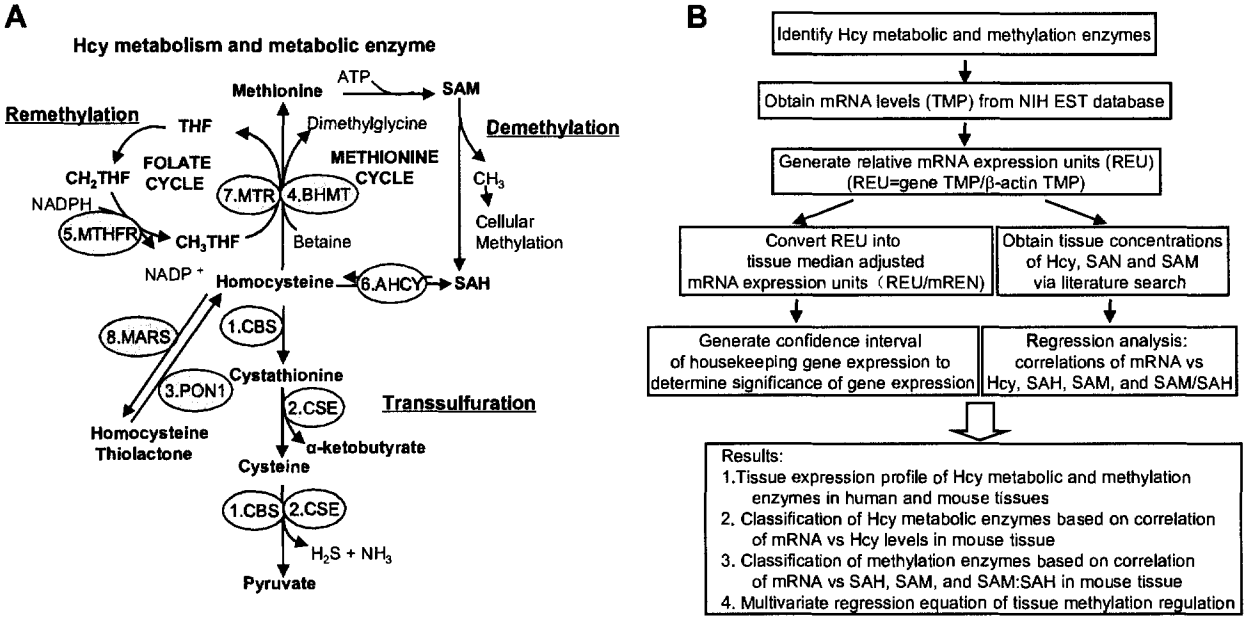

Figure 1. Hcy metabolism and study design *A*) Diagram of Hcy metabolism. Eight enzymes involved in Hcy metabolism were selected for database mining and expression profile analysis. *1*) CBS, cystathioninine-beta-synthase; *2*) CSE, cystathionine-γ-lyase; *3*) PON1, paraoxonase 1; *4*) BHMT, betaine:homocysteine methyltransferase; *5*) MTHFR, 5,10-methylenetetrahydrofolate reductase; *6*) AHCY, *S*-adenosylhomocysteine hydrolase or adenosylhomocysteinase; *7*) MTR or MS, 5-methyltetrahydrofolate: homocysteine methyltransferase or methionine synthase; *8*) MARS or AARS, methionyl-tRNA synthase. THF, tetrahydrofuran; CH_2THF, 5-methylenetetrahydrofolate; CH_3THF, 5-methyltetrahydrofolate; NADPH, reduced form of $NADP^+$; $NADP^+$, nicotinamide adenine dinucleotide phosphate; H_2S, hydrogen sulfide; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine. *B*) Strategies of database mining, analysis of gene expression profiles and regression analysis. Hcy metabolic and methylation enzymes were selected. Tissue gene mRNA levels (TMP) were retrieved from National Institutes of Health EST database. Generated relative mRNA expression units (REU) and tissue median adjusted mRNA expression units were evaluated. Confidence interval of housekeeping gene expression was determined. Finally, tissue gene expression profiles and relationship between gene expression and Hcy metabolites levels were established.

cell growth, possibly by increasing cellular SAH concentrations, consequently reducing DNA methyltransferase 1 (DNMT1) activity, and demethylating cyclin A promoter, which leads to cyclin A chromatin remodeling and transcriptional suppression (5, 10). We hypothesized that DNA hypomethylation is a key mechanism responsible for HHcy-related vascular disease (11, 12). Our hypomethylation hypothesis is supported by clinical studies demonstrating that elevated Hcy levels in patients are linked to increased SAH levels and impaired erythrocyte membrane protein methylation (13–15). Furthermore, animal studies corroborate our findings by showing that CBS-deficient mice have increased SAH levels and decreased DNA methylation (1, 16). The underlying molecular mechanism of SAM and SAH regulation in HHcy remains unknown.

It has been reported that the levels of Hcy, SAM, and SAH vary across different mouse tissues (17, 18) These data support the concept that circulating Hcy does not equilibrate Hcy levels across all tissues, and that Hcy metabolism and cellular methylation may be differentially regulated at the tissue level. We now postulate that a differential distribution of Hcy metabolic enzymes is responsible for tissue-specific Hcy metabolism, which contributes to distinct pathologies in HHcy. In this study, we examined gene expression of various Hcy-related metabolic and methylation enzymes using a bioinformatic approach and evaluated the relationship of enzyme expression and Hcy metabolism, as well as methylation status in human and mouse tissues.

MATERIALS AND METHODS

Hcy metabolic and methylation enzymes

We selected 8 key Hcy metabolic enzymes and 12 methylation enzymes for the assessment of tissue expression profiles and correlations (**Table 1**) based on their metabolic roles schematically described and numbered in Figs. 1*A* and 3*A*. Hcy metabolic enzymes include CBS, cystathionine-γ-lyase (CSE), paraoxonase 1 (PON1), betaine:homocysteine methyltransferase (BHMT), 1,5,10-methylenetetrahydrofolate reductase (MTHFR), *S*-adenosylhomocysteine hydrolase or adenosylhomocysteinase (AHCY), 5-methyltetrahydrofolate:homocysteine methyltransferase or methionine synthase (MS or MTR), and methionyl-tRNA synthetase (MARS). Methylation enzymes are glycine *N*-methyltransferase (GNMT), phosphatidylethanolamine *N*-methyltransferase (PEMT), methionine adenosyltransferase (MAT), BHMT, MTHFR, AHCY, MTR, *S*-adenosylmethionine decarboxylase (AMD), DNA methyltransferase I (DNMT1), DNA methyltransferase 3a (DNMT3a), isoprenylcysteine carboxyl methyltransferases (ICMT), and histone-lysine N-methyltransferase (HMT). Enzyme commission ID numbers and National Center for Biotechnology Information (NCBI)/UniGene ID numbers listed in Table

TABLE 1. *Enzyme identification*

Enzyme abbreviation and full name	Enzyme commission ID	NCBI/UniGene ID: human; mouse
Hcy metabolic enzymes		
1. CBS (cystathioninine-β-synthase)	EC4.2.1.22	Hs.533013; Mm.206417
2. CSE (cystathionine-γ-lyase)	EC4.4.1.1	Hs.19904; Mm.28301
3. PON1 (paraoxonase 1)	EC3.1.1.2	Hs.370995; Mm.237657
4. BHMT (betaine:homocysteine methyltransferase)	EC2.1.1.5	Hs.80756; Mm.423099
5. MTHFR (5,10-methylenetetrahydrofolate reductase)	EC1.1.99.15	Hs.214142; Mm.89959
6. AHCY (*S*-adenosylhomocysteine hydrolase or adenosylhomocysteinase)	EC3.3.1.1	Hs.388004; Mm.2203
7. MTR, MS (5-methyltetrahydrofolate:homocysteine methyltransferase or methionine synthase)	EC2.1.1.13	Hs.498187; Mm.40335
8. MARS (mthionyl-tRNA synthetase)	EC6.1.1.10	Hs.632707; Mm.19223
Methylation enzymes		
1. GHMT (glycine *N*-methyltransferase)	EC2.1.1.20	Hs.144914; Mm.29395
2. PEMT (phosphatidylethanolamine *N*-methyltransferase)	EC2.1.1.17	Hs.287717; Mm.2731
3. MAT (methionine adenosyltransferase)	EC2.5.1.6	Hs.282670; Mm.14064
4. BHMT (betaine:homocysteine methyltransferase)	EC2.1.1.5	Hs.80756; Mm.423099
5. MTHFR (5,10-methylenetetrahydrofolate reductase)	EC1.1.99.15	Hs.214142; Mm.89959
6. AHCY (*S*-adenosylhomocysteine hydrolase or adenosylhomocysteinase)	EC3.3.1.1	Hs.388004; Mm.220328
7. MTR, MS (5-methyltetrahydrofolate:homocysteine methyltransferase or methionine synthase)	EC2.1.1.13	Hs.498187; Mm.40335
8. AMD (*S*-adenosylmethionine decarboxylase)	EC4.1.1.50	Hs.159118; Mm.253533
9. DNMT1 (DNA methyltransferase 1)	EC2.1.1.37	Hs.202672; Mm.128580
10. DNMT3a (DNA methyltransferase 3a)	EC2.1.1.37	Hs.515840; Mm.5001
11. ICMT (isoprenylcysteine carboxyl methyltransferases)	EC2.1.1.100	Hs.515688; Mm.277464
12. HMT (histone-lysine *N*-methyltransferase)	EC2.1.1.43	Hs.709218; Mm.35345

Enzyme identification, enzyme commission ID numbers, and NCBI/UniGene ID numbers are from the enzyme nomenclature database (http://www.expasy.ch/enzyme/) and the NIH/NCBI UniGene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db = unigene).

1 were obtained from the enzyme nomenclature database (http://www.expasy.ch/enzyme/) and the National Institutes of Health (NIH)/NCBI UniGene database (http://www.ncbi.nlm.nih.gov/sites/entrez?db=unigene). Four enzymes (enzymes 4 to 7: BHMT, MTHFR, AHCY, and MTR) fall under both Hcy metabolic and methylation enzyme groups, and are therefore listed with identical numbers in both sections of the table.

Data mining and gene expression profiles in human and mouse tissues

An experimental data mining strategy (Fig. 1*B*) was applied to establish the expression profiles of mRNA transcripts of the selected enzymes. The mRNA levels of selected enzymes across 20 human and 19 mouse tissues were examined (Fig. 2*A*) by mining human and mouse expression sequence tag (EST) databases deposited in the NIH UniGene database. The human and mouse tissues were given tissue ID numbers in Fig. 2*A*. The EST database is created *via* cDNA cloning from various tissue cDNA libraries followed by DNA sequencing. Gene mRNA levels are described as gene transcript units per million transcripts (TPM) (19). We generated the relative mRNA expression units (REU) of the gene by normalizing the TPM of the gene of interest with that of β-actin (left-side *y* axis in Figs. 2 and 3*C*). To fairly compare gene expression across selected tissues, we further adjusted by comparison to the median REU (mREU). The mREU was determined from REU in 20 human or 19 mouse tissues selected in this study. The ratio of REU/mREU is expressed as tissue median adjusted mRNA expression units and presented in Figs. 2*B* and 3*B* (right-side *y* axis). To establish a confidence interval of gene expression, we calculated the gene expression REUs and ratio of REU/mREU of 3 randomly selected housekeeping genes [pituitary tumor-transforming 1 interacting protein (PTTG1IP), pyruvate kinase muscle (PKM2), and heterogeneous nuclear ribonucleoprotein K (HNRNPK)] in 20 human or 19 mouse tissues. The mean ratio of REU/mREU of each housekeeping gene was then determined: $x = \Sigma(\mathrm{REU}^{1-20}/\mathrm{median})/20$ for human, and $x = \Sigma(\mathrm{REU}^{1-19}/\mathrm{median})/19$ for mouse. The 3 housekeeping gene mean ratios of REU/mREU were averaged: $X = \Sigma(x^{1-3})/3$. As we described previously (20), the confidence intervals expressing the general variation of housekeeping gene expression were established by calculating the averaged mean $X \pm 2\mathrm{SD}$ of the ratio of REU/mREU of the 3 housekeeping genes (21). Tissue median adjusted mRNA expression levels that surpass the upper limit line of the confidence interval were recognized as high-level expression. Gene expression levels <1 copy of TPM for any given gene were considered as no expression.

Tissue Hcy, SAH, and SAM concentrations

The concentrations of Hcy, SAH, and SAM were determined previously by Ueland *et al.* (16) and Helland and Ueland (18, 22) in tissues from adult male mice under physiological condition. Tissue Hcy concentrations were measured using a radioactive method combined with high-pressure liquid chromatography (HPLC). Briefly, tissue extracts were prepared immediately after sacrificing the animals, deproteinized with perchloric acid, purified with dextran-coated charcoal, and incubated with ^{14}C-adenosine and SAH hydrolase to convert Hcy to ^{14}C-SAH, which is analyzed by HPLC on reversed-phase column (16). The SAM and SAH levels were measured in perchloric acid extracts by HPLC (22). SAM/SAH ratios were calculated for the current analysis based on Helland and Ueland's (18, 22) results. Tissue Hcy, SAH, and SAM concentrations and SAM/SAH ratios were used for further comparison and regression analyses.

Linear regression analysis

Simple linear regression analyses were performed using Sigma Plot 9.0 (Systat Software, Inc, San Jose, CA, USA) by plotting mRNA levels (REU) of individual enzyme against concentrations of Hcy, SAH, SAM, and SAM/SAH ratios in 6 or 7 mouse tissues including the brain, kidney, liver, spleen, heart, lung, and thymus (see Table 2).

Multivariable regression analyses

We performed multivariable regression analyses to evaluate the combined effect of methylation enzymes on tissue methylation regulation. Because tissue concentrations of SAH and SAM are at or below low micromole levels, we intend to predict SAH and SAM levels using gene transcription information. Therefore, methylation enzymes, whose expressions exhibit significant correlation with Hcy metabolites identified from linear regression analyses, were defined as the independent variables, whereas SAH and SAM concentrations were used as the dependent variables. Each variable contains 7 data points (7 mouse tissues). Multivariable regression analyses were performed between multiple independent variables, and each single dependent variable, using Sigma Plot 9.0 (Systat). Finally, significant variables were selected and used for further multivariable regression analyses to establish a finalized multivariable regression equation. The program was set to reject normality and constant variance tests if $P \geq 0.05$.

RESULTS

Hcy metabolic enzymes are differentially expressed in human and mouse tissues

mRNA expression profiles of selected genes in human and mouse tissues are presented in **Figs. 2*B, C*** and **3*B, C***. Confidence intervals expressing the general variations of housekeeping gene mRNA levels were generated based on mRNA levels of 3 housekeeping genes (PTTG1IP, PKM2, and HNRNPK), which have relatively consistent mRNA levels across the selected tissues in both humans and mice. PTTG1IP expression profiles were presented in Fig. 2*A* as representative of housekeeping genes. The confidence intervals of the house gene expression were $X \pm 2SD = 1.31 \pm 1.59$ in human tissue and $X \pm 2SD = 1.34 \pm 2.18$ in mouse tissue, and not significantly different between species. Lines denoting an upper limit of the confidence interval were placed in all gene tissue expression profile bar graphs in Figs. 2 and 3 ($X+2SD=3$ in human tissue; and $X+2SD=3.5$ in mouse tissue) to determine the significance of gene expression. Gene mRNA expression levels higher than the upper limit line of the confidence interval are recognized as high-level expression. Distribution patterns of each individual enzyme appear to differ between human and mouse tissue. Hcy metabolic and methylation enzymes were more abundantly and evenly distributed in human tissue than in mouse tissue across the tissues examined, except for BHMT (Figs. 2*B, C* and 3*B, C*).

Among Hcy metabolic enzymes, CBS transcripts were expressed in most human tissues examined, except for adipose tissue (not shown), adrenal gland (not shown), bone marrow, heart, and thymus. In human tissues (Fig. 2*B*), CBS mRNA was dominantly expressed in muscle and highly expressed in eye, liver, muscle, nerve (not shown), ovary, pituitary gland, and prostate (not shown). In mouse tissues (Fig. 2*C*), on the other hand, CBS mRNA was dominantly expressed in liver, and highly expressed in pancreas and pituitary gland.

CSE mRNA was expressed in most tissues except heart, pituitary gland, spleen, thymus, and vascular tissue. It was dominant in the liver of both humans and mice, and high in human muscle. PON1 was dominantly expressed in the liver of humans and mice, high in mouse muscle, low in brain, liver, lung, pancreas, and testis of humans, as well as mouse eye and lung. BHMT was high in the liver and kidney of humans, but high in the liver and muscle of mice. MTHFR transcripts were expressed in the majority of tissues examined, except adipose tissue (not shown), adrenal gland (not shown), bladder (not shown), connective tissue, thymus, and vascular tissue. MTHFR was dominant in the pituitary gland and high in the lymph node of humans. High levels of MTHFR mRNAs were also found in the pituitary gland, lymph node, and pancreas of mice. AHCY transcripts were expressed in most human tissues except nerve (not shown). It was high in muscle and pituitary gland of humans, while high in pituitary gland and eye of mice. MTR was also high in muscle and pituitary gland of humans, but high in the heart tissue of mice. MARS transcripts were ubiquitously expressed at low levels in tissues examined. The higher expression levels of MARS were found only in muscle tissue in humans, and in the pancreas in mice.

Methylation enzymes are differentially expressed in human and mouse tissues

In human tissues (Fig. 3*B*), both GNMT and MAT had preferential expression in the liver. DNMT1 exhibited ubiquitously low expression in most tissues, but was highly expressed in the lymph node. In comparison, DNMT3a was primarily expressed in embryonic tissue, eye, heart, and muscle. HMT exhibited high expression levels in the muscle. PEMT, ICMT, and AMD exhibited ubiquitous expression across most tissues, with comparatively low expression in the liver. In mouse tissues (Fig. 3*C*), GNMT, MAT, and PEMT had dominant expression in the liver. In addition, GNMT also had a high level of expression in the lymph node. HMT had high expression levels in the eye, lymph node, and pancreas. DNMT1 was primarily expressed in the eye and ovary. DNMT3a had significant expression in the brain, eye, pancreas, and pituitary gland. AMD was highly expressed in the lymph node, muscle, ovary, and pituitary gland. ICMT was high in the eye, lymph node, and pituitary gland.

Hcy is differentially regulated and positively correlated with SAH concentrations in mouse tissues

We summarized tissue concentrations of Hcy, SAH, and SAM in brain, heart, kidney, liver, lung, spleen, and thymus (SAM and SAH only), which were reported

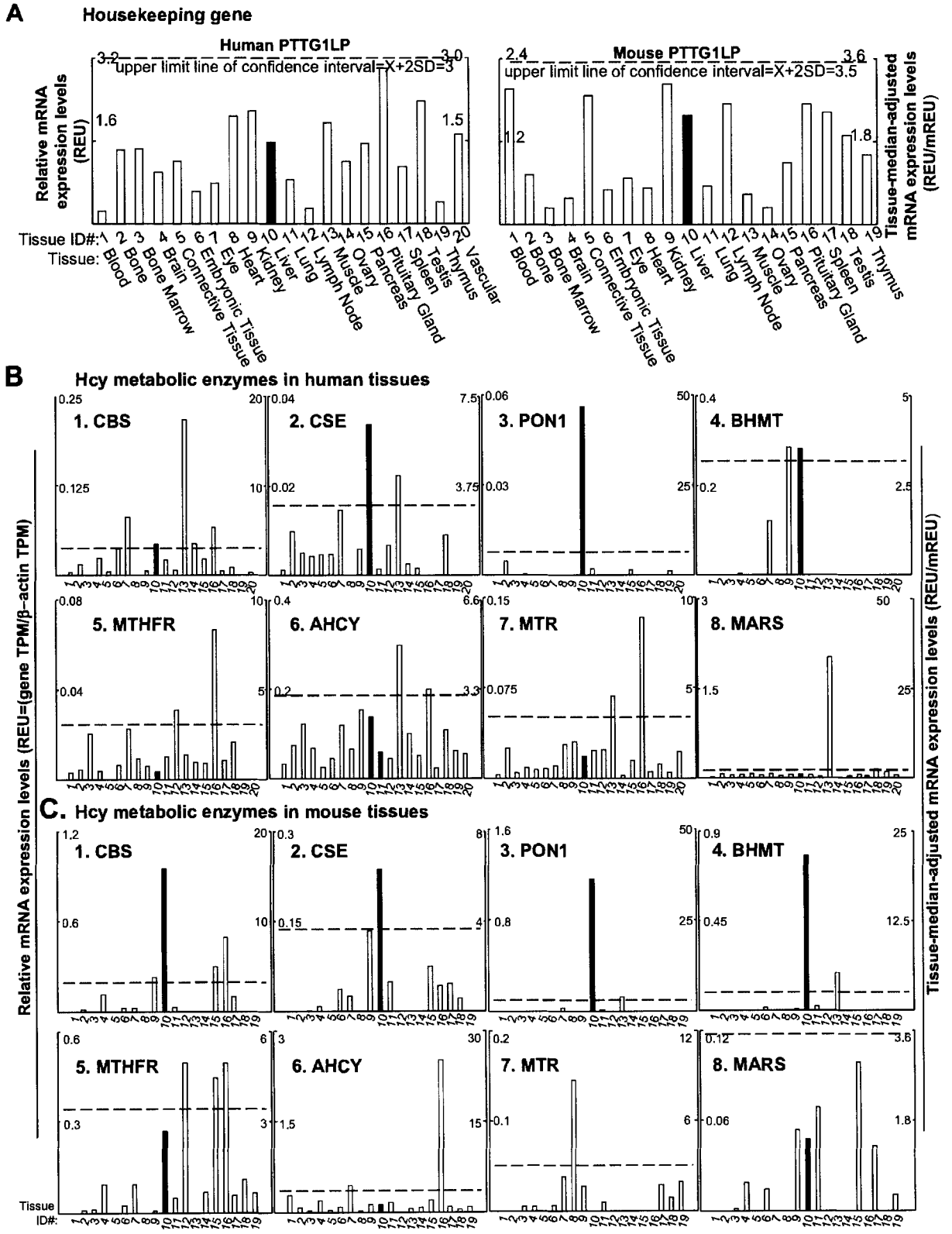

Figure 2. Tissue mRNA distribution profiles of Hcy metabolic enzymes. Twenty human and 19 mouse tissues were given tissue ID numbers and examined for mRNA expression by mining human and mouse EST databases in NCBI/UniGene site. Relative mRNA expression (REU) of the gene is obtained by normalizing gene transcripts per million (TPM) with that of β-actin. Tissue-median-adjusted mRNA expression levels (REU/mREU) were calculated for all genes. Confidence intervals of 3 housekeeping gene mRNA expression were established. Dashed lines are upper limits of the confidence intervals of the housekeeping gene. Left and right *y* axes describe REU and REU/mREU, respectively. Solid bars highlight

(*continued on next page*)

previously (16, 18). As demonstrated in **Table 2**, the concentrations of Hcy, SAH, and SAM varied across mouse tissues. Liver Hcy concentrations was 3.63 nmol/g, 4.1-fold greater than the concentrations found in the brain and spleen (0.89 nmol/g), where the lowest Hcy levels were observed. The Hcy concentration tissue gradient was liver > kidney > heart > lung > spleen/brain. SAH concentrations were more variable. The SAH level in the liver was 25.5 nmol/g, which was 63.7-fold greater than the concentration in the heart (0.4 nmol/g) .Tissue SAH levels also had a different concentration gradient in the order of liver > lung > kidney > spleen > thymus ≫ brain > heart. SAM was differentially distributed and had a tissue distribution pattern of liver > kidney > spleen > heart ≫ lung > thymus > brain. The liver SAM level was 112.8 nmol/g, 3.15-fold greater than the concentration in the brain (35.8 nmol/g). Furthermore, Hcy concentrations positively correlated with SAH levels in the tested mouse tissues (**Fig. 4**). Tissue Hcy and its metabolites have not been examined in normal human subjects and have only been tested in a very few rodent studies (17, 23, 24). Mouse tissue Hcy measured by Ueland *et al.* (16) corroborated that measured by Svardal *et al.* (17) in the same tissues from rats, with the exception of dissecting the brain into two parts (cerebrum Hcy 0.78 nmol/g and cerebellum Hcy 5.15 nmol/g). Mouse tissue SAM and SAH concentrations were also examined by the Choi group (23) and the James group (24) and exhibited identical concentration gradient, liver > kidney > brain, for both SAM and SAH. However, probably due to the technique variations, these studies (23, 24) presented concentration ranges for both SAM (19.1–112.8 nmol/g in liver, 13.51–107.4 nmol/g in kidney, and 8.7–34.9 nmol/g in brain), and SAH (20.6–25.5 nmol/g in liver, 5.0–24.5 nmol/g in kidney, and 0.2–15.1 nmol/g in brain). In this study, we used Hcy, SAH, and SAM data generated by Ueland's group (16, 18, 22) for the consideration of methodology consistency. In addition, the radioactive HPLC method used by Ueland's group provided the best sensitivity for measuring relatively low levels of Hcy in the tissue.

Transcripts of 5 Hcy metabolic enzymes (CBS, CSE, PON1, BHMT, and MTHFR), and 3 methylation enzymes (GNMT, PEMT, and MAT) are significantly correlated with tissue Hcy levels

We assessed the correlation between transcripts (REU) of Hcy metabolic/methylation enzymes and Hcy levels in 6 mouse tissues, including kidney, liver, brain, heart, lung, and spleen, to identify the relationship between enzyme expression and Hcy concentrations in these tissues. We found that the tissue mRNA levels of 5 Hcy metabolic enzymes, CBS, CSE, PON1, BHMT, and MTHFR, were positively correlated with tissue Hcy levels, with regression constants of 0.95, 0.98, 0.97, 0.98, and 0.78, respectively (**Fig. 5*A***). Similarly, transcripts of the methylation enzymes GNMT, PEMT, and MAT were well correlated with tissue Hcy levels, with regression constants of 0.98, 0.98, and 0.97 (Fig. 5*B*). In contrast, transcripts of the Hcy metabolic enzymes AHCY, MARS, and MTR (Fig. 5*A*) and the methylation enzymes AMD, DNMT1, DNMT3a, ICMT, and HMT (Fig. 5*B*) were not correlated with tissue HCY levels. We classified those enzymes, whose transcripts positively correlated with tissue Hcy levels, as type 1 enzymes, and those that did not exhibit correlation as type 2 enzymes (Fig. 5).

Transcripts of methylation enzymes GNMT, PEMT, MAT, BHMT, and MTHFR are significantly correlated with tissue SAH levels

SAM and SAH are metabolites of Hcy. SAM is the substrate and SAH is the product of methyltransferase reactions. SAM and SAH levels are important metabolic indicators of cellular methylation status (25). To determine the regulatory effect of methylation enzymes on tissue SAM and SAH metabolism, as well as tissue methylation status, we performed regression analysis with mRNA expression levels (REU) of individual enzymes against SAH, SAM levels, and SAM/SAH ratios in 7 mouse tissues (Table 2) (18). The correlations between methylation enzyme mRNA levels and tissue SAH levels exhibited similar patterns as that between methylation enzymes and Hcy, as shown in **Fig. 6*A***. Five methylation enzymes (GNMT, PEMT, MAT, BHMT, and MTHFR) exhibited positive correlations between tissue mRNA and SAH levels. In addition, BHMT and MTR mRNA levels were positively correlated with SAM concentrations and SAM/SAH ratios, respectively (Fig. 6*B, C*). AHCY expression did not vary with the change in tissue Hcy, SAH, and SAM concentrations. Enzymes that positively correlated with tissue SAH concentrations overlapped with those that correlated with Hcy concentrations described previously and were therefore classified as type 1 enzymes. Those methylation enzymes that did not correlate with SAH concentrations were classified as type 2 enzymes (Fig. 6*A*). The type 2 enzymes correlate with neither tissue Hcy nor tissue SAH concentrations. Interestingly, among all methylation enzymes, BHMT is the only one that positively correlated with SAM levels, and MTR is the only one that correlated with SAM/SAH ratios (Fig. 6*B, C*).

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gene expressions in the liver. Enzyme abbreviations are listed in Table 1. *A*) Representative tissue mRNA distribution profile of housekeeping gene PTTG1LP in human and mouse. See Methods and Materials for details. *B*) mRNA distribution profiles of 8 Hcy metabolic enzymes in 20 human tissues. *C*) mRNA distribution profiles of 8 Hcy metabolic enzymes in 19 mouse tissues.

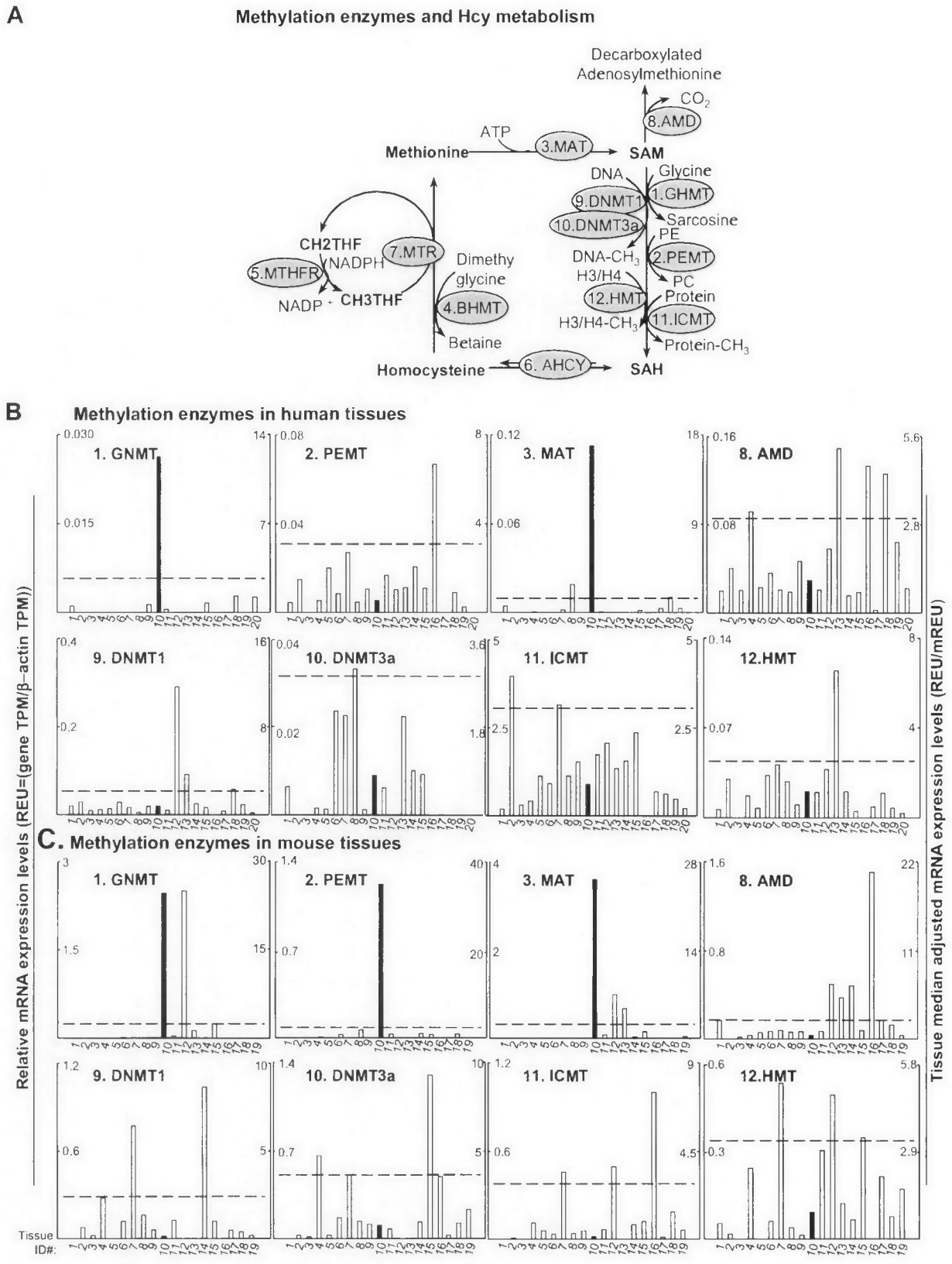

Figure 3. Methylation enzymes and tissue expression profiles. *A*) Diagram of methylation-related genes in Hcy metabolism. Twelve methylation enzymes were selected for database mining and expression profile analysis. THF, tetrahydrofuran; CH_2THF, 5-methylenetetrahydrofolate; CH_3THF, 5-methyltetrahydrofolate; NADPH, reduced form of $NADP^+$; $NADP^+$, nicotinamide adenine dinucleotide phosphate; Met, methionine; Hcy, homocysteine; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; H3/H4, histone 3/histone 4. Enzyme abbreviations are listed in

(*continued on next page*)

TABLE 2. *Hcy metabolite concentrations in mouse tissue*

Tissue	Metabolite concentration (nmol/g wet wt)			
	Hcy[a]	SAM[b]	SAH[b]	SAM:SAH
Brain	0.89 ± 0.09	35.8 ± 4.0	0.8 ± 0.1	47.1 ± 9.6
Heart	1.12 ± 0.10	58.5 ± 4.2	0.4 ± 0.3	142.7 ± 87.6
Kidney	1.29 ± 0.11	107.4 ± 5.5	4.2 ± 0.8	25.6 ± 5.0
Liver	3.63 ± 0.89	112.8 ± 12.4	25.5 ± 3.9	4.4 ± 0.8
Lung	1.02 ± 0.12	47.7 ± 3.6	5.5 ± 0.9	8.6 ± 1.5
Spleen	0.89 ± 0.09	65.2 ± 8.1	1.7 ± 0.4	38.4 ± 10.2
Thymus	NA	41.3 ± 11.5	1.2 ± 0.3	34.7 ± 13.6

Concentrations of Hcy, SAH, and SAM in mouse tissues were previously examined by Ueland *et al.* (16) and Helland and Ueland (18) using HPLC. [a]Details in Lee and Wang (11). [b]Details in Perna *et al.* (15).

MTHFR and MTR are negatively correlated with SAH, but GNMT and PEMT are positively correlated with SAH levels in mouse tissues

We selected 7 enzymes, of 12 analyzed, whose transcripts exhibited significant positive correlations with SAH, SAM, or SAM/SAH ratio, through linear regression analyses, and performed multivariable regression analyses to delineate the regulatory relationship between enzyme mRNA levels and SAH, SAM, or SAM/SAH ratios. Three multivariable regression equations were established (**Fig. 7*A***). Significant negative correlations were identified for MTHFR and MTR (P=0.042 and P=0.033), and positive correlations were identified for GNMT, PEMT, and MAT (P=0.013, collinear and interchangeable) with SAH (Fig. 7*A*, Eq. 1). The coefficients, which indicate the strength of the correlation between enzymes and SAH, appeared to be the strongest for MTHFR (99.39), less so for MTR (67.73), and mild for GNMT (18.60). A significant positive correlation was found between MTR (P=0.024) and SAM/SAH ratio (Fig. 7*A*, Eq. 3). After eliminating nonsignificant variables based on Pvalues, a working model of enzymatic methylation regulation was established and presented as a finalized multivariable regression equation (Fig. 7*B*): SAH = 6.869 − (59.267·MTHFR) − (57.087·MTR) + (28.318·GNMT). A schematic representation of this regulation is shown in Fig. 7*C*.

DISCUSSION

Hcy metabolism involves multiple enzymes, and its metabolites SAM and SAH are key modulators of cellular methylation. It is well documented that enzymatic regulation determines Hcy metabolism, as >100 HHcy-related human disorders are associated with CBS or MTHFR mutations (2, 26, 27). Moreover, cofactors of CBS and MTR (vitamins B6, B12, and folate) lower Hcy levels in patients with HHcy (27, 28). We previously reported that Hcy causes cell-type-specific hypomethylation in endothelial cells (4, 5) and hypothesized that Hcy metabolism and methylation may be differentially regulated in tissues, resulting in tissue-specific pathology in HHcy. The goal of the present study was to establish tissue expression profiles of Hcy metabolic and methylation enzymes and to identify key enzymes that contribute to tissue-specific regulation of Hcy metabolism and methylation.

We now report 4 major findings from our study: *1*) Hcy metabolic and methylation enzymes are differentially expressed in human and mouse tissues; *2*) for all enzymes examined, the enzyme transcript tissue distribution profiles of mice exhibit different patterns than those of humans; *3*) 5 Hcy metabolic and 5 methylation type I enzymes are positively correlated with tissue Hcy and SAH levels, respectively; and *4*) Hcy-related methylation is delineated by the correlations between SAH concentration and the transcripts of MTHFR, MTR, GNMT, PEMT, and MAT, which can be expressed using a multivariable regression equation (Fig. 7*B*).

We have developed a database-mining approach combined with statistical analysis to evaluate the regulatory relationship between Hcy metabolic/methylation enzyme gene expression and its metabolites using existing data sets. The EST gene expression data and the metabolic data were derived from different animals under physiological conditions. Hypotheses generated from this study shall be tested by future experiments in humans and animals under physiological and pathological conditions and should provide impor-

Table 1. *B*) mRNA distribution profiles of methylation enzymes in human tissues. *C*) mRNA distribution profiles of methylation enzymes in mouse tissues. Expression profiles of 4 of 12 methylation enzymes listed in Table 1 (enzymes 4–7: BHMT, MTHFR, AHCY, and MTR) are omitted because they are included in the Hcy metabolic enzyme group and presented in Fig. 2*B, C*. Dashed lines are the upper limits of the confidence intervals of the housekeeping genes. Solid bars highlight gene expressions in the liver. Left and right *y* axes describe REU and REU/mREU, respectively. Enzyme abbreviations are listed in Table 1.

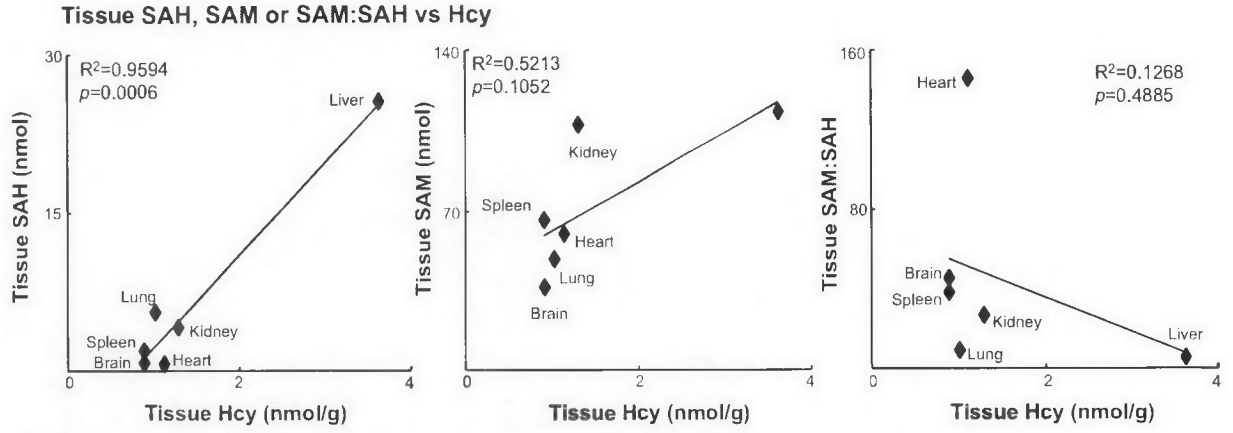

Figure 4. Correlation of tissue Hcy levels with SAH and SAM concentrations and SAM/SAH ratios in mice. Note that tissue Hcy levels are positively correlated with SAH levels.

tant insights into tissue Hcy metabolic and methylation regulation.

Gene expression was assessed through a database-mining approach utilizing the NCBI/UniGene database. The EST database has been generated *via* the continued efforts of an international consortium over the past 20 yr, based on random selection of sequencing cDNA libraries (19). It is a highly recognized and reliable resource for gene mRNA expression in normal tissues. EST represents the averaged values of different data entries and describes gene mRNA levels as the copy number of EST cDNA clones per 10^6 clones sequenced. We believe that the EST database has significant advantages over traditional methods of evaluating gene mRNA expression levels (Northern blot, dot blot, reverse transcription-polymerase chain reaction (RT-PCR), and real-time PCR), which involve RNA/DNA binding to DNA probes/primers and may introduce nonspecificity and inaccuracy.

Of all the tissues examined, liver plays an important role in the systemic regulation of Hcy metabolism (29–31). Interestingly, we found that hepatic enzyme expression differs between humans and mice, suggesting that the two species rely on systemic Hcy control to different degrees. Only CSE, PON, GNMT, and MAT exhibit liver-dominant expression patterns in humans. In comparison, more enzymes, including CBS, CSE, PON1, BHMT, GNMT, PEMT, and MAT exhibit liver-dominant expression patterns in mice (Figs. 2*B* and 3*A*). This pattern suggests that humans possess a higher level of tissue-specific regulation of Hcy metabolism, whereas mouse Hcy metabolism is dominantly controlled by the liver. This hypothesis is supported by tissue profiles of CBS, the primary Hcy clearance enzyme. We identified more tissues expressing CBS, the primary Hcy clearance enzyme, than previously reported (27) for both mice and humans. Among human tissues, CBS mRNA levels are higher in the eye, muscle, and pituitary gland than in the liver. This suggests that unlike mice, in which CBS is predominantly expressed in the liver, human Hcy metabolism requires tissue-specific regulation. It also may be of clinical relevance that homocystinuria, a multisystem disease caused by CBS deficiency, predominantly manifests in serious eye anomalies, including lens dislocation, myopia, glaucoma, and optic atrophy (32). Similarly, MTHFR and MTR, both of which are folate cycle enzymes, do not have a liver-dominant expression pattern in either mice or humans (Fig. 2*B, C*). These results suggest that folic acid metabolism is highly regulated at the tissue level for both species. Congruent with previous reports (33), however, we found that the expression of BHMT, a crucial enzyme that catalyzes the transformation of Hcy to methionine, is limited to liver, kidney, and eye in humans and to liver and muscle in mice (Figs. 2*B, C*). This aspect of Hcy metabolism is highly liver dependent for both humans and mice.

On the basis of linear regression analysis findings presented in Fig. 6, we classified Hcy metabolic and methylation enzymes into two types. Type 1 enzymes include Hcy metabolic enzymes CBS, CSE, PON1, BHMT, and MTHFR, as well as methylation enzymes GNMT, PEMT, and MAT, whose transcripts display positive correlations with tissue Hcy levels. Type 2 enzymes include Hcy metabolic enzymes AHCY, MTR, and MARS, and methylation enzymes AMD, DNMT1, DNMT3a, ICMT, and HMT, which do not correlate with tissue Hcy levels. Data suggest that the type 1 enzymes are sensitive to changes in Hcy concentrations and play important regulatory roles in tissue Hcy metabolism. Furthermore, among the 5 type 1 enzymes, spontaneous HHcy has been associated with homozygous gene deficiency of CBS (plasma Hcy ~200 μM) (34), MTHFR (plasma Hcy 33 μM) (3), and CSE (plasma Hcy 18 μM) (35), as well as inhibition of BHMT activity (plasma Hcy 18.5 μM) (36) in mouse models but has not been observed in mice deficient in PON1 (37). (As a point of reference, the plasma Hcy level in the control wild-type mouse is 3–5 μM). Hcy levels are regulated by enzyme expression levels. These data suggest that the expressions of type 1 enzymes all influence plasma Hcy levels and further support our hypothesis that Hcy metabolism is coordinated by multiple enzymes in a tissue-specific manner.

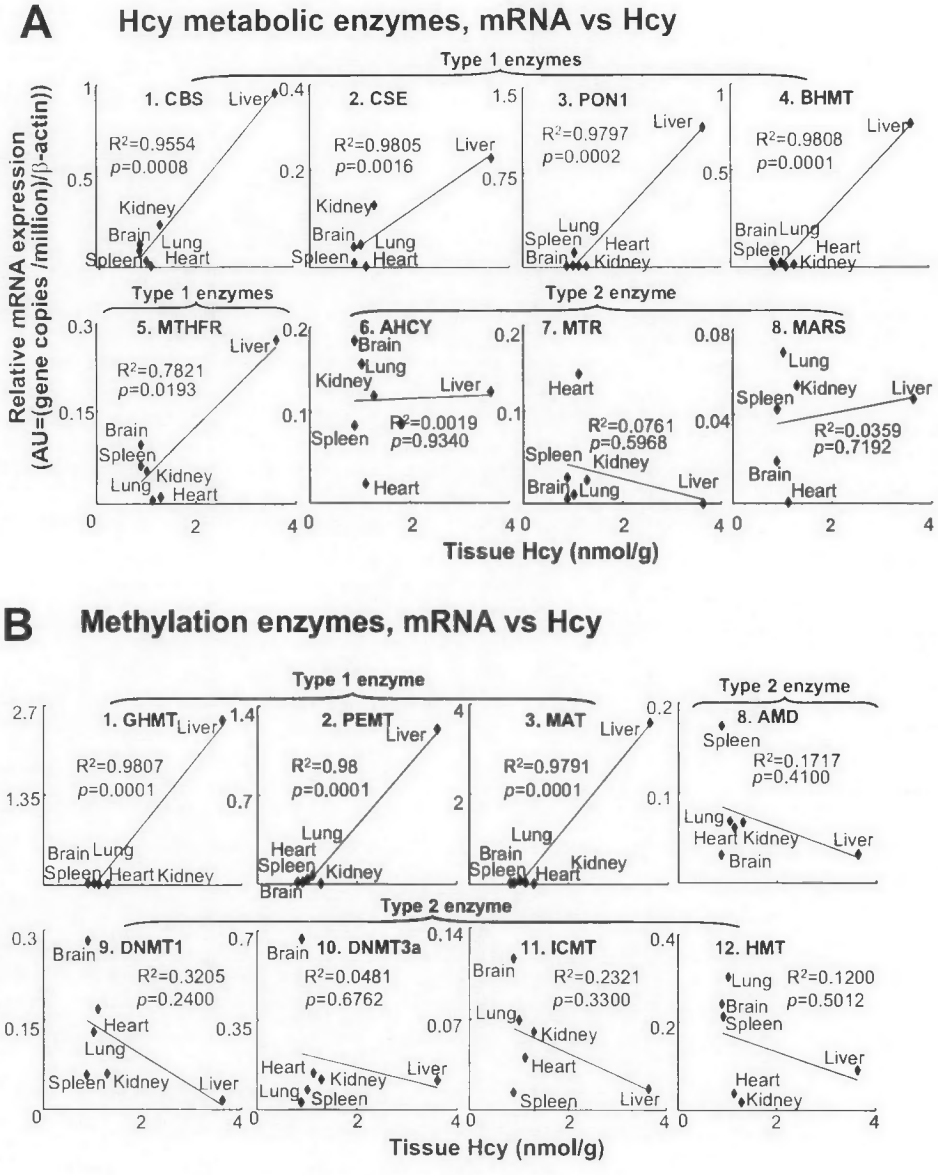

Figure 5. Correlations of Hcy metabolic enzyme expression with Hcy levels in tissues. Relative expression of selected enzymes was determined as described in Fig. 2 and expressed by relative mRNA expression levels (REU). *A*, *B*) Correlation between mRNA levels of Hcy metabolic enzymes (*A*) and methylation enzymes (*B*), and Hcy levels in mouse tissues. Tissue relative expressions of enzyme mRNA were plotted against tissue Hcy levels shown in Table 1. Linear regression analyses were performed using data points from 7 mouse tissues. Genes are classified into 2 types. Type 1 enzymes exhibited positive correlation between gene expression and Hcy levels in selected tissues. Type 2 enzymes do not correlate with tissue Hcy concentration. Enzymes 4–7 (BHMT, MTHFR, AHCY, and MTR) are members of both groups. Enzyme abbreviations are listed in Table 1.

Moreover, we identified significant positive correlations between 5 methylation enzymes (GNMT, PEMT, MAT, BHMT, and MTHFR) and SAH, between BHMT and SAM, as well as between MTR and SAM/SAH ratios (Fig. 6). The positive correlation patterns between the 5 methylation enzyme transcripts and SAH levels (Fig. 6*A*) are consistent with their correlation with Hcy concentrations (Fig. 5*B*). We classified these 5 enzymes as type 1 enzymes, which have significant positive correlations with tissue Hcy and SAH production. More interestingly, we found that tissue Hcy levels positively correlated with SAH levels, but not with SAM concentrations or SAM/SAH ratios (Fig. 4). Taken together, our data indicate that Hcy metabolic product SAH functions as the primary predictor of tissue-specific methylation. This observation is consistent with previous report on SAH as the principal indicator of cellular methylation status (25). In a cohort of young healthy women, the plasma Hcy correlated strongly with plasma SAH level, but not with either SAM level or SAM/SAH ratio. In the same patient group, plasma SAH and lymphocyte DNA hypomethylation exhibited significant correlation (38). Furthermore, in $CBS^{-/+}$ mice fed a methyl-deficient diet for 24 wk, an increase in SAH alone affected tissue DNA methylation in liver, kidney, brain, and testes, while SAM level did not correlate significantly with tissue DNA methylation status (24).

The multivariable regression analyses evaluated correlation of multiple enzyme mRNA expression with Hcy metabolites. This method simulated the biological environment, in which all examined methylation enzymes are present simultaneously. We found that the tissue expressions of MTHFR and MTR were negatively correlated with tissue SAH, but GNMT, PEMT, and MAT were positively correlated with tissue SAH levels (Fig. 7*B*). This multivariable equation suggests a dual directional regulatory relationship between gene expression and Hcy metabolites. Changes in enzyme mRNAs alter tissue SAH

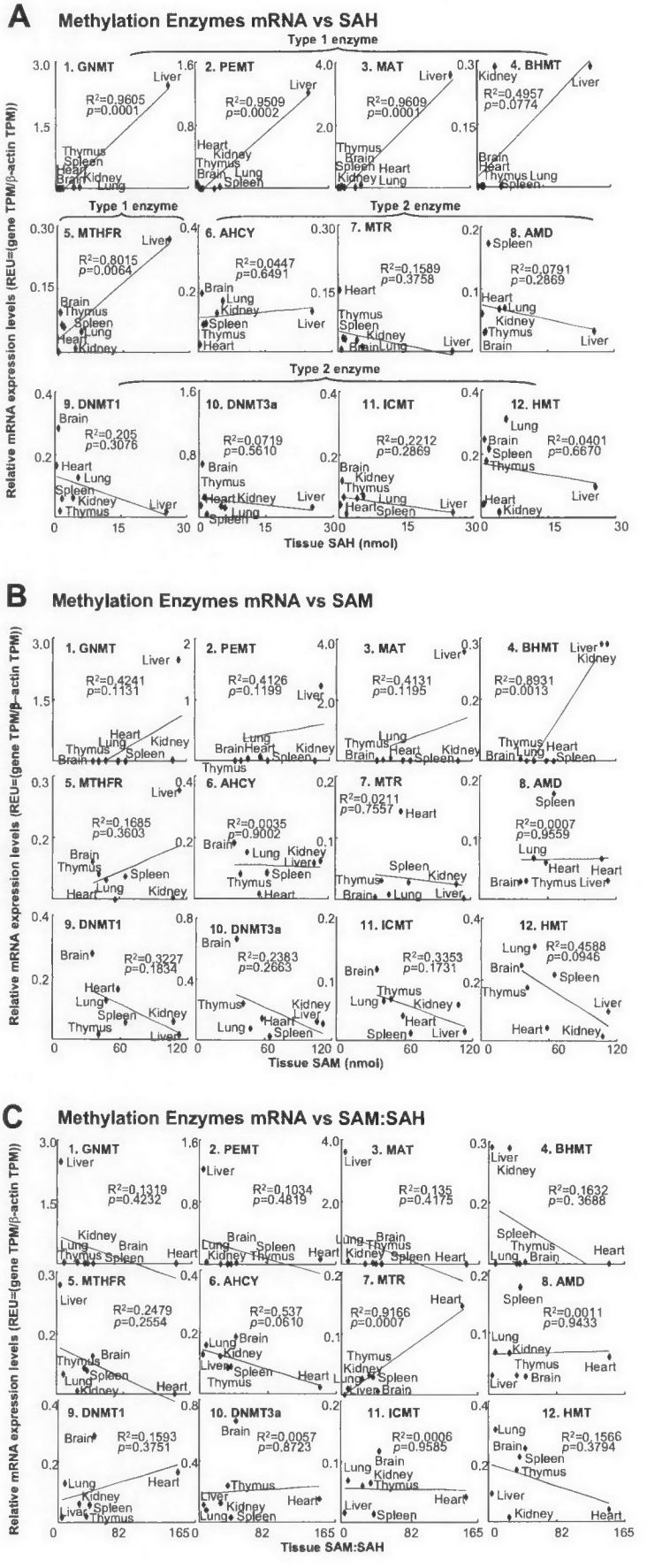

Figure 6. Correlation between methylation enzyme transcripts and Hcy, SAM, and SAH concentrations and SAM/SAH ratio. Linear regression analyses were performed between enzyme mRNA levels (Fig. 3*C*) and Hcy, SAM, and SAH concentrations, as well as SAM/SAH ratios (Table 2) in 7 mouse tissues. *A*) Correlations between enzyme mRNAs and SAH levels. *B*) Correlations between enzyme mRNA and SAM levels. *C*) Correlations between enzyme mRNAs and SAM/SAH ratio. Genes are classified into 2 types. Expression of type 1 enzymes is positively correlated with tissue SAH levels; that of type 2 enzymes is not. Enzyme abbreviations are listed in Table 1.

A **Multivariable regression equations**

1. SAH =10.106-(99.392*MTHFR)-(67.733*MTR)+(18.606*GNMT)-(12.863*BHMT)
   P values: BHMT: 0.121; GNMT: 0.013; MTHFR: 0.042; MTR: 0.033
   Adj Rsqr: 0.994
2. SAM =69.274-(63.126*MTR)-(321.446*MTHFR)+(35.816*GNMT)+(145.559*BHMT)
   P values: BHMT: 0.258; GNMT: 0.472; MTHFR: 0.502; MTR: 0.814
   Adj Rsqr: 0.821
3. SAM:SAH =-44.445-(79.135*GNMT)+(790.588*MTHFR)+(1292.410*MTR)+(109.110*BHMT)
   P values: GNMT:0.153; MTHFR: 0.147; MTR:0.024; BHMT: 0.307
   Adj Rsqr: 0.944

GNMT, PEMT and MAT are collinear and replaceable

B **Finalized multivariable regression equation**

SAH = 6.869-(59.267*MTHFR)-(57.087*MTR)+(28.318*GNMT)
P values: GNMT: 0.002; MTHFR: 0.030; MTR: 0.014
Adj Rsqr: 0.990

GNMT, PEMT and MAT are collinear and replaceable

C **Working model; enzymatic methylation regulation**

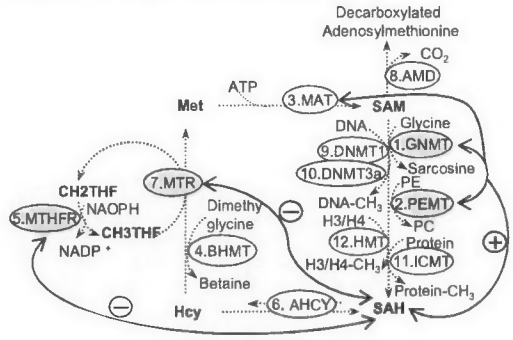

Figure 7. Multivariable regression analysis of methylation enzymes. *A*) Multivariable regression equations. Multivariable regression analyses are performed between mRNA levels of 6 methylation enzymes (MTHFR, GNMT, BHMT, PEMT, MTR, MAT) and the SAH and SAM concentrations or SAM/SAH ratio. These enzymes were significantly correlated with tissue SAH and SAM concentrations or SAM/SAH ratio in Fig. 6. *B*) Finalized multivariable regression equation. Five methylation enzymes (MTHFR, GNMT, PEMT, MTR, and MAT) and SAH were selected for multivariable regression analysis based on significance identified in *A* to establish the finalized multivariable regression equation. *C*) Working model; enzymatic methylation regulation. Arrow line with positive sign indicates positive correlation between enzymes (MAT, GNMT, and PEMT) mRNA and SAH levels identified from multivariable regression equation in *B*. Arrow line with negative signs indicates reversed correlation between enzyme (MTR and MTHFR) mRNA and SAH levels. Abbreviations of enzymes and other molecules are shown in Fig. 3*A* legend.

levels, and *vice versa*. This equation underscores the biochemical functions of the enzymes. MAT, GNMT, and PEMT promote the production of tissue SAH, while MTHFR and MTR facilitate the removal of tissue SAH. Consistent with this hypothesis, a positive relationship between SAH and GNMT was observed in $GNMT^{-/-}$ mice (39). Similarly, MAT expression and SAH concentration were both increased in the liver of rats after partial hepatectomy (40). PEMT activity positively correlated with plasma SAH in mice (41). On the other hand, multiple experimental data also demonstrated a direct effect from SAH on the expression and activities of methyltransferases. SAH inhibited DNA methylation of rat liver nuclei (42), suppressed DNMT1 activities in mouse erythroleukemia cell extracts (43), inhibited PEMT activities in rat and guinea pig liver microsomes (44) and in rabbit adrenal gland extracts (45), and reduced GNMT activities in rabbit liver extracts (46). Consistent with the negative correlation of MTHFR with SAH suggested by our equation, increased SAH levels were observed in $MTHFR^{-/+}$ mice (47). Moreover, elevated GNMT activities/expression and reduced MTHFR activities were accompanied with elevation in plasma Hcy in diabetic mice (48–50), as well as diabetic patients (51). It has been suggested that GNMT elevation and MTHFR reduction may exacerbate HHcy among diabetic patients (52).

In general, enzyme function is described as enzyme activities and mostly determined by protein levels. Changes in mRNA expression may not be the directly indicative of changes in protein and enzyme activity. However, the significant correlations between MTHFR, MTR, GNMT, PEMT, MAT, and SAH identified by both linear and multivariable regression analyses in Figs. 6*A* and 7*B* suggest that at least for these 5 genes, transcriptional regulation most likely determines their function and directly modulates SAH production. For the similar consideration, the type 1 enzymes significantly correlated with tissue Hcy levels identified by linear regression analyses in Fig. 5*A* may be regulated at the transcriptional levels as well.

In summary, we demonstrated, for the first time, a panoramic tissue gene expression profile of 8 Hcy metabolic and 12 methylation enzymes in 20 human and 19 mouse tissues. We identified 5 Hcy metabolic and 5 methylation enzymes as the type 1 enzymes, whose transcription displays significant correlation with tissue Hcy and SAH concentrations. We also established a multivariable regression equation depicting the relationship between methylation enzyme mRNA and tissue SAH levels. The gene expression profiles and the mathematical equation presented here should provide important insights for future metabolic and mechanistic studies to identify molecular targets for treating HHcy and hypomethylation-related human disease. The significant correlation between folate cycle enzymes and tissue SAH levels support the hypothesis that folate cycle modulation is a valid therapeutic strategy for treating HHcy and hypomethylation-related pathology. This is consistent with numerous findings from Hcy-lowering clinical trials, which demonstrated that folate therapy is the most effective or the only effective Hcy-lowering treatment (27, 53–55). Furthermore, the tissue-specific nature of folate cycle metabolism suggests that folate supplementation targeting on isolated pathological sites could enhance the efficacy and eliminate the associated systemic side effects.

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Hyperhomocysteinemia and Cardiovascular Risk: Effect of Vitamin Supplementation in Risk Reduction

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Abstract: Homocysteine is a sulfur-containing aminoacid produced during metabolism of methionine. Since 1969 the relationship between altered homocysteine metabolism and both coronary and peripheral atherotrombosis has been known; in recent years experimental evidences have shown that elevated plasma levels of homocysteine are associated with an increased risk of atherosclerosis and cardiovascular ischemic events. Several mechanisms by which elevated homocysteine impairs vascular function have been proposed, including impairment of endothelial function, production of Reactive Oxygen Species (ROS) and consequent oxidation of low-density lipids. Folic acid and B vitamins, required for remethylation of homocysteine to methionine, are the most important dietary determinants of homocysteinemia and daily supplementation typically lowers plasma homocysteine levels. Recently, large-scale intervention trials have been conducted to determine whether lowering homocysteine concentrations through B vitamins supplementation can decrease cardiovascular risk in healthy subjects or improve survival in patients with coronary heart disease. Some of these trials found no significant beneficial effects of combined treatment with folate and vitamin B_{12}, with or without vitamin B_6, in spite of adequate homocysteine lowering. In conclusion, it is still unclear whether decreasing plasma levels of homocysteine through diet or drugs may be paralleled by a reduction in cardiovascular risk.

Key Words: Homocysteine, MTHFR, cardiovascular disease, folate, B vitamins.

METABOLISM OF HOMOCYSTEINE

Homocysteine is a sulphur-containing aminoacid produced in the metabolism of the essential aminoacid methionine. Homocysteine can be produced by two different pathways: the trans-sulfuration pathway and the remethylation one. Fig (**1**). In remethylation, homocysteine acquires a methyl group from methyltethrahydrofolate (MTHF) to form methionine in a vitamin B_{12}-dependent reaction. The formation of the methyl donor MTHF occurs in all tissue and it depends on the presence of methylentethrahydrofolate, derived from dietary folate, and methylentethrahydrofolate reductase (MTHFR). Methionine is then activated by ATP to form S-adenosylmethionine (SAM) which is an universal methyl donor. The by-product of methylation is S-adenosylhomocysteine which is subsequently hydrolyzed regenerating homocysteine. In the transulfuration pathway, homocysteine condenses with serine to form cystathionine in an irreversible reaction catalyzed by cystathionine β-synthase (CBS). Cystathionine is hydrolyzed to form cysteine, the excess of which is excreted in the urine. Thus, the transulfuration pathway effectively catabolyzes the excess of homocysteine which is not required for methyl transfer. Homocysteine exceeding the metabolic requests of the cell, is exported in the extracellular space by an export mechanism [1]; actually, this export mechanism, together with transulfuration pathway, helps maintaining low intracellular concentration of this potentially cytotoxic sulfur amino acid, but leaves vascular tissue exposed to the possible deleterious effects of excess homocysteine. The occurrence of hyperhomocysteinemia indicates that homocysteine metabolism has been disrupted in some way and that the export mechanism is disposing into the blood excess homocysteine. Homocysteine is present in plasma in four forms: about 1% circulate as the free thiol; 70-80% is disulfide-bound to plasma proteins, chiefly albumin; the remaining 20-30% combines with itself to form the homocysteine dimer or with other thiols, including cysteine, forming the homocysteine-cysteine mixed disulfide [2]. The term "total plasma (or serum) homocysteine" (tHcy) refers to the combined pool of all four forms of homocysteine. The determination of tHcy can be performed in a fasting state or after methionine load; post-load tHcy is probably more sensitive than the fasting tHcy to disturbances in the transulfuration pathway such as those caused by CBS or vitamin B_6 deficiency [3].

CAUSES OF HYPERHOMOCYSTEINEMIA

Determinants of plasma homocysteine include genetic, physiologic and lifestyle factor, various disease and drugs (Table **1**) [4]. Among genetic factors, congenital homocystinuria, a rare congenital metabolic disease caused by a mutation in the gene encoding for CBS, is the most severe cause of hyperhomocysteinemia. This condition is unquestionably associated with precocious atherosclerosis and extensive arterial thrombosis. Other genetic causes of severe hyperhomocysteinemia include homozygous deficiency of MTHFR, deficiency of methionine synthase and methionine synthase reductase [5]. The most common genetic defect associated with moderate hyperhomocysteinemia is the point mutation

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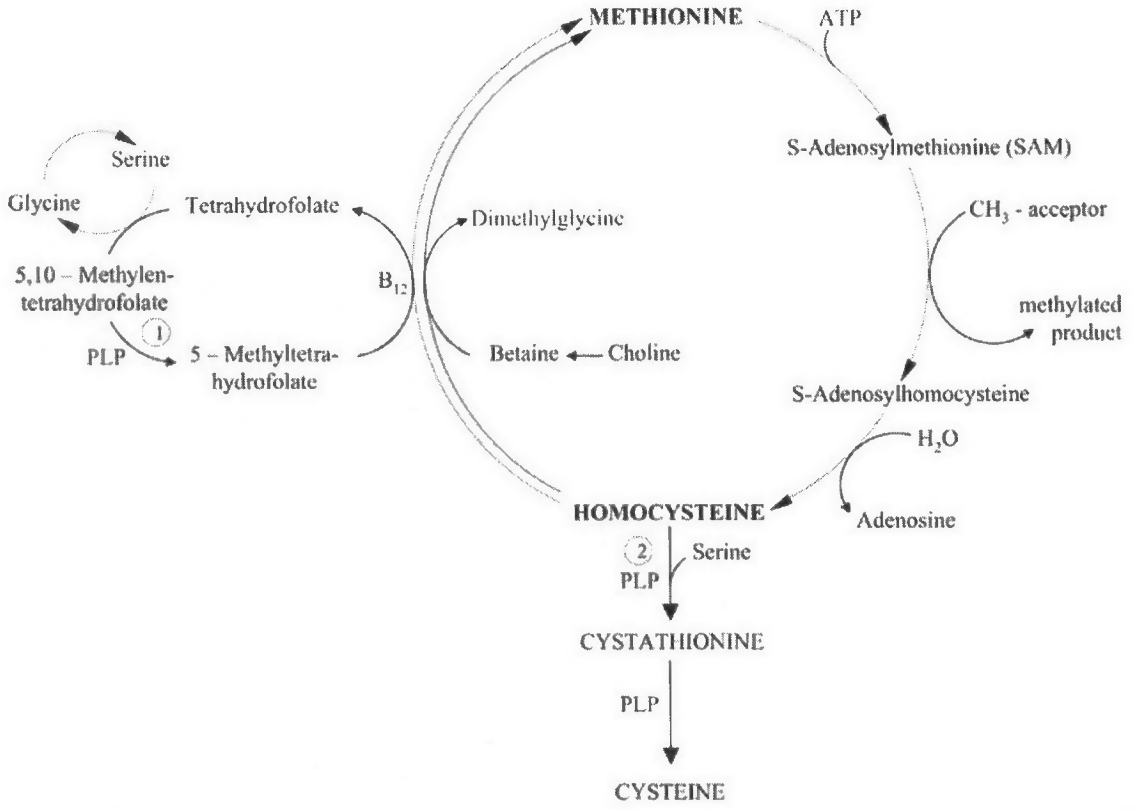

Fig. (1). Homocysteine metabolism pathways. 1: methyltetrahydrofolate reductase (MTHFR); 2: cystathionine β-synthase (CBS); PLP: pyridoxalphosphate (active form of vitamin B_6).

C677T in the gene encoding for MTHFR enzyme: this substitution is associated with a thermolabile variant of the enzyme which has about half of the normal activity [6, 7]. Individuals with the genotype 677TT usually have about 25% higher homocysteinemia than those with the CC genotype [8], but the increase depends on folate status [9]. One of the most relevant cause of hyperhomocysteinemia is folate and cobalamin deficiency, which is usually a result of poor diet, such as in vegetarians, malabsorption, alcoholism, or use of certain drugs. It is also common during pregnancy and in the elderly. Vitamin deficit predisposes to hyperhomocysteinemia with an inverse relationship [10-14]. The independent association between nutrients and homocysteinemia was studied in The Framingham Heart Study: after controlling for age, sex, and other vitamins, plasma homocysteinemia exhibited a strong, nonlinear inverse correlation with plasma folate and a weaker inverse association with vitamin B_{12} and B_6 [15]. Several clinical conditions are associated with hyperhomocysteinemia: one of the most significant is renal failure. There is an inverse relationship between homocysteinemia and renal function, ranging from normal renal function to End Stage Renal Disease (ESRD) [16]. Most dialysis patients have hyperhomocysteinemia and folic acid supplementation efficiently reduces homocysteinemia in these patients [17].

HYPERHOMOCYSTEINEMIA AND CARDIOVASCULAR DISEASE

The hypothesis that hyperhomocysteinemia has a pathogenic role in vascular disease arises from several cases of homocystinuria observed by McCully: subjects affected by homocystinuria, have a higher risk of premature, frequently fatal, thromboembolic events and autopsy evidence of vascular disease [18]. Since these seminal observations, results from a large number of clinical and epidemiologic investigations have implied a role for homocysteine in atherosclerotic cardiovascular disease (CVD), although results remain contradictory [19-21].

Pathophysiologic Mechanism

Several mechanisms have been proposed to explain the link between hyperhomocysteinemia and CVD: these include ROS generation, coagulation cascade activation, endothelial dysfunction, production of inflammatory mediators [22]. Many studies on animal models show that moderate hyperhomocysteinemia leads to accelerated development of atherosclerosis [23, 24]. The atherogenetic effect of hyperhomocysteine is quite variable and it depends on factors such as diet, sex, severity and duration of hyperhomocysteinemia. Cell culture studies demonstrate that homocysteine can promote vascular smooth muscle cell growth [25] and adhesion of leukocyte such as monocytes and T cells to endothelial cells [26]. Moreover, it has been demonstrated that homocysteine promotes inflammation by increasing production of several proinflammatory mediators, including IL-8 and MCP-1 [27]. It has also been suggested that homocysteine can affect haemostasis leading to a prothrombotic state. Homocysteine activates platelets and promote expression of the CD40/CD40L from activated platelets [28, 29]. Recently,

Table 1. Major Determinants of Plasma Homocysteine Concentration

Hyperhomocysteinemia Determinants
Genetic factors
CBS deficiency *
Methionine synthase deficiency *
Methionine synthase reductase deficiency *
MTHFR deficiency *
MTHFR 677TT genotype °
MTHFR 1298CC genotype °
Physiological determinants
Increasing age
Renal function, reduced GFR
Male sex
Pregnancy
Postmenopausal state
Increasing muscular mass
Lifestyle determinants
Folate, vitamin B_{12}, B_6, B_2 intake
Smoking
Coffee
Physical exercise
Clinical conditions
Folate, vitamin B_{12}, B_6 deficiency
Renal failure

*Involved in homocystinuria pathogenesis. °Involved in mild hyperhomocysteinemia.

Wilson *et al.* shown that ApoE-deficient mice exhibit accelerated arterial thrombosis and endothelial dysfunction when fed hyperhomocysteinemic diet [30]. In particular, there was no effect on platelet activation while results suggested that the mechanism of accelerated thrombosis may be related to diminished production of endothelium-derived nitric oxide or other prothrombotic factors. The mechanism by which hyperhomocysteinemia induces endothelial dysfunction is not completely understood, but there are strong evidence that it includes impairment of vasodilatation mediated by endothelium-derived nitric oxide. There are several evidences that hyperhomocysteinemia led to a dose dependent increase of intracellular ROS generation and to a decrease of endothelial NO release in different experimental settings [31-35]. Another potential mechanism for endothelial dysfunction during hyperhomocysteinemia is inhibition of oxide nitric production caused by asymmetric dimethylarginine (ADMA), an endogenous eNOS inhibitor [36, 37]: in human subjects plasma levels of ADMA increase rapidly after acute methionine loading and elevation of plasma ADMA correlates with impairment of endothelium-dependent relaxation [38]. Elevation of ADMA in hyperhomocysteinemia may be caused by decreased catabolism of ADMA by dimethylarginine dimethylaminohydrolase (DDAH), the enzyme that hydrolyze ADMA to citrulline and methylamines, providing a potential link between hyperhomocysteinemia and the increase in ADMA [39]. Daval *et al.* shown that hyperhomocysteinemia in mice $CBS^{-/+}$ causes tissue-specific decreases in the expression of DDHA, although in this experimental setting downregulation of DDHA do not result in a decrease of plasma ADMA levels [40].

Epidemiological Studies

The hypothesis that hyperhomocysteinemia was a cardiovascular risk factor came from several retrospective case-control studies [41-44]. After these first evidences, prospective observational studies, including cohort and nested case-control studies, have been performed, suggesting that hyperhomocysteinemia is a graded, independent risk factor for CVD. Nygard et coll. found a strong, graded association between plasma homocysteine level and overall mortality in patients with angiographically confirmed coronary artery disease, although adjustment for confounding factors, such as serum creatinine level, left ventricular ejection fraction and history with respect to myocardial infarction, weakened the predictive power of total homocysteine levels somewhat [45]. Data from a large meta-analysis revealed that stronger association was observed in retrospective studies of homocysteine measured in blood collected after the onset of CVD than in prospective studies among individuals who had no history of CVD at the time of collecting sample; however, among prospective studies of the latter subjects, and after appropriate adjustment for known cardiovascular risk factors and correction for regression dilution bias, a 25% lower usual homocysteinemia was associated with about an 11% lower ischemic heart disease and about a 19% lower stroke risk [20]. Interestingly, results from the sixth examination cycle of the Framingham Offspring Study reveal that homocysteine is included in the panel of most informative biomarkers for predicting death, whereas it is not useful for predicting major cardiovascular events [46]. In contrast, a nested case-control study involving 712 men participating in the Multiple Risk Factor Intervention Trial (MRFIT) showed no association of homocysteine plasma concentration with Coronary Heart Disease (CHD) [47]. Similarly, the Atherosclerosis Risk In Communities (ARIC) Study, a prospective case-cohort study conducted over 3.3 years, revealed that total homocysteinemia is not independently associated with CHD in men after adjustment for age and race, while only a weak association was found in women [48]. Moreover, Ridker et coll found that in a cohort of initially healthy 14916 subjects, among which 140 subsequently developed peripheral artery disease (PAD) during a 9-years follow up, non significant baseline elevation of homocysteinemia was observed [49]. Overall, inconsistent results have been obtained so it is still questionable if hyperhomocysteinemia has a causal role in the development of CVD or if it might be merely a marker of disease.

CLINICAL TRIALS: EFFECTS OF VITAMINS SUPPLEMENTATION ON CVD RISK

Given the mixed results of prospective studies, great attention was addressed to large clinical trials that unquestionably rank at the top of the hierarchy of evidence. The main trials performed in the last decade are summarized in Table **2**. VISP trial involved 3.680 individuals who experienced a non-disabling ischemic stroke; the aim of the study was to evaluate the effects of high- and low dose therapy with folate, vitamin B_6 and vitamin B_{12} on recurrent stroke considered as primary outcome and CHD and death as secondary outcome. During a 2-years follow up, authors found a moderate reduction of plasma homocysteine but no effects on vascular outcomes [50]. Authors propose that the result could be affected by the low baseline value of homocysteinemia (10.5 micromol/L, the 25th percentile for North America stroke population) and the short period of follow up. Analogous results were obtained when considering subjects with previous myocardial infarction: the NORVIT trial, a multicenter, randomised, double-blind trial, involved 3.749 subjects who had an acute myocardial infarction; participants were randomised to receive different combinations of folate, vitamin B_{12}, vitamin B_6, or placebo; the primary endpoint was a composite of recurrent myocardial infarction, stroke, and sudden death attributed to CHD during a mean follow up of 40 months. None of the treatment significantly reduced the risk of primary or secondary outcomes, despite a substantial reduction (27%) in plasma homocysteine was observed in patients receiving folic acid [51]. Overall, it seems that vitamin supplementation with folate and B vitamins is not harmful in secondary prevention of stroke or myocardial infarction. At the same time of NORVIT trial, similar results from another large-scale intervention study was published: HOPE-2 Trial included 3982 individuals with history of vascular disease (coronary, cerebrovascular, or peripheral vascular) or diabetes and additional risk factors for atherosclerosis, irrespective of their homocysteine plasma level; individuals were treated with folate, vitamin B_6 and B_{12} for an average of 5 years; a composite of death from cardiovascular causes, myocardial infarction and stroke was considered as primary outcome [52]. In accordance with previous results, authors found that the treatment decreased homocysteinemia, but it had no effects on outcome. Interestingly, when each component of the primary composite outcome was analyzed separately, there was a reduction of risk of stroke in the active-treatment group (RR: 0.75; CI: 0.59-0.97), although authors interpreted this result with caution for the low number of stroke cases included in the study and the lack of adjustment for the multiplicity of outcomes compared. In accordance to results of HOPE-2 trial on stroke risk reduction, in a recent meta-analysis Wang et coll. found that folate supplementation, regardless of B vitamins inclusion, reduces the risk in individuals without history of stroke (RR: 0.75; CI: 0.62-0.94), when the treatment is longer than 36 months (RR: 0.71; CI: 0.57-0.97) and when homocysteine plasma levels is reduced of 20% or more (RR: 0.77; CI: 0.63-0.94), suggesting an inverse relation between the degree of homocysteinemia and the RR of stroke [53]. Moreover, following studies have suggested that hyperhomocysteinemia is associ-

Table 2. Summary of the Major Clinical Trials Performed in the Last Years. See the Text for Details

Trial	Participants	Follow-up	Treatment	Primary Outcome
VISP [50]	3,680 with cerebral infarction	2 years	High dose (25 mg Vit B_6, 0.4 mg Vit B_{12}, 2.5 mg folic acid) vs low dose (200 μg Vit B_6, 6 μg Vit B_{12}, 20 μg folic acid)	Recurrent cerebral infarction
NORVIT [51]	3,748 with acute myocardial infarction	40 months	0.8 mg of folic acid, 0.4 Vit B_{12}, 40 mg Vit B_6; 0.8 mg of folic acid, 0.4 Vit B_{12}; 40 mg Vit B_6; placebo.	Recurrent myocardial infarction, stroke, sudden death attribuited to CAD
HOPE-2 [52]	5,522 with vascular disease or diabetes	5 years	2.5 mg folic acid, 50 mg Vit B_6, 1 mg Vit B_{12} vs placebo	Death from CV causes, myocardial infarction, stroke
WAFACS [54]	5442 women with history of CVD or 3 or more coronary risk factors vs 2721	7.3 years	2.5 mg folic acid, 50 mg Vit B_6, 1 mg Vit B_{12} vs placebo	Myocardial infarction, stroke, coronary revascularization, CVD mortality
BVAIT [56]	506 with Homocysteine>8.5 micromol/L and without CVD and diabetes	3.1 years	5 mg folic acid, 0.4 Vit B_{12}, 50 mg Vit B_6, vs placebo	Subclinical Atherosclerosis progression (carotid artery intima media thickness)
Jamison R, *et al.* [57]	2056 with advanced chronic kidney disease (eGFR<30ml/min) or end stage renal disease and Homocysteine > 15 micromol/L	3.2 years	40 mg folic acid, 100 mg Vit B_6, 2 mg Vit B_{12}, vs placebo	All-cause mortality
Renal HOPE-2 [58]	3,310 with eGFR<60 ml/min and at high CV risk	5 years	2.5 mg folic acid, 50 mg Vit B_6, 1 mg Vit B_{12}	Death from CV causes, myocardial infarction, stroke

ated also with stroke subtypes, especially small vessel disease, in UK black population after adjusting for age, gender, vascular risk factors, vitamin levels and renal function [54]. Both in observational studies and in randomized trial, women were underrepresented; given the paucity of data on women and the known influences of oestrogen on homocysteine plasma levels, Albert et coll. designed a randomised trial aimed at testing if therapy with folic acid, vitamin B_6 and B_{12} lowered the risk of CVD during a 7-years follow up among 2721 women who had history of CVD or at least 3 cardiac factors [55]. In this study, patients receiving active treatment had the similar cardiovascular risk compared with placebo group (RR for primary endpoint: 1.03; CI: 0.90-1.19); at the end of follow-up, authors observed also a decrease of homocysteine plasma levels by 18.5%. Most trials were designed with pre-existing cardiovascular disease as a secondary prevention strategy, but it is possible that folic acid supplementation has a greater protective effect in primary rather than secondary prevention: at this regard, authors from the BVAIT Research Group, found that in a population of about 500 subjects with a low cardiovascular risk and total homocysteinemia > 8.5 micromol/L, three years treatment with folate, vitamin B_{12} and B_6 significantly reduced progression of early-stage subclinical atherosclerosis, evaluated as carotid artery intima-media thickness, but it had no effect on progression of aortic or coronary artery calcification, measured by multidetector spiral CT [56]. It is well known that homocysteinemia is elevated in kidney disease, and renal dysfunction is recognized as a risk factor for CVD. To assess the potential benefit of homocysteinemia lowering on mortality and CVD in individuals with renal impairment, Jamison et coll. designed a randomizes controlled trial involving 2056 subjects with Advanced Chronic Kidney Disease (ACKD) or ESRD that were treated with folate and B vitamins during a 3-years follow up; primary outcome was death from any cause while secondary outcomes comprised time to myocardial infarction, stroke, amputation of lower extremity; in this experimental setting, vitamins supplementation decreased homocysteinemia but failed to decrease the risk for neither primary nor secondary endpoints, suggesting that the treatment give no benefit even in this population [57]. Analogous results were obtained from HOPE-2 when individuals with chronic kidney disease were considered in the trial: there were no significant treatment benefit on death from cardiovascular causes, myocardial infarction, and stroke; incidence of CVD increased only with decreasing GFR [58]. Moreover, in a recent post hoc analysis, Potter K et coll. found that, after adjustment for any single marker of renal function, homocysteinemia lost its significance as a predictor of the vascular measurements (carotid intima-media thickness as well as flow-mediated dilatation of the brachial artery) [59]. On the basis of these evidences, some authors suggested that renal dysfunction may account for the epidemiologic association between mild hyperhomocysteinemia and increased cardiovascular risk [60]. In a recent meta-analysis of 12 randomized controlled trials evaluating the effect of folic acid treatment on CVD risk and all-cause mortality, Bazzano *et coll* found that in individuals with prior history of vascular disease the relative risk was 0.95 for CVD, 1.04 for coronary heart disease, 0.86 for stroke and 0.96 for all-cause mortality. These results further confirm that, although vitamin supplementation significantly reduces serum homocysteine, it has no effect on CVD risk or mortality in individuals [61].

CONCLUSION

Overall, based on the present evidences, hyperhomocysteinemia should be considered only a marker of CVD and not an independent risk factor because molecular mechanisms that link homocysteinemia to CVD are not clearly understood, and because clinical trials have demonstrated that lowering homocysteinemia with vitamin supplementation is not effective in reducing CVD risk. Thus, hyperhomocysteinemia is associated with an increased incidence of cardiovascular events, as shown by prospective studies, but intervention trials failed to show a benefit from folate and B vitamins supplementation; these evidences justified many to conclude that lowering homocysteinemia is ineffective in preventing vascular disease. Although these considerations are due, we must consider that future studies must take into account renal function; moreover, there are important differences between myocardial infarction and cerebral infarction, so it is possible that homocysteine lowering could be effective in reduce the risk of stroke but not the CHD one, as supported by some trials [53, 62]. Another hypothesis is that folate therapy is not beneficial for vascular disease; if it is true, new efforts will be required to identify other therapeutic approach to low hyperhomocysteinemia, such as methionine restricted diet, to further reduce the risk of vascular events. Until these questionable points remain unresolved, lowering homocysteinemia with folate and B vitamins can't be recommended for prevention of CVD, although homocysteinemia remains an interesting field of study as unconventional cardiovascular risk factor.

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