activity in patients having diabetes mellitus but we could not confirm the finding. However, using the specific method we have demonstrated highly significantly increased activity in 100 diabetic patients (*6*). It is well known that patients having diabetes mellitus have greater tendency to develop coronary-artery and other types of atherosclerosis than do nondiabetics. Thus it is tempting to speculate that the increased activity may point to a common biochemical pathway for the genesis of atherosclerosis in nondiabetics and in patients having diabetes mellitus; such a pathway may be the glucuronic acid cycle. Winegrad *et al.* postulated that this insensitive-to-insulin cycle is hyperactive in patients having diabetes mellitus, because they demonstrated increased serum levels of L-xylulose, a component of the glucuronic acid cycle (*7*). Accelerated activity of the glucuronic acid cycle would presumably produce more uridine diphosphate glucuronic acid and in this way stimulate synthesis of mucopolysaccharides (*8*). Because beta-glucuronidase participates in the degradation in vitro of mucopolysaccharides (*9*), accumulation of excess amounts of mucopolysaccharides may secondarily induce greater beta-glucuronidase activity in order to restore the mucopolysaccharides to more normal levels.

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14 February 1966

Homocystinuria: Excretion of a New Sulfur-Containing Amino Acid in Urine

Abstract. *An unusual homocysteine-containing compound, 5-amino-4-imidazolecarboxamide-5′-*S*-homocysteinylriboside, was isolated from the urine of a child with homocystinuria and detected in the urines of six homocystinurics. Its metabolic origin is not clear, and the data suggest the existence in man of now unknown alternate pathways for the metabolism of methionine and of purines.*

Homocystinuria is an inborn error of methionine metabolism and is characterized clinically by mental deficiency, dislocated ocular lenses, and skeletal abnormalities, and by the occurrence of serious arterial and venous thromboses (*1*). Patients have elevated concentrations of methionine and homocystine in blood, and homocystine is excreted in the urine. The fundamental biochemical defect in the disease is a deficiency of the enzyme cystathionine synthase (*2*), with the normal formation of cystathionine from homocysteine and serine failing to take place both in liver and brain cells. Cystathionine, one of the major free amino acids of human brain, is absent or markedly diminished in the brain tissue of patients who have died with homocystinuria (*3*).

In homocystinuria the urinary excretion of homocystine and methionine accounts for only a small fraction of the daily dietary intake of methionine, even though the major degradative pathway in methionine metabolism is blocked at the step of cystathionine synthesis (*4*). While searching for additional sulfur-containing compounds in urine to explain this discrepancy, we found an unidentified ninhydrin-positive spot which was routinely present on two-dimensional amino acid paper chromatograms prepared from the urines of homocystinurics (Fig. 1), but which we never encountered in the urines of normal individuals.

The unknown compound migrated to a locus close to, but distinct from, that occupied by histidine when urines were chromatographed first in a mixture of pyridine, acetone, ammonium hydroxide, and water (45 : 30 : 5 : 20), and then in a mixture of isopropanol, formic acid, and water (75 : 12.5 : 12.5). Its positive reaction to the chlorplatinate spray (*5*) indicated that it contained unoxidized sulfur. Its failure to react to sodium nitroprusside alone, or to nitroprusside after prior exposure either to sodium cyanide or to methanolic NaOH, indicated that the unknown compound did not contain a sulfhydryl, disulfide, or thioester group, but was consistent with its being a thioether. The unknown compound also strongly absorbed ultraviolet light when chromatographed on paper in solvent systems not containing pyridine.

Several milligrams of the unidentified compound were isolated from a homocystinuric child's urine by ion-exchange column chromatography. A portion of urine containing 100 mg of creatinine was concentrated to a small volume by lyophilization and applied to the surface of a column (45 by 2.16 cm) of Dowex 50 × 8, buffered with pyridine acetate at *p*H 3.50 (0.1*M* in pyridine). The same buffer was then pumped through the resin column at 50°C at a flow rate of 50 ml/hr. The unknown sulfur-containing compound was eluted between 730 and 790 ml. The volatile buffer was removed from this effluent zone under reduced pressure on a rotary evaporator, and the compound was further purified by chromatography on Whatman No. 3MM paper, first in a mixture of pyridine, acetone, ammonium hydroxide, and water (45 : 30 : 5 : 20), and then in a mixture of isopropanol, formic acid, and water (75 : 12.5 : 12.5).

The isolated compound has now been tentatively identified as 5-amino-4-imidazolecarboxamide-5′-*S*-homocysteinylriboside (AICHR) (Fig. 2).

When the purified compound isolated from urine was chromatographed on the short column of the Technicon amino acid analyzer, with the buffer gradient system for protein hydrolyzate analysis (*6*), it was eluted as a single peak at the same effluent volume as ammonia and homocystine. When applied to the long column (127 cm) on the analyzer, and with the buffer gradient system recommended by Efron (*7*), the compound was eluted as a single peak 17 minutes after homocystine and 2 hours and 55 minutes before ammonia.

Repeated hydrolyses of the urinary compound in 5.7*N* HCl at 110°C for 16 hours yielded homocystine, homocys-

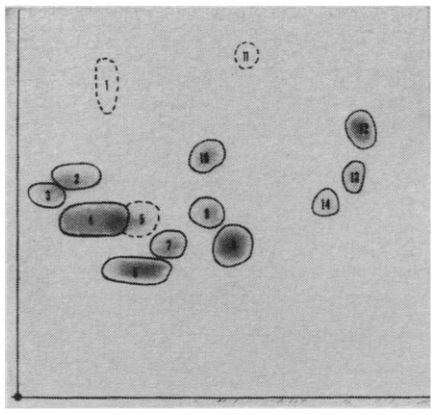

Fig. 1. Two-dimensional paper chromatogram prepared from a 50-μg creatinine equivalent of a homocystinuric's urine. Origin is at lower left hand corner. First solvent (vertical axis): pyridine, acetone, ammonium hydroxide, and water (45 : 30 : 5 : 20). Second solvent: isopropanol, formic acid, and water (75 : 12.5 : 12.5). (1) Taurine, (2) histidine, (3) the unknown compound, (4) 1-methylhistidine, (5) lysine, (6) homocystine, (7) glutamine, (8) glycine, (9) methionine sulfoxide, (10) serine, (11) threonine, (12) methionine, (13) β-aminoisobutyric acid, (14) alanine.

teine thiolactone, and glycine, but no uniform molecular ratio could be found between the fragments. After the compound had been oxidized with either hydrogen peroxide or performic acid, hydrolysis in strong acid yielded homocysteic acid and varying amounts of glycine. We then found that strong-acid hydrolysis degraded a number of authentic purines, as well as 5-amino-4-imidazolecarboxamide (AIC), to glycine. In addition, authentic homocysteine was converted by treatment with 5.7*N* HCl at 110°C into homocystine and homocysteine thiolactone.

Milder acid hydrolysis, in 1*N* HCl, at 100°C for 1 hour, degraded the urinary compound into homocystine and two other fragments. One of these fragments could be co-chromatographed on paper with authentic AIC, and it gave the same blue color as the AIC gave when sheets were sprayed with diazotized sulfanilic acid. It absorbed ultraviolet light, and the material eluted from the paper had an ultraviolet spectrum identical to that of authentic AIC. The second unidentified fragment resulting from mild hydrolysis was ninhydrin-positive, and it reacted to the *p*-anisidine spray reagent (*8*) with a color similar to that given by ribose. It co-chromatographed on paper in five different solvents with *S*-ribosylhomocysteine, and it was eluted at the same point as this compound when run on the Technicon amino acid analyzer. *S*-Ribosylhomocysteine was obtained by hydrolysis of *S*-adenosylhomocysteine in 0.1*N* HCl at 100°C for 1 hour (*9*).

The compound isolated from urine was now presumed to be AICHR. It gave a blue color fading to purple-brown, exactly like that shown by AIC riboside when chromatograms were sprayed with diazotized sulfanilic acid. It gave a negative test for phosphate when sprayed with the ammonium molybdate reagent (*5*, p. 241).

The ultraviolet absorption spectrum of the urinary compound (Bausch and Lomb Spectronic 505 spectrophotometer) was almost identical to that of authentic AIC riboside at three different *p*H's (Fig. 3). The values found agreed well with those reported for AIC riboside (*10*). At neutral *p*H, the ultraviolet maxima both of the compound isolated from urine and of AIC riboside were between 266 and 268 mμ. The properties of the urinary compound listed above all support its identification as AICHR.

The AICHR was readily detected in two-dimensional amino acid paper chromatograms of the urines of each of six homocystinuric children. The only homocystinuric patient in whose urine we have been unable to find this compound is a baby who has been fed a special low-methionine diet since early infancy, and who, as a result of this treatment, demonstrates unusually low concentrations of methionine in plasma and a markedly diminished excretion of homocystine in urine (*11*).

The amount of AICHR excreted in the urine of homocystinurics was estimated by visual comparison of the intensity of ninhydrin-developed spots on paper chromatograms of urinary amino acid with appropriate standards of the purified isolated compound. The concentration of the standard solution was determined by its ultraviolet absorption at 267 mμ, based on the assumption that the molecular extinction coefficient of AICHR and of authentic AIC riboside are the same. We found that homocystinuric children consuming normal diets excreted as much as 0.3 mmole of AICHR per gram of creatinine.

Since *S*-adenosylhomocysteine was found to co-chromatograph exactly with AICHR in the two-dimensional solvent system we used for amino acids (Fig. 1), we explored the unlikely possibility that AICHR might be an artifact produced from *S*-adenosylhomocysteine during paper chromatography. When authentic *S*-adenosylhomocysteine was chromatographed in these solvents and then eluted from paper, its ultraviolet absorption spectrum, reaction to diazotized sulfanilic acid, and behavior on the amino acid analyzer were unaltered.

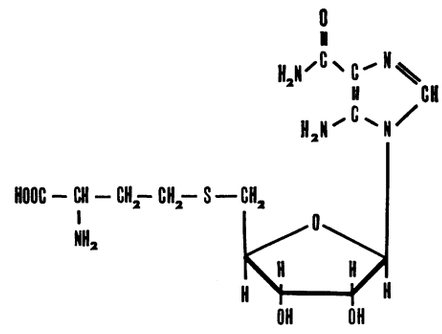

Fig. 2. Structure of 5-amino-4-imidazolecarboxamide-5′-*S*-homocysteinylriboside.

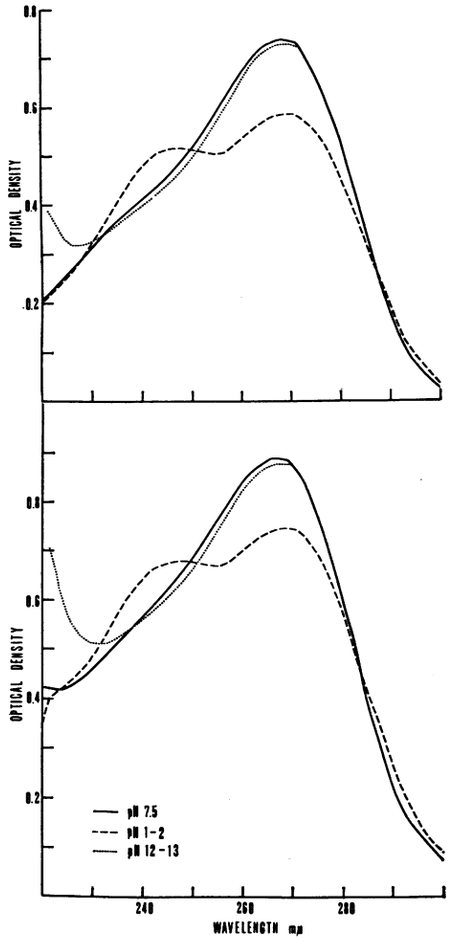

Fig. 3. Ultraviolet absorption spectra of authentic 5-amino-4-imidazolecarboxamide riboside (upper), and of 5-amino-4-imidazolecarboxamide-5′-*S*-homocysteinylriboside isolated from urine (lower).

The metabolic origin of AICHR is not clear. One possibility is that this compound is synthesized from homocysteine by reaction with AIC riboside when tissue concentrations of homocysteine are greatly increased. It is reasonable to assume that such a reaction, if it occurs, must be an enzymatic one. This assumption is rendered more valid by the observation that a normal subject (T.L.P.) who was fed a large quantity of DL-homocystine excreted large amounts of D-homocystine in urine over a 24-hour period, yet did not excrete AICHR. AIC ribotide is a normal intermediate in the biosynthesis of purines and can be dephosphorylated to AIC riboside. The mammalian liver enzyme which condenses adenosine and homocysteine to *S*-adenosylhomocysteine is thought to be quite specific with respect to both substrates (*12*), but the possibility that this enzyme, or an analogous condensing enzyme, might react with AIC riboside as well as with adenosine must be considered.

In fact, a high concentration of homocysteine in tissues might itself favor the accumulation of AIC riboside and thus promote condensation of the latter with homocysteine, if this hypothetical pathway is indeed operative. Warren *et al.* (*13*) have shown that the two biosynthetic reactions in purine synthesis leading from AIC ribotide to 5-formamido-4-imidazolecarboxamide ribotide and then to inosinic acid are reversible in vitro. These investigators found that the conversion of inosinic acid back to AIC ribotide by the liver enzymes which normally synthesize inosinic acid required a reduced folic acid compound, potassium ions, and a reducing substance. Homocysteine was by far the most effective reducing substance tested. It stimulated this reaction, in addition to its effect as a reducing substance, by a mechanism which was unexplained and which might be of importance for the same reaction sequence occurring in vivo when the concentrations of homocysteine in the tissues rise.

A second possibility for the metabolic origin of AICHR is that this compound arises from the degradation of *S*-adenosylhomocysteine. The latter might accumulate in the tissues in homocystinuria, being only one step further removed than homocysteine from the enzymatic block in methionine catabolism. However, we have not yet succeeded in identifying *S*-adenosylhomocysteine in the urine of homocystinurics. The only known mammalian pathway for the degradation of adenine and other purines results in the formation of uric acid and allantoin. Opening the purine ring to form AICHR from *S*-adenosylhomocysteine would imply the existence of a presently unknown alternate route for purine degradation. A theoretically possible degradative pathway from *S*-adenosylhomocysteine to AICHR might involve, first, deamination to *S*-inosylhomocysteine and then, facilitated by excessive homocysteine, an opening of the purine ring and removal of a formyl group by the same enzymes which have been shown to convert inosinic acid to AIC ribotide in vitro (*13*).

Homocystinuria, like other inborn errors of metabolism, may provide clues which lead to the eventual discovery of interesting new biochemical pathways in man. The compound which we have tentatively identified appears to involve the metabolism of purines as well as that of methionine, and the precise manner in which it is formed needs to be determined. It also will be important to explore what part, if any, AICHR and other still unidentified homocysteine metabolites play in producing the pathological manifestations of homocystinuria.

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Retention of Potential to Differentiate in Long-Term Cultures of Tooth Germs

Abstract. *When tooth germs derived from 14-day mouse embryos were cultured on gelatin sponges in vitro for 37 days, they lost their characteristic morphology, appearing as a layer of undifferentiated epithelium on the sponge surface, with the mesenchymal cells scattered throughout the interstices. These cultures were then transplanted subcutaneously into isologous, newborn recipients and, over a period of 56 days, developed into incisor teeth that were almost perfect in shape and structure.*

In experiments primarily designed to investigate viral oncogenesis in vitro, a series of control, uninfected organ cultures were set up as follows.

Mandibular and maxillary incisor tooth buds were dissected under sterile conditions from 14-day mouse embryos (C3H/Bi) and placed on gelatin sponges in Leighton tubes (*1*), five tooth buds to each tube. The medium used was Eagle's minimal essential medium (*2*) supplemented with calf serum (10 percent); the medium was changed every 2nd day; in all 23 tubes were set up.

Ten of the sponges were fixed in a Zenker-formalin mixture, embedded in paraffin, serially sectioned, and stained with hematoxylin and eosin after 7, 10, 14, 21, 28, 42, and 50 days in vitro. The remaining 13 sponges were removed from the Leighton tubes after 37 days in vitro, cut into small pieces, and transferred to 38 newborn syngenic recipients by a fine pipette introduced through an incision at the nape of the neck and passed down to the base of the tail, where the fragments were gently deposited.

Seven of the host mice died early owing to the effects of the operation or to maternal cannibalism, but 31 survivid, and these were killed after 8 weeks.

The incisor tooth buds from 14-day mouse embryos are at the early