column was washed by passing through 25 ml of 0.4*M* saline phosphate buffer (0.4*M* NaCl, 0.05*M* sodium phosphate buffer, *p*H 6.7) and then through 25 ml of 0.55*M* saline phosphate buffer. The DNA was eluted in steps with 2-ml portions of 1.1*M* saline phosphate buffer. A large amount of the ultraviolet absorbing materials (unidentified) came out in a 0.4*M* fraction. The fraction with the highest absorbance (at 260 mμ) eluted with 1.1*M* saline buffer was used for density gradient centrifugation.

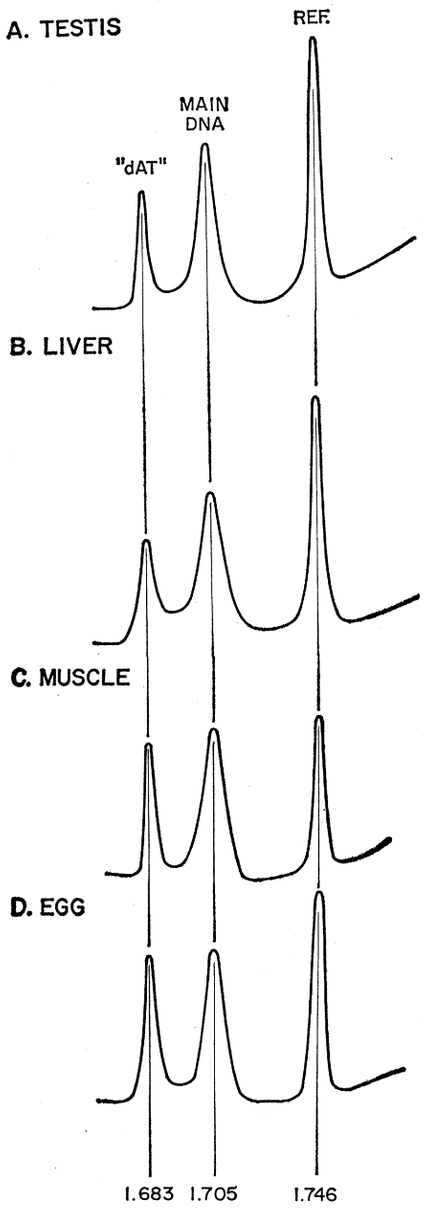

Fig. 1. Distribution of *Cancer borealis* DNA from various tissues in CsCl buoyant-density gradient. DNA samples (2 to 3 μg) were centrifuged with 1 μg N^{15}-*Pseudomonas aeruginosa* DNA as a reference at 44,770 rev/min in 7.7 molal CsCl solution at 25°C for 20 hours. Ultraviolet absorption photographs of equilibrated DNA were traced by a microdensitometer. Densities of the peaks were calculated (*1*). The density values were calculated relative to the density of *Escherichia coli* B DNA (1.713). *A*, DNA from testes; *B*, DNA from liver; *C*, DNA from claw muscle; *D*, DNA from eggs. The horizontal axis indicates buoyant density.

The isolation procedure for liver DNA was somewhat different from the method described for muscle. The frozen liver (5 g) was dipped in heated EDTA-saline (50 ml) and kept at 70°C for 15 minutes. After heating, the fragments of liver were collected by centrifugation, frozen in liquid nitrogen, and then ground in a mortar with a continuous supply of liquid nitrogen. The frozen powder was resuspended in 20 ml of the EDTA-saline buffer and 2 ml of duponol solution (25 percent), and ground in a teflon-pestle tissue grinder. The homogenate was treated with the mixture of isoamyl alcohol, chloroform, and phenol and with ribonuclease in the manner described for muscle. Apparently, a large amount of material other than DNA was precipitated by ethanol which could not be separated from the DNA by shaking with the organic solvents. The precipitates were dissolved in 3 ml of standard saline citrate, and a portion (1 ml) equivalent to 80 absorbance units at 260 mμ was diluted to 20 ml with standard saline citrate and applied to a 10-ml MAK column. The DNA was eluted as before. The column fractionation removed nonnucleic acid material and about 270 μg of DNA was obtained. The method used for isolation of DNA from eggs is similar to the method described for muscle DNA. From 15 g of eggs, approximately 1 mg of DNA was obtained.

The DNA samples from testes, muscle, liver, and eggs of *Cancer borealis* were centrifuged in 7.7 molal CsCl solution (*8*) at 25°C, 44,770 rev/min for 20 hours. The tracings of the ultraviolet absorption pictures are shown in Fig. 1, and relative amounts of the main DNA and light DNA ("dAT") in Table 1. The results show that muscle, liver, and egg samples as well as that of testes have a light DNA band whose density is identical with the light DNA previously described in testes.

In conclusion, the natural "dAT" first found in the testes and vas deferens of *Cancer borealis* exists in muscle, liver, and eggs as well, in approximately the same relative amount (30 percent). Consequently, it exists also in both male and female.

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9. Supported by grant G-18745 from the National Science Foundation and grant GM 10923-02 from the National Institutes of Health.
* Predoctoral fellow supported by training grant #5T1 GM 962 from the Division of General Medical Sciences, U.S. Public Health Service.

24 January 1964

Homocystinuria: An Enzymatic Defect

Abstract. *A deficiency, or absence, of cystathionine synthetase activity has been demonstrated in liver obtained from a mentally retarded child with homocystinuria.*

Homocystinuria was added recently to the list of biochemical abnormalities known to be associated with mental deficiency in man (*1*, *2*). Carson *et al.* (*2*) studied two homocystinuric sisters

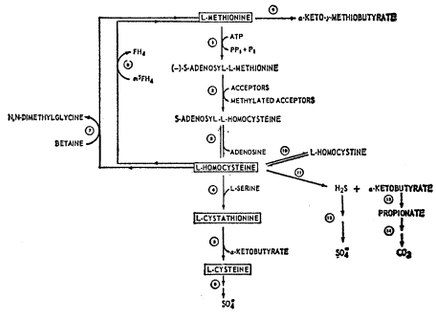

Fig. 1. Known pathways of mammalian metabolism of methionine and homocysteine. Abbreviations: ATP, adenosine triphosphate; PP_i, inorganic pyrophosphate; P_i, inorganic phosphate; FH_4, tetrahydrofolic acid; $^5FH_4$, N^5-methyltetrahydrofolic acid.

and concluded that the disorder probably reflects an inborn error of metabolism. The finding of an elevated concentration of homocystine in the plasma suggested that the aminoaciduria is attributable to an "overflow" into the urine rather than to a primary defect in renal tubular reabsorption. In addition, these authors observed an elevated concentration of methionine in the plasma without an associated increase in urinary methionine excretion. In their initial hypothesis they considered the possibility that the inborn error might involve the reaction catalyzed by cystathionine synthetase (Fig. 1, enzyme 4), but they interpreted the results of their studies as failing to confirm this suggestion and turned their attention to an alternative hypothesis, a generalized defect in methionine transport systems. A consideration of the known routes of mammalian metabolism of methionine and homocysteine (Fig. 1) suggested to us that the accumulation of both methionine and homocyst(e)ine might be best and most simply explained by a defect in cystathionine synthetase. To test this possibility directly, specimens of liver were obtained from a homocystinuric child and from several control subjects and assayed for cystathionine synthetase activity and for the activity of another enzyme which participates in methionine metabolism, methionine activating enzyme (*3*) (Fig. 1, enzyme 1). The results suggest that homocystinuria can, indeed, be attributed to a deficiency, or absence, of cystathionine synthetase activity.

Our homocystinuric patient is an 8-year-old white girl with clinical features similar to those of the other reported cases—mental retardation; bilateral posterior dislocation of the lenses; fine, blond hair; blue-gray eyes and genu valgum. Her aminoaciduria was first detected by Spaeth and Barber who are preparing a detailed description of the case (*4*). We have confirmed their observation that she excretes 40 to 50 mg of homocystine in her urine daily. Her liver is not palpable and tests of liver function produced normal results.

Sensitive methods were developed to assay enzymes 1 and 4 (Fig. 1). By these methods, no cystathionine synthetase activity could be detected in human skin (stratum corneum from fingertips), erythrocytes, or leukocytes from control patients, but activity could be demonstrated in liver obtained postmortem. The sensitivity of the assays permitted replicate determinations of both enzymes in extracts of the relatively small amount (20 to 30 mg) of tissue obtainable by needle biopsy of the liver.

The pertinent properties of human hepatic cystathionine synthetase and methionine activating enzyme were determined and will be described in a separate communication together with details of the assay methods (*5*). The results of assays of a number of liver specimens are summarized in Table 1. The data from control patients do not establish a range of normal values since some of them may be lower than those that would be expected for fresh, healthy tissue. Nevertheless, it is apparent that our methods allow ready detection of the two enzymes and that they are both present in the liver of children comparable in age to the homocystinuric patient. In marked contrast to the control specimens, the extract of the liver from the homocystinuric patient had no detectable cystathionine synthetase activity. This activity would have been demonstrable had it been 10 percent or more of the lowest control value. The extract of

Table 1. Activities of methionine activating enzyme and cystathionine synthetase in extracts of human liver. To assay methionine activating enzyme, the following, in micromoles, were incubated for 45 minutes at 37°C in 0.45 ml with enzyme extracts containing 0.1 to 0.6 mg of protein: Tris-HCl buffer, *p*H 7.6, 60; KCl, 80; $MgCl_2$, 120; adenosine triphosphate (ATP), 7.2; neutralized, reduced glutathione, 1.6; (—)-S-adenosyl-L-methionine, 0.12; L-methionine-$C^{14}H_3$, 0.059, containing 150×10^3 count/min. After incubation the reaction was stopped by dilution with ice-cold water; methionine-$C^{14}H_3$ and S-adenosylmethionine-C^{14} were separated by column chromatography, and the enzyme activity was measured by appearance of radioactivity in the S-adenosylmethionine fraction. Values from controls incubated with boiled enzyme were subtracted from the experimental values. Methionine activating activity in all cases was completely dependent upon addition of ATP. To assay cystathionine synthetase, the following, in micromoles, were incubated for 135 minutes at 37°C in 0.40 ml with enzyme extracts containing 0.1 to 0.5 mg of protein: Tris-HCl buffer, *p*H 8.3, 60; ethylenediaminetetraacetate, 1; pyridoxal phosphate, 0.015; L-cystathionine, 0.07; L-homocysteine, 5; L-serine-3-C^{14}, 1, containing 74.7×10^3 count/min. Portions of the deproteinized reaction mixture were taken, serine-3-C^{14} and cystathionine-C^{14} were separated by column chromatography, and the enzyme activity measured by appearance of radioactivity in the cystathionine fraction. Values from control mixtures incubated with boiled enzyme or with enzyme added after trichloroacetic acid were comparable and were subtracted from the experimental values. In all instances cystathionine synthetase activity was completely dependent on the addition of L-homocysteine. The values for neither enzyme are maximal since, under the conditions used, the enzymes were unsaturated with regard to L-methionine and L-serine, respectively. Protein was determined by the Lowry method (*8*). The cystathionine synthetase activity of the extract in study F was assayed three times, once without added ethylenediaminetetraacetate. The methionine activating activity of this extract was determined three times. All other values are the average of at least two determinations.

Study	Age (years), race, and sex*	Diagnosis	Acquisition and storage of tissue	Enzyme activities (mμmole/mg protein)	
				Methionine activating enzyme	Cystathionine synthetase
		Control patients			
A	7/NF	Traumatic death, normal child	3 hours postmortem, frozen 5 months	1.9	231
B	12/WF	Cystic fibrosis of pancreas	2 hours postmortem, frozen 2 weeks	4.1	163
C	64/WM	Adenocarcinoma of pancreas with liver metastases—biopsy grossly free of tumor	During laparotomy, assayed immediately	Not tested	610
D	55/WM	Psoriasis, alcoholism, mild fatty infiltration of liver	Biopsy†, assayed immediately	3.8	204
E	43/WF	Hypoalbuminemia, BSP‡ retention, normal liver histology	Biopsy†, assayed immediately	3.5	240
		Homocystinuric patient			
F	8/WF	Homocystinuria	Biopsy†, assayed immediately	**7.7**	**0**
		Recombination experiment			
G		Extract of liver A			258
		Extracts A + F			283

* N, Negro; W, white; F, female; M, male. † Liver biopsies performed with Menghini needle. ‡ Bromsulphalein.

homocystinuric liver did not inhibit the cystathionine synthetase activity of control liver (Table 1). Radioactivity from authentic L-cystathionine-C^{14} incubated with the extract of homocystinuric liver under the standard assay conditions was recovered entirely with cystathionine. It was demonstrated by means of paper chromatography that the substrates for the assay of cystathionine synthetase, serine-3-C^{14} and homocysteine were still present after incubation with the extract of homocystinuric liver. The specificity of the deficiency of cystathionine synthetase activity is suggested by the adequate activity of methionine activating enzyme. Since the reaction mixture for assay of cystathionine synthetase contained added pyridoxal phosphate, the failure to detect cystathionine synthetase activity cannot be attributed to a deficiency of this cofactor.

A major metabolic pathway for methionine is thought to be conversion to cyst(e)ine and its end products (*6*). The homocystine (and methionine) excreted in the urine of homocystinuric patients accounts for only a small portion of the total intake of methionine. If the block in cystathionine synthetase is complete in these patients, they must metabolize the major portion of the ingested methionine by alternate pathways.

Because cystathionine formation is a necessary step in the major mammalian pathway for cysteine biosynthesis, there is a strong likelihood that a homocystinuric child will suffer from cysteine deficiency during the neonatal period when dietary intake of protein is much lower relative to the body's anabolic needs than it is in later years. It is conceivable that a homocystinuric infant fed human milk may receive adequate cystine whereas such a child fed cow's milk, known to be much poorer in this amino acid (*2*), would not receive adequate amounts. In either case, early dietary cyst(e)ine supplementation may prevent irreparable damage. Another possibility which should be considered is that cystathionine, reported to be present in high concentration in human brain (*7*), serves some necessary function in addition to that of precursor of cysteine. If this is so, dietary cystathionine supplementation might be helpful. Whether abnormal accumulation of homocyst(e)ine, methionine, or one of their metabolites contributes to the pathological manifestations of homocystinuria and would thus warrant methionine restriction also remains to be determined.

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9. We are grateful to G. L. Spaeth and G. W. Barber for permitting us to study their patient.

24 February 1964

Erythromycin: Mode of Action

Abstract. *Erythromycin, a specific inhibitor of protein biosynthesis, inhibited the incorporation of phenylalanine by a cell-free ribosomal system prepared from* Escherichia coli.

The antibiotic, erythromycin, inhibits the biosynthesis of protein in susceptible bacteria but permits continued synthesis of nucleic acids (*1*); this mode of action has been compared by Brock and Brock (*2*) to that of chloramphenicol. Antibiotics which are specific inhibitors of protein synthesis in vivo have been shown to inhibit the incorporation of radioactive amino acids into trichloracetic acid–insoluble polymers by cell-free ribosomal systems prepared from antibiotic-sensitive bacteria; this has been demonstrated for chloramphenicol (*3*), puromycin (*4*), tetracyclines (*5*), and streptomycin (*6*). Inhibition of amino acid incorporation in a cell-free system by chloramphenicol is generally regarded as circumstantial evidence that such a system represents a valid model of protein biosynthesis.

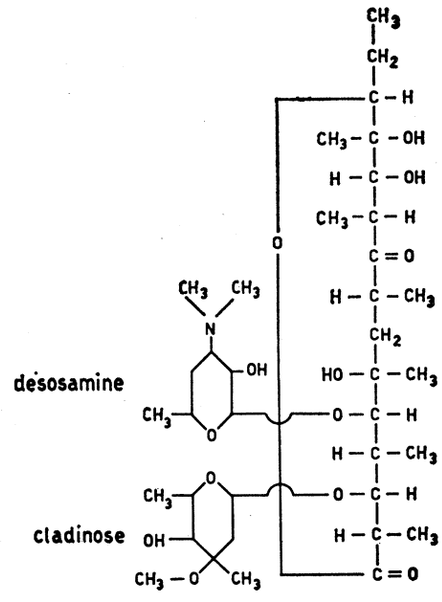

Fig. 1. Erythromycin.

We wish to report that erythromycin also inhibits the formation of polyphenylalanine in a cell-free ribosomal system prepared from *Escherichia coli* after the method of Matthaei and Nirenberg (*7*). Experimental details and results are given in Table 1.

Erythromycin is a member of the macrolide group of antibiotics. Its chemical structure (Fig. 1) offers no ready clue to its mechanism of action at the molecular level with the possible exception that there is a general structural relationship to other antibiotics containing amino sugar, such as streptomycin.

Two observations, however, may offer leads to the elucidation of the mechanism of action of erythromycin: (i) erythromycin has been found by Vasquez (*8*) to inhibit the interaction between radioactive chloramphenicol and sedimented ribosomal fractions of bacteria; we have readily confirmed this observation and have also shown that this inhibition is competitive in nature (*9*); and (ii) we have also recently observed that erythromycin is capable