# *AN RNA-HISTONE COMPLEX IN MAMMALIAN CELLS: THE ISOLATION AND CHARACTERIZATION OF A NEW RNA SPECIES**

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Many lines of evidence indicate that all specialized cells contain the total genetic information characteristic of the organism, but the mechanisms involved in the selective expression of this total information are unknown. Stedman and Stedman[1] suggested that the histones, the basic proteins of the nucleus, could serve as the controlling mechanism. Most cells of an organism, however, have the same histones,[2, 3] and since the histones of individual organs such as liver and brain do not change appreciably during development[4] or during carcinogenesis,[5, 6] it seems unlikely that the histones alone possess sufficient specificity for the control of gene function.

Sypherd and Strauss[7] suggested that an associated RNA species complementary to particular sequences of DNA could provide a mechanism for the histones to act as repressors by conferring additional specificity to these proteins. Huang and Bonner[8] have recently described an RNA-histone complex in the pea seedling. We have concurrently carried out experiments on the nucleoprotein fraction isolated from normal rat liver nuclei and have found an RNA species with a high adenine and uridine content which is intimately associated with the nucleoprotein fraction and is resistant to ribonuclease. After extraction from the basic protein with phenol and detergent, this RNA species is RNase-sensitive and is heterogeneous by sucrose density gradient analysis.

*Materials and Methods.*—Male Wistar rats weighing 100–120 gm from the Columbia University Colony were used in these experiments. They were fed rat pellets (A. E. Staley Manufacturing Co., Rockland Ill.) *ad libitum.*

*Labeled nucleoprotein:* In the experiments to be reported, either the $P^{32}$-labeled RNA-nucleoprotein complex or the nucleoprotein labeled with $C^{14}$-lysine was used. For each preparation 2–5 animals were injected intraperitoneally with neutral isotonic solutions containing either 2 mc of carrier-free $P^{32}$ (Radiochemical Centre, Amersham, England) or 50 $\mu$c lysine-UL-$C^{14}$ (Nuclear-Chicago Corp., Des Plaines, Ill.) per 100-gm rat. The animals were allowed free access to food and water at all times. Three hours following $P^{32}$ administration and 4 hr after the injection of lysine, liver nuclei were prepared and purified by a modification of the method of Maggio *et al.*[9]

With a Potter-Elvehjem homogenizer 9–12 gm of liver were homogenized in 3 vol of ice-cold buffer, pH 7.5, which consisted of $5 \times 10^{-3}$ *M* Tris, 0.25 *M* sucrose, 5 mM $MgCl_2$, 1 mM $CaCl_2$, and 30 mM KCl. The homogenate was filtered through cheesecloth and centrifuged at $800 \times g$ at 0° for 10 min. The crude nuclear pellet was washed two times with buffer and was repelleted after each wash at $800 \times g$ for 10 min. After washing, the pellet was resuspended in a small volume of similar buffer containing 2 *M* sucrose and then layered over additional hypertonic buffer in a centrifuge tube. The contents of the upper third of the tube were stirred to distribute the nuclear suspension, which was then centrifuged for 1 hr in an SW 25:1 head at 25,000 rpm in a model L2 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, Calif.) at 5°. The supernatant was discarded, and the walls of the tube were cleansed of debris. The nuclear pellet was resuspended in buffer containing 0.25 *M* sucrose and then pelleted at $800 \times g$ for 10 min. Following an additional wash and centrifugation, the pellet was washed twice with 0.15 *M* NaCl, 0.01 *M* sodium citrate, pH 6.5.

The preparation of purified nuclei was then extracted for 30 min with ice-cold 2 *M* sodium chloride. After centrifugation at 15,000 × *g* for 20 min, the debris was again extracted with 2 *M* salt. To facilitate the second extraction, the pellet was disintegrated by a 10-sec period of sonication at the lowest energy level with the microprobe of the Branson sonifier (Branson Sonic Power, Danbury, Conn.). After centrifugation the gel was combined with the first extract and was designated the deoxynucleoprotein-DNA complex (DNP).

*CsCl density gradient centrifugation:* The deoxynucleoprotein-DNA complex (DNP) was separated into its two main components, the protein fraction and the DNA moiety, by equilibrium centrifugation. About 15–25 $OD_{260\ m\mu}$ units of the DNP were made up to 5 ml final volume in 2 *M* NaCl and sufficient CsCl to give an over-all sp.g. of 1.2. This was centrifuged in polyallomer tubes (Beckman Instrument Co.) in a SW 39 rotor at 37,000 rpm for 48–60 hr at a rotor temperature of 12°. The contents of the tubes were dripped from the bottom by driving an 18-gauge needle into the tube just above the gelatinous DNA pellet. Three-drop samples were collected, and 1 ml of 2 *M* NaCl was added to each sample. The optical density was determined at either 230, 260, or 280 mμ. Radioactive samples were precipitated at a concentration of 15% trichloroacetic acid at 0° and the precipitate was collected on membrane filters (Schleicher and Schuell Co., Keene, N. H., type B6). The filters were dried, placed in scintillation vials, and counted in a model 4233 Packard liquid scintillation spectrometer using a phosphor composed of 20% ethanol, 5% 2,5-diphenyloxazole, 0.03% 1,4-bis-2-(5-phenyloxazolyl)-benzene in toluene.

*Disk electrophoresis of the nucleoproteins separated after CsCl centrifugation:* The protein which banded in the middle of the gradient was dialyzed against 0.01 *M* HCl to remove the salts. The dialysand was concentrated and 40–70 μg of protein were analyzed by the disk electrophoresis method of Reisfeld *et al.*,[10] as modified by Neidle and Waelsch.[4] Electrophoresis was carried out in the cold at a constant current of 2 mA per tube for 2 hr. A solution of 1% buffalo black in 7% acetic acid was used for staining. After 2 hr, dye not associated with protein was removed from the gel by electrophoresis in 7% acetic acid. The gel was prepared for counting by freezing on dry ice and slicing the bands into defined disks of approximately 2 mm thickness. These were placed in liquid scintillation vials and 0.25 ml of 30% $H_2O_2$ added. After standing for 17 hr at 20°, the disks dissolved. The vials were placed in ice, and 2 ml of cold Hyamine (Packard Instrument Co., Downers Grove, Ill.) were added and mixed. Before foaming, 2.5 ml of absolute alcohol were added, followed by 12 ml of phosphor in toluene. The gels remained in solution and counted at 75% efficiency for $C^{14}$ with a background of 25 cpm after dark adaptation for at least 6 hr.

*RNA chemistry:* RNA from rat liver fractions of either the DNP, the histone, or the ribosomes was extracted as described previously,[11, 12] but with the following modifications. The nucleoprotein fraction containing CsCl and NaCl was first dialyzed against pH 5 acetate buffer and concentrated. The RNA was extracted with a mixture of pH 9.5 glycine buffer containing 0.1 *M* NaCl and 0.01 *M* EDTA and 2 vol of water-saturated phenol. In addition, 0.2% Bentonite[13] and 1% sodium desoxycholate was added. After shaking for 20 min the phases were separated by centrifugation, the upper buffer phase washed 2 times with cold ethyl ether, and the RNA precipitated with 2 vol of cold 95% ethanol. The RNA was collected by centrifugation and further purified by dissolving in buffer and reprecipitating two more times with ethanol.

The phenol of the lower phase was removed, and the pellet and interphase were saved. New phenol was added, as was carrier RNA if needed, and pH 9.5 buffer containing 0.5% sodium dodecyl sulfate (SDS). This was heated at 60° with shaking for 15 min. The phases were separated by centrifugation at 20° at 15,000 × *g* for 1 min, and the upper buffer phase was washed 2 times with ethyl ether. The RNA was precipitated with 2 vol of cold ethanol and further purified as described for the RNA extracted at 0°. Additional buffer containing SDS was added to the phenol phase and extraction now carried out at 85°. The phases were separated and the RNA was extracted as before.

RNA fractions were studied by the sucrose density gradient technique. RNA layered over linear 2–20% w/w sucrose gradients in glycine buffer was centrifuged in a SW 25.3 rotor at 25,000 rpm in a model L-2 centrifuge for 18–20 hr at 4°. The gradients were analyzed using the Gilford spectrophotometer (Gilford Instruments Labs., Oberlin, Ohio) to record absorbance at 260 mμ. RNA in the fractions was precipitated with 10% trichloroacetic acid at 0°. The precipitates were collected on filters, and the radioactivity was measured in a liquid scintillation counter.

Base analysis on TCA-precipitable material was determined on aliquots containing carrier nucleoside-2′(3)-monophosphates (Pabst Labs., Milwaukee, Wis.) after overnight hydrolysis in 0.3 *M* KOH at 37°, and precipitation of the protein in perchloric acid. The mononucleotides were purified by adsorption on charcoal followed by elution with ammonia-ethanol (50:49:1: ethanol: water: concentrated $NH_3$). The purified mononucleotides were separated by high-voltage paper electrophoresis for 2 hr at 2,500 v in 0.4 *M* acetate buffer, pH 3.78.[14] The spots were detected with ultraviolet light, cut out, and counted by liquid scintillation spectroscopy. Radioactive orthophosphate migrated ahead of UMP and always represented less than 10% of the total counts.

*Results.—Basic nuclear protein:* Figure 1 shows the ultraviolet absorption spectrum of the deoxynucleoprotein fraction extracted from rat liver nuclei. The spectrum is similar to that obtained by Atchley and Bhagavan[15] and exhibits a maximum at 260 mμ and a minimum at 240 mμ. The nuclei obtained from each gram of liver yielded approximately 24 $OD_{260}$ units of the DNP complex which consisted of about 1 mg of DNA, 3 mg of protein, and 100 μg of RNA.

*Fractionation of DNP into histone and DNA:* In order to avoid extraction of the histone with mineral acid, the protein fraction (histone) was separated from the DNA of the DNP by cesium chloride density centrifugation. The histone was found to band near the center of the gradient (density = 1.286), while the less-buoyant DNA pelleted to the bottom of the gradient tube. The pattern shown in Figure 2 demonstrates the inversion in the 260/280 mμ readings when the first fractions above the gelatinous DNA pellet were collected. This change in the 260 to 280 ratio through the gradient indicates the presence of nucleic acid in the first tubes followed by protein. The contents of the tubes containing the protein were found to be soluble in 0.2 *M* $H_2SO_4$. The protein was dialyzed against 0.01 *M* HCl, and analyzed by disk gel electrophoresis (Fig. 3). The pattern is identical to that obtained by an acid extraction procedure for histones as reported by Neidle and Waelsch[4] and therefore the protein is assumed to be histone.

*Biosynthesis of electrophoretically separable histones:* To determine if this protein is synthesized *de novo*, rat liver slices were incubated with lysine-$C^{14}$, and the histones obtained from the nuclei by the cesium chloride technique. Figure 3

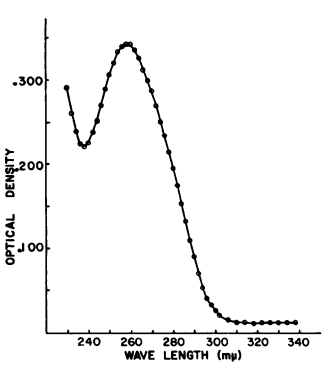


FIG. 1.—Ultraviolet absorption spectrum of rat liver deoxynucleoprotein.

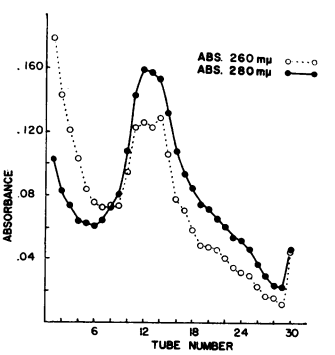


FIG. 2.—Cesium chloride equilibrium centrifugation of deoxynucleoprotein from rat liver nuclei. Gradients achieved by centrifugation for 60 hr at 39,000 rpm in a SW 39 centrifuge head. The DNA of the nucleohistone is pelleted at the bottom.

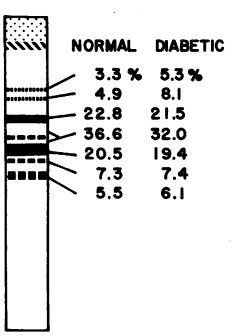


FIG. 3.—Incorporation of lysine-UL-$C^{14}$ into the protein bands of rat liver histone. In the gel electrophoresis diagram the anode is at the top.

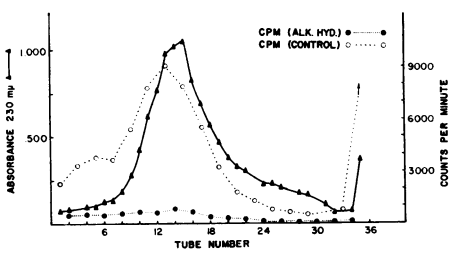


FIG. 4.—Cesium chloride equilibrium centrifugation of deoxynucleoprotein labeled with $P^{32}$ from rat liver nuclei. The DNA of the nucleohistone is pelleted at the bottom and is not recovered.

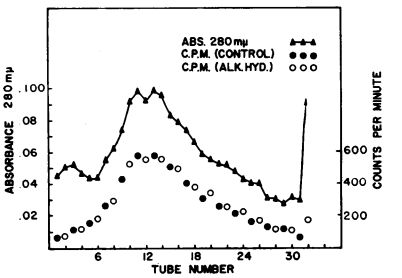


FIG. 5.—Cesium chloride equilibrium centrifugation of deoxynucleoprotein labeled with lysine-UL-$C^{14}$ from rat liver nuclei.

shows the percentage of the label incorporated into each band after gel electrophoresis of the histones. All bands were labeled with $C^{14}$ but the percentage of incorporation was not proportional to the intensity of staining with dye. As previously reported,[16] the distribution of label in the histone bands was similar in the basic protein obtained from normal and diabetic rats, which indicates that in these markedly different physiologic states rat liver histone synthesis does not appear to vary.

*Histone-bound RNA:* To determine if an RNA species were associated with the histone, the protein of the DNP fraction from liver nuclei of rats injected with $P^{32}$ was banded by cesium chloride density gradient centrifugation. Figure 4 demonstrates that the radioactive label is largely coincident with the protein band although the count pattern is somewhat skewed to the more dense part of the gradient. The $P^{32}$ in the protein region was made acid-soluble by overnight hydrolysis in 0.3 *N* KOH, which suggested that the label might be present in RNA. RNase treatment at pH 7 at 37° or after boiling and quick cooling in 0.4 *M* NaCl did not make the radioactivity associated with the protein acid-soluble. As another control, we determined if alkaline treatment extensively degraded the protein. Histone labeled with lysine-$C^{14}$ was banded, collected in fractions, and hydrolyzed overnight in 0.3 *N* KOH. As shown in Figure 5, alkaline treatment did not convert the $C^{14}$ into an acid-soluble product, and therefore it can be concluded that the $P^{32}$ counts which were rendered acid-soluble by this treatment (Fig. 4) were not made so because of extensive protein degradation.

As $P^{32}$ was found in portions of the gradient

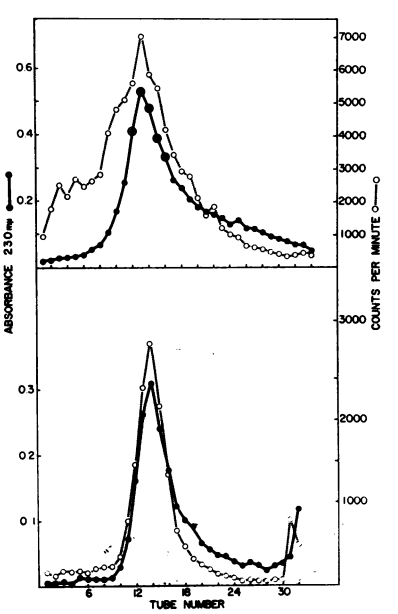


FIG. 6.—Rebanding of rat liver histone in cesium chloride equilibrium density gradients. *Upper graph:* initial banding of histone after centrifugation of rat liver DNP for 60 hr at 39,000 rpm in a SW 39 head. *Lower graph:* rebanding of histone from 5 peak tubes of upper graph after additional 60-hr centrifugation.

TABLE 1

BASE ANALYSIS OF RNA FROM RAT LIVER DNP AND RNA ASSOCIATED WITH HISTONE

| | Per cent of total $P^{32}$ | | | |
|---|---|---|---|---|
| | UMP | GMP | AMP | CMP |
| DNP-RNA | 26.0 (25.3–26.7) | 23.9 (23.3–24.4) | 26.3 (25.9–26.9) | 23.8 (23.2–24.3) |
| Histone-RNA | 25.4 (24.9–25.7) | 23.1 (22.3–24.4) | 27.9 (27.6–28.3) | 23.6 (21.6–25.2) |

TCA fractions were hydrolyzed in 0.3 *N* KOH overnight and after perchloric acid precipitation of the protein and the addition of carrier mononucleotides, the samples were analyzed in triplicate by high-voltage paper electrophoresis as described in the text. The results are expressed as the mean value of three separate experiments with the range given in parentheses.

not associated with the histone band, we determined if the label and the histone would reband on recentrifugation. Figure 6 shows the result obtained when the contents of the tubes containing the protein were pooled, mixed again with cesium chloride, and centrifuged for another 60 hr. The gradient in the upper part of Figure 6 shows the optical density pattern and the count profile of the histone on first banding; the lower pattern shows the results after rebanding. The protein rebands sharply at the same density of cesium chloride, and the radioactive label is precisely associated with the protein moiety.

*Base analysis:* The $P^{32}$-labeled RNA bound to histone was separated from DNA by equilibrium centrifugation, precipitated with TCA, and hydrolyzed in alkali. The nucleotides were separated by electrophoresis, and base analysis was calculated by the distribution of $P^{32}$ in the nucleotides. The data for the nucleotide composition of the RNA in the hypertonic salt extract of the nuclei and of the histone-associated RNA are given in Table 1. They were found to be high in their adenine and uridine contents and in this respect are different from ribosomal and transfer RNA and similar to messenger RNA.[17] When nonhistone portions of the gradient were hydrolyzed in alkali, only inorganic $P^{32}$ was recovered, which indicates that the RNA was associated only with the histone band.

*Ribosomal RNA synthesis:* In order to determine whether $P^{32}$ was incorporated into cytoplasmic ribosomes during the 3-hr period of our experiments, rat liver microsomes were isolated, treated with desoxycholate, and pelleted to obtain the ribosomal fraction. The RNA was extracted at 0° using buffer, phenol, and desoxycholate. Figure 7 shows the pattern of incorporation of $P^{32}$ into the three species of RNA separated by the sucrose density gradient technique. The radioactivity precisely parallels the optical density pattern which indicates that ribosomal RNA was synthesized in the nucleus and incorporated into the ribosome within the 3-hr period of the experiment.

*Isolation of histone-bound RNA:* The fraction of the gradient containing the radioactive RNA-histone complex was first dialyzed to remove the salts. Unlabeled ribosomal carrier RNA was added to the dialysand. This added carrier, but not the histone-bound RNA, was extracted by phenol at 0°. However, when the interphase and pellet were extracted at 60° with phenol and SDS, a method similar to that described by Greenman *et al.*,[18] the labeled RNA species associated with the histones was extracted. Very little additional RNA was isolated from the interphase material by further extraction at 85°. Figure 8 shows the distribution of $P^{32}$-labeled RNA isolated from histone following sucrose density centrifugation with marker ribosomal RNA. The pattern of $P^{32}$ counts is heterogeneous and does not follow the ribosomal RNA peaks. Figure 9 shows the pattern obtained after sucrose

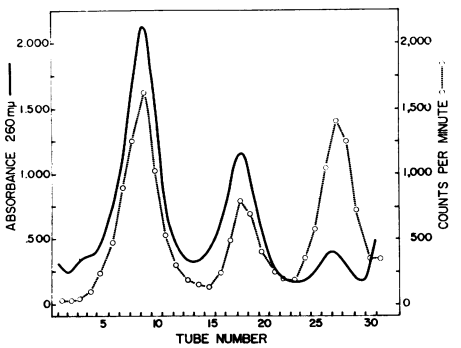


FIG. 7.—Sedimentation of purified RNA from the ribosomes of rat liver labeled *in vivo* with $P^{32}$ for 3 hr. Isolation of RNA and conditions of sucrose density centrifugation as described in text.

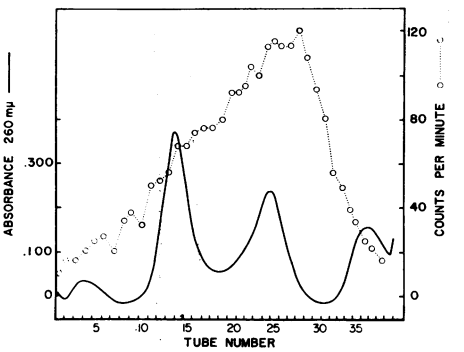


FIG. 8.—Sedimentation of $P^{32}$-labeled RNA extracted from histone by hot phenol and SDS; nonradioactive ribosomal RNA added at time of sedimentation. Isolation of the RNA and conditions of sucrose density centrifugation as described in the text.

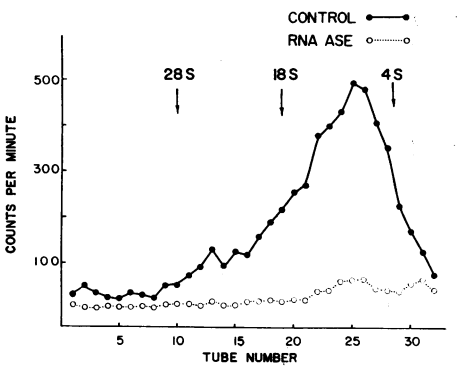


FIG. 9.—Sedimentation of $P^{32}$-labeled RNA extracted from histone by hot phenol and SDS. Each gradient fraction was split, and one half was subjected to enzymatic degradation with RNase, 20 μg/ml for 90 min at 37°, while the other half served as a control.

density centrifugation when carrier was not added to the gradient. Again, the count profile is distributed throughout the gradient. The figure also shows that following RNase treatment, the label was rendered soluble. These results indicate that histone-bound RNA is not similar to ribosomal RNA, even though ribosomal RNA was synthesized during the period of our observations.

*Discussion.*—The biological role of the histone-bound RNA described in the present report is unknown. It is possible that this trace component is an intermediate in the synthesis of messenger RNA. As messenger RNA is transcribed off the DNA template, it may be bound to nuclear basic protein prior to its attachment to the ribosome.

Another possibility is that the histone-bound RNA may play a primary role in the control of gene function. Sypherd and Strauss[7] postulated the existence of a hypothetical repressor substance containing both protein and polyribonucleotide moieties. The protein portion of the molecule was presumed to have specificity for

small effector substances, whereas the RNA component was thought to have base-pairing specificity for the DNA of the gene.

Paigen[19] suggested a system of gene regulation based on the concept that any given RNA is transcribed along only one strand (up strand) of RNA and that the function of the unread strand (down strand) of DNA is to displace the RNA product from the DNA by competitive hydrogen bonding. If the RNA of the histone complex were transcribed from a regulator portion of the genome and had areas complementary to some portion of the down strand of the structural gene, the complex could act as a repressor of genetic information by base pairing with the unread strand of DNA, preventing messenger RNA synthesis. However, the regulator RNA of the histone complex could also control gene action by a different mechanism. Native DNA is believed to exist as a coiled helix complexed with histone.[20, 21] If the regulator RNA base paired with sites on the unread strand of the DNA and interacted with histone, the read strand of the DNA could uncoil and thus be available for messenger RNA synthesis. In this scheme, which is similar to that of Frenster,[22] regulator RNA acts as a specific derepressor of gene function. At present it is not known whether the RNA-histone complex is involved in the control of gene function and, if it is, whether it acts as a derepressor or repressor of genetic information. However, as this complex is now a defined entity, its place in mammalian cell metabolism may be investigated.

*Summary.*—Basic proteins of rat liver nuclei isolated by the cesium chloride density gradient method had a sharp banding profile on equilibrium centrifugation. The density at which the protein banded was the same reported by Huang and Bonner in their studies on pea seedling histones.[8] We have found an RNA species intimately associated with the nuclear histone fraction. The RNA of the RNA-histone complex was RNase-insensitive when bound to histone but sensitive to the enzyme when removed from the protein. The RNA was heterogeneous when studied by the sucrose density gradient technique and its calculated base ratio indicated a high adenine and uridine content. These observations indicate that this RNA is not ribosomal or transfer RNA.

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† Postdoctoral fellow, National Cancer Institute grant CA 5011.

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## *DEFICIENCIES OF CYSTATHIONASE AND HOMOSERINE DEHYDRATASE ACTIVITIES IN CYSTATHIONINURIA*

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Congenital cystathioninuria is a familial disease first reported in 1958.[1] Too few patients with this condition have been studied to allow definition of the clinical syndrome, but it may include various congenital defects and mental retardation or aberration.[1-3] Harris *et al.*,[1] and subsequently Brenton *et al.*,[4] demonstrated abnormally high concentrations of cystathionine in tissue extracts from a cystathioninuric patient. Cystathionine was detected also in the serum of a patient reported by Frimpter *et al.*[2] The presence of increased tissue cystathionine content led Harris *et al.* to advance the hypothesis that this syndrome resulted from a deficiency of the enzyme cystathionase,[5] which normally cleaves cystathionine (reaction 1):[1]

$$\text{Cystathionine} + H_2O \rightarrow \text{cysteine} + \alpha\text{-ketobutyrate} + NH_3. \quad (1)$$

To test this hypothesis directly we assayed hepatic cystathionase, using the methods developed in the course of our studies of human cystathionine synthase (E.C.4.2.1.21)[5] deficiency.[6] Cystathionase activity was markedly reduced in the extract prepared from the liver of a patient with cystathioninuria.

Crystalline rat liver cystathionase also catalyzes homoserine deamination[7] [L-homoserine hydro-lyase(deaminating) E.C.4.2.1.15] (reaction 2):

$$\text{Homoserine} \rightarrow \alpha\text{-ketobutyrate} + NH_3. \quad (2)$$

Patients lacking cystathionase offer a unique opportunity to determine whether the human enzyme, like that of the rat, has this second activity. We developed a microassay for homoserine dehydratase and found that the extract shown to be deficient in cystathionase activity was also markedly deficient in homoserine dehydratase activity. This suggests that cystathionase and homoserine dehydratase are catalyzed by a single enzyme in man and indicates that cystathioninuria is associated with deficiences of at least two enzymatic activities.

*Experimental Procedures and Methods.*—Assays were performed on extracts of human liver because we failed to detect cystathionase activity in human skin, erythrocytes, or leucocytes.