Journal of Clinical Investigation
Vol. 45, No. 3, 1966

Cystinuria: Biochemical Evidence for Three Genetically Distinct Diseases *

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In the early 1950's, Harris, Mittwoch, Robson, and Warren (1, 2) investigated the mode of inheritance of cystinuria in 27 families by using quantitative determinations of cystine and dibasic amino acids as the genetic marker. Homozygotes were identified by the formation of urinary tract calculi composed of cystine and by gross hyperexcretion of cystine, lysine, arginine, and ornithine. Investigation of known heterozygotes (parents and children of affected subjects) revealed distinct phenotypic heterogeneity and identified two types of families. In one, comprising about two-thirds of the pedigrees studied, heterozygotes uniformly excreted normal quantities of cystine and dibasic amino acids, and genetic analysis was compatible with autosomal recessive inheritance. In the second, smaller group of pedigrees, an intermediate phenotype was found. All heterozygotes tested excreted moderate excesses of cystine and lysine. The urinary excretion pattern in these detectable heterozygotes was interpreted as evidence for incompletely recessive inheritance, and hence suggested that cystinuria was more than a single inherited disease process.

During the past three years, *in vivo* and *in vitro* studies (3–6) have provided convincing evidence for an intestinal as well as a renal transport defect in cystinuria. We reported previously (6) that jejunal mucosa from ten of twelve cystinuric subjects lacked the ability to accumulate cystine, lysine, or arginine. However, two other patients with cystinuria and renal lithiasis were noted to transport cystine normally and to accumulate lysine and arginine to approximately half normal values. The present study was initiated to clarify the biochemical and genetic nature of the intestinal transport differences observed and to relate our findings to those of Harris and co-workers. The results indicate that cystinuria represents at least three biochemically and genetically distinct diseases.

Methods

Fifteen patients (nine female, six male) with documented cystinuria, ranging in age from 19 to 47 years, and 29 normal volunteer subjects were admitted to the Clinical Center of the National Institutes of Health for study. Each of the cystinuric patients had formed numerous cystine stones and excreted gross excesses of cystine, lysine, arginine, and ornithine in the urine.

In vitro studies. Jejunal mucosa was obtained by peroral biopsy using a Rubin tube placed at the ligament of Treitz under fluoroscopic control. Kidney cortex was obtained by open biopsy in patients undergoing renal surgery for removal of stones or unroofing of benign cysts. The techniques of incubating jejunal mucosa and kidney cortex slices with ^{14}C- and ^{35}S-labeled L-amino acids have been described extensively (6, 7), as have estimations of tissue water, inulin space, and distribution ratios for labeled amino acid concentration in cell water to that in the incubation medium (6, 7). L-Cystine-^{35}S, L-lysine-^{14}C, and L-arginine-^{14}C were obtained commercially as described previously (6). In studies designed to determine the effect of removal of sodium ion from the incubation medium, equimolar Tris hydrochloride (buffered to pH 7.4) was substituted for sodium chloride and sodium bicarbonate.

Oral loading studies. After an overnight fast, L-cystine [1] (0.5 mmole per kg body wt) was mixed with 100 ml distilled water and ingested quickly. The glass container was then rinsed with four 25-ml washes of distilled water to insure complete ingestion of the amino acid load. Ten-ml samples of venous blood were obtained before and 1, 2, 3, and 5 hours after the cystine load. The blood

* Submitted for publication July 29, 1965; accepted November 30, 1965.

Presented in part at the Annual Meeting of The American Society for Clinical Investigation, Atlantic City, N. J., May 2, 1965.

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[1] Fisher Scientific Co., Washington, D.C.

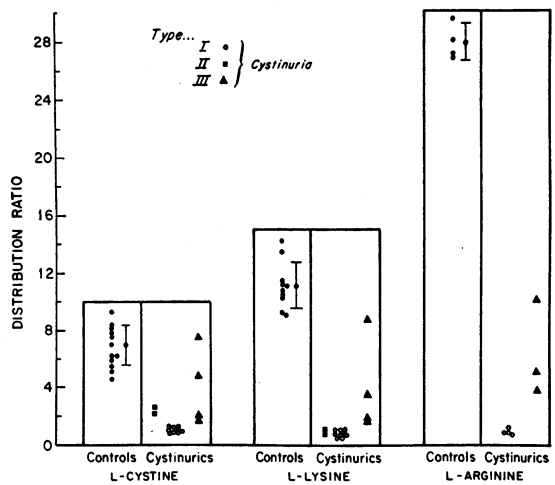

FIG. 1. UPTAKE OF CYSTINE, LYSINE, AND ARGININE BY JEJUNAL MUCOSA FROM CONTROL AND CYSTINURIC SUBJECTS. Mean ± 1 SD for control values is shown in bracket adjacent to individual points. All data were obtained from 45-minute incubation studies. Cystinuric subjects are separated into types I, II, and III as described in the text. Concentrations of cystine, lysine, and arginine were those given in Table I.

samples were immediately placed in 10-ml volumetric flasks containing 0.1 M phosphate buffer (pH 6.8), iodoacetate, and sodium bicarbonate to prevent oxidation of cysteine to cystine (8). Plasma cystine was determined by column chromatographic techniques with a Phoenix amino acid analyzer.

Genetic survey. First morning, fasting urine samples were obtained from 24 patients and children of 10 of the affected cystinuric subjects. Urine samples were frozen immediately until analyzed for cystine, dibasic amino acids, and creatinine. Cystine concentration was estimated by photometric sulfhydryl determination after electrolytic reduction as described by Crawhall, Saunders, and Thompson (9), and is reported as milligrams cystine per gram creatinine to standardize the results. Urinary creatinine was determined by the method of Taussky (10). Lysine and arginine were identified in urine samples by monodirectional high-voltage electrophoresis (4,000 v) with a 1.6% formic acid solvent. Creatinine determinations were performed on each urine sample before electrophoresis and a volume equivalent to 20 μg of creatinine was spotted in 120-cm sheets of Whatman 3 MM paper in each case. Appropriate lysine and arginine standards were run with each group of samples. Electrophoresis was carried out for 120 minutes after which papers were dried and stained with Ninhydrin.

Results

Uptake of cystine, lysine, and arginine by intestinal mucosa. The results of 45-minute incubation studies with cystine, lysine, or arginine as labeled substrate are shown in Figure 1. As reported previously (4, 6), intestinal mucosa from normal subjects accumulated these amino acids in the cell water to concentrations much greater than those in the incubation medium, yielding mean distribution ratios of 7.0 for cystine, 11.2 for lysine, and 28.1 for arginine. However, amino acid uptake by gut from cystinuric subjects revealed distinct heterogeneity. Jejunal mucosa from nine of these patients (type I), including two identical twins, exhibited complete inability to accumulate lysine and cystine in excess of the concentration in the incubation medium, yielding distribution ratios of approximately 1. Similar results were noted with arginine in four of these nine patients. Six cystinuric subjects accumulated these amino acids quite differently from the nine just described. In two subjects (type II) ratios of 1 were noted for lysine, but cystine was accumulated to ratios of 2 or more. In the four remaining cystinurics (type III) from three families cystine, lysine, and arginine were taken up by gut mucosa distinctly in excess of the concentration in the incubation medium.

It should be pointed out that the uptake results in the latter group (type III) revealed significant scatter. Two subjects accumulated cystine and lysine almost normally; two others (siblings) accumulated both amino acids to ratios of approximately 2. When gut mucosa from the latter two patients was incubated with arginine, distribution ratios greater than 4 were noted in each, in marked contrast to the results with the type I cystinuric subjects. Furthermore, additional biochemical and

TABLE I

*Uptake of cystine, lysine, and arginine by gut mucosa from normal subjects and patients with cystinuria**

Subjects	Distribution ratio + 1 SD: Cystine 0.03 mmole per L†	Lysine 0.065 mmole per L	Arginine 0.065 mmole per L
Control	7.0 ± 1.4 (12)‡	11.2 ± 1.6 (10)	28.3 ± 1.3 (4)
Cystinuric§			
Type I	1.1 ± 0.2 (9)	1.0 ± 0.3 (9)	0.9 ± 0.2 (4)
Type II	2.4 (2.3, 2.5)‖ (2)	1.0 (0.9, 1.1)‖ (2)	
Type III	4.1 ± 2.8 (4)	4.2 ± 3.0 (4)	6.6 ± 3.3 (3)

* Tissues were incubated for 45 minutes in Krebs-Ringer bicarbonate buffer, pH 7.4, at 37° C.
† Initial medium concentration.
‡ Value in parentheses refers to number of subjects studied (each study was performed in duplicate).
§ Distribution ratios in all three cystinuric groups differed significantly from values for control subjects ($p < 0.01$).
‖ Results in both subjects given in parentheses.

genetic studies, to be presented subsequently, indicated that the latter two subjects could best be grouped with the type III cystinurics. These *in vitro* results are summarized in Table I. Throughout the remainder of this report, the cystinuric patients will be identified as type I, type II, or type III, respectively, based on the uptake results noted above.

These results, unexpected for a single inherited disease entity, raised four questions, which we have attempted to answer: Were all of these patients homozygous? Did the *in vitro* findings reflect qualitative or quantitative differences within the cystinuric population? Did the *in vitro* findings reflect *in vivo* pathophysiologic differences? Were the three types of cystinurics observed in this study related to the two groups identified by Harris?

Demonstration of homozygous state. Each cystinuric subject had formed numerous urinary tract calculi composed of cystine, and exhibited great excesses of urinary lysine and arginine by high voltage electrophoresis. As shown in Figure 2, urinary excretion of cystine in several type II and type III subjects was in the range observed for the patients with the most severe gut lesion (type I). Furthermore, lysine uptake (Figure 2) by kidney cortex obtained at surgery from one of the type III subjects was distinctly impaired, as was that observed in three type I subjects (7). Thus, all three groups of cystinuric subjects fulfilled the previously accepted (1, 2) criteria for the homozygous state and could not be separated by urinary cystine values or by present results of *in vitro* studies with kidney cortex.

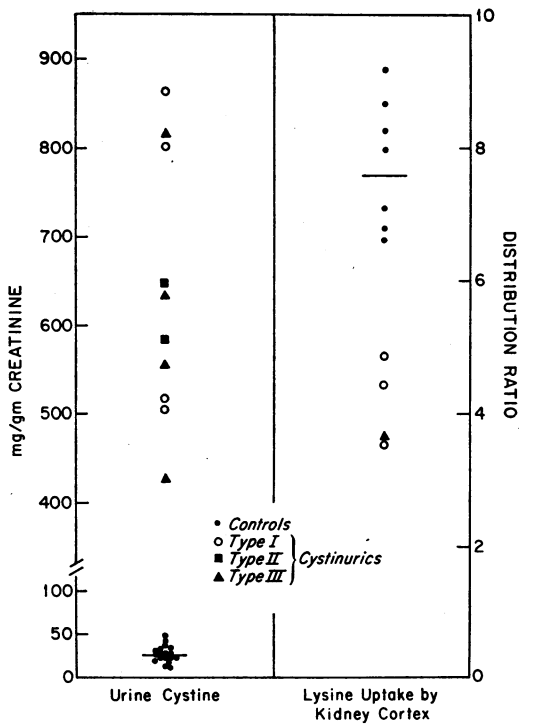

FIG. 2. DETERMINATION OF URINARY CYSTINE AND UPTAKE OF LYSINE BY KIDNEY CORTEX SLICES. Cystine determinations were performed on first morning, fasting urine samples. Kidney slices were incubated for 30 minutes in Krebs-Ringer bicarbonate buffer, pH 7.4 at 37° C. Lysine concentration was 0.065 mmole per L.

TABLE II

Characterization of lysine and cystine uptake by gut mucosa from normal and cystinuric subjects

Substrate	Experimental conditions	Minutes of incubation	Per cent inhibition*			
			Control	Cystinuric		
				Type I	Type II	Type III
L-Lysine 0.065 mmole per L	Anaerobic†	45	52–68	0	0	54–65
	Na^+ free	45	46–60	0	0	45–50
	L-Arginine (0.8 mmole per L)	15	45–54	0	0	35–47
L-Cystine 0.03 mmole per L	Anaerobic	45	50–70	0	64	70–80
	Na^+ free	45	68–80	0	79	67–75
	L-Arginine (0.8 mmole per L)	15	25–45	0	23	38–50

* Calculated from results in three subjects from control group, cystinuria type I, and cystinuria type III. Range of values presented for controls and type III cystinurics. None of the type I cystinurics showed inhibition under the conditions used. The results for type II cystinuria were obtained from a single patient.

† Anaerobic conditions were achieved by gassing Krebs-Ringer bicarbonate buffer, pH 7.4, with 95% N_2-5% CO_2. The incubation flasks were regassed with the N_2-CO_2 and stoppered for the duration of the experiment.

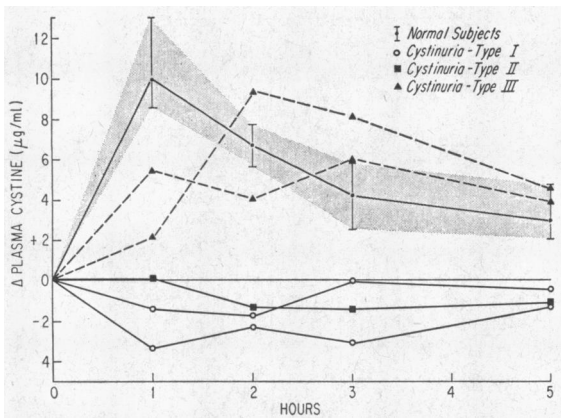

FIG. 3. RESPONSE OF PLASMA CYSTINE CONCENTRATION TO ORAL CYSTINE LOAD. Oral cystine load was 0.5 mmole per kg. Control values represent mean and range of results in three subjects.

Evidence for qualitative differences. In studies of amino acid transport *in vitro,* a distribution ratio of 1 may result from uptake by nonmediated, nonenergy-dependent processes conforming to the laws of physical diffusion, and may thus represent the absence of active transport mechanisms. This possibility was explored in repeated studies of amino acid uptake by gut mucosa from representative subjects of the three groups of cystinurics. These results are summarized in Table II. As expected, anaerobic conditions and removal of Na^+ from the incubation medium produced distinct inhibition of lysine and cystine uptake by mucosa from normal subjects. In addition, arginine, known to share a common mediated transport process with lysine and cystine in human gut (6), competed with lysine for uptake by normal mucosa. However, neither anaerobic conditions, removal of Na^+, nor the presence of arginine was noted to influence lysine or cystine uptake by mucosa from type I subjects, in contrast to the results of identical experiments performed with jejunal mucosa from type III cystinurics, all of whom responded to anaerobiosis, Na^+ deletion, and arginine competition as the controls did. The results in a single type II subject fell midway between the two extremes, that is, cystine uptake was sensitive to the altered experimental conditions employed, but lysine uptake was not. Finally, lysine uptake by mucosa from type I and type II cystinurics yielded distribution ratios of unity when the medium concentration of lysine was reduced from 0.065 to 0.003 mmole per L and failed to exhibit saturation kinetics over a 400-fold range of substrate concentrations.

In vivo differences. The physiologic significance of these *in vitro* results was investigated by oral loading studies with cystine. As is noted in Figure 3, three normal subjects showed a distinct rise in plasma cystine within 1 hour after ingestion of this amino acid, but two type I and one type II cystinurics exhibited no increase in plasma cystine during the 5-hour study, their blood levels actually falling slightly in each case. However, the two type III cystinurics who showed the most normal intestinal uptake of cystine *in vitro* also evidenced a distinct rise in plasma cystine concentration after the oral load that approached that in control subjects. Hence, the *in vitro* transport differences were also reflected in the ability of the cystinuric patients to absorb cystine from the intestinal tract *in vivo.*

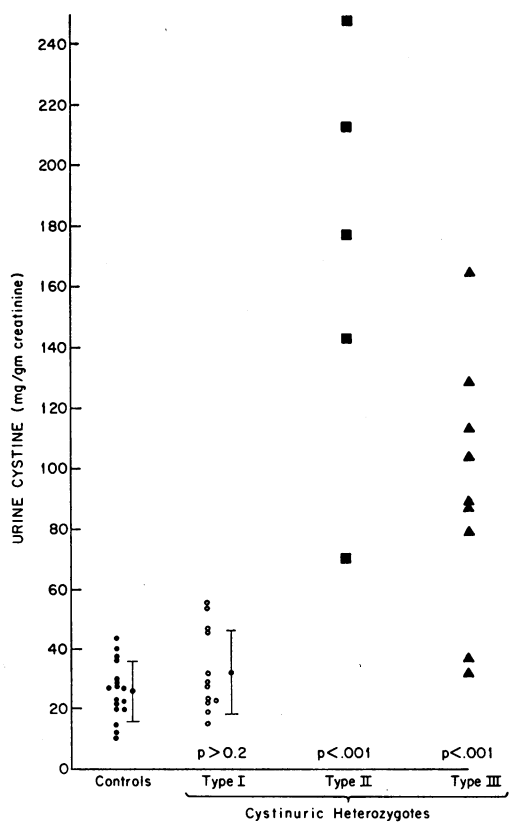

FIG. 4. DETERMINATION OF URINARY CYSTINE CONCENTRATION IN CONTROL AND CYSTINURIC HETEROZYGOUS SUBJECTS (PARENTS AND CHILDREN OF AFFECTED SUBJECTS). First morning urine samples were analyzed throughout.

TABLE III

Summary of evidence for three genetically distinct types of cystinuria

Type	Mediated intestinal transport			Urinary excretion of cystine and lysine by heterozygous subjects
	Lysine	Cystine	Arginine	
I	Absent	Absent	Absent	Normal
II	Absent	Present	Not tested	Excessive
III	Present	Present	Present	Excessive

Genetic survey. These results suggested that there are, in fact, at least three types of homozygous cystinuric subjects and led us to re-examine the pedigrees of affected subjects for additional evidence of genetic difference. First morning urine samples were obtained from 20 normal subjects and from parents and children of type I, II, or III cystinuric subjects. Quantitative determinations of cystine and qualitative estimation of lysine and arginine were performed on each sample from the heterozygotes. As noted in Figure 4, all type I heterozygotes (five families) excreted normal quantities of cystine, and high voltage electrophoresis revealed no excess of lysine or arginine. All type II heterozygotes (one family) excreted distinctly increased quantities of cystine and lysine. Six of eight type III heterozygotes (three families) excreted excessive quantities of cystine and lysine. Two parents of type III subjects (from two different pedigrees) exhibited distinctly increased quantities of lysine while excreting normal quantities of cystine. Though the population is small, statistical analysis indicated that the extent of cystine hyperexcretion observed in type II heterozygotes was significantly greater than that seen in type III heterozygotes ($p < 0.02$).

There are striking similarities between these results and those reported by Harris and co-workers (1, 2). They described one group of heterozygous subjects who could not be distinguished from control subjects, as we have with type I heterozygotes. He described a second group of heterozygous subjects with abnormal urine patterns, identical in kind to those observed in both our type II and type III heterozygotes. Hence, it is quite likely that the type I designation of this report is identical to the "recessive" notation of Harris, and that our type II and type III subjects were both included in his "incompletely recessive" group.

Discussion

These studies indicate that cystinuria is indeed a group of biochemically and genetically distinct inherited diseases. The evidence for this hypothesis is summarized in Table III. There can be no doubt that the *in vitro* transport results, the *in vivo* cystine loading studies, and the results of the family survey distinguish between types I and III. The type II patients require comment. In these subjects, the transport and absorption results resembled the data from type I cystinurics, with the important exception that in the type II patients cystine uptake was accomplished by a mediated process apparently absent in type I subjects. It might still have been possible to include these individuals as a subtype within the type I population, but the urinary findings in heterozygous subjects made this untenable. Similarly, to have included these individuals in the type III population would have required dismissal of both the results of lysine uptake *in vitro* and oral cystine loading *in vivo*. For these reasons, a third group was deemed mandatory.

It is apparent from Figure 1 and Table I that the standard deviation noted in the uptake results

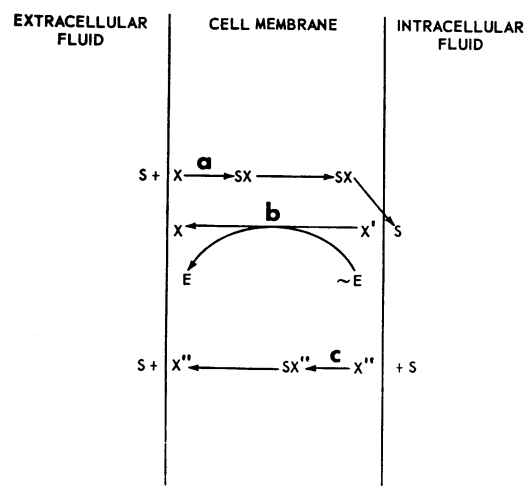

FIG. 5. SCHEMATIC REPRESENTATION OF MEDIATED TRANSPORT SYSTEM AND POSSIBLE SITES OF GENETIC LESION IN CYSTINURIA. S = substrate; X = carrier or enzyme; X′ = inactivated carrier; E = source of energy; and X″ = carrier required for efflux that may or may not be identical with X. Sites a, b, and c represent possible locations for defect, as discussed in the text.

with gut from type III cystinuric subjects is considerably greater than that seen in either the control group or the other two cystinuric populations. This may indicate still greater heterogeneity within this cystinuric population, but there is no additional evidence from the genetic survey to support this idea at present.

Intestinal mucosa from type I cystinuric subjects appears to lack completely the mechanisms for mediated or active transport of cystine, lysine, and arginine. What is responsible for this absence of active transport? If we consider (Figure 5) that active transport mechanisms result from binding of substrates to specific enzymes or carriers within the cell membrane (site a, Figure 5) coupled to the expenditure of metabolic energy (site b, Figure 5), then disruption of either of these crucial steps could abolish uphill movement.

If one postulates that the mechanism for coupling transport of cystine and the dibasic amino acids is defective in Type I cystinuria and that the membrane carrier is intact, arginine should inhibit still further the already reduced uptake of cystine and lysine, a finding not observed in type I subjects. Furthermore, since other amino acid transport systems are not defective in cystinuric gut (6), such a postulate would imply the existence of a coupling mechanism or mechanisms specific for cystine and the dibasic amino acids, a thesis for which there is no evidence.

It seems more likely that the transport defect in this recessive disease results from defective synthesis of a specific enzyme or carrier protein in the region of the cell membrane that normally binds cystine and the dibasic amino acids. This hypothesis would explain the distribution ratios of 1, the results with Na^+ free media and anaerobiosis, and the failure of large excesses of arginine to compete with lysine or cystine for uptake by gut mucosa from these subjects. We may extend this thesis to include the type II and III cystinuric subjects by suggesting that the same carrier is affected in these patients, but in a less damaging way, so that mediated transport of either cystine (type II), or of cystine, lysine, and arginine (type III) is retained, at least in part. The results in type III cystinurics could also be explained by postulating a genetically controlled acceleration of efflux (site c, Figure 5) rather than a slowing of influx, since both would result in lower steady state distribution ratios. Such a hypothesis implies that efflux of amino acids is a mediated process rather than a simple "leak," an idea for which there is growing experimental evidence (11, 12).

In contrast to the distinct heterogeneity observed between cystinuric families, pedigree analysis within each family has shown a remarkable consistency. Urinary amino acids have been used as the genetic marker in nearly 40 pedigrees studied by Harris and co-workers (1, 2), Crawhall, Saunders, and Thompson (13), and ourselves. In each case, either all heterozygous subjects (parents and children of affected subjects) excreted abnormal quantities of cystine, lysine, or both, or none of them did. There is, to use present terms, not a single instance of a cystinuric subject resulting from the union of one parent with a normal amino acid pattern in the urine (type I) and one with excessive urinary cystine or lysine (type II or III). If this pattern persists as more families are studied, it will lend strong support to the thesis, originally suggested by Harris (2), that the heterogeneity in cystinuria results from mutations at different loci. Allelic, but complementary, mutations could also explain the results.

Summary

Results of *in vitro* transport studies with gut mucosa, oral loading studies with cystine, and patterns of urinary excretion of cystine and dibasic amino acids in heterozygous subjects indicate that cystinuria is at least three biochemically distinct inheritable diseases.

Evidence is presented which suggests that in one form of cystinuria active or mediated transport of cystine, lysine, and arginine by gut mucosa is totally abolished, a finding compatible with functional or structural absence of a specific enzymatic carrier protein.

The pattern of urinary amino acid excretion seen in known heterozygous subjects suggests that the phenotypic heterogeneity observed results from autosomal mutations at different loci or from mutations that are allelic and complementary.

Acknowledgment

We would like to thank the following physicians for allowing us to study their patients: Dr. Donald Beard, Emory University, Atlanta, Ga.; and Drs. Fred Bartter and Myron Lotz, National Heart Institute, Bethesda, Md.

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