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Studies of Glucuronidation. IV. Evidences of Different Processes for *o*-Aminophenol and *p*-Nitrophenol* (33003)

ANTHONY R. TEMPLE, MICHAEL S. CLEMENT, AND ALAN K. DONE
Department of Pediatrics, University of Utah College of Medicine, Salt Lake City, Utah 84112

In recent years several authors have suggested that there may be more than one, perhaps several, hepatic glucuronyl transferases. Isselbacher *et al.*(1,2) reported that an enzyme solubilized from rabbit liver microsomes readily conjugated ether and ester aglycones, but not aniline. The Gunn rat, which has an inherited inability to conjugate bilirubin, has been found to conjugate aniline(3) and *p*-nitrophenol (PNP)(4), but not *o*-aminophenol (OAP)(3,5,6,7). Certain differences between OAP and PNP have been found in their reactions to various inhibitors of glucuronidation(8), and in their rates of development in the immature mouse(9).

The studies reported herein provide additional evidence that OAP and PNP are conjugated with glucuronic acid by different means, probably involving different enzymes.

Materials and Methods. The basic procedures were as described elsewhere(10). "Adult" Holtzman rats, Gunn rats, Hartley guinea pigs and postmortem human liver samples were used. The rats ranged in age from 3 to 6 months, and the guinea pigs 2–4 months. Animals were decapitated, after being stunned by a blow to the head, and their livers were excised and washed in cold (approximately 4°C) 0.15 *M* KCl containing 3.2×10^{-4} *M* $KHCO_3$, pH 7.0. Human postmortem liver specimens were obtained as soon after death as possible (usually several hours) and then were frozen immediately until used.

Portions of liver were weighed, placed in five volumes of the cold KCl–$KHCO_3$ solution and homogenized using a motor-driven Teflon homogenizer in an ice bath. This homogenate was then used in the "whole homogenate" assays for glucuronyl transferase activity. The "microsomal fraction" was obtained by centrifuging the whole homogenate at 8500*g* for 20 min and then centrifuging the supernatant at 109,000*g* for 30 min. The sedimented fraction, resuspended in 5 volumes of the KCl–$KHCO_3$ solution, was used for assaying microsomal activity.

Except where stated otherwise, the incubation mixture contained liver homogenate or microsomes equivalent to 200 mg (wet weight) of liver, 0.1 ml of 0.5 *M* Tris buffer at pH 7.4, 0.15 *M* $MgCl_2$, .07 m*M* OAP or

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TABLE I. Comparative Enzyme–Substrate Affinities (liver whole homogenates).

	OAP	PNP
Holtzman rat		
Percentage conjugation[a]	42 ± 2.4 (30)[b]	85 ± 1.1 (22)[b]
K_m[c]	3.1×10^{-4} *M*	2.0×10^{-4} *M*
V_{max}	12.5×10^{-9} *M*/min	25.0×10^{-9} *M*/min
Guinea pig		
Percentage conjugation[a]	82 ± 3.6 (11)[b]	96 ± 1.2 (9)[b]
K_m[c]	$.72 \times 10^{-4}$ *M*	$.48 \times 10^{-4}$ *M*
V_{max}	26.3×10^{-9} *M*/min	31.3×10^{-9} *M*/min

[a] In 30 min (see "Methods").
[b] Mean ± SEM (no. of assays in parentheses).
[c] Michaelis constant.

PNP, and uridine diphosphate glucuronic acid (UDPGA) in a concentration of 3 m*M* when rat liver was used or 0.3 m*M* when guinea pig liver was employed; incubation was in a volume of 1.5 ml at 37°C for 30 min for the quantitative assays, while for kinetic studies the incubation time used to determine initial velocity (V_0) was 10 min for the rat and 5 min for the guinea pig. All of the aforementioned conditions have been determined to be optimum(10). Lineweaver-Burk and Hofstee plots of the kinetic data suggested that the Michaelis-Menten relationship existed under the conditions mentioned above (notably including the presence of nonlimiting quantities of UDPGA).

The OAP-glucuronide was measured by the method of Dutton and Storey(11). The PNP-glucuronide formation was estimated indirectly by measuring the disappearance of free PNP using the method of Isselbacher(12), and directly by the method of Clement *et al.*(13). In those experiments employing a solubilized preparation of glucuronyl transferase, the method of Isselbacher(1,2) employing venom of the Habu snake (*Trimeresurus flavoviridis*) was used. Experiments employing a breast milk inhibitor were as described by Arias(14); the volume of milk used was 0.2 ml.

Results. Enzyme–substrate affinities. Under optimal assay conditions for liver whole homogenates, it appears that PNP is more readily conjugated than is OAP by both the rat and the guinea pig (Table I). Over a 30-min period of incubation (by which time the reaction has gone to completion), the rat conjugated twice as much PNP as OAP. The difference was less in the case of the guinea pig, which conjugated only 14% more PNP than OAP. This species difference was attributable to a relatively poor affinity of the rat liver enzyme for OAP.

Kinetic studies employing initial rate measurements and variations of substrate concentrations to the point of enzyme saturation provide confirmatory results (Table I). In both the rat and guinea pig the Michaelis constant (K_m) with OAP as the acceptor substrate suggests an approximately 1.5 times greater affinity for PNP as the acceptor than for OAP. However, V_{max}, the maximum achievable velocity (approached asymptotically with increasing quantities of substrate), is half as great for OAP as for PNP in the rat. In contrast, there is only a slight difference in V_{max} for the two substrates in guinea pig liver. These findings suggest that the differences between PNP and OAP conjugation by rat liver are due less to differences in reactivity of the substrates than to differences in the enzymatic processes per se. The curves are interpreted as indicating that the two substrates saturate the enzyme(s) at similar concentrations, but at lower levels of activity in the case of OAP, especially in the rat. Permeability differences at the microsomal membrane may have played a role, but this factor unfortunately could not be evaluated because we have been unable to obtain any activity for OAP-glucuronide formation in our soluble preparations (see below).

TABLE II. Glucuronide Formation by Homogenates of Gunn Rat Liver with and without Subsequent Hydrolysis of β-Glucuronidase.

	Mean glucuronide (%)[a]	
	OAP	PNP
Without added UDPGA	1.5 (5)	1.5 (8)
With 3 m*M* UDPGA	14 (5)	76 (8)
Hydrolysis experiment		
Control (no hydrolysis)		83
β-glucuronidase hydrolysis[b]		3.0
β-glucuronidase hydrolysis[b] and saccharate inhibition		67

[a] Number of multiple assays in parentheses.

[b] Remaining glucuronide measured after 60-min incubation with 50 U of β-glucuronidase (Warner-Chilcott Co.) at pH 4.5 and 37°C in fresh 0.1 *M* acetate buffer; saccharo-1,4-dilactone concentration was 0.6 m*M* (total incubation vol., 6.2 ml).

Gunn rat. Both whole homogenate and microsomal preparations from Gunn rats actively conjugated PNP but not OAP. Table II presents the whole homogenate data. The conjugation of 76% of the PNP compares favorably with the figure of 85% obtained with nomal rat liver. By contrast, the Gunn rat conjugated only one-third as much OAP as did normal rats. Microsome preparations from Gunn rat liver (data not shown) conjugated 64% of PNP in 30 min compared with 80% for Holtzman rats. To confirm that glucuronides of PNP were being formed, β-glucuronidase was added to the incubation mixture after 30 min (by which time the full complement of glucuronide presumably had been formed) under conditions appropriate for hydrolysis of β-glucuronides(13). In the representative "hydrolysis experiment" shown in Table II it can be seen that of the 83% PNP–glucuronide apparently formed only 3% remained after β-glucuronidase hydrolysis unless the glucuronidase inhibitor, saccharo-1,4-dilactone, was present in which case there remained 67% of the PNP in the form of the glucuronide.

Inhibition studies. (*a*) *Breast-milk inhibition.* A breast milk sample known to contain an inhibitor of bilirubin glucuronide formation (i.e., one which had produced clinical jaundice in a neonate) was found to inhibit glucuronide formation in both the rat and guinea pig with both OAP and PNP as acceptor substrates. It was elected to employ this inhibitor in comparisons of OAP and PNP glucuronidation.

The effects of the inhibitor on the kinetics of glucuronidation by rat liver homogenates are illustrated in Fig. 1 for OAP and in Fig. 2 for PNP; similar data for the guinea pig are shown in Fig. 3 for OAP and in Fig. 4 for PNP. In both species, the inhibition of glucuronidation is noncompetitive in the case of OAP, but competitive in the case of PNP.

(*b*) *Mutual inhibition.* In order to assess further the relationship between the conjugation of the two substrates under study, mutual inhibition studies were performed in which the glucuronidation of one substrate was measured with and without the presence of the other. These data were also analyzed kineti-

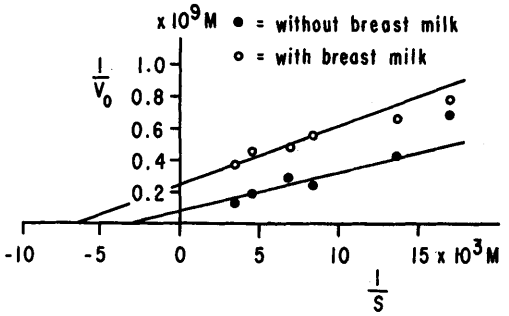

FIG. 1. Lineweaver-Burk plot of kinetic data for the glucuronidation of OAP by rat liver homogenate, with and without the addition of a breast-milk inhibitor. Inhibition is noncompetitive.

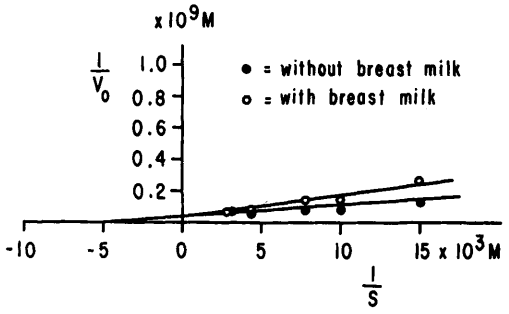

FIG. 2. Lineweaver-Burk plot of kinetic data for the glucuronidation of PNP by rat liver homogenate, with and without the addition of a breast-milk inhibitor. Inhibition is competitive.

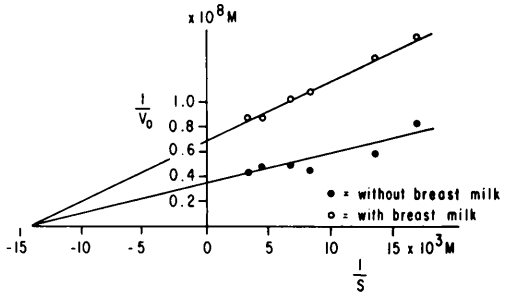

FIG. 3. Lineweaver-Burk plot of kinetic data for the glucuronidation of OAP by guinea pig liver homogenate, with and without the addition of a breast-milk inhibitor. Inhibition if noncompetitive.

cally according to the Lineweaver-Burk method of graphical analysis. The OAP in a 2:1 to 5:1 molar excess over PNP inhibited PNP glucuronide formation only very slightly in a noncompetitive fashion, as is shown in Fig. 5. By contrast, PNP in a 2:1 to 5:1 molar ratio over OAP inhibited glucuronide formation quite markedly and the inhibition again appeared to be noncompetitive (Fig. 6).

Temperature effects. By varying the incubation temperature it was found that OAP conjugation had a marked optimum of activity at 37°C, being only two-thirds as active at either 25 or 45°C (Fig. 7). On the other hand, PNP conjugation had approximately the same activity at 25 or 37°C, but greatly diminished activity at 45°C.

Intracellular localization. Assays of liver at optimal conditions revealed striking differences in intracellular localization and response to solubilization of UDP-glucuronyl transferase activity toward the two substrates (Table III). Here, again, the lesser activity of rat liver homogenate toward OAP than PNP is evident. Even more striking, however, is the paucity of activity toward OAP in microsomes of rat liver, only 21% of the whole homogenate activity being demonstrable there. This is contrasted with 89% of activity toward PNP being microsomal. In the guinea pig the difference between the two substrates

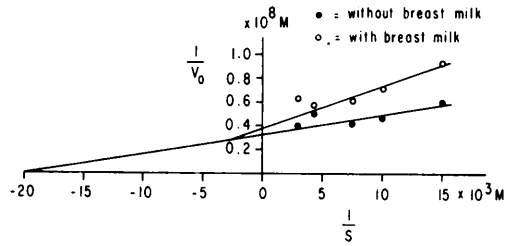

FIG. 4. Lineweaver-Burk plot of kinetic data for the glucuronidation of PNP by guinea pig liver homogenate, with and without the addition of a breast-milk inhibitor. Inhibition is competitive.

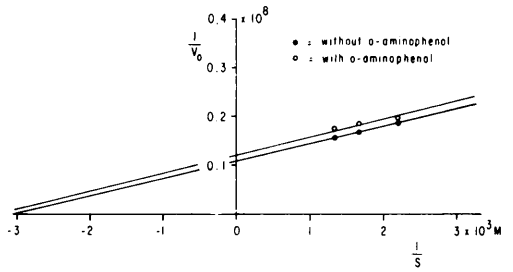

FIG. 5. Lineweaver-Burk plot of the conjugation of PNP by liver homogenate, in the presence or absence of OAP, 0.67 m*M*.

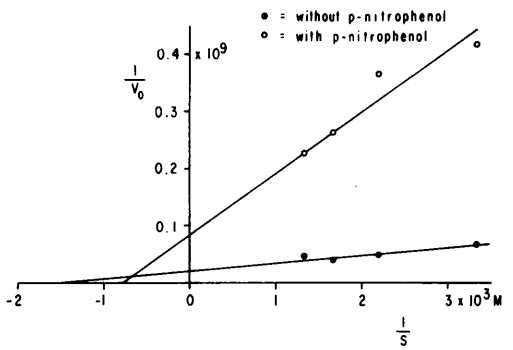

FIG. 6. Lineweaver-Burk plot of the conjugation of OAP by liver homogenate, in the presence or absence of PNP, 0.33 m*M*.

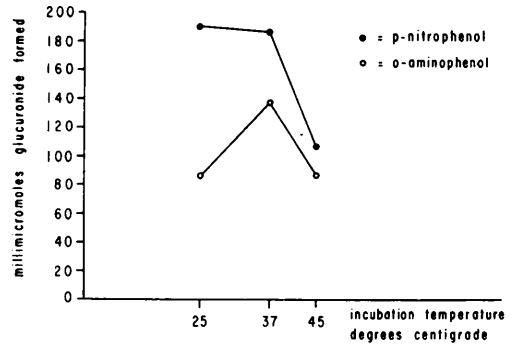

FIG. 7. Effects of temperature on the glucuronidation of OAP and PNP by rat liver homogenate (30-min incubation).

TABLE III. Representative Comparisons of the Distribution of Glucuronidation Activity among Various Fractions of Liver.

	Glucuronide formed (%)	
	OAP	PNP
Holtzman rat		
Whole homogenate	34	90
Microsomes	7	80
Solubilized	0	63
Guinea pig		
Whole homogenate	91	95
Microsomes	64	97
Solubilized	0	32

was less striking; the microsomal preparation accounted for 70% of the whole homogenate activity toward OAP and 100% toward PNP. In neither species could the enzyme concerned with OAP conjugation be solubilized, while solubilization of that concerned with PNP conjugation was accomplished.

Human liver activity. Whole homogenates of postmortem human liver were very active in the conjugation of PNP but conjugated very little OAP (Table IV). In each instance the experiments were duplicates; i.e., identical preparations were run simultaneously with the two substrates. There appeared to be no correlation between the extents of conjugation of the two substrates and no apparent relationship to age or the interval since death, although the data are too few to be certain of a lack of correlation. From the available data it cannot be determined whether the differences noted are related to more rapid deterioration of the PNP system, since no living or early postmortem specimens were studied. That such may be the case, however, is suggested by additional experiments which showed that the liver preparations from experimental animals retained their activity toward PNP, but not OAP, with aging or freezing.

TABLE IV. Relative Activity of UDP-Glucuronyltransferase toward OAP and PNP in Human Postmortem Liver Whole Homogenates.

Liver sample	Age of deceased (years)	Elapsed time between death and autopsy (hours)	Glucuronide formed (%)	
			OAP	PNP
1	70	6	8.0	97.0
2	90	11	3.0	78.0
3	57	13	15.0	95.0
4	5	14	2.5	80.0
5	74	18	27.0	97.0
Means			11.1	89.4

Discussion. With increasing investigations of glucuronidation, the question of multiplicity of UDP-glucuronyl transferase enzyme(s) has become critical. Historically, because of the difficulties of employing bilirubin in assays of glucuronidation activity, more readily assayable compounds such as OAP and PNP have been employed. In using such nonphysiological compounds, the question always arises as to the extent to which their metabolism reflects the physiological situation. The question is always a difficult one, but is made even more so by the prospect of there being different enzyme systems for different processes. As mentioned in the introduction, there has already accumulated considerable circumstantial evidence of the existence of more than one, perhaps several, glucuronyl transferase systems. The present studies provide strong, albeit indirect, evidence of the presence of different enzymes for the conjugation of the two substrates under study, both of which form ether glucuronides.

At the present time it is necessary to depend upon indirect, circumstantial evidence to make such an assessment, since the enzyme(s) has (have) not yet been isolated and purified. Final proof of heterogeneity must await characterization of purified preparations; in the meantime, a multiplicity of differences in characteristics of the reactions involving at least OAP and PNP would seem to be fairly conclusive evidence of there being separate systems, at least for these two substrates.

The results of kinetic studies are difficult to evaluate whenever an impure system is employed. It was surprising, however, that in the present studies the reactions followed quite closely the Michaelis-Menten (or perhaps more properly the Briggs-Haldane) relationships. This at least introduced the possibility of characterization by kinetic means. In

any event, within the limitations posed by such an impure system, the kinetic studies suggest that there are striking differences in the conjugation of the two compounds under study which are more likely to be related to enzyme differences than to characteristics of the substrates per se.

Perhaps the strongest evidences of heterogeneity provided by the present studies are the findings of striking discordance between the activities toward OAP and PNP in the Gunn rat and in human postmortem liver specimens. In both instances PNP was conjugated actively, while OAP was not. The findings in the Gunn rat are in agreement with those of Van Leusden *et al.*(4). The present studies offer the advantage of simultaneous determinations of activities of preparations from the same animal toward the two substrates. A number of additional examples of discordance in the ability of Gunn rats to conjugate various glucuronide acceptors are summarized by Dutton (15). To our knowledge, this is the first demonstration of discordance in this system in human liver. The present data do not enable us to differentiate between enzyme deterioration postmortem and actual differences in enzyme–substrate affinities as causes for this discordance. However, the question is irrelevant for present purposes since this finding is nonetheless indicative of the presence of differing systems.

The results of the mutual inhibition studies are generally in agreement with the findings of Storey(8) except that we found the minor inhibition of PNP conjugation by OAP to be noncompetitive rather than competitive. It is difficult to place much weight on this discrepancy, however, because the amount of inhibition exerted is so small that the Lineweaver-Burk type of characterization of inhibition is likely to be relatively unreliable. Be that as it may, a difference might be expected on the basis of the quantity of UDPGA utilized. Other studies from this laboratory(10) have demonstrated an extraordinary requirement for UDPGA, especially of rat liver, and our studies employed more of the glucuronide donor than has usually been used. Most suggestive of heterogeneous systems is the finding that PNP inhibited OAP conjugation far more strikingly than vice versa and that the inhibition was noncompetitive, rather than competitive as would be expected to be the case were the two substrates competing for the same active enzyme sites. Similar significance can be placed upon the results of the breast-milk inhibition studies. Although conjugation of both substrates was inhibited, the type of inhibition was clearly different for the two substrates in both the rat and the guinea pig.

Both the studies of intracellular localization of the enzyme activity and the kinetics of the reaction(s) suggest discordance not only between the conjugation of the two substrates, but also perhaps between the two species studied, the rat and the guinea pig. The reduced activity toward OAP as contrasted with PNP in whole homogenates and in microsomes is strikingly evident only in the rat. There are enough additional differences between handling of the two substrates in the guinea pig, however, to rule out the possibility that there are different enzymes for the two substrates only in the rat. The data are nonetheless indicative that there are interspecies, as well as intersubstrate, difficulties when one attempts to interpolate the results of glucuronidation studies. It is not known whether the apparent differences in localization of the systems for the two substrates are due to actual differences in the residences of enzymes within the cell or to differential effects of the procedures for demonstrating "localization." For instance, the failure to solubilize activity toward OAP may reflect destruction or inactivation of the enzyme by the solubilization procedure, perhaps by incubation at pH 9. Inactivation of the enzyme for bilirubin glucuronidation has been shown to occur at pH 9(16). Here again, however, the question does not diminish the weight of evidence of heterogeneity. Whatever the mechanism, these findings are suggestive of differing enzyme characteristics.

Any evidence of the existence of different processes for the glucuronidation of different substrates can only be as good as the proof that one is dealing with a glucuronide as the final product and that it is formed by enzymatic means. Such proof has generally been

lacking in studies which have suggested the presence of heterogeneous systems. The proof of enzymatic glucuronide formation in the present studies, although indirect, seems formidable. Details of the verification of PNP glucuronidation are described elsewhere(13). Briefly enumerated, they include comparison of the ultraviolet absorption spectrum with that of authentic PNP–glucuronide, hydrolysis of the formed product and reappearance of free PNP upon hydrolysis with β-glucuronidase, inhibition of such hydrolysis by saccharo-1,4-dilactone which is a known inhibitor of β-glucuronidase, the demonstration that the forward reaction is dependent upon UDPGA, and the failure of the reaction to proceed when the enzyme preparation was previously subjected to boiling. It should be emphasized that each of these various means of verification was employed in each of the experiments described herein, and with OAP as well as with PNP.

The existence of more than one glucuronidation system would not be particularly surprising in view of the fact that there are a number of different types of glucuronide linkages. Thus, it could reasonably be anticipated that different enzymes might be involved in the formation of ether, ester and N-linked glucuronides. Indeed, there is evidence(15) that substrates representative of these various types of linkages do involve different enzymatic processes. The finding that two substrates, both of which form ether glucuronides, are apparently handled by different systems is not only less readily explicable, but also opens to question the basis for heterogeneity of glucuronyl transferases, if such exists.

Any attempt to correlate the handling of various endogenous and exogenous materials is thwarted by insufficient information. It would seem to be clear on the basis of evidence summarized elsewhere(15) as well as from the present findings of active PNP conjugation by congenitally jaundiced (Gunn) rats and other studies from this laboratory that PNP is not metabolized by the same system as is bilirubin. It is less clear whether OAP and bilirubin are conjugated by the same or different system(s). The possibility that one or the other of these two foreign substrates can be correlated with corticosteroid conjugation remains to be explored fully. In any event, it is evident that caution must be exercised in basing conclusions regarding physiologic processes on studies of the glucuronidation of exogenous materials.

Summary. Evidences are presented that *p*-nitrophenol (PNP) and *o*-aminophenol (OAP) utilize different enzyme systems for their glucuronide conjugation. Included are differences between the two substrates in the kinetics of the reaction, the response to mutual inhibition and to inhibition by a breast-milk inhibitor of bilirubin metabolism, intracellular localization, ability of the responsible enzyme to be solubilized, activity of the conjugating processes in the Gunn rat and in postmortem human liver specimens, and the effects of temperature upon the rate of conjugation. These findings support a concept of multiplicity of UDP-glucuronyl transferase enzymes.

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Excretion of Delta-Aminolevulinic Acid in the Absence of Demonstrable Erythropoiesis (33004)

N. T. SHAHIDI AND M. L. EFRON (Introduced by K. J. Isselbacher)
Department of Pediatrics, University of Wisconsin; and Massachusetts General Hospital, Harvard Medical School

Several studies (1,2) have indicated that delta-aminolevulinic acid (ALA) is an early intermediate in the biosynthesis of porphyrins and heme in that the condensation of 2 molecules of this amino acid by a Knorr type of reaction results in the formation of porphobilinogen which, in turn, is converted to porphyrins (3). The development of sensitive colorimetric methods for quantitative estimation of ALA has led to the discovery of this amino acid in biological material (4, 5). Several investigators have shown that ALA is present in significant amounts in urine from healthy individuals (5–7). It is well known that in hepatic prophyria, the liver dysfunction is responsible for the enhanced excretion of ALA. In the absence of porphyria, however, no information as to the origin of urinary ALA is available. Since the erythrocyte precursors are by far the major site of porphyrin and heme synthesis, it is generally assumed that the source of urinary ALA is the nucleated erythron. To verify this assumption the urinary concentration of ALA was estimated in patients with no demonstrable erythropoiesis. These were 3 patients with congenital hypoplastic anemia (pure red cell anemia), aged from 8 to 12 years and 6 patients with aplastic anemia (bone marrow aplasia and peripheral pancytopenia), aged from 4 to 16 years. The patients with congenital hypoplastic anemia were known to have had the disease since infancy, and in no instance did their bone marrow specimens and peripheral blood reveal signs of erythropoiesis. They were all refractory to corticosteroids and required regular blood transfusions to maintain adequate hemoglobin concentration. Patients with aplastic anemia had pancytopenia of long standing. The examination of their bone marrow specimen on several occasions had revealed complete absence of erythroid and myeloid precursors. Only a few scattered lymphocytes were present.

The method used for the determination of ALA in the urine was that described by Mauzerall and Granick (5) with slight modification. The measurements were made on urine specimens from two 24-hour urine collections. The results as shown in Table I are expressed as the mean values for the two determinations. As seen in Table I, it is obvious that ALA-like substance is present in the urine from patients with severe aplastic anemia and congenital hypoplastic anemia in significant amounts. However, as pointed out by Mauzerall and Granick, the Ehrlich-positive products resulting from condensation of Dowex 50 eluate of normal urine may contain organic-solvent-insoluble material such as might be formed from glucosamine or pyrrole corresponding to aminoacetone. To ascertain whether or not the products measured in the urine of the patients with no demonstrable erythropoiesis was due to the presence of these compounds, the following experiments were performed:

(i) The eluates from Dowex 50 column from urine of 1 of these patients and 1 patient with known lead intoxication were compared with commercially pure ALA. After heating in 1 M sodium acetate buffer, pH 4.6, in the presence of 2,4-pentanedione to condense ALA, these eluates were subjected to high-