4. Close similarity is found to the types of protein reported in extracts from other plants. However, the monodisperse fraction from clover differs from that described from spinach and tobacco in that it is not associated with ribonucleic acid.

5. Leaves of both monocotyledonous and dicotyledonous plants appear to contain the same types of soluble cytoplasmic protein.

The author wishes to acknowledge the expert technical assistance of Miss J. Reid.

REFERENCES

Allen, R. J. L. (1940). *Biochem. J.* **34**, 858.
Ceriotti, G. (1955). *J. biol. Chem.* **214**, 59.
Chibnall, A. C. (1939). *Protein Metabolism in the Plant.* New Haven: Yale University Press.
Crook, E. M. (1946). *Biochem. J.* **40**, 197.
Eggman, L., Singer, S. J. & Wildman, S. G. (1953). *J. biol. Chem.* **205**, 969.
Lugg, J. W. H. (1949). *Advanc. Protein Chem.* **5**, 229.
Neish, A. C. (1939). *Biochem. J.* **33**, 293.
Ogur, M. & Rosen, G. (1950). *Arch. Biochem.* **25**, 262.
Parker, G. (1952). *Biochem. J.* **51**, 389.
Pirie, N. W. (1950). *Biochem. J.* **47**, 614.
Singer, S. J., Eggman, L., Campbell, J. M. & Wildman, S. G. (1952). *J. biol. Chem.* **197**, 233.
Vesselinovitch, S. D. & Funnell, H. S. (1954). *Canad. J. Biochem. Physiol.* **32**, 567.
Vischer, E. & Chargaff, E. (1948). *J. biol. Chem.* **176**, 715.
Wildman, S. G. & Bonner, J. (1947). *Arch. Biochem.* **14**, 381.
Wildman, S. G., Cheo, C. C. & Bonner, J. (1949). *J. biol. Chem.* **180**, 985.
Wildman, S. G. & Gordon, S. (1942). *Proc. nat. Acad. Sci., Wash.*, **28**, 217.
Wildman, S. G. & Jagendorf, A. T. (1952). *Annu. Rev. Pl. Physiol.* **3**, 131.

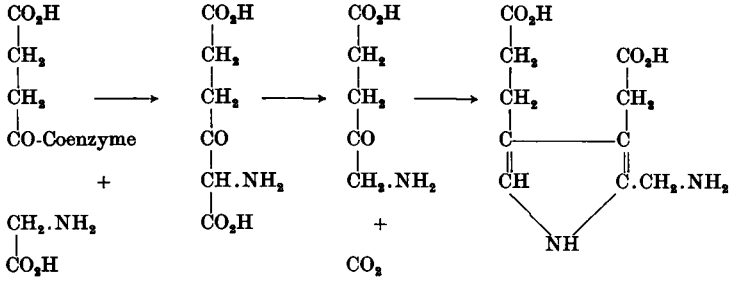
The Metabolism of δ-Aminolaevulic Acid

1. NORMAL PATHWAYS, STUDIED WITH THE AID OF ^{15}N

BY N. I. BERLIN,* A. NEUBERGER† AND J. J. SCOTT†
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 4 *January* 1956)

On the basis of results obtained with *in vitro* systems, δ-aminolaevulic acid (ALA) has been found by several groups of workers to be a biological precursor of porphyrins and haem. The following reactions are believed to occur (Shemin & Russell, 1953; Neuberger & Scott, 1953):



The last compound, porphobilinogen (PBG), has been shown by Falk, Dresel & Rimington (1953) to be converted enzymically into porphyrins *in vitro*. Employing isotopically labelled glycine or ALA, Shemin & Russell (1953) and Shemin, Russell & Abramsky (1955) have demonstrated that ALA is used in preference to glycine and succinate for haem synthesis by avian red cells. Shemin, Abramsky & Russell (1954) showed that a soluble enzyme system, obtained by centrifuging lysed avian red cells, would convert ALA into haemin. Gibson, Neuberger & Scott (1954, 1955) have isolated a widely distributed enzyme which catalyses the conversion of ALA into PBG.

Net synthesis of haem or porphyrin from glycine in a lysed avian red-cell system is very slight, but large quantities of porphyrins are formed when PBG is added in place of glycine (Falk *et al.* 1953). ALA also was found to give rise to free porphyrins under these conditions (Neuberger & Scott, 1953; Dresel & Falk, 1953), and was shown by the latter

* Special Research Fellow, National Heart Institute, U.S. Public Health Service. Present address: Lieut.-Com. N. I. Berlin, U.S.N. 775 North Van Dorn Street, Alexandria, Virginia, U.S.A.

† Present address: Department of Chemical Pathology, St Mary's Hospital Medical School, St Mary's Hospital, London, W. 2.

authors to be equivalent to PBG in this respect. At the same time, however, Neuberger & Scott stated that when [^{15}N]ALA was tested *in vivo*, its incorporation into the haem of circulating haemoglobin was much less than that resulting from an equivalent dose of [^{15}N]glycine. This finding is now reported in full, together with the results of subsequent studies *in vivo*. The experiments were designed to throw light on this low incorporation of ALA into haem, and also to investigate the overall metabolism of ALA in the intact animal. Preliminary accounts of some of these studies have already appeared (Berlin, Neuberger & Scott, 1954; Berlin, Gray, Neuberger & Scott, 1954; Scott, 1955).

Shemin & Russell (1953) postulated a succinate–glycine cycle (see also Shemin, 1955) involving ALA, and have indeed obtained some evidence for its existence. Operation of this postulated cycle could explain many of the metabolic inter-relationships involving glycine, and would place ALA, at least qualitatively, in a rather central position in intermediary metabolism. Quantitative aspects of the metabolism of ALA in intact animals acquire therefore an enhanced importance.

EXPERIMENTAL

Animals. Albino rats of the National Institute for Medical Research strain were employed. For preparation of haemin, the animals were killed 6 days after injection of [^{15}N]ALA.

Urine flow. Whenever urinary ALA and PBG were to be determined, physiological saline (2 ml.) was injected into each rat every 2 hr. for 8 hr., in order to overcome an apparent antidiuretic effect of ALA. For the same reason, human subjects were given 100 ml. of water hourly for 8 hr. after swallowing a solution of ALA.

Administration of δ-aminolaevulic acid. The [^{15}N]ALA (Neuberger & Scott, 1954) contained 30·6 atoms % excess ^{15}N. Immediately before injection, a solution of ALA hydrochloride was mixed with one equivalent of NaOH. Solutions of ALA allowed to stand after neutralization give a positive test with Ehrlich's reagent. For addition of ALA to the diet, a solution of the unneutralized hydrochloride was used. In the tests with human subjects, ALA was given orally as the hydrochloride dissolved in 200 ml. of water.

Glutathione (GSH) and ascorbic acid. Immediately before injection of these compounds they were dissolved in one equivalent of 0·2 N-NaOH.

Fractionation of urinary nitrogen

It was necessary to treat small urine samples, produced by rats which had received [^{15}N]ALA, in such a way that simultaneous values for the percentage excess of ^{15}N in the free NH_3, urea and residual N, together with the overall amounts of nitrogen (i.e. $^{14}N + ^{15}N$) in these three fractions, could be obtained. The principle of the first method employed (method *A*) was to determine the total N, and its mean percentage of excess ^{15}N after Kjeldahl digestion and distillation of a measured fraction of urine. The remainder of the urine sample was then treated as described below to yield comparable figures for the free ammonia N and for the sum of free ammonia N and urea N. The values for the urea N and for the residual N were then calculated by difference, since the addition of urease in the last operation precludes a subsequent direct determination of residual N. All values reported (Fig. 1 and Tables 3–6) represent the means of complete determinations of N and of ^{15}N carried out in triplicate. Tests, using a mixture of known quantities of NH_4Cl, urea and ALA at concentrations similar to those found in the urine samples, indicated that recovery of the N in each fraction was not less than 96% with method *A*.

Total nitrogen. Two measured fractions of the urine sample, each equivalent to about 1 mg. of N, were digested for 24 hr. with 1·5 ml. of conc. H_2SO_4 containing SeO_2, $HgSO_4$ and K_2SO_4. One sample was used for determination of the excess of ^{15}N by the procedure of Sprinson & Rittenberg (1949). The other sample was distilled from a Kjeldahl apparatus into a known volume of 0·02 N-HCl; N was then estimated by back-titration with NaOH, Tashira's indicator (Cole, 1933) being used.

Ammonia nitrogen (method A). The remainder of the urine sample was divided accurately into two parts. The smaller part (one-fifth) was set aside for estimation of urea N. The larger part was transferred to a Kjeldahl flask and a saturated aqueous solution of NaOH (0·25 vol.) was added. Air or nitrogen was then bubbled through the mixture for 2 hr. at room temperature. Ammonia was trapped and treated as before, except that a known volume of the distillate was withdrawn for titration, the remainder being used for ^{15}N determination.

Ammonia plus urea nitrogen. The pH of the smaller fraction mentioned above was adjusted to neutrality, if necessary, and the urine was transferred to a Kjeldahl flask. The urea was then decomposed with urease (British Drug Houses Ltd.), added in tablet form. After standing for 2 hr., a saturated aqueous solution of NaOH (0·25 vol.) was added and the resulting ammonia was collected and treated as described for ammonia N (method *A*).

Other methods for isolation of nitrogenous constituents of urine. For reasons which are made clear when the Results are described, it was necessary to see whether the strong alkali used in the procedure described was causing any redistribution of ^{15}N among the three fractions referred to. For this purpose two procedures were developed; in both, the free NH_3 was removed without the use of strong alkali, leaving a solution from which the urea N could then be isolated directly. Since only the excess of ^{15}N in each fraction was to be measured, the fact that neither of these procedures permitted such accurate recoveries as did the original was immaterial.

Method B for urine NH_3. This method was elaborated from the data given by Folin & Bell (1917). A column was prepared in the following manner: dry Decalso F (Permutit Co., Gunnersbury Avenue, London, W. 4; 2 g. for 10 ml., or less, of diluted urine) was placed in a glass tube having an H3 sintered filter disk at the lower end; distilled water was then added, air bubbles were removed, and most of the water was allowed to run through under gravity. The urine sample was diluted with water (1 vol.) and the pH was adjusted to between 6 and 7 if necessary; the diluted urine was then added to dry Decalso F (0·2 g./ml. of diluted urine) in a small flask, which was agitated gently for 0·5 hr. The resulting slurry was poured on to the column, and washed in with water (1 vol.), the flow of liquid out of the tube being

limited throughout all the operations to just below 3 ml./min. When all the liquid had been run on, the column was washed with water (1 vol.). The column effluent was set aside for isolation of urea N as described below. The absorbed ammonia was then displaced from the column by elution with a saturated aqueous solution of KCl (2 vol.). Sufficient of the KCl eluate was then transferred to a Kjeldahl flask, and the excess of ^{15}N was determined as before.

Method C *for urine* NH_3. The procedure was essentially similar to that of Van Slyke (1915). The pH of the urine sample was adjusted to 3 with HCl, and an equal volume of 20% (w/v) phosphotungstic acid was added. The precipitated ammonium salt was separated by centrifuging, and washed by resuspension and centrifuging in 1% (w/v) phosphotungstic acid (2 × 10 ml.). The supernatant fluid left after the first separation was extracted with 50% (v/v) 'amyl alcohol' in ether until small samples of the aqueous layer no longer gave any precipitate with NH_3. After one further extraction with ether to remove the amyl alcohol, air was blown through the solution to remove the ether. This solution was set aside for isolation of urea N. Ammonia N and excess of ^{15}N were then determined by method *A*, the whole of the washed ammonium phosphotungstate precipitate being used in place of a measured fraction of a urine sample.

Urea nitrogen (*methods* B *and* C). A measured fraction of the column effluent (method *B*) or of the supernatant solution (method *C*) was treated with urease as described in method *A*.

Hippuric acid. This was isolated by the method of Arnstein & Neuberger (1951).

ALA. This was estimated by the picrate method of Shuster (1956); the results were frequently checked by his acetoacetate method.

Porphobilinogen. PBG was estimated by a modification of the method of Cookson & Rimington (1954). Before reaction with Ehrlich's reagent, the urine was so diluted that the optical density at 552 mμ. was less than 0·4 after addition of the reagent. In the human experiments, four drops of a saturated solution of $CuSO_4$ were added to every 10 ml. of diluted urine. This treatment was found to stabilize the colour subsequently obtained, particularly when the fading of the colour was due to the presence of thiols. PBG was isolated from human urine by the method of Cookson & Rimington (1954). The identity of the crystalline material so obtained was checked chromatographically, with the system described below. It was applied to the paper as a solution in dilute ammonia.

Chromatographic separation of urine constituents. Rat urine (10 ml.) was freeze-dried after adjusting to pH 2 with HCl. The residue was then extracted with 2 ml. of 90% (v/v) ethanol, and the ethanolic solution was applied to Whatman no. 1 paper. The chromatogram was run for 16 hr. in *n*-butanol–water–acetic acid (63:27:10 by vol.) at 25°; this is similar to the solvent system employed by Cookson & Rimington (1954). Under our conditions the following R_F values were observed: glycine 0·13, ALA 0·23, PBG 0·48. The first two compounds were located by spraying with ninhydrin (0·1% solution in *n*-butanol) and drying at 60°. Under these conditions ALA gives a brown–yellow spot, which later turns purple. PBG was located by spraying with Ehrlich's reagent.

Faecal nitrogen

The combined 24 hr. faeces from two rats were digested with 20 ml. of conc. H_2SO_4 and catalysts referred to under 'Total N' above. When digestion was complete (3–4 days) the acid solution was cooled and made up to a known volume. For total faecal N, 0·02 vol. was removed and treated as described above.

Amide nitrogen from liver protein

The washed liver was ground in a mortar with 80% (v/v) ethanol; the suspension was boiled and filtered. The residue was washed by centrifuging in ethanol and then in ether, and dried. The precipitate (1·0 g.) was then boiled under reflux for 3 hr. with 2·5 N-HCl (25 ml.). On cooling, the solution was made alkaline to phenolphthalein with $Ba(OH)_2$; the ammonia thus liberated was then distilled *in vacuo* at room temperature into an excess of 2 N-H_2SO_4. This solution was then transferred to a Kjeldahl flask, made strongly alkaline with 10 N-NaOH, and distilled with heating into an excess of HCl for subsequent determination of ^{15}N.

RESULTS

Incorporation of ALA into haem

The incorporation into haem of isotope from different labelled compounds may be satisfactorily compared by calculating the 'corrected dilution coefficient', C.D.C. (Arnstein & Neuberger, 1951). This was defined by the relationship

$$\text{C.D.C.} = Q(C_0/C - 1),$$

Table 1. *Incorporation of* [^{15}N]*ALA or* [^{15}N]*glycine into haem*

The [^{15}N]ALA containing 30·6 atoms % excess ^{15}N was either given in the diet or injected intraperitoneally (IP), as described in the text. GSH (rat 34) or ascorbic acid (rat 38), at a dose level of 0·2 m-mole/100 g., was injected simultaneously with the [^{15}N]ALA. For definition of 'corrected dilution coefficient' (C.D.C.) see text.

Rat (nos.)	Dose of ALA (m-mole/100 g.)	Route of administration	% excess ^{15}N in haem	C.D.C.
19 + 20	0·20	Fed	0·013	466
17 + 18	0·20	IP	0·018	340
34	0·20 (+ GSH)	IP	0·017	360
36	0·20 (+ ascorbic acid)	IP	0·017	360
30 + 31	0·50	IP	0·045	339
12	Glycine 0·20	Fed	0·105	60*

* Calculated from the data of Muir & Neuberger (1949).

Table 2. *Utilization of ALA-N for hippuric acid and urea synthesis*

In the experiments in which hippuric acid was isolated, each animal received 0·20 m-mole of sodium benzoate/100 g. at the same time as the labelled compound. Abbreviations are the same as in Table 1.

Rat (nos.)	Substance administered: [^{15}N]ALA (m-mole/100 g.)	Substance administered: [^{15}N]glycine (m-mole/100 g.)	% excess ^{15}N	% excess ^{15}N in hippuric acid	% excess ^{15}N in urea	C.D.C.
21 + 22	0·165 (IP)	—	30·6	0·075	—	67·1
*	—	0·033 (IP)	15·5	1·278	—	0·366
19 + 20	0·160 (fed)	—	30·6	—	0·042	117
17 + 18	0·160 (IP)	—	30·6	—	0·044	111
23	—	0·160 (IP)	31·4	—	0·047	106

* Data of Arnstein & Neuberger (1951).

where Q is the dose (m-moles/100 g. body weight) and C_0 and C are the isotope concentrations of the labelled atoms in the starting material and in the product isolated, respectively. C.D.C. is expressed in the same units as the dose. Since glycine N or ALA nitrogen is the sole source of haem N, this equation required no modification to take into account the fact that more than one glycine molecule is required in order to form one haem molecule.

Table 1 shows that [^{15}N]glycine was incorporated 5 to 6 times as readily as [^{15}N]ALA in the intact rat. Slightly less was incorporated when ALA was fed, rather than injected, owing presumably to the ready oxidation of neutral solutions of ALA exposed to the air. Considered alone, these findings would have suggested that ALA was behaving more as a precursor of glycine than as a porphyrin precursor. This possibility was eliminated by isolating hippuric acid formed from benzoate administered either with [^{15}N]glycine or with [^{15}N]ALA. The ^{15}N content of the hippuric acid formed from [^{15}N]ALA was of the same order as that of the urea isolated in a parallel experiment. [^{15}N]Glycine, while it gave rise almost to the same labelling in the urea as [^{15}N]ALA, was used for hippuric acid synthesis to a very much higher degree (Table 2).

Urinary excretion of ^{15}N

Attention was next turned to the distribution of isotope amongst the urinary constituents. It was found that from one-third to two-thirds of the isotope administered as [^{15}N]ALA was eliminated by this route, and that the bulk of the ^{15}N was in the 'residual' urinary nitrogen, that is the fraction remaining after free ammonia and urea had been removed (Fig. 1). The excretion was rapid, since 75 % of the ^{15}N excreted in this way during the first day had already been eliminated in the first 6 hr. When glutathione (GSH) or ascorbic acid was injected simultaneously with [^{15}N]ALA, an increased ^{15}N excretion was observed, while the distribution of isotope in the three fractions of urinary nitrogen was little altered (Table 3). This increase cannot, however, be considered significant as subsequent experiments, discussed below, have shown. The reasons for injecting GSH or ascorbic acid are explained below. Neither GSH nor ascorbic acid increased the incorporation of ALA into haem (Table 1). The isotope content of liver-protein amide nitrogen, relative to the size of the dose, was higher in rat 36, which had ascorbic acid, than in rats 30 + 31 (Table 3).

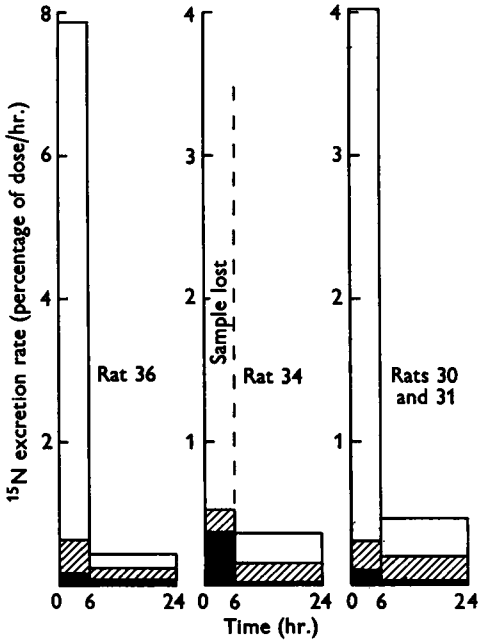


Fig. 1. Rates of urinary excretion of ^{15}N by rats which had received [^{15}N]ALA. The rats were injected intraperitoneally with a neutralized solution of [^{15}N]ALA hydrochloride, containing 30·6 atoms % excess ^{15}N. The amounts of [^{15}N]ALA received were as follows: rats 30 + 31, 0·125 m-moles/100 g.; rats 34 and 36, 0·05 m-mole/100 g. ■, $NH_3{}^{15}N$; ▨, urea ^{15}N; □, residual ^{15}N.

It may be seen from Table 4 that the percentage of excess ^{15}N in the ammonia was extremely high, and was still apparently greater than that in the urea on the day after the last injection of [^{15}N]ALA. Previous work in which ^{15}N-labelled L-amino acids or $^{15}NH_4Cl$ (see Discussion) have been administered has shown that little of the isotope is found in the urinary free ammonia, and has indicated that free ammonia in the body is rapidly converted into urea. Wu (1950) pointed out that the ^{15}N concentration in the ammonia and the urea excreted during such experiments was time-dependent, but that the ratio of percentage excess ^{15}N in the NH_3 and urea had generally fallen below unity after a few hours. In view of these well established results, discussed more fully below, we therefore subjected our own findings with [^{15}N]ALA to a closer scrutiny. It was necessary to establish that the NH_3 isolated by method *A* did in fact represent the original urinary free NH_3, and that its ^{15}N content had not been increased during isolation by exchange with another urinary constituent, such as ALA, which would be expected to have a high ^{15}N content. Similar experiments were therefore performed, after which the urinary free NH_3 was isolated either by adsorption on Decalso F (method *B*) or by precipitation with phosphotungstic acid (method *C*). The results of these experiments, shown in Table 5, fully substantiate those of Table 4. By dilution with $^{14}NH_4Cl$ it was found, in experiments not reported here, that the [^{15}N]ALA contained a small amount of $^{15}NH_4Cl$ (approximately 5%, w/w), which resisted removal by repeated recrystallization from dry methanol–ether and dry methanol–ethyl acetate. On injection, this impurity would be converted rapidly into urea, as shown by Schoenheimer (1946), and would not therefore give rise to a high ^{15}N concentration in the free urinary NH_3 many hours after administration.

Nature of 'residual' nitrogen

The ^{15}N excretion data showed that the largest contribution was from the 'residual' fraction of the urine N; components of this fraction were therefore sought. It was believed that a major component would be unchanged ALA, but the first component identified and isolated was PBG (Berlin, Neuberger & Scott, 1954). Our finding that PBG is excreted after injection of ALA has recently been confirmed (Shemin, Gatt, Schmid & Weliky, 1955).

Porphobilinogen. The amount of metabolized ALA excreted as PBG reaches a maximum in the rat at a dose level of about 0·5 m-mole of ALA/100 g.; the absolute amount excreted also reaches a maximum at about this value, indicating saturation of the capacity of the organism for converting ALA into PBG under these conditions. This is illustrated in Fig. 2 (see also Table 8). Since completion of the experiments summarized in Fig. 2,

Table 3. *Urinary ^{15}N excretion pattern of rats which received* [^{15}N]*ALA*

In each case the [^{15}N]ALA was injected intraperitoneally in four equal parts on successive days. The values for ^{15}N content of haemin prepared from these rats are shown in Table 1. Rats 30+31, 34 and 36 weighed 491, 240 and 244 g. respectively. Amide N was obtained from liver protein as described. NH_3 was isolated by method *A*.

Rat (nos.)	Compound injected (m-moles/100 g.)		Urine N component	Totals after 1 day				Totals after 5 days		Amide ^{15}N in liver protein (% excess) after 6 days
	ALA	Other compounds		$^{14}N+^{15}N$ (mg./100 g.)	% excess ^{15}N	% of 1-day dose ^{15}N	% of total urine excess ^{15}N	% of total dose ^{15}N	% of total urine excess ^{15}N	
30+31	0·50	None	Urea	52·2	0·048	4·4	12·9	4·0	13·6	0·017
			NH_3	2·74	0·226	1·1	3·2	1·3	4·5	
			Residual	13·8	1·18	28·4	83·8	24·2	81·9	
			Total (determined)	68·7	0·283	33·9	—	29·6	—	
34	0·20	0·20 GSH	Urea	49·0	0·012	2·6	?	4·5	10·1	Not determined
			NH_3	2·73	0·222	2·6	?	2·0	4·5	
			Residual	Not calculable				37·9	85·2	
			Total (determined)	Lost				44·5	—	
36	0·20	0·20 ascorbic acid	Urea	90·5	0·016	6·3	11·1	6·9	13·4	0·017
			NH_3	4·15	0·068	1·2	2·2	2·2	4·3	
			Residual	10·5	1·08	49·4	86·5	42·5	82·3	
			Total (determined)	105	0·124	57·0	—	51·6	—	

Table 4. *Ratios of* ^{15}N *content and* ^{15}N *concentration in urea and* NH_3 *(by method* A)

The total dose given to each rat is shown in Table 3; it was given in four equal parts on four successive days. Ammonia N was isolated and determined by method *A*.

Days after start of experiment	Rats 30 + 31		Rat 34		Rat 36	
	Total excess urea ^{15}N (mg.)/ total excess NH_3 ^{15}N (mg.)	NH_3 ^{15}N % excess/ urea ^{15}N % excess	Total excess urea ^{15}N (mg.)/ total excess NH_3 ^{15}N (mg.)	NH_3 ^{15}N % excess/ urea ^{15}N % excess	Total excess urea ^{15}N (mg.)/ total excess NH_3 ^{15}N (mg.)	NH_3 ^{15}N % excess/ urea ^{15}N % excess
0·25	2·1	8·2	0·4	27·4	3·0	8·7
1	6·7	2·9	7·1	3·0	11·1	1·9
2	2·2	8·3	1·1	8·2	2·1	8·6
3	2·7	4·8	5·8	5·6	2·1	10·3
4	2·9	5·0	1·2	10·4	5·2	2·8
5	7·4	4·2	2·8	9·9	11·1	2·0

Table 5. *Concentration of* ^{15}N *in urea and* NH_3 *determined by method* B *or* C

[^{15}N]ALA was injected intraperitoneally in a single dose. Free urinary NH_3 was isolated by adsorption on to Decalso F (method *B*) or by precipitation with phosphotungstic acid (method *C*). Values for ^{15}N percentage excess below 0·005 were not considered significant when the ratios in the final column were calculated.

Rat (nos.)	Dose of ALA (m-mole/100 g.)	Period of urine collection (hr.)	Method of isolation	NH_3 ^{15}N (% excess)	Urea ^{15}N (% excess)	NH_3 ^{15}N % excess/urea ^{15}N % excess
49 + 50	0·05	0–24	*B*	0·386	0·050	7·5
		24–48	*B*	0·010	0·004	>2
51	0·15	0–24	*C*	0·928	0·158	5·9
		24–48	*C*	0·010	0·002	>2
54	0·15	0–24	*C*	0·854	0·157	5·4
		24–48	*C*	0·009	0·006	1·5

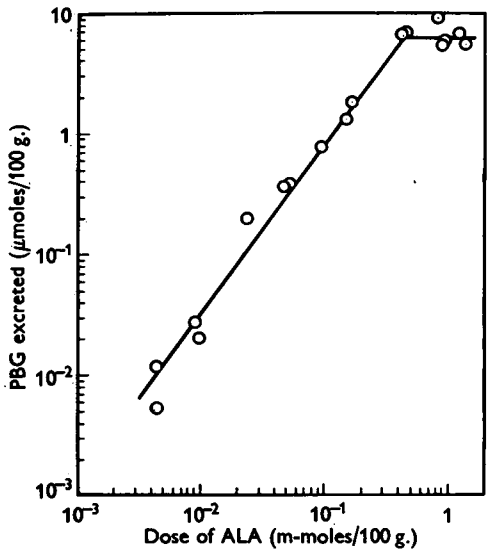


Fig. 2. Relationship between the dose of ALA and the amount of urinary PBG excreted by rats. Both axes are measured on a logarithmic scale. No rat was used more than once, and the body weights were all within the range 180–250 g.

we have found that addition of Cu^{2+} ions to the urine before reaction with Ehrlich's reagent prevents the colour from fading (see Experimental section). With a stable Ehrlich colour, the optical density may be as much as 10% higher; the percentage of ALA converted into PBG was therefore somewhat greater than would appear from Fig. 2.

δ-Aminolaevulic acid. Urine voided in the first 6 hr. after injection of ALA was subjected to chromatography as described. After spraying with ninhydrin solution there appeared, in addition to the usual amino acid spots, a large yellow spot (R_F 0·24) which was found to be unchanged ALA by comparison with the pure compound. This procedure, while indicating that a large quantity of unchanged ALA was being excreted, was not, however, suitable for estimation. ALA was later determined by methods developed by Shuster (1956). As shown in Table 6, the excretion of ALA was sufficiently great to account for most of the residual N fraction, assuming that the [^{15}N]ALA excreted was not greatly diluted by endogenous ALA. Proof of the validity of this assumption is supplied in the next paper (Berlin, Neuberger & Scott, 1956).

Other components. No gross increase in free urinary porphyrins was detected after administration of [^{15}N]ALA. The urine was darker than normal, and became dark brown on standing, but no pink fluorescence was observed in ultraviolet light. The pigment could not be extracted into organic solvents at any pH. The absorption spectrum of this urine, with a band at 490–496 mμ., which sharpened without change in position after acidification of the urine with HCl, suggested the presence either of 'porphobilin' (Waldenström & Vahlquist, 1939; Prunty, 1945) or of the mixture of pigments formed from PBG and described by Brockman & Gray (1953).

Excretion of ALA and PBG

In view of the apparent increase in aminolaevulic-dehydrase activity found in the livers of rabbits poisoned by repeated doses of Sedormid (Gibson *et al.* 1955), it was of interest to see whether repeated doses of ALA would provoke any change in the amounts of ALA and PBG excreted. Gibson *et al.* (1954) also described the activation of ALA dehydrase by GSH and tests were therefore made to ascertain whether the enzyme was further activated in the body by injecting GSH at the same time as large doses of ALA. Results of these experiments are summarized in Table 6. Repeated daily injections of ALA had no demonstrable effect on the amounts of ALA and PBG subsequently excreted. When GSH was injected at the same time as [^{15}N]ALA, the amount of residual ^{15}N appeared to be increased (Table 3). But this observed increase cannot be taken as significant in view of the considerable variations found among animals not so treated (Table 6). The percentage of ALA excreted unchanged was not higher when GSH was given simultaneously than the upper value observed after injection of the same quantity of ALA alone; nor was the amount of PBG excreted significantly increased by administration of GSH.

Attempts were made to determine and isolate ALA-N in urine for ^{15}N analysis by oxidation with periodate, a method which has also been described by Shemin, Russell & Abramsky (1955). A 95–100% recovery was obtained when the oxidation was carried out with pure ALA, but the method was found to be unsatisfactory with urine, owing to the presence of other substances which evolve NH_3 under these conditions.

Faecal excretion of ALA nitrogen

The total faecal N and ^{15}N after injection of [^{15}N]ALA is shown in Table 7, the total urinary N and ^{15}N excreted during the same period are also given, since the ALA excreted has been shown to vary between such wide limits (Table 6).

Table 6. *Effect of repeated doses of ALA and of GSH on the excretion of ALA and PBG*

Rat (nos.)	Days after start of experiment	Compound administered intraperitoneally (m-mole/100 g.)		Percentage of daily dose of ALA excreted in 24 hr. in the form indicated		
		ALA	GSH	ALA	PBG	Total
101 + 102	1	0·20	—	37·7	3·0	40·7
	2	0·20	—	60·0	2·4	62·4
	3	0·20	—	56·7	2·5	59·2
	4	0·20	—	61·1	2·9	64·0
	5	0·20	0·20	45·7	3·0	48·7
103 + 104	1	0·20	0·20	58·4	2·8	61·2
105 + 106	1	—	0·20	—	—	—
	2	0·20	0·20	44·9	3·3	48·2

Table 7. *Faecal and urinary excretion of ALA nitrogen*

Rats 52 + 53, of total weight 406 g., were given 0·15 m-mole of [^{15}N]ALA (30·6 atoms % excess)/100 g. by intraperitoneal injection. The urine and faeces were separated on voiding.

Time after injection (hr.)	Faecal N (mg.)	Faecal ^{15}N (% excess)	Percentage of the dose of ^{15}N	Total percentage excreted
24	123	0·161	7·6	—
48	103	0·090	3·6	11·2
	Urine N (mg.)	Urine ^{15}N (% excess)		
24	434	0·431	70·2	—
48	504	0·013	2·5	72·7
				83·9

Effects of ALA in man

Urinary excretion products. The rates of excretion of ALA and PBG in the urine, after ingestion of ALA by a human subject, are shown in Fig. 3. There was little variation ($< \pm 1\%$) between different subjects at the same dose level. The PBG was isolated from the urine and crystallized. It was chromatographically indistinguishable from PBG isolated similarly from the urine of an acute porphyric.

As in the rat, the percentage of the dose converted into PBG increased with the dose within the range tested (Table 8), indicating an increasing saturation of aminolaevulic dehydrase in the tissues concerned.

Photosensitization. Administration of ALA to man was followed, in all normal subjects tested, by an erythema of the exposed skin. The severity of the

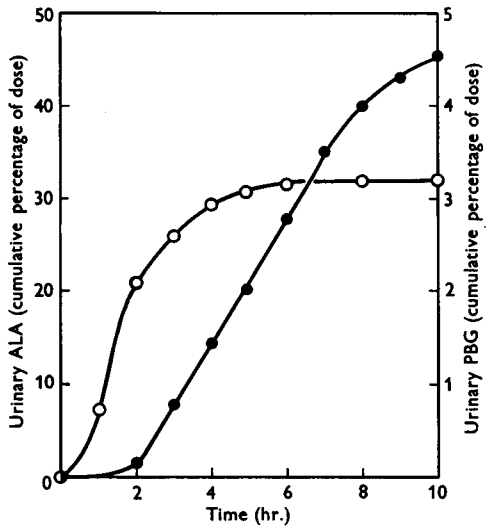


Fig. 3. Excretion of ALA and PBG by a human subject after ingestion of ALA. This subject (B.A.A.) received 0·0095 m-mole of ALA/100 g., and excreted 4·6% of the dose as PBG in 24 hr. ○, ALA (left-hand axis of ordinates); ●, PBG (right-hand axis of ordinates). Similar curves were obtained by plotting the data from other subjects (Table 8).

Table 8. *Percentage of administered ALA excreted as urinary PBG in human subjects*

Subject	Dose (m-moles/100 g.)	Percentage of the dose excreted as PBG
J.J.S.	0·0075	4·76
J.J.S.	0·0075	4·10
N.I.B.	0·0091	3·29
B.A.A.	0·0095	4·62
J.J.S.	0·0150	6·76
K.D.G.	0·0210	8·54

Table 9. *Photosensitivity following ingestion of ALA by human subjects*

Subject	Sex	Dose (m-moles/100 g.)	Appearance of erythema (hr. after dose)	Date	Weather	Exposure to sun	Nature of symptoms	Duration of symptoms (days after dose)
J.J.S.	m	0·0075	10	29. iii. 54	Foggy	None	Erythema and tingling sensation (face); eyes smarting and bloodshot (sunglasses not worn)	1
J.J.S.	m	0·0075	11	11. v. 54	Cloudy	None	Erythema and tingling sensation (face)	1
J.J.S.	m	0·0150	8	14. iv. 54	Cloudy	None	Erythema and burning sensation (face and back of hands)	2
N.I.B.	m	0·0091	10	22. iv. 54	Cloudy	None	Erythema and burning sensation (face)	1
B.A.A.	f	0·0095	7	24. vi. 54	Sunny	20 min. in sun after 5 hr.	Erythema (face and hands), replaced after the first day by suntan	1 30
K.D.G.	m	0·0210	2	25. ii. 55	Sunny with snow on ground	7 min. in sun after 5 hr.	Erythema, with severe and painful burning (face only) lasting 4–24 hr. Exfoliative eruption all over face Desquamation Recovery	1–2 3–10 21

response increased with the dose, but variation in response between subjects appeared to be considerable; this may have been due in part to the variation of light intensity to which the subjects were exposed. Except during the first test, sun-glasses were worn throughout the day; this is an advisable precaution. The preliminary findings are reported in Table 9, pending a fuller investigation of this phenomenon.

DISCUSSION

Excretion pattern of aminolaevulic acid nitrogen

After administration of [^{15}N]aminolaevulic acid to rats a proportion of the labelled nitrogen, which varied between 30 and 70%, was excreted in the urine within 24 hr. after administration (Tables 3 and 7). Between 75 and 87% of the urinary ^{15}N was found in the residual fraction, i.e. the fraction which remains after removal of urea and ammonia. These findings are in striking contrast with those obtained with ^{15}N-labelled L-α-amino acids, where it was found that, with doses similar to those used here, 25–40% of the labelled nitrogen was excreted by 25 or 48 hr. Moreover, 90% of the ^{15}N excreted was in the form of urea (Schoenheimer, Ratner & Rittenberg, 1939*a*; Ratner, Rittenberg, Keston & Schoenheimer, 1940; Weissman & Schoenheimer, 1941). With α-amino acids only about 3–6% of the administered ^{15}N appeared in the faeces. The larger proportion observed after giving aminolaevulic acid (Table 7) is probably due to the presence of labelled bile pigment and porphyrins (Berlin *et al.* 1956).

A small part, probably 5% (Table 6), of the ^{15}N of the residual nitrogen fraction is due to porphobilinogen, but most of it is in the form of unchanged aminolaevulic acid. This deduction is based on the close similarity in the order of values of the proportion of the dose excreted in the urine and measured colorimetrically as aminolaevulic acid (Table 6) and that of ^{15}N present in the residual fraction of the urinary nitrogen (Table 3). A similar parallelism was observed in the time relationship of the excretion of ^{15}N and of that of aminolaevulic acid; by either criterion it was found that most of the excretion occurred in the first 6 hr. (Fig. 1), and almost none was excreted after 24 hr. (Fig. 3).

Another unusual finding is the very high ratio of isotope content (in terms of atom excess ^{15}N) of the urinary ammonia to that of urea (Table 4). With almost all L-amino acids this ratio was found to be 1·2–1·5 during the first few hours after administration, to decrease later to 1·0 or slightly below 1·0 (for review see Wu, 1950). With L-aspartic acid an initial value of 0·55 has been observed (Wu & Rittenberg, 1949). The values found for this ratio with aminolaevulic acid (Tables 4 and 5) were almost always appreciably higher than 1·0, even many hours after administration, were frequently of the order of 8–11, and in one experiment a figure of 27·4 was obtained. These high values cannot be explained by contamination of [^{15}N]aminolaevulic acid with $^{15}NH_4Cl$, since ammonium salts are greatly inferior to L-amino acids as precursors of urinary ammonia; for the rat, the rabbit and man, ratios of 0·46 (Schoenheimer, 1946), 0·19 (Waelsch & Rittenberg, 1942) and 0·15 (Sprinson & Rittenberg, 1949) respectively have been reported. It is also most unlikely that the high ^{15}N content of the ammonia fraction results from chemical manipulations involved in the isolation procedure, since three different methods yielded similar high values (Table 5). It thus follows either that aminolaevulic acid is deaminated in the kidney and is, like glutamine, a specific precursor of urinary ammonia, or that ammonia is removed from aminolaevulic acid or porphobilinogen or some other metabolite of aminolaevulic acid after the labelled substance has left the kidney tubules.

These findings with aminolaevulic acid resemble closely those obtained with racemic or D-[^{15}N]amino acids. Thus with DL-tyrosine (Schoenheimer, Ratner & Rittenberg, 1939*b*), D-leucine (Ratner, Schoenheimer & Rittenberg, 1939) and D-lysine (Ratner, Weissman & Schoenheimer, 1943) 60–70% of the administered ^{15}N was excreted within 24 hr., and a large proportion of the urinary ^{15}N was found in the residual nitrogen fraction. The ratio of isotope content of the ammonia fraction to that of the urea varied with the D-amino acids mentioned between 4 and 6, but with DL-serine a ratio of 10 was found (Stetten, 1942). These findings have been explained by assuming that a large fraction of the D-amino acid is excreted as such, whilst another portion is deaminated by the D-amino acid oxidase of the kidney.

Tables 6 and 8 show the amounts of porphobilinogen excreted in the urine but, owing to the simultaneous formation of porphyrins and bile pigment (Berlin *et al.* 1956), the quantities formed must have been much greater. Granick & Vanden Schrieck (1955) reported the presence of a zinc porphyrin in the urine of a rat given aminolaevulic acid. In the present work no significant amounts of porphyrins or metallo-porphyrins could be detected in the urine.

Although a large proportion of the aminolaevulic acid was excreted as such or converted into pyrrolic metabolites, some of the nitrogen was used for the synthesis of amide groups of proteins (Table 3), the formation of urea (Tables 2 and 3) and the synthesis of amino acids (Table 2), indicating that an appreciable proportion of the nitrogen has become incorporated into the general 'nitrogen pool' of the body. This must arise, at least partly, from the liberation of ammonia occurring during the con-

version of porphobilinogen into porphyrins, but it may also be due to degradation. The possibility of a deamination of aminolaevulic acid itself has already been discussed above in relation to the ammonia results.

Incorporation of aminolaevulic acid nitrogen into haem

The rapid elimination in the urine of a large part of the aminolaevulic acid suggests that this substance is poorly, if at all, reabsorbed by the kidney tubules, resembling in this respect porphobilinogen (Goldberg & Rimington, 1954). The latter is assumed to be a necessary intermediate in haem synthesis; it would therefore appear likely that neither aminolaevulic acid nor porphobilinogen escapes from the cells of the haemopoietic system, since they are not normally excreted in significant amounts relative to the rate of haem synthesis.

The question next arises as to whether ALA or PBG can *enter* the cells of the haemopoietic system. Aminolaevulic acid N is incorporated into haem by the intact animal (Table 1). Comparison of the C.D.C. values shows that glycine is incorporated into haem 5–6 times as readily as aminolaevulic acid, but that aminolaevulic acid N gives rise practically to no glycine N as measured by hippuric acid (Table 2). The haem N labelling must therefore have come directly by penetration of aminolaevulic acid or porphobilinogen into the haemopoietic sites and not by a breakdown followed by non-specific re-utilization of its nitrogen. Immature red blood cells cannot therefore be completely impermeable both to ALA and to PBG. Further evidence on this point is brought forward in the subsequent paper (Berlin *et al.* 1956); it is convenient to defer further consideration of these results until they can be discussed in the light of the findings there described.

Experimental photosensitivity in man, induced by aminolaevulic acid

The marked photosensitivity of human subjects who have been given aminolaevulic acid is of particular interest in view of the severe photogenic blistering which is the main clinical feature of congenital porphyria. The nature of the reaction in the experimentally induced condition is, however, different from that usually observed in the disease, where exposure to light will have occurred over long periods. In none of the human subjects was there any vesicular eruption. Only in the subject who received the largest dose was there any desquamation; in all subjects the condition in fact resembled most closely that of sunburn. The photosensitivity of congenital porphyria has long been assumed to be due to porphyrins, particularly uroporphyrin I, known to be present in the blood and tissues of patients with this disease. Preliminary experiments have shown that protoporphyrin is excreted in relatively large quantities in the bile of rats after administration of ALA (Scott, 1955). Circulating porphyrins may therefore be responsible for the experimentally induced sensitivity; the skin reaction is, however, very mild in the latter condition, compared with that in the disease, where exposure with porphyrinaemia has been prolonged.

SUMMARY

1. [^{15}N]Aminolaevulic acid (ALA) was fed or administered parenterally to normal human subjects and to rats. Incorporation of ^{15}N into the haem of the circulating haemoglobin of rats was six to seven times less than was reported for an equivalent amount of [^{15}N]glycine.

2. Administration of [^{15}N]ALA produced labelling in the urinary urea to the same extent approximately as [^{15}N]glycine, but the latter was much more efficient as a precursor of hippuric acid than the former. [^{15}N]ALA also gave rise to significant labelling in the amide groups of liver proteins.

3. A large proportion of the ALA is excreted unchanged in the urine, both in man and in the rat within a few hours of administration. It is concluded that ALA is poorly, if at all, reabsorbed by the tubules of the kidney.

4. Most of the ^{15}N present in the urine was found in the fraction remaining after removal of urea and ammonia, and must be presumed to have been mainly in the form of unchanged ALA. In almost all experiments it was observed that the ^{15}N concentration in the urinary ammonia was considerably greater than in the urea, even after 2 days. The possibility of a specific deamination of ALA by the kidney is discussed, but alternative explanations, involving a post-renal release of labelled ammonia, have not been excluded.

5. Administration of ALA to man or rat has been found to give rise to excretion of appreciable amounts of porphobilinogen (PBG) in the urine. The conversion of ALA into PBG increased with the dose level up to a maximum.

6. Administration of ALA to human subjects produced skin reactions suggesting photosensitization. The bearing of this experimentally induced sensitivity on the explanation of the clinical condition associated with congenital porphyria is discussed.

REFERENCES

Arnstein, H. R. V. & Neuberger, A. (1951). *Biochem. J.* **50**, 154.

Berlin, N. I., Gray, C. H., Neuberger, A. & Scott, J. J. (1954). *Biochem. J.* **58**, xxx.

Berlin, N. I., Neuberger, A. & Scott, J. J. (1954). *Abstr. 5th Int. Congr. Haemat., Paris*, p. 279.

Berlin, N. I., Neuberger, A. & Scott, J. J. (1956). *Biochem. J.* **64**, 90.
Brockman, P. E. & Gray, C. H. (1953). *Biochem. J.* **54**, 22.
Cole, S. (1933). *Practical Physiological Chemistry*, 9th ed., p. 327. Cambridge: Heffer.
Cookson, G. H. & Rimington, C. (1954). *Biochem. J.* **57**, 476.
Dresel, E. I. B. & Falk, J. E. (1953). *Nature, Lond.*, **172**, 1185.
Falk, J. E., Dresel, E. I. B. & Rimington, C. (1953). *Nature, Lond.*, **172**, 292.
Folin, O. & Bell, R. (1917). *J. biol. Chem.* **29**, 329.
Gibson, K. D., Neuberger, A. & Scott, J. J. (1954). *Biochem. J.* **58**, xli.
Gibson, K. D., Neuberger, A. & Scott, J. J. (1955). *Biochem. J.* **61**, 618.
Goldberg, A. & Rimington, C. (1954). *Lancet*, **2**, 172.
Granick, S. & Vanden Schrieck, H. G. (1955). *Proc. Soc. exp. Biol., N.Y.*, **88**, 270.
Muir, H. M. & Neuberger, A. (1949). *Biochem. J.* **45**, 163.
Neuberger, A. & Scott, J. J. (1953). *Nature, Lond.*, **172**, 1093.
Neuberger, A. & Scott, J. J. (1954). *J. chem. Soc.* p. 1820.
Prunty, F. T. G. (1945). *Biochem. J.* **39**, 446.
Ratner, S., Rittenberg, D., Keston, A. S. & Schoenheimer, R. (1940). *J. biol. Chem.* **134**, 655.
Ratner, S., Schoenheimer, R. & Rittenberg, D. (1939). *J. biol. Chem.* **134**, 653.
Ratner, S., Weissman, N. & Schoenheimer, R. (1943). *J. biol. Chem.* **147**, 549.
Schoenheimer, R. (1946). *The Dynamic State of Body Constituents*, 2nd ed., p. 51. Cambridge: Harvard University Press.
Schoenheimer, R., Ratner, S. & Rittenberg, D. (1939*a*). *J. biol. Chem.* **127**, 333.
Schoenheimer, R., Ratner, S. & Rittenberg, D. (1939*b*). *J. biol. Chem.* **130**, 703.
Scott, J. J. (1955). In *The Biosynthesis of Porphyrins and Porphyrin Metabolism, Ciba Foundation Symp.*, p. 43. London: Churchill.
Shemin, D. (1955). In *The Biosynthesis of Porphyrins and Porphyrin Metabolism, Ciba Foundation Symp.*, p. 4. London: Churchill.
Shemin, D., Abramsky, T. & Russell, C. S. (1954). *J. Amer. chem. Soc.* **76**, 1204.
Shemin, D., Gatt, S., Schmid, R. & Weliky, I. (1955). *Fed. Proc.* **14**, 279.
Shemin, D. & Russell, C. S. (1953). *J. Amer. chem. Soc.* **75**, 4873.
Shemin, D., Russell, C. S. & Abramsky, T. (1955). *J. biol. Chem.* **215**, 613.
Shuster, L. (1956). *Biochem. J.* **64**, 101.
Sprinson, D. B. & Rittenberg, D. (1949). *J. biol. Chem.* **180**, 707.
Stetten, D. (1942). *J. biol. Chem.* **144**, 501.
Van Slyke, D. D. (1915). *J. biol. Chem.* **22**, 281.
Waelsch, H. & Rittenberg, D. (1942). *J. biol. Chem.* **144**, 53.
Waldenström, J. & Vahlquist, B. (1939). *Hoppe-Seyl. Z.* **260**, 189.
Weissman, N. & Schoenheimer, R. (1941). *J. biol. Chem.* **140**, 779.
Wu, H. (1950). *J. gen. Physiol.* **34**, 403.
Wu, H. & Rittenberg, D. (1949). *J. biol. Chem.* **179**, 847.

The Metabolism of δ-Aminolaevulic Acid

2. NORMAL PATHWAYS, STUDIED WITH THE AID OF ^{14}C

BY N. I. BERLIN,* A. NEUBERGER† AND J. J. SCOTT†
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 4 *January* 1956)

In the preceding paper (Berlin, Neuberger & Scott, 1956) are described the results of investigations of the metabolism of δ-aminolaevulic acid (ALA) by administration of this compound to rats and to humans, and by studying the fate of the nitrogen of [^{15}N]ALA in rats. It was shown that a large proportion of the ALA was rapidly excreted as such, together with a small amount of porphobilinogen (PBG). The incorporation of isotope into haem was lower than expected, being less than that from an equivalent dose of [^{15}N]glycine.

* Special Research Fellow, National Heart Institute, U.S. Public Health Service. Present address: Lieut.-Com. N. I. Berlin, U.S.N., 775 North Van Dorn Street, Alexandria, Virginia, U.S.A.

† Present address: Department of Chemical Pathology, St Mary's Hospital Medical School, St Mary's Hospital, London, W. 2.

In this paper is described a more detailed investigation into the fate of administered ALA. Because of the greater dilution detectable, and because it affords information about other parts of the molecule, [1:4-$^{14}C_2$]ALA was employed. The labelling was thus in the positions corresponding to the carboxyl carbon atoms of succinic acid, from which ALA is believed to be derived biologically.

The object of those experiments in which rats were used was twofold. It was desired to obtain data comparable with those resulting from our experiments with [^{15}N]ALA. It was also necessary to study the distribution of radioactivity from [1:4-$^{14}C_2$]ALA before administering it to a human subject.

There have already appeared preliminary accounts of parts of this work in which [1:4-$^{14}C_2$]ALA was administered to rats and to a human subject