methionine and the synthesis of methionine by cell suspensions of *Esch. coli* (Wijesundera & Woods, 1953; Cohn, Cohen & Monod, 1953). However, the addition of indole to the growth medium stimulated rather than depressed indole synthesis, therefore the effect of tryptophan remains unexplained.

SUMMARY

1. Indole is formed from glucose and ammonium salts by washed-cell suspensions of a mutant strain of *Escherichia coli* (strain 7-4) unable to convert indole into tryptophan.

2. The medium on which the cells are grown influences the amount of indole formed by the harvested cells.

3. Magnesium ions greatly stimulate indole formation by cells grown on either a synthetic medium or on various complex media. Manganous and ferrous ions stimulate to a lesser extent.

4. Serine appears to be concerned in indole formation. This is shown by experiments with cell suspensions of both *Esch. coli* 7-4 and a further mutant (*Esch. coli* T2-15) which requires tryptophan plus serine or glycine for growth.

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Synthesis and Metabolism of some Substances Related to δ -Aminolaevulic Acid

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In a preliminary communication (Neuberger & Scott, 1953) reasons were given which had led us to believe that δ -succinamidolaevulic acid might be an intermediate in the biosynthesis of porphyrins. But this succinamido compound, unlike δ -aminolaevulic acid (ALA) itself, was not converted into porphyrins by the intact cells of *Rhodospseudomonas spheroides*, as observed by Dr J. Lascelles, nor did it show any activity, according to the experiments of Drs E. Dresel and J. E. Falk, in their system of lysed fowl red cells. ALA itself is the immediate precursor of porphobilinogen (PBG), which in turn gives rise to porphyrins (Shemin & Russell, 1953; Neuberger & Scott, 1953; Dresel & Falk, 1953). In the present paper further observations with rats are presented which indicate that succinamidolaevulic

acid is not converted into ALA to a significant extent. Experiments are also reported which indicate that *N*-succinylglycine and *N*- β -carboxypropionylglycine, unlike *N*-acetyl glycine or *N*-propionylglycine, are not converted into glycine in the rat. This suggested that the negative results obtained with succinamidolaevulic acid might be due to a general inability of the organism to split the succinamido bond. δ -Acetamidolaevulic acid was therefore synthesized, but biological tests indicate that this compound is not hydrolysed in the body to a significant extent.

It is now assumed that the condensation of glycine and 'active' succinate gives rise in the first place to α -amino- β -oxoadipate, which loses one of its carboxyl groups to yield ALA. Free β -keto acids containing an α -amino group have so far not been prepared, and nothing is known of the stability of compounds of this type. As a preliminary to a possible synthesis of the α -amino- β -keto acid itself, several esters of the acid have been synthesized, and

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the behaviour of these substances in different biological systems has been compared with that of ALA.

Synthesis of compounds

The synthesis of δ -aminolaevulic acid by two routes, and that of δ -succinamidolaevulic acid, of δ -succinimidolaevulic acid and of N - β -carboxypropionylglycine, have already been described (Neuberger & Scott, 1954). Independently, Shemin, Russell & Abramsky (1955, see also Shemin & Russell, 1953) have synthesized aminolaevulic acid by three methods, one starting from β -oxoadipic acid, the second with a Gabriel condensation similar to that used in one of our two methods, and the third consisting of a Bamberger fission of β -imidazolylpropionic acid. Succinylglycine was prepared according to Scheiber & Reckleben (1913).

δ -Acetamidolaevulic acid was synthesized in two ways: β -oxoadipic acid (I; Eisner, Elvidge & Linstead, 1950) was converted by treatment with NaNO_2 into δ -oximinolaevulic acid (II); the latter was reduced by catalytic hydrogenation to δ -aminolaevulic acid (III) or by reductive acetylation to δ -acetamidolaevulic acid (IV). However, the yield in the latter step was not good, and direct acetylation of (III) with acetic anhydride in acetic acid was more satisfactory.

In order to acquire more knowledge of the chemistry of α -amino- β -oxoadipic acid a number of its esters were synthesized, and their hydrolysis has been studied under a variety of conditions. The diethyl ester has also been obtained by Shemin & Weliky (unpublished results quoted by Shemin *et al.* 1955),

Ethyl β -oxoadipate (Va), which is most conveniently prepared from ethyl α -carbethoxy- β -oxoadipate by the method of MacDonald & Stedman (1955), was converted by treatment with ethyl nitrite into the α -oximino derivative, and the latter

was catalytically reduced to the amino acid ester (VI) following the direction of Harington & Randall (1931), who had used the next lower homologue. The hydrochloride of the diester (VI) had m.p. 155–156° and was readily converted by boiling with concentrated HCl into δ -aminolaevulic acid hydrochloride (III). Treatment with acid under milder conditions gave in good yield a monoethyl ester. This half-ester is assumed to have the structure (VII) rather than (VIII) for the following reasons: (a) in the partial hydrolysis of (VI) the approach of the catalytically active proton to the δ -ester group will be less hindered by Coulomb forces than that to the α -ester group, which is adjacent to the positively charged ammonium group. It might thus be expected that, if the possible effect of the β -keto group is disregarded, the δ -ester group should be hydrolysed by acid at a greater rate than the α -ester group. (b) The half-ester was found to be stable up to a temperature of about 140°. This is not surprising for a compound of structure (VII), but (VIII), being a free β -keto acid, would be expected to be decarboxylated fairly readily. (c) The $\text{p}K_1$ of the half-ester was found to be 4.05; the magnitude of this $\text{p}K$ which corresponds with the ionization of the carboxyl group is similar to that of the $\text{p}K_1$ of α -ethyl hydrogen glutamate (3.84; Neuberger, 1936). If the half-ester had the structure (VIII), a $\text{p}K_1$ value of about 1.8–2.1, slightly lower than that of γ -ethyl hydrogen glutamate (2.15), would have been expected. The half-ester (VII), on treatment with ethanolic HCl, gave the diethyl ester (VI), and on treatment with methanolic HCl under mild conditions gave the ethyl methyl diester, which must have structure (IX) (isomer A).

An ethyl methyl diester of α -amino- β -oxoadipic acid that was not identical with (IX) was unintentionally obtained in the attempted synthesis of the dimethyl ester. Methyl β -oxoadipate (Vb), made by treatment of (I) with diazomethane, was allowed to react with ethyl nitrite, and the oximino derivative was reduced to an amino acid ester as described above. Analysis showed that the product was not, as expected, a dimethyl ester, but apparently an ethyl methyl ester. The compound was a derivative of α -amino- β -oxoadipic acid, since it

yielded (III) or glycine on hydrolysis under suitable conditions. The m.p. of this diester hydrochloride was similar to that of (IX), but the mixed m.p. was greatly depressed. Moreover, whilst the new diester (isomer B, X) gave readily a crystalline *N*-benzyloxycarbonyl derivative (see below), the isomer A (IX) gave only an oily product, which could not be induced to crystallize by seeding with the crystalline derivative of (X). It thus appeared that the new diester must have structure (X). Transesterification must have occurred either during the reduction of the oximino compound, which was done in ethanolic-aqueous HCl, or during the recrystallization of the amino acid ester hydrochloride from ethanol-ether. The preferential transesterification which occurred in the ω -position must be due to the electrostatic factors already discussed.

Heating the diester (X) with 5*N*-HCl at 100° for 1 hr. gave only δ -aminolaevulinic acid hydrochloride, with evolution of carbon dioxide. Treatment of the diester at room temperature with 2*N*-HCl and 7*N*-HCl for different periods gave mixtures of crystalline substances which, on paper-chromatographic analysis, were shown to contain varied proportions of starting material, a half-ester (probably the α -methyl ester) and aminolaevulinic acid. Alkaline hydrolysis under a variety of conditions produced mixtures of glycine and aminolaevulinic acid, the proportion of the former increasing with alkalinity. There was no evidence that significant amounts of the desired α -amino- β -keto acid accumulated in any of these experiments. It would appear that with

acid the ester groups are split to give α -amino- β -oxoadipic acid (XI) but that the latter readily loses carbon dioxide under the conditions required for the hydrolysis. In alkaline solution there is, in addition to the ester hydrolysis and decarboxylation, an attack of the hydroxyl ion on the ester (X) which results in a C₂-C₃ fission, producing in the first place glycine ester and ethyl hydrogen succinate. Further attack by hydroxyl ions then yields the free acids. Similar results were obtained with the half-ester (VII).

It appeared possible that the ease with which (XI) loses carbon dioxide in acid solution might be due to the presence of the α -amino group, which carries a positive charge in an acidic or neutral medium. *N*-Benzyloxycarbonyl derivatives of the esters (VII) and (X) were therefore prepared, and it was hoped that these might be converted into the free acyl derivative of (XI) under mild conditions. Catalytic reduction would then yield the desired compound (XI). However, treatment of these acyl derivatives in aqueous ethanol with 2 or 3 equivalents of alkali at room temperature yielded only unchanged starting material. The use of larger quantities of alkali at various temperatures gave a compound melting at 103–104°, afterwards identified as δ -benzyloxycarbamidolaevulinic acid, and *N*-benzyloxycarbonylglycine. The former was identified by comparison with a specimen synthesized from δ -aminolaevulinic acid and *N*-benzyloxycarbonyl chloride. This acylamidolaevulinic acid, after melting at 103–104°, was found to solidify and to remelt at 124–125°. It was thought that this might be caused by a conversion of the acid into either an enolic lactone, i.e. a butenolide, or a hydroxylactone. The former possibility was excluded, since elementary analysis indicated that the conversion was not associated with a loss of water. The second possibility is unlikely, since solution of both materials in aqueous ethanol gives the same pH. Moreover, dissolving material of m.p. 124–125° in alkali, followed by acidification, yielded a specimen which after air-drying had unchanged m.p. It thus appears that δ -benzyloxycarbamidolaevulinic acid

occurs in two crystalline modifications. The lower-melting material is the more unstable, since our recent preparations have yielded directly the higher-melting form.

EXPERIMENTAL

Synthesis of compounds

Melting points are uncorrected. Paper chromatography was done throughout with Whatman no. 1 paper and *n*-butanol-water-acetic acid (63:27:10, by vol.).

***δ-Oximinolaevulinic acid* (II).** To a solution of *β*-oxoadipic acid (0.82 g.) in 10 ml. of water was added at 0° 2*N*-NaOH (4.75 ml., 1.95 equiv.), followed by NaNO₂ (0.3 g. in 3 ml. of water). To the mixture after standing for 0.5 hr. at 0° was added slowly with shaking 2*N*-H₂SO₄ (7.5 ml.). The solution was then allowed to warm up to 20° and concentrated *in vacuo* to one-third of its volume. A precipitate which formed on addition of ethanol (60 ml.) was filtered off and the solution was concentrated *in vacuo* to dryness. The residue was extracted with several portions of anhydrous ether. The combined ether extracts were concentrated to dryness and the residue was recrystallized first from anhydrous ether and then from chloroform. *δ-Oximinolaevulinic acid* had m.p. 115–116°. The yield was 35% of the theoretical. (Found: C, 41.3; H, 5.1; N, 9.0, 8.9, 9.1. C₅H₇O₄N requires C, 41.4; H, 4.8; N, 9.6%.)

A solution of the oximino acid (0.97 g.) in 15 ml. of *N*-HCl containing palladium-charcoal (0.5 g.) was shaken in an atmosphere of hydrogen. The theoretical amount of gas was taken up in 25 min. The solution remaining after removal of the catalyst was concentrated *in vacuo*. The residue was crystallized from dry ethanol-ether and from dry ethanol-ethyl acetate. The compound had m.p. 142–143°; the mixed m.p. with an authentic sample of *δ*-aminolaevulinic acid hydrochloride (m.p. 145°) was 143–144°. The *R_F* value of the compound was 0.23, which is identical with that reported for aminolaevulinic acid (Berlin, Neuberger & Scott, 1956).

Catalytic reduction of the oximino acid with palladium-charcoal in glacial acetic acid containing acetic anhydride was sluggish and incomplete after 5 hr. A small amount of material of m.p. 97° was obtained, which was later shown to be *δ*-acetamidolaevulinic acid. The yield was only 5%. It was found more convenient to prepare this compound by acetylation of aminolaevulinic acid.

δ-Acetamidolaevulinic acid. To a solution of *δ*-aminolaevulinic acid hydrochloride (0.5 g.) in acetic acid (10 ml.) were added successively acetic anhydride (0.3 ml.) and anhydrous sodium acetate (246 mg.). The mixture was shaken for 0.5 hr. and then allowed to stand for 18 hr. A precipitate was removed by centrifuging and the solution was concentrated *in vacuo* to dryness. The residue was dissolved in ethanol (10 ml.) and anhydrous ether (10 ml.) was added. The resulting precipitate was discarded. The ethanol-ether solution was concentrated to dryness and the residue was dissolved in a very small volume of hot ethanol. On addition of ether a crystalline precipitate appeared which was recrystallized from ethyl acetate. The *δ*-acetamidolaevulinic acid had m.p. 97°. Yield was 55%. (Found: C, 48.5; H, 6.6; N, 7.9. C₇H₁₁O₄N requires C, 48.5; H, 6.4; N, 8.1%.)

δ-Benzoyloxycarbamidolaevulinic acid. To a solution of *δ*-aminolaevulinic acid hydrochloride (0.335 g.) in water (7 ml.), cooled to about 5° and containing MgO (0.2 g.), was added in portions and with intermittent shaking benzyloxycarbonyl

chloride (0.4 ml.) over a period of 20 min. The mixture was then allowed to stand at room temperature for 1 hr. and filtered. The filtrate was extracted with ethyl acetate and acidified. The precipitate which formed was filtered off and dried. On recrystallization from ethyl acetate-light petroleum (b.p. 60–80°) *δ*-benzyloxycarbamidolaevulinic acid was obtained. The yield was 55%. [Found: C, 58.6; H, 5.5; N (Kjeldahl), 5.4; C₁₃H₁₅O₅N requires C, 58.9; H, 5.7; N, 5.3%.] The substance had m.p. 103–104°, resolidified at about 110° and remelted at 124–125°. Material of m.p. 103–104°, which was heated *in vacuo* at 80° in the presence of P₂O₅ for 2 hr., did not melt at 103–104°, but had m.p. 125°. The analysis of the heated material showed that no change in elementary composition had been produced. (Found: C, 58.7; H, 5.5%.) Samples of materials having m.p. 103–104° and 125° respectively were titrated electrometrically in 50% (by vol.) ethanol with a glass electrode. The apparent initial pH in both cases was 3.7; both materials gave normal titration curves on addition of 0.1 *N*-NaOH, the end-points corresponding to equivalent weights of 263 and 268 respectively. (C₁₃H₁₅O₅N requires equiv. wt. 265.) Material of m.p. 125° (50 mg.) was dissolved in a slight excess of aqueous alkali. Acidification gave a crystalline precipitate which was filtered off and air-dried; m.p. was unchanged. Three later preparations yielded only material having m.p. 125°.

Ethyl α-amino-β-oxoadipate hydrochloride. Ethyl α-ethoxycarbonyl-β-oxoadipate (Eisner *et al.* 1950) was converted into β-oxoadipate by the method of Riegel & Lilienfeld (1945), or later, more conveniently, by the method of MacDonald & Stedman (1955). Ethyl β-oxoadipate (25 g.) in dry ether (200 ml.) was treated with a slight excess of ethyl nitrite and dry HCl, following the directions of Harington & Randall (1931) for β-oxoglutarate. At the end of the reaction the ethereal solution was concentrated until almost all the ether had been removed. To the residue, ether (20 ml.) was added, and the cooled solution was extracted three times with 100 ml. portions of 0.5 *M*-Na₂CO₃ previously cooled to 2°. Each Na₂CO₃ extract was acidified to pH 3 as quickly as possible. The combined aqueous solution was extracted four times with ether. The combined ether extracts were dried and concentrated *in vacuo*. The residue was left in a vacuum desiccator over KOH for 18 hr.; it weighed 28 g.

Palladium-charcoal catalyst was prepared according to Harington & Randall (1931). The nitroso compound (28 g.) in 350 ml. of ethanol containing HCl (17.5 g.) was reduced catalytically at atmospheric pressure in the presence of palladium-charcoal (7.5 g.). Hydrogen uptake became very slow after 2 hr. and amounted to 78% of the theoretical. The catalyst was removed by filtration and washed with ethanol. The ethanolic solution on concentration *in vacuo* yielded ethyl α-amino-β-oxoadipate hydrochloride in a yield of 65% (based on wt. of the impure nitroso compound). Repeated recrystallization from ethanol-ether gave material with m.p. 155–156° (with decomp.). [Found: C, 44.6; H, 6.6; N (Kjeldahl), 4.9. C₁₀H₁₇O₅N.HCl requires C, 44.9; H, 6.8; N, 5.2%.] On refluxing a solution of the hydrochloride in 5 *N*-HCl for 3 hr. there was obtained in a yield of 80% a crystalline substance which was identified by m.p., mixed m.p. and chromatography as being *δ*-aminolaevulinic acid hydrochloride. The diester had an *R_F* value of 0.58, the spot being located with the aid of ninhydrin, the picrate reagent (Shuster, 1956), and immediate reduction of AgNO₃ in the presence of NH₃. On

hydrolysis with HCl in the cold there appeared on chromatograms a spot with an R_F value of 0.39 giving colour reactions identical with those shown by the diester. Material with this R_F was also formed during the early stages of hydrolysis by 2N-HCl at 100°, whilst prolonged treatment produced mainly aminolaevulinic acid. Pilot experiments with chromatography suggested that the new substance might be obtained in a high yield by treatment with 2N-HCl for 1 min. at 100°.

Ethyl hydrogen α -amino- β -oxoadipate hydrochloride (VII). A solution of the diester (0.9 g.) in 2N-HCl (5 ml.) was heated at 100° for 1 min. The solution was cooled and concentrated *in vacuo* to dryness; the residue was dried over KOH at 12 mm. for 18 hr. The resulting glass was dissolved with slight warming in dry ethanol; on addition of dry ether a crystalline substance was obtained with m.p. 151–154°, and which on chromatography appeared almost homogeneous; in addition to material of R_F 0.39, there was a trace of aminolaevulinic acid (R_F , 0.23). Repeated recrystallization from ethanol-ether raised the m.p. to 156–157° (with decomp. but without marked evolution of gas) and led to an apparently complete removal of aminolaevulinic acid. (Found: C, 39.9; H, 6.1; N, 5.7. $C_8H_{13}O_5N \cdot HCl$ requires C, 40.0; H, 5.8; N, 5.8%). The mixed m.p. with the diester was 140–145°. The yield of *monoester* (VIII) was 55%. Electrometric titration of the ester (120 mg.) with 0.1N-NaOH indicated an apparent pK_1 4.05, and pK_2 6.4 (approx.). Heating the half-ester to 140° for 45 min. yielded material of unchanged m.p. and R_F value. The half-ester could easily be re-esterified to yield the original diester. A solution of the half-ester (50 mg.) in dry ethanol (5 ml.) containing HCl (1 g.) was allowed to stand at 20° for 3 days. Evaporation of the solution to dryness and crystallization of the residue from ethanol-ether yielded the original diethyl ester, as shown by chromatography and mixed m.p.

Ethyl methyl α -amino- β -oxoadipate hydrochloride (isomer A, IX). The half-ester (VII; 0.3 g.) was dissolved in 10 ml. of dry methanol containing HCl (6%, w/v). After standing at 20° for 1 hr. the solution was concentrated to dryness under reduced pressure, and the residue was dried over KOH *in vacuo*. The material was crystallized and recrystallized from methanol-ether. The yield was 60%. The m.p. of the *ethyl methyl ester* was 142–5°. (Found: C, 42.6; H, 6.0. $C_9H_{15}O_5N \cdot HCl$ requires C, 42.6; H, 6.3%). Treatment of the mixed ester with benzyloxycarbonyl chloride under a variety of conditions did not yield crystalline material.

α -Ethyl δ -hydrogen α -benzyloxycarbamido- β -oxoadipate. The half-ester (VII; 0.46 g.) was dissolved in water at 4°, and to the solution was added in portions with intermittent shaking and cooling over 25 min. benzyloxycarbonyl chloride (0.4 ml.) and MgO (0.2 g.). A heavy precipitate formed during the reaction, and more precooled water (5 ml.) was added as required. The mixture was allowed to stand for 10 min. at 0° after the last addition and filtered. The precipitate was washed with cold, saturated $MgSO_4$ and ethyl acetate. On suspending the precipitate in a slight excess of 0.3N-HCl an oil appeared which soon crystallized. The material was dried and recrystallized successively from ethyl acetate–light petroleum (b.p. 60–80°) and chloroform–light petroleum (b.p. 60–80°). A further small amount of this material was obtained from the mother liquor of the magnesium-salt precipitate. The alkaline solution gave, on acidification, an oil which was extracted into ethyl acetate. Some crystalline material was obtained from this fraction which melted over a wide range. The

amount was small and this fraction was discarded. The main product, which had m.p. 122°, was identified as *α -ethyl δ -hydrogen α -benzyloxycarbamido- β -oxoadipate*. [Found: C, 56.7; H, 5.6; N (Kjeldahl), 4.5; $C_{16}H_{19}O_7N$ requires C, 57.0; H, 5.8; N, 4.2%.] A portion (145 mg.) was dissolved in methanol (1 ml.) and titrated electrometrically, after addition of water (3 ml.), with 0.1N-NaOH. Alkali consumption to an apparent pH of 7.0 indicated an equivalent weight of 321 ($C_{16}H_{19}O_7N$ requires equiv. wt. 337). On further addition of alkali there appeared to be buffering in the region of pH 8–11, presumably caused by the ionization of an enolic group. After neutralization a further 14.0 ml. of 0.1N-NaOH (approx. 3 equiv.) was added and the solution was left for 2 days. Acidification gave a crystalline precipitate of m.p. 98–100°. Recrystallization and drying at 80° raised the m.p. to 122–123°, which was not lowered by admixture with δ -benzyloxycarbamidolaevulinic acid. The material was hydrogenated with H_2 and Pd and the resulting amino acid was identified as δ -aminolaevulinic acid. Treatment of the acylamidoadipate with 20 equiv. of 2N-NaOH gave the benzyloxycarbonyl derivative of glycine (m.p. and mixed m.p. 119–120°) in good yield; the corresponding derivative of aminolaevulinic acid could not be isolated in this experiment. The product was hydrogenated and glycine was identified by chromatography.

Ethyl methyl α -amino- β -oxoadipate hydrochloride (isomer B, X). β -Oxoadipic acid (Eisner *et al.* 1950) (10 g.) in ether (250 ml.) was treated at 0° with an ethereal solution of diazomethane (2.5 equiv.). After removal of the solvent the residue was distilled. The main fraction distilled between 92° and 98° (0.02 mm. Hg) and was methyl β -oxoadipate. Bardham (1936) records b.p. 122° (0.5 mm. Hg). Yield was 45%. Nitrosation of the methyl ester with ethyl nitrite and reduction of the oximino group were carried out as described above for the corresponding ethyl ester; in particular catalytic reduction was carried out in 5% (w/v) ethanolic HCl. The yield of *ethyl methyl α -amino- β -oxoadipate hydrochloride* of m.p. 140–145° (with decomp.) was 60% (based on the weight of methyl β -oxoadipate). (Found: C, 42.9; H, 6.4; N, 5.6; Cl, 13.9. $C_9H_{15}O_5N \cdot HCl$ requires C, 42.6; H, 6.3; N, 5.5; Cl, 14.0%). The mixed m.p. with isomer A (m.p. 142–5°, structure IX) was 127–128°. Hydrolysis by boiling with 5N-HCl for 1 hr. yielded CO_2 , isolated as $BaCO_3$, and aminolaevulinic acid hydrochloride, identified by mixed m.p. determination, chromatography and conversion into porphobilinogen by means of the specific dehydrase (Gibson, Neuberger & Scott, 1955). Hydrolysis with acid under mild conditions has so far not yielded material with properties expected for free α -amino- β -oxoadipic acid. Hydrolysis with alkali under a great variety of conditions produced, as measured by chromatography, mainly aminolaevulinic acid and glycine.

Ethyl methyl α -benzyloxycarbamido- β -oxoadipate. The mixed ester (isomer B, 1.2 g.) dissolved in water (4 ml.) was treated at about 4° with benzyloxycarbonyl chloride (1.0 g.) and MgO (0.4 g.), both added in portions over 25 min. with intermittent shaking. The mixture was then allowed to stand for 25 min. and extracted with two portions of ethyl acetate, after the addition of 3 ml. of water. The combined ethyl acetate extracts were washed successively with water, saturated $KHCO_3$ and saturated NaCl. The residue was dissolved in warm benzene; on addition of light petroleum a crystalline precipitate appeared which was recrystallized from the same solvent mixture (m.p. 75–76°). The yield of *ethyl methyl α -benzyloxycarbamido- β -oxoadipate*

was 66%. (Found: C, 58.0; H, 6.1; N, 4.1. $C_{17}H_{21}O_2N$ requires C, 58.1; H, 6.3; N, 4.0%.) Hydrolysis of this material, with both acid and alkali under different conditions, was carried out. Apart from unchanged ester the only products which could be identified were aminolaevulinic acid, glycine and their *N*-benzyloxycarbonyl derivatives.

BIOLOGICAL METHODS

Animals. Albino rats of the National Institute for Medical Research strain were used. All compounds were administered by intraperitoneal injection and, where urinary excretion of the injected compound or its metabolite was measured quantitatively, 0.9% NaCl (2 ml.) was injected intraperitoneally every 2 hr. for 8 hr.

Organism. A culture of *Rhodopseudomonas spheroides* was kindly supplied by Dr June Lascelles, Microbiology Unit, Department of Biochemistry, Oxford; the cells were grown in the light and harvested as described by Lascelles (1956). Incubations were at 35° under aerobic conditions, either in the light or in the dark, the detailed directions of Lascelles (1956) being followed. Cell-free extracts were obtained as follows: cells (0.5 g.) were harvested after 48 hr., washed with 0.15 M-KCl and ground in a precooled mortar containing alumina and 0.1 M potassium phosphate (pH 6.8; 15 ml.). The mixture was centrifuged (*c* 10 000 g) for 30 min. to yield 8 ml. of cell-free extract.

Liver homogenate. Rat liver was homogenized for 2 min. with 2 vol. of 0.15 M-KCl in a Potter-Elvehjem apparatus at a temperature close to 0°.

Isolation or estimation of compounds. Hippuric acid was isolated as described (Arnstein & Neuberger, 1951). Porphobilinogen was estimated by the method of Cookson & Rimington (1954) as modified by Berlin *et al.* (1956). Porphyrin formation in the microbiological experiments was measured by the method used by Lascelles (1956).

Aminolaevulinic acid was estimated by the picrate method of Shuster (1956).

Although free aminolaevulinic acid requires heating with alkali and acetoacetate to give a product which yields a colour with Ehrlich's reagent (Granick & Vanden Schriek, 1955; Shuster, 1956), acetamidolaevulinic acid was found to give a chromogen by alkali treatment, in the absence of acetylacetone or acetoacetate, in a similar manner to *N*-acetylglucosamine (Morgan & Elson, 1934). However, the colour was found to be less intense than that produced under the conditions described for aminolaevulinic acid (Shuster, 1956). The latter method was therefore used for the estimation of the acetamido compound in the urine samples of rats given acetamidolaevulinic acid. This was justified since the absence of coloration with picrate (Shuster, 1956) indicated that the urines did not contain free aminolaevulinic acid. The relationship between acetamidolaevulinic acid content and colour intensity was linear between 0.2 and 2.0 μ moles under the conditions of Shuster (1956).

Radioactivity measurements. Counting was done in 1 cm.² disks at infinite thickness with an end-window Geiger counter.

RESULTS

Availability of certain N-acyl derivatives for glycine formation

The incorporation of radioactivity from succinyl- and *N*- β -carboxypropionyl- $[\alpha\text{-}^{14}\text{C}]$ glycine into hippuric acid and haem was compared with that from free $[\alpha\text{-}^{14}\text{C}]$ glycine (Table 1). The results supported the assumption that no incorporation took place until the labelled carbon was in the α -position in free glycine, and the data of Table 1 indicate the slight extent to which this process took place. To facilitate comparison, the corrected dilution co-

Table 1. *Availability of succinyl- $[\alpha\text{-}^{14}\text{C}]$ glycine (SG) or β -carboxypropionyl- $[\alpha\text{-}^{14}\text{C}]$ glycine (CPG) for formation of $[\alpha\text{-}^{14}\text{C}]$ glycine*

It is assumed that no incorporation of isotope took place until the test compounds had been converted into glycine (Gly), hence the percentage apparent hydrolysis is $100 \times \text{c.d.c. (Gly)}/\text{c.d.c. (observed)}$; for definition see text. In Expts. 1-5, rats received sodium benzoate (0.2 m-mole/100 g.) intraperitoneally either at the same time as the labelled compound (Expts. 1 and 2) or 6 hr. later; hippuric acid was isolated from the urine voided during 24 hr. after the first injection. In Expts. 6 and 7, haemin was prepared from the blood of rats 5 days after injection of the labelled compound.

Expt. no	Labelled compound			Specific activity of compound isolated ($\mu\text{C}/\text{m-mole}$)	c.d.c.	Apparent hydrolysis to glycine (%)
		Dose (m-mole/100 g.)	Specific activity ($\mu\text{C}/\text{m-mole}$)			
(a) Hippuric acid						
1	Gly	0.033	0.075	6.04×10^{-3}	0.378 (0.356)*	(100)
2	CPG	0.033	3×0.075	3.16×10^{-5}	235	0.2
3	Gly	0.044	5.0	3.92×10^{-2}	5.56 (5.24)*	(100)
4	SG	0.044	5.0	8.71×10^{-4}	253	2.2
5	CPG	0.044	5.0	7.91×10^{-4}	278	2.0
(b) Haemin						
6	Gly	0.20	5.0	1.21×10^{-1}	66	(100)
7	CPG	0.20	5.0	1.54×10^{-2}	5500	1.2

* Data of Arnstein & Neuberger (1951).

efficients (C.D.C.; Arnstein & Neuberger, 1951) were calculated. $C.D.C. = Q(C_0/C - 1)$, where Q is the dose of labelled compound, C_0 and C are the molar isotope contents of the compound administered and the compound re-isolated, respectively. For haemin, which contains eight glycine α -carbon atoms, C is one-eighth of the isotope content measured.

Availability of certain N-acyl derivatives for aminolaevulinic acid formation

If hydrolysis of an *N*-acetylated derivative of δ -aminolaevulinic acid were to take place *in vivo*, then porphobilinogen should be detectable in the urine (Berlin *et al.* 1956). In fact no porphobilinogen or free aminolaevulinic acid was detectable after giving δ -succinimido-, δ -succinamido-, or δ -acetamidolaevulinic acid; the rate of excretion of the last compound was measured (Fig. 1). Its excretion was rapid and almost quantitative; further evidence that unchanged acetamidolaevulinic acid was being excreted was supplied by the fact that the urine, on heating with alkali in the absence of ethyl acetoacetate, gave then a red colour with Ehrlich's reagent (see Experimental).

Pyrrole formation from esters of amino-oxoadipic acid

There was clear evidence that both the di- and the (α)mono-ethyl esters of α -amino- β -oxoadipic acid were hydrolysed and decarboxylated *in vivo*. Porphobilinogen formation from these two compounds was readily measurable (Table 2), but was much less than from aminolaevulinic acid itself.

Fig. 1. Urinary excretion of δ -aminolaevulinic acid (ALA) or of δ -acetamidolaevulinic acid (AcALA). Rats were given either ALA or AcALA (0.2 m-mole/100 g.). No porphobilinogen or free ALA was detected in the urine of the rats given AcALA. Free ALA was determined by the picrate method and AcALA by the acetoacetate method. ○, AcALA after AcALA injection; ●, ALA after ALA injection.

Urine samples from rats which had received the diester were subjected to chromatography on paper as described by Berlin *et al.* (1956). Spots corresponding to aminolaevulinic acid (R_F 0.23) were detectable in urine samples produced 2, 4, 6 and 8 hr. after injection. In addition, spots corresponding to a monoethyl ester of amino-oxoadipic acid were detectable after 2 and 4 hr. but not later.

The availability of diethyl α -amino- β -oxoadipate for porphyrin formation was demonstrated with cell suspensions (Fig. 2) or cell-free extracts (Table 3) of *Rps. spheroides*. This organism has been shown by Lascelles (1956) to produce different amounts of porphyrins from any one substrate, according as incubation is in the light or in the dark.

The ability of whole cells to utilize the diester was largely lost when they were broken up (Table 3).

Table 2. Excretion of porphobilinogen (PBG) after administration of esters of α -amino- β -oxoadipic acid to rats

Diethyl amino-oxoadipate or the (α)mono-ethyl ester (0.15 m-mole/100 g.) was injected. At this dose level 1.7–1.8% of ALA given has been found to be converted into urinary PBG (Berlin *et al.* 1956).

Time after injection (hr.)	PBG excreted (% of dose)	
	After diester	After monoester
2	0.027	0.027
4	0.052	0.066
6	0.023	0.046
8	0.012	0.015
Totals	0.114	0.154

Fig. 2. Porphyrin formation from diethyl α -amino- β -oxoadipate, or from ALA, by *Rps. spheroides*. The incubation mixtures contained washed-cell suspension (2 mg. dry wt./ml.), potassium phosphate buffer (200 μ moles, pH 6.8) and substrate (20 μ moles) in a total vol. of 7 ml. Incubation was either in the dark or in the light, with shaking. At intervals, 1 ml. was removed and added to 3 ml. of 1.4N-HCl; after centrifuging, the porphyrin content of the supernatant was measured as described. ○, ALA in the light; ●, ALA in the dark; □, diester in the light; ■, diester in the dark.

Table 3. *Porphyrin formation in a cell-free extract of Rps. spheroides*

Cell-free extract (1 ml.) was incubated either in the light or in the dark with potassium phosphate buffer (pH 6.8, 100 μ moles) and substrate (10 μ moles) in a total vol. of 2 ml.

Substrate	Time (hr.)			
	In the light		In the dark	
	1.25	18	1.25	18
Porphyrin formed (μ moles)				
Aminolaevulinic acid	285	32	35	390
Diethyl α -amino- β -oxoadipate	6	7	6	21

Fig. 3. Formation of porphobilinogen (PBG) in liver homogenates. The incubation mixture contained liver homogenate (4 ml.), 0.1M potassium phosphate buffer (2 ml., pH 6.8) and substrate (20 μ moles). Incubation was at 37°, with gentle agitation. At intervals, 0.1 ml. was removed and added to 10% trichloroacetic acid (2 ml.) for assay of PBG after centrifuging. Substrates: $\square$, ALA; $\bullet$, monoester, and $\circ$, diester, of α -amino- β -oxoadipic acid.

Porphobilinogen formation from both the di- and the (α)mono-ethyl ester of amino-oxoadipic acid took place in rat-liver homogenates; as expected, the monoester was more effective than the diester, but both were rather less effective than aminolaevulinic acid (Fig. 3).

DISCUSSION

It appears that *N*-succinyl- or β -carboxypropionyl-glycine are hydrolysed to a negligible extent, if at all, in the body. Tests with these two compounds labelled in the position corresponding to the α -carbon atom of glycine indicated (Table 1) that they enter into anabolic pathways of glycine to an extent which suggests that they may be excreted or oxidized at least as readily as they are hydrolysed. In oxidation their labelled α -carbon atoms would be

only indirectly available for glycine formation. Such behaviour is in sharp contrast to that of *N*-formyl-, *N*-acetyl- and *N*-propionyl-glycine (Abbott 1942) and to similar derivatives of other amino acids (for review see Jackson & Chandler, 1939). A similar failure to hydrolyse *N*-succinyl and *N*- β -carboxypropionyl derivatives has been observed in succinimido- and succinamido-laevulinic acids, but in this case the *N*-acetyl derivative is also not split in the body (Fig. 1); in contrast to the latter finding, Neuberger & Sanger (1943) found that ϵ -*N*-acetyllysine can replace the parent amino acid in deficient diets. The failure of the body to hydrolyse these two *N*- β -carboxypropionyl amino acids recalls the finding of Christman & Lewis (1921) that esterases will hydrolyse only the first ester group of dialkyl succinates, being apparently inhibited by the close proximity of the charged carboxyl group previously liberated.

Tables 2 and 3 and Figs. 2 and 3 show that diethyl or, better, (α)mono-ethyl α -amino- β -oxoadipate can give rise to δ -aminolaevulinic acid both *in vivo* and, to a lesser extent, in cell-free biological systems. The observation that the diester gives rise to urinary porphobilinogen after injection into rats was also made by Shemin *et al.* (1955). Although these esters of amino-oxoadipic acid have been shown to give rise to aminolaevulinic acid, porphobilinogen and porphyrins, it cannot be claimed on this evidence alone that the free acid is in fact a normal intermediate in biosynthesis, since similar findings would have resulted from spontaneous decarboxylation of this β -keto acid after saponification by an esterase.

SUMMARY

1. The diethyl ester and the (α)mono-ethyl (δ)hydrogen ester of α -amino- β -oxoadipic acid hydrochloride have been synthesized. The two ethyl methyl esters of this acid have also been prepared and identified. *N*-Benzyloxycarbonyl derivatives of certain of these compounds were prepared.

2. The hydrolysis of esters of amino-oxoadipic acid, or of the corresponding *N*-benzyloxycarbonyl derivatives, has been studied. It has not so far been possible to isolate the free amino acid. Under acid conditions the above-mentioned half-ester and aminolaevulinic acid were the main products. Under alkaline conditions aminolaevulinic acid and glycine, or their *N*-acyl derivatives, were the main nitrogenous products. δ -Benzyloxycarbamidolaevulinic acid was also prepared from aminolaevulinic acid hydrochloride; the compound occurs in two crystalline modifications.

3. δ -Acetamidolaevulinic acid has been synthesized from aminolaevulinic acid and was also prepared by reductive acetylation of δ -oximinolaevulinic acid.

4. In contrast to simple unbranched *N*-acyl derivatives of glycine, *N*- β -carboxypropionyl- and *N*-succinyl-glycine are hydrolysed *in vivo* to a negligible extent only. Corresponding derivatives of aminolaevulinic acid also are not hydrolysed, nor is, in this case, the *N*-acetyl amino acid. With the latter compound rapid excretion may account for the failure of hydrolytic systems to yield the free amino acid.

5. Both the diester and the (α)mono-ethyl ester of amino-oxoadipic acid have been shown to give rise to porphobilinogen or porphyrins in biological systems. These included rats, liver homogenates, and suspensions or cell-free extracts of *Rhodospseudomonas spheroides*. This is not, however, sufficient evidence to establish that amino-oxoadipic acid is the biological precursor of aminolaevulinic acid.

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The Concentration of Glucose in Rat Tissues

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Paper chromatography has been used in the present investigation for the determination of the true glucose content of eight tissues of the rat. This method is more effective than older methods in separating glucose from other reducing substances in tissue extracts, but it does not appear to have been used before for systematic investigations of the glucose content of animal tissues, though Wallenfels, Bernt & Limberg (1953) used it for measuring glucose in blood. Previous authors have determined the yeast-fermentable reducing substances in some tissues, and the values obtained by chromatography in the present work are of the same order.

EXPERIMENTAL

Preparation of tissues. Male albino rats, weighing 105–160 g., were used, after having been kept without food for 20–28 hr. but supplied with water *ad lib*. The animals were killed instantaneously either by immersion in liquid oxygen or by decapitation. In the latter method tissues were put in liquid oxygen after a specified time. The time interval between death and freezing is given in the first column of Tables 2–9. The tissues were broken up in the liquid oxygen with a pair of pliers and quickly weighed on a torsion balance while still frozen. The weighed tissue (up to 450 mg.) was ground in an ice-cold mortar with acid-washed sand in 1 ml. of 0.3 N-Ba(OH)₂ (Somogyi, 1945). The following additions were then made: 1 ml. of 0.3 N-Ba(OH)₂, water (2 ml. minus an allowance for tissue water, taken to be 80% of the