resembles that of the coenzyme. Omission of 5'-deoxyadenosylcobalamin resulted in a spectrum resembling Spectrum 3; this spectrum did not change in the first two minutes.

The occurrence of a spectral change on incubation of a coenzyme B_{12}-dependent enzyme with substrate has been demonstrated previously in the case of diol dehydrase[5]. However, the spectrum of actively functioning diol dehydrase could not be interpreted because of the large excess of unbound coenzyme present in the reaction mixture. In the present experiments, in which enzyme and coenzyme were present in equivalent amounts, it was possible not only to demonstrate the occurrence of a spectral change but also to establish that this change represented the appearance of a species of cobalamin which closely resembled cob(II)alamin in its spectral characteristics. It is reasonable to postulate that this species is involved in the catalytic process. Although it is not possible to interpret such a spectral change in terms of a unique mechanism, the characteristics of the new species raise the possibility that it is generated by a homolytic cleavage of the carbon–cobalt bond of the coenzyme. If so, this result would imply that ethanolamine deaminase operates by a free radical mechanism, as was suggested by EGGERER *et al.*[9] in connection with the mechanism of action of methylmalonyl-CoA isomerase.

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Intramitochondrial localization of 5-aminolaevulinate synthase induced in rat liver with allylisopropylacetamide

The synthesis of haem in higher organisms involves a close cooperation of mitochondria and cell sap. Synthesis of 5-aminolaevulinate is a mitochondrial process, its

conversion into coproporphyrinogen takes place in the cell sap, and the last steps from coproporphyrinogen to haem again occur in the mitochondria[1,2]. Recent work in three laboratories[3–5] has shown that the enzyme catalysing the terminal step in this sequence, ferrochelatase (protohaem ferro-lyase, EC 4.99.1.1), is firmly bound to the mitochondrial membrane, while in yeast it even requires phospholipids for maximal activity[5]. We have determined the intramitochondrial localization of the enzyme catalysing the first step in haem synthesis, 5-aminolaevulinate synthase, in rat-liver mitochondria. Our experiments show that it is actually a matrix enzyme, defined in the customary sense, *i.e.*, an enzyme either present free in the matrix space or so loosely bound to the inner membrane that it is released by sonic fragmentation of the mitochondria.

5-Aminolaevulinate synthase was induced with allylisopropylacetamide in rats, essentially as described by NARISAWA AND KIKUCHI[6]. After 24 h fasting, female rats of about 150 g were subcutaneously injected with 40 mg allylisopropylacetamide; food was withheld and after 24 h the same amount of allylisopropylacetamide was injected and 16 h later the rat was killed. Liver mitochondria were prepared by differential centrifugation in 0.25 M sucrose[7]. For the determination of 5-aminolaevulinate synthase activity in intact mitochondria they were incubated aerobically for 4 min at 38° and pH 7.4 in manometer flasks with vigorous shaking in a reaction mixture containing 75 mM glycine, 10 mM succinate, 50 mM Tris chloride, 20 mM sodium–potassium phosphate, 15 mM KCl, 5 mM $MgCl_2$, 2 mM sodium ethylenediaminetetraacetate and 0.1 mM pyridoxal phosphate. The reaction was stopped by adding trichloroacetic acid to a final concentration of 2.5%. 5-Aminolaevulinate synthase in mitochondria, disrupted by sonication or digitonin, was determined by the same procedure but the succinate was replaced by 0.3–0.4 mM succinyl-CoA prepared according to SIMON AND SHEMIN[8]. The 5-aminolaevulinate formed was determined by the procedure of GRANICK[9]. With both assays the amount of 5-aminolaevulinate formed was proportional to enzyme concentration within the range used.

Intact mitochondria isolated from rats injected with allylisopropylacetamide had an activity varying between 0.2 and 1.3 nmoles 5-aminolaevulinate synthesized per min per mg protein, compared with 0.05 for non-induced rats. Mitochondrial preparations in which the specific activity was at least 0.5 were used for further studies. Induction did not affect other mitochondrial properties tested. In four different mitochondrial preparations from well-induced rats the respiratory rates and respiratory control with glutamate *plus* malate, succinate, or tetramethylparaphenylenediamine *plus* ascorbate as substrate were not significantly different from the values obtained for non-induced rats.

For fractionation studies the final mitochondrial pellet obtained in the isolation procedure was taken up in 5 ml 10 mM sodium phosphate (pH 7). The suspension was then sonicated at 0° for 3 periods of 30 sec in the MSE 100-W sonic disintegrator, at maximal energy output. The sonicate was fractionated by differential centrifugation. The distribution of 5-aminolaevulinate synthase compared to that of cytochrome *c* oxidase (EC 1.9.3.1, marker for the mitochondrial inner membrane) and glutamate dehydrogenase (EC 1.4.1.3, marker for the mitochondrial matrix space) is shown in Table I. Recovery of 5-aminolaevulinate synthase was lower than recovery of protein, and this was also observed in a second experiment. The activity lost was not retrieved by combining Fractions P_2 and S_2 and therefore the loss was probably due to inacti-

TABLE I

THE INTRAMITOCHONDRIAL LOCALIZATION OF 5-AMINOLAEVULINATE SYNTHASE; FRACTIONATION OF MITOCHONDRIA AFTER SONICATION

Mitochondria were sonicated for 90 sec. The sonicated mitochondria were centrifuged at 20 000 × *g* for 15 min in Rotor 9A of the Lourdes Beta-Fuge, yielding residue P_1 and supernatant S_1. The latter was centrifuged for 60 min at 100 000 × *g* in the Rotor 50 of the Spinco ultracentrifuge, yielding residue P_2 and supernatant S_2. 5-Aminolaevulinate synthase activity was assayed as described in the text. The specific activity is expressed as nmoles 5-aminolaevulinate synthesized per mg protein per min. Cytochrome *c* oxidase was measured by the procedure of COOPERSTEIN AND LAZAROW[10] as described previously[11]. The specific activity is expressed in arbitrary units. Glutamate dehydrogenase was measured as described by BEAUFAY *et al.*[12]. The specific activity is expressed as μmoles NADH oxidized per mg protein per min. Total activities are expressed as percentage of the values found in the sonicated mitochondrial fraction.

	Mitochondria	*Sonicated mitochondria*	P_1	S_1	P_2	S_2
Protein	103	100	9	88	22	56
5-Aminolaevulinate synthase						
Specific activity	0.68	0.54	0.31	0.47	0.20	0.49
Total activity	138	100	5	81	9	54
Cytochrome oxidase						
Specific activity	110	108	215	99	325	7
Total activity	105	100	18	82	67	4
Glutamate dehydrogenase						
Specific activity	0.65	0.65	0.20	0.83	0.25	1.15
Total activity	103	100	2.5	110	8.5	98

vation of enzyme during the fractionation. Nevertheless, the results of this fractionation experiment clearly show that the synthase behaves like glutamate dehydrogenase in being released from the mitochondrial membranes after sonication of mitochondria. Under these conditions both the enzymes from the matrix space and from the inter-membrane space become non-sedimentable at 100 000 × *g*. To exclude that 5-amino-laevulinate synthase belongs to the latter category, mitochondria were treated with digitonin, as described by SCHNAITMAN *et al.*[13]. Under these conditions the mitochondrial outer membrane (marker monoamine oxidase, EC 1.4.3.4) peels off, the inter-membrane enzymes are released, while the inner membrane with most of its complement of matrix enzymes can be sedimented at low speed[13]. The outcome of such a fractionation is presented in Table II. The distribution of the synthase closely parallels that of both cytochrome oxidase and glutamate dehydrogenase with the bulk of the activity being present in the low-speed pellet. On the other hand, 81% of the monoamine oxidase activity remained in the supernatant, showing that most of the mitochondria had shed their outer membrane. We conclude, as did MCKAY *et al.*[3] in a recent abstract, that the 5-aminolaevulinate synthase, induced by allylisopropyl-acetamide, is either loosely bound to the mitochondrial inner membrane or present free in the matrix space.

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TABLE II

THE INTRAMITOCHONDRIAL LOCALIZATION OF 5-AMINOLAEVULINATE SYNTHASE; FRACTIONATION OF MITOCHONDRIA AFTER DIGITONIN TREATMENT

Rat-liver mitochondria (184 mg protein in 7 ml 0.25 M sucrose) were incubated with 0.22 mg digitonin per mg protein for 20 min at 0°. The suspension was then centrifuged for 10 min at 10 000 × *g* to separate a precipitate fraction P_3 (inner membrane *plus* matrix) from a supernatant fraction, S_3 (outer membrane *plus* inter membrane enzymes). P_3 was taken up in 30 mM sodium phosphate (pH 7). Before the enzyme assays the fractions were briefly sonicated. Enzyme assays and presentation of the data as in Table I; monoamine oxidase was determined by a procedure similar to that of SCHNAITMAN *et al.*[13], and the specific activity is expressed as nmoles benzaldehyde formed per min per mg protein.

	Mitochondria	P_3	S_3
Protein (% of total)	100	77	21
5-Aminolaevulinate synthase			
Specific activity	0.47	0.54	0.26
Total activity	100	88	11
Cytochrome oxidase			
Specific activity	73	80	54
Total activity	100	85	15
Glutamate dehydrogenase			
Specific activity	0.69	0.69	0.49
Total activity	100	77	15
Monoamine oxidase			
Specific activity	15.5	3	58
Total activity	100	15	81

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