N-Dimethylaminoethyl- $\beta\beta$ -dimethylacrylate. The above anhydride (12.5 g., 0.07 mole) was refluxed overnight with dimethylaminoethanol (6.25 g., 0.07 mole); the reaction mixture was distilled *in vacuo* to give 15 g. of crude ester, b.p. 60–65° at 0.5 mm. Hg.

$\beta\beta$ -Dimethylacrylylcholine. A mixture of the above crude ester (10 g.) and methyl iodide (20 g., 0.14 mole) in dry ether (125 ml.) was allowed to stand for 3 days at 25°; the precipitate was collected by filtration and crystallized from ethanol to give $\beta\beta$ -dimethylacrylylcholine iodide as a light-yellow powder (m.p. 160°), yield, 5 g. (0.015 mole, 32% of dimethylaminoethanol) (Found, C, 38.1; H, 6.5; I, 40.4. $C_{10}H_{20}O_2NI$ requires C, 38.3; H, 6.4; I, 40.5%). The iodide (36 mg., 0.12 m-mole) in water (1 ml.) was converted into the *chloride* by passing it through De-Acidite FF as described above; 75% of the ester emerged in the first 5 ml. of effluent. This was treated with excess of sodium aurichloride to give a yellow precipitate, which on recrystallization from water yielded the ester *aurichloride* as yellow needles, m.p. 97°, yield 12 mg. (0.023 m-mole, 19%) (Found Au, 37.5. $C_{10}H_{20}O_2NAuCl_4$ requires Au, 37.5%). The chloride was also obtained from this salt by passing it through De-Acidite FF as described for the naturally occurring ester.

Spectroscopic comparison of esters

Infrared light. Infrared spectroscopy was carried out in a Perkin-Elmer double-beam recording spectrophotometer with the KBr-window technique. The window contained 0.34% of the ester aurichloride. The spectra of the synthetic and natural esters are plotted side by side in Fig. 1 and will be seen to be essentially identical.

Ultraviolet light. Ultraviolet spectroscopy was carried out in a Hilger Uvispek spectrophotometer. Dilutions were made under standard conditions in relatively large volumes

to minimize errors due to absorption of the ester on glassware. All molar-extinction coefficients (ϵ) were calculated from the optical density of a 1 cm. thickness of solution and the molar concentration of ester, determined for the chlorides by the ferric hydroxamate method (Hestrin, 1949) at 520 $m\mu$ with the synthetic ester iodide as standard. The results are plotted in Fig. 2. It will be seen that the aurichlorides (upper curve) have a higher ϵ_{max} and lower λ_{max} than the chlorides (lower curve), and that the spectra of the synthetic and natural esters are essentially identical.

SUMMARY

1. The biologically active choline ester, $\beta\beta$ -dimethylacrylylcholine, has been isolated from the hypobranchial gland of the marine snail *Thais floridana* as the pure, crystalline aurichloride.

2. The identity of the naturally occurring compound with the synthetic ester is demonstrated by physical and chemical methods.

The synthesis of $\beta\beta$ -dimethylacrylylcholine iodide was carried out by Dr L. E. Tammelin and Ing. I. Enander, and the infrared spectroscopy by Dr L. Larsson, Research Institute of National Defence, Department 1, Sundberg 4, Sweden. I am most grateful to them for permission to include their results.

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The *in vitro* Enzymic Hydroxylation of Steroids

5. HYDROGEN TRANSPORT IN OX-ADRENOCORTICAL MITOCHONDRIA IN RELATION TO STEROID HYDROXYLATION*

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It has been shown by Grant & Brownie (1955) and by Sweat & Lipscomb (1955) that reduced triphosphopyridine nucleotide (TPNH) is required for the hydroxylation of steroids in the 11 β -position by enzymes in ox-adrenocortical mitochondria. Subsequently, Grant (1956*a*) demonstrated that fumarate, added to enzyme reaction mixtures for steroid 11 β -hydroxylation, effects triphosphopyridine (TPN⁺) reduction according to the reactions: (1) fumarate $\rightarrow$ L-malate; (2) L-malate +

TPN⁺ $\rightarrow$ pyruvate + carbon dioxide + TPNH + H⁺. Sweat & Lipscomb (1955) suggested the following sequence of reactions for the formation of TPNH: (3) Fumarate $\rightarrow$ L-malate; (4) L-malate + diphosphopyridine nucleotide (DPN⁺) $\rightarrow$ oxaloacetate + DPNH; (5) DPNH + TPN⁺ $\rightarrow$ DPN⁺ + TPNH. Reaction (5) requires the presence of a pyridine nucleotide transhydrogenase, known to occur in certain animal tissues (Kaplan, Colowick & Neufeld, 1953), but not previously reported in the adrenal cortex. The presence of the transhydrogenase in the adrenal cortex was not demonstrated

* Part 4: Grant (1956*a*).

directly, but was inferred from the observations that when either TPN^+ or DPNH is added to the mitochondrial suspensions in the absence of fumarate or malate only traces of corticosterone are formed from 11-deoxycorticosterone, but when traces of TPN^+ are added with the DPNH a significant amount of 11-deoxycorticosterone 11 β -hydroxylation occurs. Both Brownie & Grant (1954) and Sweat & Lipscomb (1955) found that citrate was a relatively feeble 'activator' of steroid 11 β -hydroxylation, although its addition to the reaction mixture might be expected to result in reduction of TPN^+ coupled to D-isocitrate oxidation. The discovery of a DPNH-specific isocitric dehydrogenase in animal tissues by Plaut & Sung (1954) and the investigations of isocitrate oxidation in rat-liver mitochondria by Ernster & Navazio (1957) suggested that a study of the oxidation of isocitrate in ox-adrenocortical mitochondria might help to explain the relatively feeble steroid 11 β -hydroxylation in the presence of this tricarboxylic acid.

A preliminary account of some of the experiments described here has been published (Grant & Mongkolkul, 1958).

EXPERIMENTAL

Enzyme preparations, materials and methods

Suspensions of mitochondria were prepared from finely minced, fresh ox-adrenocortical tissue as described by Brownie & Grant (1954). TPN^+ (about 95% pure) and crystalline adenosine triphosphate were purchased from Sigma Chemical Co. (U.S.A.); TPN^+ was assayed by the glucose 6-phosphate dehydrogenase method of Horecker & Smyrniotis (1955); DPNH (about 95% pure) and DPNH were purchased from Boehringer and Sons, Mannheim-Waldhof, Germany, and their purities checked by alcohol dehydrogenase from the same firm. TPNH was prepared by the method of Conn, Kraemer, Pei-Nan & Vennesland (1952). DL-isoCitric lactone was purchased from L. Light and Co., and converted into isocitrate by the method of Krebs & Eggleston (1944). Cytochrome *c* was purchased from L. Light and Co. and assayed by the method of Potter (1949). 2:6-Dichlorophenolindophenol was purchased from British Drug Houses Ltd.

Mitochondria, suspended in 0.25 M-sucrose containing 0.05 M-potassium phosphate, pH 7.4, were depleted of native pyridine nucleotides by pre-incubation, termed 'aging', at 37°, with gentle shaking (Huennekens & Green, 1950). Mitochondria from about 1 g. of adrenal cortex were contained in 1 ml. of suspension.

Oxygen-consumption measurements were made by the usual Warburg technique.

Changes in optical density were observed by using a Unicam SP. 500 spectrophotometer with a water-jacketed cuvette holder for the maintenance of constant temperatures.

For the determination of the pyridine nucleotide specificity of the mitochondrial isocitric dehydrogenase the

cytochrome system was blocked with cyanide and 2:6-dichlorophenolindophenol was used as a terminal hydrogen acceptor according to the method of Ernster & Navazio (1957). The reaction was initiated by the addition of isocitrate, and readings were taken at 600 m μ at intervals against a blank containing all additions except pyridine nucleotides.

For the determination of activities of reduced pyridine nucleotide oxidases and pyridine nucleotide transhydrogenase cytochrome *c* was employed as electron acceptor and observations were made at 340 m μ with occasional stirring of the cuvette contents for aeration.

RESULTS

Comparison of rates of oxidation of citrate, isocitrate and fumarate by ox-adrenocortical mitochondria

The results, illustrated in Fig. 1, show that citrate and DL-isocitrate are readily oxidized by ox-adrenocortical mitochondria at rates which are not significantly different from that observed with fumarate as substrate.

Fig. 1. Oxidation of citrate, DL-isocitrate and fumarate by mitochondria from ox-adrenal cortex. Each Warburg flask contained, in 3 ml. final volume, 0.5 ml. of a suspension in 0.25 M-sucrose of mitochondria, which had not been aged, from 1 g. of adrenal cortex, 150 μ moles of potassium chloride, 12 μ moles of magnesium sulphate, 120 μ moles of potassium phosphate buffer, pH 7.4, 3 μ moles of adenosine triphosphate and 30 μ moles of potassium citrate or potassium fumarate or 60 μ moles of potassium DL-isocitrate. The gas phase was air, and the flasks were shaken in a water bath at 37°. The tricarboxylic acids and fumarate were tipped in at zero time.

Pyridine nucleotide specificity of isocitric dehydrogenase in ox-adrenocortical mitochondria

Fig. 2 shows the effects of added pyridine nucleotides on the oxidation of *isocitrate* by aged mitochondria from ox-adrenal cortex. These results suggest that oxidation of *isocitrate* in the mitochondria proceeds mainly by way of a DPN^+ -specific *isocitric* dehydrogenase. The possibility, however, remains that the oxidation involves a TPN^+ -linked enzyme working with traces of TPN^+ remaining in the aged mitochondria, and that the observed DPN^+ reduction results from the activity of a pyridine nucleotide transhydrogenase according to the reaction

suggested by Kaplan, Swartz, Frech & Ciotti (1956). This possibility was investigated in the following experiments.

Activity of reduced pyridine nucleotide oxidases of pyridine nucleotide transhydrogenase in ox-adrenocortical mitochondria

The results of a typical experiment shown in Fig. 3 indicate that aged mitochondria from ox-

adrenal cortex contain an active DPNH oxidase, that TPNH is not oxidized at an appreciable rate in this system and that there is no significant reduction of added DPN^+ by TPNH . In a similar experiment added TPN^+ was found to be without significant effect on the rate of oxidation of DPNH . It may therefore be concluded that under these experimental conditions the activity of pyridine nucleotide transhydrogenase in these mitochondria is negligible and that it cannot account for the reduction of DPN^+ observed on addition of *isocitrate* in the experiments described above.

DISCUSSION

From the results described here it is obvious that mitochondria from ox-adrenal cortex can oxidize citrate and *isocitrate* at approximately the same rate as fumarate. This is in agreement with the observations of Sourkes & Heneage (1952) on whole homogenates of ox-adrenal glands. Rates of oxidation by mitochondria, of the same order and under similar conditions, are observed with succinate as substrate (Brownie, Grant & Davidson, 1954). Thus permeability barriers to the entry of

Fig. 2. Effect of added pyridine nucleotides on the oxidation of *isocitrate* by mitochondria from ox-adrenal cortex. The mitochondria were aged for 1.5 hr. at 37° as described in the Experimental section. Each cuvette of 5 mm. light path contained mitochondria from about 50 mg. of adrenal cortex, 1 μmole of manganese chloride, 10 μmoles of magnesium sulphate, 120 μmoles of potassium phosphate buffer, pH 7.4, 3 μmoles of potassium cyanide, 0.5 μmole of TPN^+ or DPN^+ , as indicated, 0.19 μmole of 1:6-dichlorophenolindophenol and 20 μmoles of DL-potassium *isocitrate* in a final volume of 1.5 ml. The final pH was 7.4 and the temperature 20° . The blank cuvette contained all substances other than pyridine nucleotide. The *isocitrate* was added at zero time.

Fig. 3. Oxidation of reduced pyridine nucleotides and the activity of pyridine nucleotide transhydrogenase in mitochondria from ox-adrenal cortex. The mitochondria were aged for 1.5 hr. at 37° . Each cuvette of 5 mm. light path contained mitochondria from about 50 mg. of adrenal cortex, 60 μmoles of potassium phosphate buffer, pH 7.4, 0.02 μmole of cytochrome c, 0.25 μmole of DPNH , TPNH and DPN^+ , as indicated, in a final volume of 1.5 ml. The final pH was 7.4 and the temperature 20° . The blank cuvette contained all substances other than pyridine nucleotides, which were added to the other cuvettes at zero time. Aeration was maintained by occasional stirring with glass rods.

citrate into the mitochondria (Johnson & Lardy, 1958) or the deficiency of these particles in aconitase (Dickman & Speyer, 1954) are unlikely to be limiting factors which might account for the relatively feeble 'stimulation' of 11-deoxycorticosterone 11 β -hydroxylation by citrate as compared with fumarate (Grant & Brownie, 1955; Sweat & Lipscomb, 1955). The results now obtained suggest that isocitrate oxidation in ox-adrenocortical mitochondria is linked predominantly with the reduction of DPN⁺ rather than TPN⁺. Since TPNH is required for steroid hydroxylation this would explain the relatively feeble effect of citrate. The possibility has not been excluded that a selective destruction of a TPN-specific isocitric dehydrogenase occurs during ageing at 37°, but this is not thought to be likely since under some circumstances the TPN⁺-linked enzyme from pig heart may be heated to 56–57° for 15 min. with only slight loss of activity (Hogeboom & Schneider, 1950) and the thermal lability of the DPN⁺-linked enzyme may be used as a means of freeing a yeast TPN⁺-isocitric dehydrogenase of this activity (Kornberg, 1955).

The feeble oxidation of TPNH by the mitochondrial flavoproteins and cytochrome system as compared with that of DPNH is in keeping with the view first expressed by Colowick (1951) that TPNH oxidation is not normally coupled with phosphorylation and with numerous observations of biosynthetic reactions which require TPNH. Examples of such reactions are steroid hydroxylations (Grant, 1956b), the concerted cyclization and hydroxylation of squalene in sterol formation (Clayton & Bloch, 1956), the reversal of fatty acyl-coenzyme A dehydrogenase activity in fatty acid synthesis (Lynen & Decker, 1957), the synthesis of fructose in male reproductive organs (Hers, 1956) and the incorporation of C₁ units into serine (Hatefi, Osborn, Kay & Huennekens, 1957). These observations suggest a special role for TPNH as a specific reductant in biosynthetic reactions rather than as a substrate for oxidations via flavoproteins and cytochromes coupled with phosphorylations. In the adrenal cortex the necessity of conserving TPNH for steroid-hormone biosynthesis is obvious.

Oxidation of TPNH in the adrenal cortex via DPN⁺ with the aid of a pyridine nucleotide transhydrogenase offers the possibility of controlling the TPNH level and through it steroid biosynthesis. Transhydrogenase activity was not, however, demonstrated under the present experimental conditions, which were designed to investigate the pyridine nucleotide specificity of the mitochondrial isocitric dehydrogenase. Sweat & Lipscomb (1955), however, demonstrated transhydrogenase activity in ox-adrenocortical mitochondria in-

directly by their observations that 11 β -hydroxylation of 11-deoxycorticosterone by these mitochondria is negligible in the presence of TPN⁺ or DPNH alone, but that hydroxylation occurs equally well in the presence of either TPNH or DPNH along with small amounts of TPN⁺. Failure to observe transhydrogenase activity in the present investigation may be associated with the ageing of the mitochondria, and it is tempting to speculate that this ageing may result in loss of minute traces of 17-hydroxysteroids, notably oestradiol-17 β , which according to Talalay & Williams-Ashman (1958) may function as co-enzymes for pyridine nucleotide transhydrogenase. This possibility is at present under investigation.

SUMMARY

1. The feeble 'stimulation' of 11-deoxycorticosterone 11 β -hydroxylation in mitochondria from ox-adrenal cortex by citrate is not due to failure of this substance to enter the mitochondria or to be oxidized via isocitrate, but may be related to the fact that isocitrate oxidation which appears to be predominantly diphosphopyridine nucleotide linked in these mitochondria fails to produce adequate amounts of reduced triphosphopyridine nucleotide.

2. The reduction of diphosphopyridine nucleotide by the mitochondrial enzymes in the presence of isocitrate, under the conditions described, cannot be attributed to the activity of a pyridine nucleotide transhydrogenase.

3. The failure to oxidize reduced triphosphopyridine nucleotide by flavoprotein-cytochrome systems in the mitochondria and the possible significance of the transhydrogenase reaction in relation to biosynthetic reactions are discussed.

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The Effect of Haemorrhage on the Incorporation of [α - 14 C]Glycine into Stercobilin

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It was shown by Tarchanoff (1874) that injected haemoglobin gave rise to bile pigments. This observation led eventually to an approximate correlation of the rate of haemoglobin breakdown with the daily amounts of bile pigment produced (Eppinger & Ranzi, 1920; Hawkins, Shribhishaj, Robschit-Robbins & Whipple, 1931; Miller, Robschit-Robbins & Whipple, 1945). From the nature and arrangement of the β -substituents of biliverdin, from which the other naturally occurring bile pigments are derived, it is evident that, of the free porphyrins, only protoporphyrin IX could theoretically give rise directly to biliverdin. Evidence for such a conversion has been obtained by using isotopically labelled protoporphyrin (London, Yamasaki & Sabella, 1951); however, when a rabbit's liver was perfused with a solution of protoporphyrin only 2% of the porphyrin was recovered, while the remainder did not give rise to bile pigments (Watson, Pass & Schwartz, 1941; Salzburg & Watson, 1941). Since it is known that bile pigments are formed in the liver, it may be inferred that they arise directly only from haem (London, 1950) or from haem proteins.

Isotope studies have shown that bile pigment is derived not only from red cells at the end of their life span, but also from a second source, responsible for the labelling of faecal stercobilin during the first

few days after feeding isotopically labelled glycine. The origin of this second fraction of bile pigments, indistinguishable chemically from the catabolic fraction produced from the breakdown of red cells at the end of their life span, is unknown but the increase in percentage of this fraction from about 10% in the normal person to about 80 or 90% of the bile pigments in patients with congenital porphyria, pernicious anaemia and sickle-cell anaemia, conditions associated with increased haemopoiesis, has suggested that this fraction may arise in association with haemopoiesis. Since this suggestion has been confirmed by our findings described in the present paper, the early labelled fraction may conveniently be called the haemopoietic fraction. It has been suggested that the haemopoietic fraction may arise in the bone marrow from premature breakdown of haemoglobin from intact red cells (London, Shemin, West & Rittenberg, 1949; Gray, Neuberger & Sneath, 1950), or from haem in excess of globin (Neuberger, Muir & Gray, 1950). On the other hand, direct synthesis from pyrrolic compounds without the formation of haemoglobin as an intermediate has been considered (London, West, Shemin & Rittenberg, 1950), and some support for this was obtained by Berlin, Neuberger & Scott (1956), who showed that a proportion of δ -aminolaevulinic acid admin-