Some Aspects of the Zimmermann Reaction

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The formation of a coloured product in the reaction of acetone with *m*-dinitrobenzene and alkali was first noted by Janovsky & Erb (1886), described in more detail by Janovsky (1891) and the wide scope of the reaction was recognized by Bittó (1892). Zimmermann (1935, 1937) first applied the reaction to keto steroids and used it for the colorimetric determination of 17-oxo steroids occurring in human urine. Since then the reaction has been extensively used for the routine assay of urinary 17-oxo steroids and, more recently, for the assay of urinary 17-hydroxy corticosteroids after their conversion into 17-oxo steroids (Norymberski, 1952; Appleby, Gibson, Norymberski & Stubbs, 1955). Numerous workers varied the conditions of the reaction on a purely empirical basis in order to render the assay of 17-oxo steroids more sensitive and more specific (for reviews of the subject, see Mason & Engstrom, 1950; Munson & Kenny, 1954; Zimmermann, 1955). The present work started with the aim of isolating the product of the Zimmermann reaction of a 17-oxo steroid, of establishing its structure and properties and examining the efficacy of established methods for the assay of 17-oxo steroids in the light of that information.

Kellie & Smith (1956) showed that the violet product obtained from dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) in the Zimmermann reaction is in a pH-dependent equilibrium with an apparently colourless compound which they isolated as a paper-chromatographic fraction. The latter compound has now been obtained in yellow prismatic needles, m.p. 186–187°, of the composition $C_{25}H_{30}N_2O_6$. Its ultraviolet spectrum in ethanol (Fig. 1) with maximal absorption at 236 mμ (ϵ 12 000) is consistent with the structure of a monosubstituted *m*-dinitrobenzene (Canbäck, 1949*a*). In ethanolic potassium hydroxide the compound gives rise to the characteristic absorption in the visible spectral region (Fig. 1) with maximal absorption at 520–525 mμ (ϵ 30 000). Its infrared spectrum shows all the absorption bands exhibited by dehydroepiandrosterone and by *m*-dinitrobenzene.

The violet product formed in alkaline solution was isolated as an amorphous solid containing 55% of the amount of potassium calculated for $C_{25}H_{29}KN_2O_6$. In ethanolic potassium hydroxide the compound exhibited maximal absorption at 520 mμ with an extinction accounting for 55% of the yellow parent compound. It seems likely that the isolated salt was highly solvated. Its infrared spectrum showed unusually strong absorption in the region 1750–1000 cm.$^{-1}$, which could be resolved into a number of individual peaks including one at 1743 cm.$^{-1}$ and a considerably stronger one at 1699 cm.$^{-1}$; the parent compound exhibited a much stronger peak at the former position but lacked the last-named peak. This is consistent with the view that on treatment with alkali the 17-oxo group (1743 cm.$^{-1}$) enters into conjugation with an unsaturated system (1699 cm.$^{-1}$). The above-mentioned properties require that the steroidal and aromatic moieties of the yellow product are linked by a C–C bond in the α-position to the carbonyl group. Consequently, the yellow product is formulated as 3β-hydroxy-17-oxoandrost-5-en-16ξ-yl-2,4-dinitrobenzene (I*a*) and the violet product as the potassium salt of the *para*-quinonoid aci-form (I*b*), presumably involving the contributing structures of the *ortho*-quinonoid aci-form, of the enolic form and of the 16-carbanion. Further evidence in support of these structures is discussed below.

The yield of (I*b*) under varying conditions of the reaction (Table 1) was calculated on the assumption

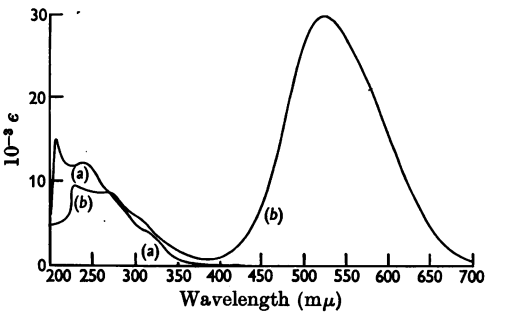
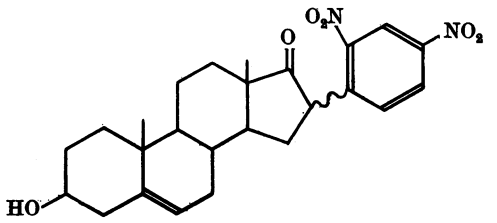
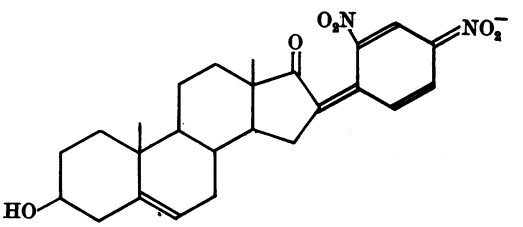


Fig. 1. Spectra of 3β-hydroxy-17-oxoandrost-5-en-16ξ-yl-2,4-dinitrobenzene in ethanol (curve *a*) and in ethanolic 0·1 N-potassium hydroxide (curve *b*).



(Ia)



(Ib)

Table 1. *Reaction of dehydroepiandrosterone with* m-*dinitrobenzene under varying conditions*

T_1 is the time (hr.) at which maximal absorption at 520 mμ was attained, T_2 the time (hr.) at which half that absorption was attained and T_3 the time (hr.) at which absorption dropped significantly (≥10%) from its maximal value. Relative blank is defined as $100E_{520} \times v_1/v_2$, where v_1 is the final volume and v_2 the initial volume of the reaction mixture.

		At 0°					At 25°				
Base	Solvent	Yield (%)	T_1	T_2	T_3	Rel. blank	Yield (%)	T_1	T_2	T_3	Rel. blank
2·5N-Potassium hydroxide	Ethanol	41	6	1·8	>24	80	42	0·6	0·15	4	130
2·1N-Benzyltrimethylammonium methoxide	Methanol	42	40	12	>50	30	45	4·5	1	6	50
2·5N-Tetramethylammonium hydroxide	Water	—	—	—	—	—	67	3	0·5	4·5	150
2·4N-Tetraethylammonium hydroxide	Ethanol	63	3	0·7	>6	60	73	0·7	<0·1	3	120
1·8N-Tetraethylammonium hydroxide	Ethanol	62	10	2	>28	90	65	1·5	<0·3	>5	130
1·7N-Tetraethylammonium hydroxide	Water	—	—	—	—	—	55	2·5	0·5	5·5	30
2·1N-Benzyltrimethylammonium hydroxide	Water	60	17	3	>25	30	61	1·5	—	—	50
2·4N-Benzyltrimethylammonium hydroxide	Water	—	—	—	—	—	68	1	0·25	>4	60

that the light-absorption at 520 mμ of the reaction mixture (corrected by subtraction of the reagent blank) is entirely due to the pure compound (I*b*) with a molecular extinction of 30 000. The experiments were carried out by dissolving dehydroepiandrosterone in an ethanolic solution of *m*-dinitrobenzene (0·5%, w/v) and admixture of the appropriate base (1:2, v/v). With ethanolic potassium hydroxide (2·5N) at 25° (Callow, Callow & Emmens, 1938) a yield of 42% was obtained and only a slightly higher yield with methanolic 2·1N-*N*-benzyl-*NNN*-trimethylammonium methoxide (Bongiovanni, Eberlein & Thomas, 1957), though the blanks were much lower with the latter base. Considerably higher yields were obtained with *NNNN*-tetramethyl-, -tetraethyl- and *N*-benzyl-*NNN*-trimethyl-ammonium hydroxide. An aqueous solution of the last-named base proved most satisfactory, since it gave high yields (60–70%) and reasonably low blanks. Further results of this series of experiments are summarized in Table 1.

A group of modifications of the Zimmermann reaction involves the extraction of the purple product with an organic solvent (Cahen & Salter, 1944; Henry & Thevenet, 1951; Zimmermann, Anton & Pontius, 1952; Masuda & Thuline, 1953; Werbin & Ong, 1954; Crépy, Meslin & Desgrez, 1956; Migeon & Plager, 1955). We found that this procedure substantially lowered the blanks when potassium hydroxide was used in the reaction but only slightly so when benzyltrimethylammonium hydroxide was used. Recently, James & de Jong (1961) reported that negligible blanks are obtained by the use of aqueous tetramethylammonium hydroxide and extraction of the product with ether.

In the following experiments the reaction was performed with commercial aqueous (approx. 40%, w/w) benzyltrimethylammonium hydroxide for 1·5 hr. at 25°. The relative colour equivalents of several keto steroids were determined (Table 2). Linear relations were established between extinctions and (i) varying amounts of dehydroepiandrosterone, (ii) varying amounts of dehydroepiandrosterone added to an extract of acid-treated urine and (iii) varying amounts of that extract (Fig. 2). The volume of ethanol used for dilution of the reaction mixture could be reduced without significantly affecting the intensity or stability of colour (Table 3). A microtechnique (dilution: 0·15 ml. to 0·65 ml.) was adopted which permits the accurate measurement of 17-oxo steroids in the range 0·5–4·0 μg. (Table 4).

Table 2. *Relative molar colour equivalents of some keto steroids in the Zimmermann reaction*

	Calc. from E_{520}	Calc. from $2E_{520}-(E_{460}+E_{580})$
3β-Hydroxyandrost-5-en-17-one	100*	100*
3α-Hydroxy-5α-androstan-17-one	101	97
3α,11β-Dihydroxy-5β-androstan-17-one	86	60
Androst-4-ene-3,17-dione	116	86
11β-Hydroxyandrost-4-ene-3,17-dione	110	61
Androst-4-ene-3,11,17-trione	132	95
3α-Hydroxy-5β-androstane-11,17-dione	114	137
5α-Cholestan-3-one	14	2
Cholest-4-en-3-one	20	6
3β-Hydroxypregn-5-en-20-one	8	0·5
3α,17α,21-Trihydroxy-5β-pregnane-11,20-dione	4	3
11β,21-Dihydroxypregn-4-ene-3,20-dione	16	1·5

* Arbitrary values.

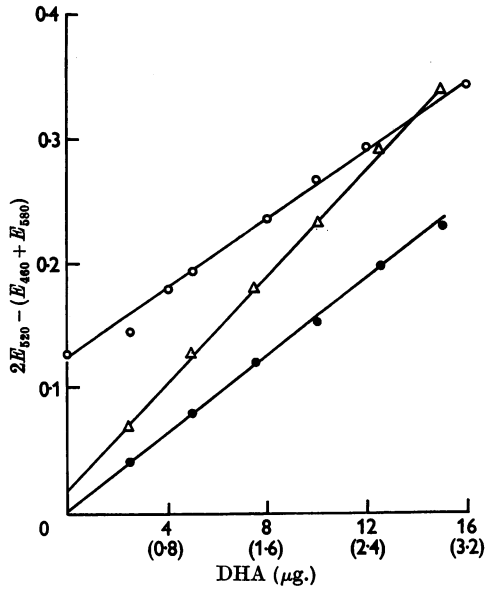


Fig. 2. Proportionality in the determination of dehydroepiandrosterone (DHA) (●); DHA added to an extract of acid-treated urine (○); an extract of acid-treated urine (△). Figures in parentheses on the abscissa are volumes of urine (ml.).

Table 3. *Effect of dilution in the determination of dehydroepiandrosterone*

v_1 represents the final and v_2 the initial volume of the reaction mixture. The blank is $100E_{520}\times v_1/v_2$. Figures in parentheses derive from measurements carried out 1 hr. after dilution.

v_1/v_2	Yield (%)	Blank
2	46 (46)	38 (39)
4	50 (49)	41 (36)
8	53 (53)	43 (37)
20	51 (52)	68 (40)

Table 4. *Proportionality and accuracy in the micro-determination of dehydroepiandrosterone*

The means are those of six determinations. DHA, dehydroepiandrosterone.

DHA (μg.)	E_{520} (μg. of DHA) Mean	S.D.	$2E_{520}-(E_{460}+E_{580})$ (μg. of DHA) Mean	S.D.
0·25	0·073	0·033	0·064	0·007
0·5	0·082	0·018	0·072	0·004
1·0	0·087	0·008	0·072	0·003
2·0	0·090	0·007	0·072	0·002
3·0	0·095	0·004	0·072	0·001
4·0	0·093	0·004	0·072	0·003

EXPERIMENTAL

General

A Hilger Uvispek and a Unicam SP. 600 spectrophotometer were used, the former for the determination of ultraviolet and visible spectra and for all measurements requiring the use of micro-cells, and the latter for other colorimetric determinations. Unless otherwise indicated, infrared spectra were taken with the Perkin–Elmer Infracord spectrophotometer. Ethanol was purified by treatment with calcium hydride under reflux followed by distillation. Di-isopropyl ether was freed from peroxides and distilled. All other solvents were of AnalaR grade and were used without further purification. Aqueous solutions of tetramethyl-, tetraethyl- and benzyltrimethyl-ammonium hydroxide and methanolic benzyltrimethylammonium methoxide were used as supplied by British Drug Houses Ltd. However, since this work was completed, batches of benzyltrimethylammonium hydroxide were received which gave very high reagent blanks; they were purified by shaking the base with calcium hydroxide for a few hours, allowing the mixture to stand overnight and filtering through sintered glass. An ethanolic solution of tetraethylammonium hydroxide was prepared by concentrating the aqueous base in an evacuated desiccator over potassium hydroxide and by dissolving the residue in ethanol. Ethanolic potassium hydroxide was prepared according to Hamburger (1952).

Preparation and properties of 3β-hydroxy-17-oxoandrost-5-en-16ξ-yl-2,4-dinitrobenzene

Dehydroepiandrosterone (100 mg.) and *m*-dinitrobenzene (1 g.) in purified ethanol (200 ml.) were mixed with aqueous tetraethylammonium hydroxide (100 ml.; 25%, w/w). After 16 hr. at 0–4° the purple solution was diluted with water (150 ml.) and extracted with three portions of ether (2 × 250 ml., 1 × 100 ml.). The extracts were washed with 0·5 N-sodium hydroxide in 20% (v/v) ethanol (6 × 100 ml.) and the washings discarded. The combined ethereal phases were diluted with light petroleum (500 ml.; b.p. 60–80°), extracted with 0·2 N-sodium hydroxide in 20% (v/v) ethanol (5 × 100 ml.) and the extracts were back-washed with ether–light petroleum (1:1, v/v; 3 × 100 ml.). The aqueous phases were combined, acidified with hydrochloric acid and extracted with ether (2 × 100 ml.); the extract was washed with water (50 ml.), dried over anhydrous sodium sulphate and evaporated to dryness. The crude product (90 mg.) was obtained as a yellow gum; in ethanolic 0·1 N-potassium hydroxide it had $\lambda_{max.}$ 520 mμ ($E^{1\%}_{1\,cm.}$ 430). The preparation was repeated four times, the crude products were combined and crystallized from chloroform–di-isopropyl ether. Pure *3β-hydroxy-17-oxoandrost-5-en-16ξ-yl-2,4-dinitrobenzene* (I*a*) was obtained in yellow prismatic needles, m.p. 186–187° (Kofler stage), $[\alpha]_D$ −170° (*c* 0·69 in $CHCl_3$); in ethanol it had $\lambda_{max.}$ 236 mμ (ε 12000), in ethanolic 0·1 N-potassium hydroxide it had $\lambda_{max.}$ 520–525 mμ (ε 30000) (see Fig. 1), $\nu_{max.}$ (in potassium bromide) 3550 (OH), 1730 (CO), 1610 (arom.), 1540–1550 (NO_2) and 1340–1350 (NO_2) cm.$^{-1}$ (Found: C, 66·2; H, 6·7; N, 5·6. $C_{25}H_{30}N_2O_6$ requires C, 66·1; H, 6·7; N, 6·2%). *Potassium salt*: a solution of (I*a*) (23·4 mg.) in ethanolic 0·1 N-potassium hydroxide (0·5 ml.) was diluted with benzene (20 ml.) and centrifuged and the supernatant decanted. The violet solid was washed with a little benzene and then dried *in vacuo* (0·1 mm. Hg) for 6 hr. at room temperature. $\lambda_{max.}$ (in ethanolic 0·1 N-potassium hydroxide) 520 mμ ($E^{1\%}_{1\,cm.}$ 333), $\lambda_{max.}$ (in pyridine) 515 mμ ($E^{1\%}_{1\,cm.}$ 240). Its concentrated solutions in water and in ethanol were violet but became colourless on dilution. Its infrared spectrum taken in a potassium bromide pellet (2%, w/w) showed $\nu_{max.}$ 975, 915, 860, 835, 810, 765, 740 cm.$^{-1}$ and extremely strong absorption between 1750 and 1000 cm.$^{-1}$, obscuring any detail in that region; an identical spectrum was obtained by mixing equimolar amounts of (I*a*) and potassium hydroxide. An expanded spectrum taken at lower concentration (approx. 0·2%, in potassium bromide), with the Unicam SP. 100 spectrophotometer fitted with the SP. 130 prism-grating double monochromater, showed $\nu_{max.}$ 1743, 1699, 1602, 1556, 1537, 1462, 1436, 1392, 1377, 1350, 1303, 1217, 1137, 1033 cm.$^{-1}$ (Found: K, 4·4. $C_{25}H_{20}KN_2O_6$ requires K, 7·9%).

Analytical procedure

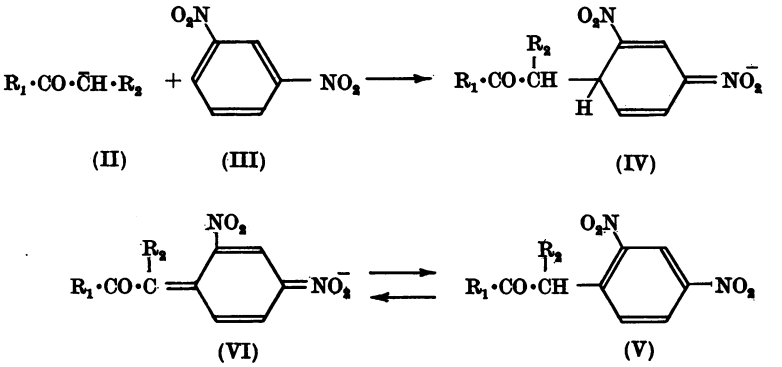
Modified Zimmermann reaction: general procedure. (*a*) Range 3–20 μg. The analytical sample, a solution of *m*-dinitrobenzene in purified ethanol (0·1 ml.; 0·5%, w/v), and aqueous benzyltrimethylammonium hydroxide (0·05 ml.; 40%, w/w) are well mixed. After 1·5 hr. at 25° the mixture is diluted with ethanol (3·0 ml.) and colorimetric readings are taken against a reagent blank at 460, 520 and 580 mμ. The measurements are evaluated according to Allen (1950) and expressed in terms of dehydroepiandrosterone used as standard. (*b*) Range 0·5–4·0 μg. The test is performed as described above except that the reaction mixture is diluted with a smaller volume (0·5 ml.) of ethanol and that colorimetric readings are taken in a micro-cell of 1 cm. light-path. Commercial ethanol may be used to dilute the reaction mixture in procedure (*a*) but it is desirable to use purified ethanol in procedure (*b*).

Variation of conditions (*Table* 1). (*a*) Base, time and temperature. With dehydroepiandrosterone used as model compound and one of the bases listed in Table 1, the reaction was performed at 0° or at 25° or both as under 'Modified Zimmermann reaction' but with 10- to 20-fold amounts of reagents. At suitable time intervals a sample (0·2 ml.) of the reaction mixture was removed and diluted with ethanol (3·0 ml.); extinction was determined at 520 mμ only. (*b*) Dilution (Table 3). Samples of dehydroepiandrosterone (20 μg. each) were treated respectively with 0·2, 0·5, 1·0 and 2·0 ml. of a mixture (2:1, v/v) of ethanolic *m*-dinitrobenzene (0·5%, w/v) and of aqueous benzyltrimethylammonium hydroxide (40%, w/w) for 1 hr. at 25°. Each reaction mixture was then diluted with ethanol (4·0 ml.) and its extinction at 520 mμ determined. (*c*) Extraction of colour. Dehydroepiandrosterone (40 μg.) was treated as under 'Modified Zimmermann reaction' but with double amounts of reagents. From the extinction at 520 mμ of the diluted reaction mixture a 67% yield of the violet product was calculated: the corresponding relative blank (for definition see Table 1) was 70. Another sample of the same reaction mixture was diluted with aqueous ethanol (5·4 ml.; 50%, v/v), extracted with chloroform (3·0 ml.) and the lower phase (4·7 ml.) separated and diluted with ethanol (0·2 ml.). The extract contained 57% of the theoretical yield of the violet product; the corresponding relative blank was 50. When an analogous experiment was performed with ethanolic 2·5 N-potassium hydroxide in place of the organic base, a 42% yield was obtained (rel. blank 190) and after extraction with chloroform a yield of 37% (rel. blank 65).

Other experiments. All other experiments were carried out by the general procedures under 'Modified Zimmermann reaction' (*a*) and (*b*). Results are given in Tables 2 and 4 and in Fig. 2.

DISCUSSION

Early interpretations of the reaction between ketones (–CO·CH_2–), *m*-dinitrobenzene and alkali were reviewed by Zimmermann (1936) and Canbäck (1949*b*). Zimmermann (1936, 1937) assigned structure (IV) to the coloured reaction product. Canbäck (1949*c*) adopted the same formal presentation of structure, but considered the new C–C bond as one between a dipole and an ion. He also pointed out that structure (IV) is that of the intermediate in the nucleophilic aromatic substitution of *m*-dinitrobenzene (III) by the carbanion (II), leading to structure (V) by elimination of a hydride ion, the latter step being facilitated by the presence of an oxidizing agent. Since aromatic nitro compounds can act as oxidizing agents in this type of reaction (cf. Ingold, 1953), it is likely that when the reaction is carried out with a large excess of the ketone (Canbäck's conditions) the



main product will have structure (IV) and when it is carried out with a large excess of *m*-dinitrobenzene (Zimmermann's conditions) the main product will have structure (V). Klyne (1957) suggested that the Zimmermann reaction involves a dehydrogenation step and formulated the product as (V). Neunhoeffer, Thewalt & Zimmermann (1961) demonstrated the occurrence of dehydrogenation in the reaction of androsterone (3α-hydroxy-5α-androstan-17-one) with *m*-dinitrobenzene and alkali and isolated an amorphous violet product of composition $C_{25}H_{31}KN_2O_6, \frac{1}{2}KOH$, convertible by acid into a yellow compound, $C_{25}H_{32}N_2O_6$. The cited properties of the two compounds give little indication of their purity or structure and therefore the evidence of Neunhoeffer *et al.* (1961) for structures (V) and (VI) rests largely on considerations of the reaction mechanism. In contrast, the evidence here presented is based on the properties of the pure product obtained from dehydroepiandrosterone. Together the two types of evidence afford convincing proof of structure.

The present modification of the analytical Zimmermann test yielded up to 70% of the violet reaction product, which is an improvement on other variations of the reaction. A promising approach to increase the yield further, i.e. the sensitivity of the test, is to perform the reaction in the presence of an oxidizing agent other than *m*-dinitrobenzene, as indicated by the work of Neunhoeffer *et al.* (1961).

SUMMARY

1. The structure of 3β-hydroxy-17-oxoandrost-5-en-16ξ-yl-2,4-dinitrobenzene was assigned to a yellow crystalline compound isolated by acidifying the reaction mixture obtained from dehydroepiandrosterone, *m*-dinitrobenzene and alkali. The yellow compound was converted by alkali into a violet product formulated as its *para*-quinonoid aci-form.

2. The yields of the quinonoid compound were determined in the analytical Zimmermaun test carried out under different conditions. Replacement of alkali by quaternary-ammonium hydroxides gave rise to higher yields of the quinonoid compound and lower reagent blanks. The analytical test was accordingly modified and adapted to the accurate determination of 0·5 μg. or more of 17-oxo steroids.

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Incorporation of Amino Acids into the Protein of Isolated Mitochondria

A SEARCH FOR OPTIMUM CONDITIONS AND A RELATIONSHIP TO OXIDATIVE PHOSPHORYLATION

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Most of the protein biosynthesis occurring in rat-liver cells takes place in the microsomes (Keller, Zamecnik & Loftfield, 1954), but some amino acid incorporation into protein also occurs in nuclei (Rees & Rowland, 1961) and in mitochondria (McLean, Cohn, Brandt & Simpson, 1958; Roodyn, Reis & Work, 1961). The amount of amino acid incorporation into protein which occurs in isolated mitochondria is very small compared with that found with microsomes (McLean *et al.* 1958), but nevertheless the mitochondrial system is of special interest because of the importance of the mitochondria in the general metabolism of the cell.

Since only a slight amount of incorporation occurs in mitochondria it seemed desirable to confirm the earlier reports on mitochondrial amino acid incorporation (McLean *et al.* 1958) and to establish that the mitochondria themselves are responsible for the incorporation of amino acids into protein. The optimum conditions for the incorporation of amino acids into the proteins of isolated rat-liver mitochondria and the effect of variation of the conditions on the rate of incorporation were also investigated.

METHODS

Animals. Female albino rats, weighing about 200 g., were used in the experiments.

Radioactive compounds. DL-[1-^{14}C]Leucine and generally (G) labelled L-[G-^{14}C]leucine were used, and were obtained from The Radiochemical Centre, Amersham, Bucks.

Materials. Free acid AMP, the sodium salts of ATP, ADP, and AMP, tris and NAD were obtained from the Sigma Chemical Co. Ribonuclease (recrystallized five times) was obtained from both the Sigma Chemical Co. and Nutritional Biochemicals Corp. Phosphocreatine was prepared by the method of Ennor & Stocken (1948), and phosphocreatine kinase by the method of Kuby, Noda & Lardy (1954). Bovine serum albumin prepared by Armour Pharmaceuticals was used and rat serum albumin was prepared by the method of Korner & Debro (1956). All other reagents were of AnalaR grade, except amino acids, which were obtained from Roche Products Ltd. Sucrose was further purified by passing solutions through a column of Amberlite MB-1 mixed-bed ion-exchange resin, and the solutions were then boiled to remove dissolved carbon dioxide and to reduce bacterial contamination. All solutions were made in glass-distilled water.

Preparation of mitochondria. The rats were killed by decapitation and were bled. The liver was rapidly removed and transferred to ice-cold 0·25M-sucrose, in which it was cut into small pieces with scissors. All subsequent operations in the preparation of the mitochondria were carried out between 0° and 2°. The pieces of liver were blotted, the volumes measured by displacement in fresh 0·25M-sucrose and the liver was homogenized in 0·25M-sucrose, a hand-operated homogenizer of the type described by Dounce, Witter, Monty, Pate & Cottone (1955), which was kept in an ice bath, being used.

Homogenization was carried out in three stages as described by de Duve, Pressman, Gianetto, Wattiaux & Appelmans (1955). The liver was first homogenized in 2·5 vol. of sucrose, with three strokes of the homogenizer, and was then filtered through Terylene net (1 mm. × 1 mm. mesh) to remove fibrous connective tissue. The homogenate was then centrifuged at 600*g* for 10 min. to remove nuclei and intact cells. The supernatant fluid was kept at 0° and the pellet was rehomogenized in 1·5 vol. of 0·25M-sucrose and spun again at 600*g* for 10 min. This step was repeated once more with 1 vol. of 0·25M-sucrose, so that the liver was homogenized three times in all, in a total volume of 0·25M-sucrose equal to five times the volume of liver used, and on all occasions the supernatant fluid was removed and kept. The combined supernatants were then centrifuged at 5000*g* for 10 min. to give a mitochondrial pellet. The super-