It is a pleasure to express my indebtedness to those mentioned above who supplied essential compounds for this work. In addition I wish to thank Professor A. H. Ennor for helpful advice and criticism.

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# Biosynthesis of Fatty Acids in Cell-free Preparations

## 4. SYNTHESIS OF FATTY ACIDS FROM ACETATE BY A PARTIALLY PURIFIED ENZYME SYSTEM FROM RABBIT MAMMARY GLAND*

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It was shown in previous papers of this series (Popják & Tietz, 1955; Tietz & Popják, 1955) that cell-free extracts prepared from lactating-rat mammary gland were able to synthesize long- and short-chain fatty acids from [$^{14}$C]acetate. This synthesis was dependent on the addition of adenosine triphosphate (ATP), the concomitant oxidation of pyruvate, oxaloacetate or α-oxoglutarate, and the presence of coenzyme A (CoA). This last requirement could be demonstrated only if the extracts were depleted of endogenous CoA by treatment with Dowex-I resin.

Before commencing a detailed study of the steps involved in fatty acid synthesis, it was necessary to simplify the original assay conditions used by Popják & Tietz (1955), and to attempt the purification of the mammary gland extracts. In this communication the preparation of a partially purified enzyme system from rabbit mammary gland is described. This system can synthesize fatty acids from acetate in the presence of stoicheiometric amounts of reduced diphosphopyridine nucleotide (DPNH) as electron donor. The results have been presented in a preliminary form (Hele & Popják, 1955).

## MATERIALS AND METHODS

### *Preparation of the fatty acid-synthesizing fraction from rabbit mammary gland*

Lactating rabbits, about 17 days after parturition, were killed by the intravenous injection of Nembutal (10%, w/v; 1·5 ml./kg.) and the mammary glands excised. The average yield was 100 g./animal. The glands were cooled in crushed ice, freed from fat and connective tissue, and finely minced with scissors. The mince was homogenized for 2 min. in a blender (M.S.E. Ato-mix 800), run at top speed, with 2 vol. of buffer (0·154 M-KCl, 100 parts; 0·154 M-$MgCl_2$, 10 parts; 0·1 M potassium phosphate buffer, pH 7·4, 34 parts). This and all subsequent operations were carried out at 0–4°. The homogenate was centrifuged at 400 *g* for 10 min., and the supernatant collected with filtration through a pad of gauze to remove fat. This supernatant was centrifuged at 44 000 *g* for 1 hr. in the Spinco model L preparative ultracentrifuge. In the earliest preparations the supernatant was centrifuged once again, this time at 100 000 *g* for 1 hr., before precipitation with $(NH_4)_2SO_4$ was carried out. In later preparations, when much larger volumes of material were being handled, this second centrifuging was dispensed with and precipitation with $(NH_4)_2SO_4$ carried out in the following manner with the material centrifuged at 44 000 *g*.

The supernatant was brought to 90% saturation by the addition of 70 g. of $(NH_4)_2SO_4$/100 ml. at 0–4°. The precipitate (F 90) was collected by filtration on a large fluted paper (Whatman no. 12) overnight, followed by press-drying between filter papers the next day. This precipitate could be stored in solid $CO_2$ for at least 6 months with no loss of fatty acid-synthesizing activity.

Refractionation of this material was carried out as follows. Sufficient cold water was added to each 21 g. of F 90 precipitate to bring the volume to 100 ml., and saturated with $(NH_4)_2SO_4$ to about 21%. A smooth suspension was achieved by mechanical stirring. Ammonium sulphate (9·8 g./100 ml.) was added to give a final saturation of about 35%; the precipitate was removed by centrifuging at 100 000 *g* for 10 min. in the Spinco ultracentrifuge. This high-speed centrifuging was necessary for the removal of finely particulate material which could not be removed in 20 min. at 40 000 *g*.

A clear supernatant was obtained, which was brought to about 60% saturation by the addition of 17·4 g. of $(NH_4)_2SO_4$/100 ml. of supernatant, and the precipitate collected by centrifuging at 22 000 *g* for 10 min. This precipitate, which

* Part 3: Tietz & Popják (1955).

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contained the fatty acid-synthesizing system, was dissolved in 0·05M-$KHCO_3$ to give a final protein concentration of 50–70 mg./ml. and will be referred to as the fatty acid-synthesizing fraction (FASF). About 1 g. of protein was obtained in this fraction from 100 g. of mammary-gland tissue. These preparations, if undialysed, retained their fatty acid-synthesizing activity for 2–3 months if kept frozen at −15°.

For certain experiments the FASF was dialysed for 15–18 hr. at 0–4° against 0·05M aminotrishydroxymethylmethane (tris) buffer, pH 7·5, containing $10^{-3}$M cysteine; 1 l. of buffer was used/ml. of FASF. Some precipitation of protein occurred, and the dialysed preparations were centrifuged at 20 000 *g* for 10 min. immediately after dialysis to remove this material. Such dialysed preparations lost 10–30% of their activity immediately after dialysis. They became totally inactive after 1–2 weeks, even when stored at −15°, at a protein concentration of about 50 mg./ml. and in the presence of small amounts of $(NH_4)_2SO_4$ added immediately after dialysis.

The fractions obtained from different batches of mammary gland were far more constant in their fatty acid-synthesizing ability than were the rat mammary-gland extracts previously employed.

### *Assay systems*

The following basic system was used with little modification both for experiments in which DPNH utilization was studied in the spectrophotometer (Unicam SP. 500), and for work upon the incorporation of $^{14}$C-labelled acetate into fatty acids and fatty acylhydroxamates. Each 3 ml. of reaction mixture contained: 30 $\mu$moles of ATP, 2 $\mu$moles of CoA, 0·4–0·8 $\mu$mole of DPNH, 30 $\mu$moles of cysteine, 100 $\mu$moles of tris buffer, pH 9·4; 20 $\mu$moles of $MgCl_2$; 5–60 $\mu$moles of acetate (labelled or unlabelled) and 5–10 mg. of FASF. The final pH was 8. All substances were neutralized with KOH or HCl as indicated. All the reagents were incubated together without acetate, or without FASF, at 38° for 5 min. and the reaction was started by the addition of the missing component. All experiments were performed in test tubes, small conical flasks, Warburg flasks, or spectrophotometer cells, with no precautions taken to obtain anaerobic conditions. Most experiments done in the spectrophotometer were carried out at room temperature after the initial incubation, but a few experiments were performed at 38° with an electrically heated, thermostatically controlled cell-carrier to be described elsewhere. Additions to spectrophotometer cells were made with the adder-mixer described by Boyer & Segal (1954).

### *Extraction of fatty acids*

This was done as previously described (Popják & Tietz, 1954; 1955).

Chromatographic fractionation of the fatty acids from $C_8$ to $C_{18}$ was done by the reversed-phase technique of Howard & Martin (1950) and, when separation of butyric, crotonic (*trans*-but-2-enoic), hexanoic and octanoic acids was required, by the liquid–vapour chromatography of James & Martin (1952).

[*carboxy*-$^{14}$C]Acetate was obtained from the Radiochemical Centre, Amersham, Bucks., and was usually diluted with unlabelled potassium acetate to a specific activity of 1 $\mu$c/$\mu$mole. The methods of purifying the fatty acids from radioactive acetate, the methods of $^{14}$C assay and calculations employed, have already been described (Popják & Tietz, 1954, 1955).

### *Preparation and chromatography of hydroxamates from acyl-CoA derivatives*

The method of Lipmann & Tuttle (1945) was used, except that salt-free hydroxylamine prepared by the method of Beinert *et al.* (1953) was employed. A solution (1 vol.) of 2M hydroxylamine in methanol (pH 6·5) was mixed with 1 vol. of 0·1M acetate buffer (pH 5·4), and added to 1 vol. (usually 3 ml.) of the enzymic incubation mixture. After 1 hr. at about 20°, the mixture was evaporated to dryness under reduced pressure on a hot-water bath; the hydroxamates were extracted from the dry residue with warm ethanol (5 ml.). The ethanolic extract was evaporated to dryness under reduced pressure in a small tube and the hydroxamates were taken up in 0·2–0·3 ml. of ethanol; insoluble salts were removed by centrifuging at 0°. Samples (0·01–0·05 ml.) were placed in small spots on strips of Whatman no. 1 paper and chromatographed according to Thompson (1951) with either butanol–acetic acid–water (4:1:5) or octanol–formic acid–water (3:1:3) solvent systems. Marker hydroxamates were prepared from methyl or ethyl esters of the various acids or from the acid anhydrides. With the two solvent systems it has been possible to achieve satisfactory resolution (in order of increasing $R_F$ values) of the hydroxamates of acetoacetic, $\beta$-hydroxybutyric, acetic, (?$\beta$-oxohexanoic), crotonic, butyric, hexanoic, $\beta$-hydroxyoctanoic and octanoic acids. Only the acetohydroxamate and $\beta$-hydroxybutyrohydroxamate were present in sufficient quantity in the samples applied to the paper to give intensely coloured spots on the paper after spraying with 5% $FeCl_3$ soln. in 0·1N-HCl. Hydroxamate prepared from ethyl acetoacetate gives no colour with the acid $FeCl_3$ soln.; it reacts, however, with neutral $FeCl_3$ but the intensity of colour on a molar basis is only about one-fiftieth of that given by acetohydroxamic acid.

The position and amounts of [$^{14}$C]hydroxamates on the paper strips were determined by an automatic recording scanning device in which the chromatographic paper strips were driven between two thin mica-window Geiger–Müller counters. The pulses from the counters were taken to a ratemeter and were recorded simultaneously at four different levels of sensitivity varying from $3^0$ to $3^3$; i.e. if the counting rate at the lowest sensitivity of the instrument gave a galvanometer deflexion of 1 cm., the same counting rate was recorded simultaneously also as 3, 9 and 27 cm. deflexions. The chart of the recording galvanometer ran synchronously with the movement of the chromatogram between the Geiger counters. Thus the position of each spot on the paper, even in the absence of a colour reaction, could easily be determined. For identification purposes hydroxamates prepared from the enzyme incubations were mixed with authentic samples of hydroxamates and chromatographed; the chromatograms were sprayed with a 5% $FeCl_3$ soln. in 0·1N-HCl and were then scanned for radioactive spots. It was found experimentally that the areas under the curves obtained in these records were proportional to the concentration of $^{14}$C-labelled material in the spots. From the measurement of the areas under the curves the total radioactivity of each spot could be determined with an accuracy of about 20%. For more accurate evaluations

appropriate portions of the chromatograms were cut into equal squares and their $^{14}C$ content was measured individually by counting under a mica-window Geiger counter.

### *Materials*

CoA (about 75% pure, batches 405, 407, 408) and diphosphopyridine nucleotide (DPN, 92% β-isomer) were obtained from the Pabst Laboratories. DPNH (about 64% of reduced coenzyme) and highly purified yeast alcohol-dehydrogenase were obtained from Boehringer & Soehne. ATP was obtained from L. Light and Co. or from the Sigma Chemical Co. (98–99% pure). Triphosphopyridine nucleotide (TPN, 98% pure), adenosine 5-phosphate (AMP, 98–100% pure), glucose 6-phosphate (98–100% pure), TPN-specific glucose 6-phosphate dehydrogenase and hypoxanthine were also Sigma preparations. Tris buffer (L. Light and Co.) was purified by recrystallization from aqueous ethanol. Diol buffer (2-amino-2-methylpropane-1:3-diol; L. Light and Co.) was not recrystallized.

Reduced triphosphopyridine nucleotide (TPNH) was prepared from Sigma TPN, by the method of Conn, Kraemer, Liu & Vennesland (1952). Assays of this preparation showed, per mg.: 0·48 μmole of total TPN nucleotide (about 0·36 mg.) by adenine determination at 260 mμ.; 0·23 μmole of TPNH, from the optical density at 340 mμ. and confirmed by TPNH–glutathione reductase assay; 0·28 μmole of TPN, determined by glucose 6-phosphate dehydrogenase assay. The nucleotide was therefore 48% reduced. No DPN or DPNH could be detected by alcohol dehydrogenase assay.

Oxidized glutathione (GSSG) was prepared by the oxidation with iodine of the partially reduced substance (Hopkin and Williams) followed by precipitation with an acetone–ether mixture and drying *in vacuo*. TPNH–glutathione reductase was prepared from peas, according to Mapson & Goddard (1951), and refractionated with $(NH_4)_2SO_4$ (Kaplan, Colowick & Neufeld, 1953). Dowex resins, nos. 1 and 50, came from the Dow Chemical Co., Midland, Michigan, U.S.A.

### *Chemical methods*

Protein was determined by the biuret procedure of Gornall, Bardawill & David (1949) which agreed to within 10% with the dry weight method used by Popják & Tietz (1954). Hydroxamates were determined according to Lipmann & Tuttle (1945) and orthophosphate according to Allen (1940).

*Pyridine nucleotides.* In studies of interconversions and destructions of the reduced and oxidized forms of these nucleotides TPN was determined by the use of glucose 6-phosphate dehydrogenase and TPNH by the TPN-specific glutathione reductase (Mapson & Goddard, 1951) present in the FASF. DPN and DPNH were assayed with alcohol dehydrogenase (Racker, 1950) with ethanol or acetaldehyde respectively as the substrate. In the latter case the pH of the incubation mixture (which under conditions of fatty acid synthesis was pH 8·0) was lowered by carefully controlled addition of M phosphate buffer, pH 6·5, in order to shift the equilibrium of the alcohol dehydrogenase reaction further towards the oxidation of DPNH. In all these instances the change observed in the optical density of the solutions at 340 mμ. was used as a quantitative measure of the assays. The molar extinction coefficient of DPNH (and TPNH) was taken to be $6{\cdot}22 \times 10^6$ cm.$^2$ × mole$^{-1}$ (Horecker & Kornberg, 1948).

## RESULTS

### *Incorporation of [$^{14}C$]acetate into fatty acids by preparations of rabbit mammary gland*

The rabbit mammary-gland extract, when tested in a system similar to that used by Popják & Tietz (1954) and before treatment with ammonium sulphate, showed a fatty acid-synthesizing activity (Table 1) close to that obtained with rat mammary-gland extracts. The same requirements for cofactors and other additions were shown. α-Oxoglutarate and malonate did not stimulate synthesis by the rabbit preparation to the extent obtained with rat mammary-gland extracts. Since it was thought that the stimulating action of α-oxoglutarate in the presence of DPN on fatty acid synthesis was due to the generation of DPNH (Tietz & Popják, 1955) we simplified the assay system by using DPNH alone. As is shown in Table 2 the use of DPNH with a dialysed and undialysed enzyme preparation was nearly as effective as DPN + α-oxoglutarate + malonate in promoting fatty acid synthesis. An almost complete dependence of fatty acid synthesis on

Table 1. *Fatty acid synthesis from* [$^{14}C$]*acetate by mammary-gland extract of rabbits*

Each flask contained: potassium acetate, 60 μmoles (5 μc of $^{14}C$); CoA, 0·3 μmole; DPN, 1 mg.; undialysed mammary-gland extract, 2 ml. Final vol., 3 ml. Incubation for 1 hr. at 38°.

| Additions | Acetate used for fatty acid synthesis/100 mg. of protein (μm-moles) |
|---|---|
| None | 7·37 |
| ATP, 0·01 M | 173·00 |
| α-Oxoglutarate, 0·01 M | 25·40 |
| Malonate, 0·05 M | 42·20 |
| α-Oxoglutarate + malonate | 23·80 |
| α-Oxoglutarate + ATP | 126·00 |
| Malonate + ATP | 234·00 |
| α-Oxoglutarate + malonate + ATP | 286·00 |

Table 2. *Fatty acid synthesis in dialysed and undialysed rabbit mammary-gland extracts*

Each flask contained: ATP, 30 μmoles; potassium acetate, 60 μmoles (5 μc of $^{14}C$); CoA, 0·3 μmole. Final vol., 3 ml. Incubation for 1 hr. at 38°. Fatty acid synthesis expressed as μmole of acetate incorporated into fatty acids/100 mg. of protein.

| Additions | Fatty acid synthesis: Undialysed enzyme | Fatty acid synthesis: Dialysed enzyme |
|---|---|---|
| DPN (1 mg.) | 0·270 | 0·072 |
| DPN + α-oxoglutarate (30 μmoles) + malonate (150 μmoles) | 0·438 | 0·256 |
| DPNH* (1 mg.) | 0·357 | 0·192 |

* Prepared by chemical reduction of DPN.

DPNH was demonstrated after refractionation of the mammary-gland extracts with ammonium sulphate.

As is shown in Table 3 the refractionation of F 90 to FASF increased the synthesizing activity some eight- to ten-fold, and at the same time removed endogenous DPN, so that a marked requirement for this coenzyme could be demonstrated. In all FASF preparations there was a small, yet significant, utilization of acetate for fatty acid synthesis (about 0·005 μmole/10 mg. of protein/60 min.) even in the absence of added DPNH. This blank could not be reduced further either by dialysis or by treatment of dialysed preparations with Dowex-1 resin (cf. Table 4). This suggested that small amounts of DPN or DPNH might be added with the CoA or ATP, but assay with alcohol dehydrogenase failed to reveal such contamination.

Table 3. *Fatty acid synthesis from* [$^{14}$C]*acetate by ammonium sulphate fractions of mammary-gland extract in the presence and absence of DPNH*

The incubation mixture, in a final vol. of 3 ml., contained: ATP, 30 μmoles; CoA, 1·5 μmoles; cysteine, 30 μmoles; potassium acetate, 60 μmoles (= 11 μc of $^{14}$C); $MgCl_2$, 20 μmoles; tris buffer, pH 9·4, 100 μmoles. In the experiment with F 90, 18·5 mg. of protein, and in the other two (FASF) 10·8 mg. of protein, were used. DPNH (0·5 μmole) was added. Incubation at 38°. Fatty acid synthesis is expressed in terms of acetate μm-moles utilized/10 mg. of protein.

| Ammonium sulphate fraction | Length of incubation (min.) | Fatty acid synthesis: DPNH added | Fatty acid synthesis: No DPNH |
|---|---|---|---|
| F 90 | 27 | 3·56 | 1·90 |
| FASF* | 10·5 | 10·60 | 0·49 |
| FASF* | 21 | 23·05 | 3·04 |

* FASF was made from the F 90 shown in this table.

Langdon (1955) suggested that the optimum incorporation of [$^{14}$C]acetate into fatty acids by rat-liver extracts is dependent upon TPNH rather than upon DPNH. The synthesis achieved in Langdon's experiments in the presence of TPNH (0·6 μmole/3 ml. of reaction mixture) was double that obtained in its absence. This raised the possibility that the FASF might also show a requirement for TPN or TPNH and that the fatty acid synthesis observed by us on addition of DPNH might be accounted for by the presence of a transhydrogenase (Kaplan *et al.* 1953) which would reduce endogenous TPN with DPNH. These possibilities were tested in the experiments presented in Table 4. In one set of experiments chemically prepared TPNH was used, and in another TPNH was generated from TPN by the action of the TPN-specific glucose 6-phosphate dehydrogenase present (in non-rate-limiting amounts) in the FASF. These experiments demonstrated that the addition of stoicheiometric amounts of TPNH had no stimulatory effect upon fatty acid synthesis by the rabbit preparation, and provided no evidence for the presence of transhydrogenase in the FASF.

The breakdown of TPN or TPNH either through dephosphorylation, or by other pathways, was small under the experimental conditions (i.e. in the presence of all the cofactors required for fatty acid synthesis). It was insufficient to account for the negative effect of TPNH on fatty acid synthesis on the basis of the destruction of TPN or TPNH; but a small conversion into DPN or DPNH was observed in control experiments, sufficient to account for the [C$^{14}$]acetate incorporations in the absence of DPNH. DPN could be reduced to DPNH by

Table 4. *Fatty acid synthesis from* [$^{14}$C]*acetate. Tests for specificity of pyridine nucleotides*

The standard assay system described under Methods was used with 10 μmoles (10 μc of $^{14}$C) of potassium acetate and 0·8 μmole of the pyridine nucleotides. The reaction mixtures were incubated with 5 or 10 mg. of protein at 38° for 30 min. (Expts. 1 and 2) and 45 min. (Expt. 3). The FASF preparations were obtained at different times from the same F 90.

| Pyridine nucleotide added | Acetate (μmoles) incorporated into fatty acids: Expt. 1, Undialysed FASF (10 mg.) | Expt. 1, Dialysed FASF (10 mg.) | Expt. 2* Dialysed FASF (10 mg.) | Expt. 3* Dialysed FASF (5 mg.) |
|---|---|---|---|---|
| None | 0·006 | 0·005 | 0·006 (0·010)† | — |
| DPNH | 0·342 | 0·233 | 0·162 (0·132)† | 0·344 |
| DPNH + TPN | 0·334 | 0·248 | 0·200 | — |
| TPNH | — | — | 0·023 | 0·029 |
| DPNH + TPNH | — | — | 0·150 | 0·310 |
| DPN + TPNH | — | — | 0·024 | 0·018 |
| TPN | — | — | — | 0·007 |
| DPN | — | — | — | 0·030 |

* In Expt. 2 the TPNH was prepared by chemical reduction; in Expt. 3 the TPNH was generated in the FASF by the addition of glucose 6-phosphate (1 μmole) at the start of incubation.

† The figures in parentheses relate to results obtained with dialysed and Dowex-treated enzyme.

AMP, itself generated from ATP during the activation of acetate (Lipmann, Jones, Black & Flynn, 1952; Beinert *et al.* 1953). The relevant data are presented below.

*Utilization of DPNH associated with fatty acid synthesis*

The FASF contained a small amount of reducible substrate, which caused a blank utilization of DPNH in the absence of acetate. Because this substrate (or substrates) could not entirely be removed by dialysis and because the FASF was partially inactivated by dialysis, it was finally decided to exhaust the reducible material, at the expense of the loss of a certain amount of DPNH, by pre-incubation of the reaction mixture at 38° for 5 min. in the absence of acetate. The loss of DPNH during such period, as measured by the fall in optical density at 340 mμ., varied in different preparations from 5 to 20% of the amount of DPNH added. After incubation the optical density of the reaction mixture was read in the spectrophotometer until the fall ceased; this usually occurred within 5 min. When the optical density readings were steady, acetate was added, whereupon a rapid, progressive drop in optical density occurred. A drop of this magnitude was not observed in the control, to which water had been added (Fig. 1). For the first 5 min. the rate of DPNH utilization was linear with time; then the reaction slowed down, reaching a steady state when about 60% of the DPNH had been utilized. The reaction was resumed when more DPNH was added, and, as before, reached a steady state at 60% DPNH utilization. The addition of more CoA, acetate, or ATP did not lead to a similar resumption of the reaction. Addition of more FASF was either without effect, or resulted in a slow, indecisive drift forwards or backwards.

In Fig. 1 it will be noted that upon the addition of acetate or water a small rise in optical density (about 0·04 unit) occurred. This was due to the disturbance of fine particles added with the FASF, which were not completely removed by centrifuging and were believed to be fatty in nature. This rise was observed in many experiments, but in as many others a fall in optical density occurred. Since it was difficult to ascertain the exact time at which DPNH utilization commenced, the reading observed 30 sec. after making the additions was taken as that for zero time, and DPNH utilization was measured in terms of decrease in optical density from this point. A small error was inevitably introduced, but this could be rendered proportionately smaller by using larger amounts of DPNH (about 0·8 μmole), thereby extending the linear part of the curve for up to 20 min. The percentage utilization of DPNH was calculated on the basis of the amount of DPNH present at zero time, obtained by subtracting a similarly treated blank (which contained all reagents except DPNH) from the zero-time reading.

DPNH utilization in the presence of acetate could also be demonstrated in the F 90 precipitate (Table 5). Refractionation with ammonium sulphate led to a fourfold increase in acetate-de-

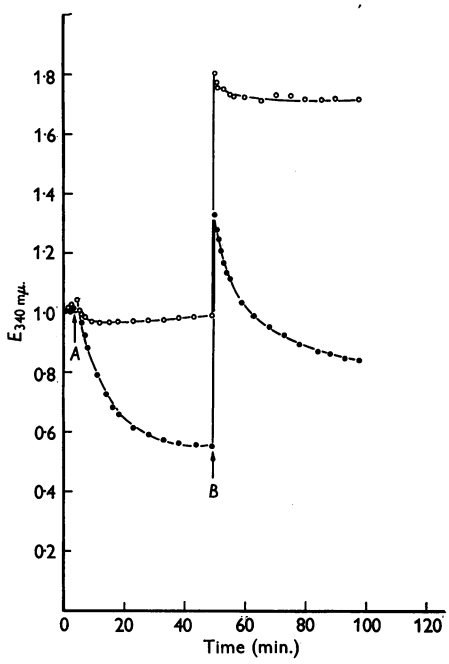


Fig. 1. Utilization of DPNH by the FASF. Each cell contained the standard assay system with 0·4 μmole of DPNH. At *A* 50 μmoles of acetate was added to ●, and an equivalent volume of water to ○. At *B* a further 0·4 μmole of DPNH was added to both cells.

Table 5. *Purification of the fatty acid-synthesizing system based on acetate-dependent utilization of DPNH*

The assay system, in a final vol. of 3 ml., contained: ATP, 30 μmoles; CoA, 1·5 μmoles; DPNH, 0·6 μmole; cysteine, 30 μmoles; tris buffer, pH 9·4, 100 μmoles; potassium acetate, 50 μmoles; $MgCl_2$, 20 μmoles; F 90, 19 mg. or FASF, 10·8 mg. (both undialysed). Assays at room temperature. 1 unit = 1 μmole of DPNH utilized in the presence of acetate/15 min.

| Ammonium sulphate fraction | Total protein (mg.) | Total units | Units/10 mg. of protein | Recovery (%) | |
|---|---|---|---|---|---|
| | | | | Protein | Units |
| F 90 | 860 | 2·56 | 0·03 | 100 | 100 |
| FASF | 190 | 2·42 | 0·13 | 22 | 95 |

pendent DPNH utilization per mg. of protein, with a 95 % recovery of this activity in the FASF.

The utilization of DPNH by the FASF was dependent on the presence not only of acetate, but also of CoA and ATP (Table 6). In the absence of cysteine only 30 % activity was observed. Little stimulation by magnesium was observed with the undialysed preparation used here.

The rate of DPNH utilization, when measured over the linear part of the curve, was dependent on the concentration of acetate, protein, CoA and ATP (Fig. 2). At low concentrations of protein or of CoA, lag phases were observed at the beginning of the reaction but the rates then increased, and linear portions were always found. Variation in protein concentration was without effect upon the point at which the steady state was reached; but this point varied with the concentrations of CoA and ATP in a complex manner that was not fully investigated.

The steady state was always reached when the same percentage of DPNH had been utilized, independent of the amount of DPNH present at zero time. Typical curves obtained with 0·290 and 0·656 $\mu$mole of DPNH (amounts present at zero time) are shown in Fig. 3. The steady state was reached when 0·179 and 0·395 $\mu$mole of DPNH had disappeared, giving 61·6 and 60·2 % utilization respectively. The rate of the reaction within the first 5 min. was independent of DPNH concentration.

It was thought possible that the steady state might represent the setting up of a true equilibrium of the reaction catalysed by the $\beta$-hydroxybutyryl dehydrogenase. (For terminology of enzymes of fatty acid metabolism see Beinert *et al.* 1956). This equilibrium is known to have a marked dependence upon pH (Lynen, Wessely, Wieland & Rueff, 1952; Wakil, Green, Mii & Mahler, 1954), and therefore the effect of pH upon the point at which the steady state

Table 6. *Component requirements of the spectrophotometric-assay system*

The standard assay system was employed with omissions as indicated. Undialysed FASF (10 mg.) was used and observations were made at room temperature.

| | DPNH used/10 min./ 10 mg. of protein ($\mu$mole) |
|---|---|
| Complete system | 0·147 |
| No $MgCl_2$ | 0·127 |
| No acetate | 0·002 |
| No CoA | 0·004 |
| No ATP | 0·017 |
| No cysteine | 0·058 |
| No enzyme | 0·020 |

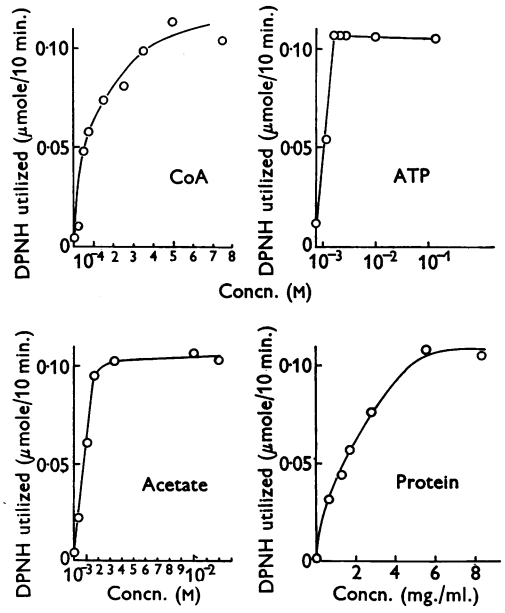


Fig. 2. Effect of the concentration of CoA, ATP, acetate and FASF upon the rate of DPNH utilization by the FASF. The standard assay system was used, with 0·8 $\mu$mole of DPNH.

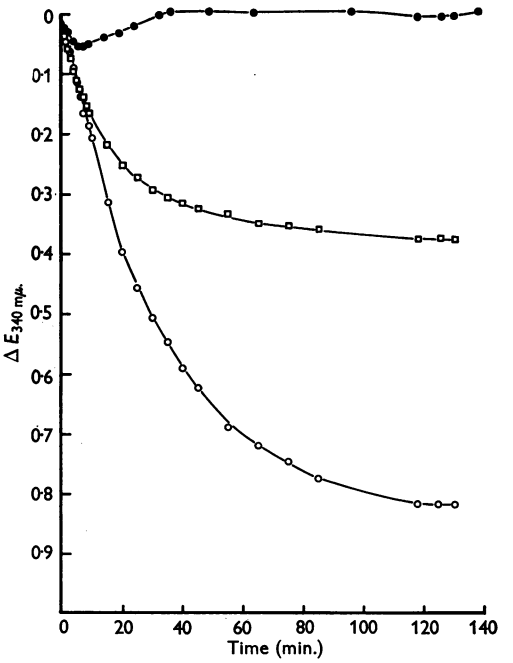


Fig. 3. Effect of DPNH concentration upon DPNH utilization by the FASF. The standard assay system was used, with 0·4 and 0·8 $\mu$mole of DPNH added initially. These amounts fell to 0·290 $\mu$mole (□) and 0·656 $\mu$mole (○) after the preliminary incubation at 38°. ●, 0·275 $\mu$mole of DPNH, no acetate, water added at zero time.

was established was investigated (Fig. 4). The experiments were run until no further drop in optical density occurred for at least 20 min. In a number of experiments slow re-reduction of DPN occurred after this time, possibly at the expense of AMP. The point at which the steady state occurred was expressed in terms of the percentage of DPNH unchanged (based on the DPNH present at zero time, as described above) when the reaction came to a standstill. This was done so that a direct comparison with the data of Wakil *et al.* (1954) could be obtained. The relevant equilibrium data of these workers is expressed in terms of percentage substrate (β-hydroxybutyryl-CoA) oxidized. Control experiments showed that tris and diol buffers were without effect upon DPNH utilization, other than the effect due to pH. All experiments were accompanied by a blank to which water was added in place of acetate. The pH of the reaction mixture was determined with the glass electrode at the end of the experiment.

The point at which the steady state was reached varied over the range pH 8–9·8. At pH 8, the percentage of DPNH remaining unchanged when the reaction came to a standstill was less than one-half of the percentage of DPNH unchanged at pH 9·3. Below pH 8, the blank oxidation of DPNH interfered with the interpretation of the results, but as the significance of this blank is difficult to assess, curves both corrected and uncorrected for it are shown.

These experiments show that between pH 8 and 9·8 a correlation exists between the effect of pH upon the steady state in the FASF system and upon the equilibrium of the reaction catalysed by the β-hydroxybutyryl dehydrogenase. It is concluded that the point at which the steady state is established is determined in the first place by the equilibrium of the reaction catalysed by β-hydroxybutyryl dehydrogenase present in the FASF. Other slower reactions, either utilizing or regenerating DPNH, are probably superimposed upon this, when the rate of DPNH utilization in the fatty acid-synthesizing system is sufficiently slowed down by the approach of the true equilibrium of the β-hydroxybutyryl dehydrogenase reaction.

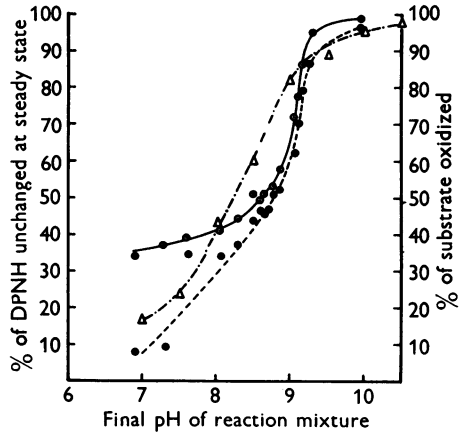


Fig. 4. pH dependence of DPNH–DPN steady state catalysed by the FASF, as compared with the pH dependence of the equilibrium of the reaction catalysed by β-hydroxybutyryl dehydrogenase. The data for the latter are redrawn from Wakil *et al.* (1954). Standard assay conditions, with 0·8 μmole of DPNH and 10 mg. of undialysed FASF, at room temperature. △, β-Hydroxybutyryl dehydrogenase; ⊙, FASF corrected for blank DPNH utilization; ●, FASF uncorrected.

*Relationship between DPNH utilization and* [$C^{14}$]*-acetate incorporation into fatty acids and fatty acid precursors*

In a number of experiments an attempt was made to examine the stoicheiometric relationship between DPNH utilization and [$^{14}$C]acetate incorporation into saturated and unsaturated fatty acids. For this purpose reaction mixtures of 30–60 ml. were employed. A sample was removed before the addition of [$^{14}$C]acetate, and the optical density at 340 mμ. was observed throughout the experimental period. After the addition of acetate, 3 ml. samples were withdrawn at various intervals, read in the

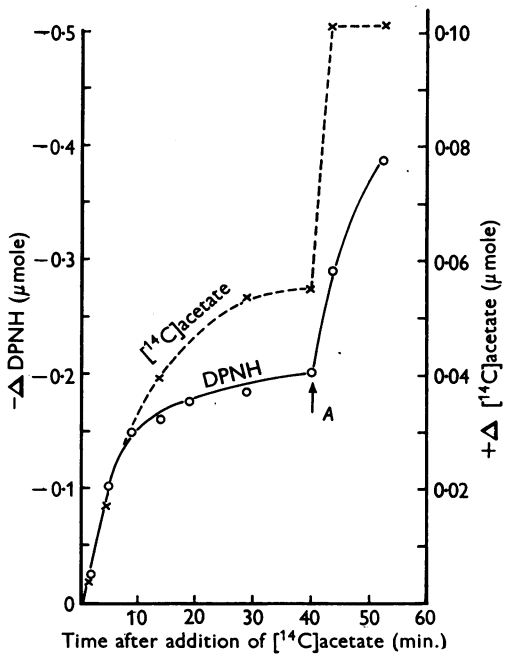


Fig. 5. Utilization of DPNH, and the incorporation of [$^{14}$C]acetate into fatty acids. The standard assay with undialysed enzyme and with 0·4 μmole of DPNH was used. At *A* a further 0·4 μmole of DPNH/3 ml. of reaction mixture was added. Room temperature.

spectrophotometer, and immediately saponified with KOH in preparation for the extraction and assay of labelled fatty acids. In some experiments further samples were taken for the estimation of hydroxamates by paper chromatography (see below).

The course of [$^{14}$C]acetate incorporation into saturated and unsaturated fatty acids followed closely the course of DPNH utilization (Fig. 5). During the early part of the experiment the ratio of DPNH utilized to [$^{14}$C]acetate incorporated was 5·7:1. This ratio fell to 3·3:1 when the steady state had been established. After the further addition of DPNH the ratio fell further to 2·4:1 and then rose again to 2·8:1.

Experiments of this type were of little use in obtaining data on the stoicheiometry of the reaction, although they suggested strongly that a precursor of fatty acids was being formed at the expense of DPNH and that this precursor was not extracted and estimated as fatty acid. The rate of formation of this precursor probably fell off as the steady state appeared, whereas the rate of fatty acid synthesis fell off more slowly. Further addition of DPNH, as is shown in Fig. 5, shifted the whole system in the direction of fatty acid synthesis.

*Identification of fatty acids and their precursors by chromatography as their* [$^{14}$C]*hydroxamates*

Two experiments were carried out, similar in principle to that shown in Fig. 5, except that the samples withdrawn at intervals were treated with the hydroxylamine reagent of Lipmann & Tuttle (1945), chromatographed, and scanned for radioactivity. The hydroxylamine reagent is expected to react with all fatty acids or their precursors present as their acyl–CoA derivatives.

In the first large-scale experiment, performed at room temperature, the chromatograms were developed with the octanol–formic acid solvent system. Samples were removed at the following intervals after the addition of acetate: at 5 min. during the linear phase of DPNH utilization; at 15 min. at the inflexion of the DPNH-utilization curve; at 45 min., some 15 min. after the steady state in DPNH utilization had appeared; at 55 min., i.e. 5 min. after the second addition of DPNH, and on the linear portion of the utilization curve; at 115 min., 65 min. after the second addition of DPNH and 15 min. after the setting up of the second steady state. DPNH utilization was observed throughout the experiment on a 3 ml. sample withdrawn from the main digest immediately after the addition of acetate, and was accompanied by a blank sample withdrawn before the addition of acetate. The data on the distribution of radioactivity amongst the fatty acyls* and their precursors were expressed as $\mu$moles of these substances formed, and published in a preliminary communication (Hele & Popják, 1956). To facilitate the present discussion of this experiment figures from this preliminary communication will be included in the text where necessary.

The octanol–formic acid solvent system used in this experiment gave excellent separation of the hydroxamates of the short-chain fatty acids, but did not differentiate between $\beta$-hydroxybutyryl and acetoacetyl. The percentage of $\beta$-hydroxybutyryl present in this mixed component was later calculated to be about 30, on the basis of information obtained from chromatograms from a second experiment, which were developed with the butanol–acetic acid solvent system. This system gives a very satisfactory separation of the two acids, and a direct measure of the ratio of $\beta$-hydroxybutyryl to acetoacetyl (Table 8). Percentages arrived at in this manner are considered preferable to those given in the preliminary communication (Hele & Popják, 1956), which were obtained by calculation from the overall utilization of DPNH and incorporation of [$^{14}$C]acetate, and based on the arbitrary assumption that perfect stoicheiometric agreement between these two could be obtained experimentally.

The first experiment showed that acetyl, and $\beta$-hydroxybutyryl plus acetoacetyl, were present throughout in large excess over the higher fatty acyls and their precursors. Even if $\beta$-hydroxybutyryl represents only 30% of the mixed component it is still present in considerable excess over crotonyl. This may be illustrated by making a comparison between the figures for $\beta$-hydroxybutyryl and acetoacetyl in Table 7, and the figures for higher fatty acyls also taken from the preliminary communication (Hele & Popják, 1956). It is unlikely that the amounts of acetyl, $\beta$-hydroxybutyryl and acetoacetyl present are at any time low enough to be rate-limiting. This excess of $\beta$-hydroxybutyryl over fatty acyls, particularly during the early part of the experiment, but falling off as more fatty acyls are formed, probably accounts for the excess of DPNH utilization over [$^{14}$C]acetate incorporation observed in experiments of the type shown in Fig. 5, and strongly suggests that the postulated precursor in these experiments was $\beta$-hydroxybutyrate.

These chromatograms also showed clearly the stepwise synthesis of fatty acids, as originally postulated by Lynen (1954). After 5 min. crotonyl (0·005 $\mu$mole), butyryl (0·01 $\mu$mole), and a substance identified by its $R_F$ as $\beta$-hydroxyoctanoyl (0·0025 $\mu$mole), appeared. No higher fatty acyls

* Since these acids are present as their hydroxamates, which must in turn have been derived from acyl–CoA derivatives, these acids are referred to as their acyl radicals.

were found. After a further 10 min. the amounts ($\mu$moles) of crotonyl (0·017), butyryl (0·032) and $\beta$-hydroxyoctanoyl (0·0065) present had approximately trebled, and a trace of octanoyl (0·0015) had appeared. After 45 min. further increases in these substances had occurred, amounting to 0·026, 0·082, 0·03 and 0·0065 $\mu$mole respectively. Five minutes after the second addition of DPNH the amount of butyryl and octanoyl had increased to 0·111 and 0·013 $\mu$mole, the amount of crotonyl decreased to 0·018 $\mu$mole, and the amount of $\beta$-hydroxyoctanoyl increased to 0·013 $\mu$mole. These amounts were relatively unchanged by the time the second steady state had become established, but hexanoyl had appeared for the first time, suggesting that this system preferentially metabolized $C_6$ derivatives, thus preventing their accumulation.

Table 7. *Stoicheiometry of fatty acid synthesis from DPNH and* [$^{14}$C]*acetate*

The standard assay system was used with 10 mg. of undialysed FASF and 0·8 $\mu$mole of DPNH/3 ml. of incubation mixture; 50 min. after addition of acetate another 0·4 $\mu$mole of DPNH/3 ml. was added. (A): $\mu$mole of $\beta$-hydroxybutyryl + acetoacetyl formed (Hele & Popják, 1956). (B): $\mu$mole of $\beta$-hydroxybutyryl formed, obtained by correcting the data in column (A) (see text). These figures are taken as equivalent to the $\mu$moles of DPNH utilized in the formation of $\beta$-hydroxybutyryl. (C): $\mu$mole of DPNH utilized in the formation of butyryl, crotonyl, $\beta$-hydroxyoctanoyl, and octanoyl, calculated from data given by Hele & Popják (1956). (D): DPNH utilized ($\mu$mole), as measured at 340 m$\mu$., and corrected for blank utilization in a system without acetate. (E): DPNH calc. [columns (B) + (C)]/DPNH utilized, 340 m$\mu$.

| Time after addition of acetate (min.) | (A) | (B) | (C) | (D) | (E) |
|---|---|---|---|---|---|
| 15 | 0·31 | 0·122 | 0·123 | 0·300 | 0·82 |
| 45 | 0·57 | 0·150 | 0·379 | 0·362 | 1·46 |
| 55 | 0·58 | 0·153 | 0·393 | 0·406 | 1·34 |
| 115 | 0·55 | 0·152 | 0·328 | 0·698 | 0·69 |

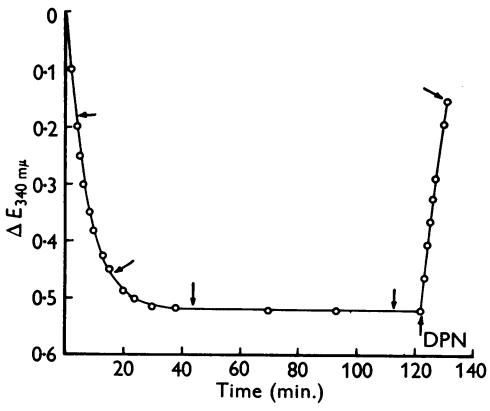


Fig. 6. Progress of DPNH utilization during an extended period of fatty acid synthesis. Experiment from which results shown in Table 8 have been derived. Standard conditions of assay with 10 mg. of undialysed FASF, 0·4 $\mu$mole of DPNH and 10 $\mu$moles (10 $\mu$c of $^{14}$C) of [$^{14}$C]acetate/3 ml. were employed. At 122 min. 4 $\mu$moles of DPN/3 ml. of incubation mixture was added. The times of sampling are indicated by arrows.

The first experiment provided sufficient data for the demonstration of a stoicheiometric relationship between [$^{14}$C]acetate incorporation and DPNH utilization in fatty acid synthesis (Table 7). This was done by comparing the DPNH utilization observed in the spectrophotometer with the expected DPNH utilization calculated from the $\mu$moles of radioactive fatty acyls found on the chromatograms. The agreement obtained was very satisfactory in view of the fact that the data for $\beta$-hydroxybutyryl were supplied from two separate experiments, that an error of 20 % is involved in the determination of the amounts of fatty acyls present, and that the utilization of DPNH, although predominantly related to $\beta$-hydroxybutyryl formation, is also complicated by side reactions described elsewhere in the text.

The second large-scale experiment was similar to the first, except that after the steady state had become established for 70 min. 4 $\mu$moles of DPN/3 ml. of reaction mixture was added in an attempt to reverse the reaction in the direction of fatty acid oxidation. The expected reduction of DPN occurred, and 0·182 $\mu$mole of DPNH had been formed in 9 min. (cf. Fig. 6) when the last sample was taken. In this experiment chromatograms were developed with both octanol–formic acid and butanol–acetic acid solvent systems. After scanning, the paper strips were cut into sections, and the radioactivity of the spots was determined by direct counting. The progress of this experiment is shown in Fig. 6. As in general the results from this experiment resembled both quantitatively and qualitatively those of the first experiment, only the additional information gained will be discussed. All fractions increased steadily up to the time of the steady state (44 min.) except acetohydroxamate, which reached its maximum value in 5 min. from the start. The amount of acetoacetyl was always about three times greater than that of $\beta$-hydroxybutyryl (Table 8). During the steady-state period, when no DPNH utilization was observed in the spectrophotometer, the amount of $\beta$-hydroxybutyryl increased nearly twofold and this was accompanied by more than a twofold increase in acetoacetyl. Similar increases in the saturated fatty acids also occurred during this period. Up to this point this experiment correlated well with the experiment of Fig. 5. Even when the apparent utilization of DPNH ceased, $\beta$-hydroxybutyrate continued to be synthesized. This occurred

Table 8. *Amounts of acetoacetyl–CoA and β-hydroxybutyryl–CoA formed in FASF from* [$^{14}$C]*acetate under standard conditions of fatty acid synthesis*

Results were obtained from radioactive counts made on paper chromatograms of the hydroxamates developed in the butanol–acetic acid–water system. The progress of DPNH utilization in this expt. is shown in Fig. 6. At 122 min. 4 μmoles of DPN/3 ml. of incubation mixture were added.

| Time of sampling after start of reaction (min.) ... | 4 | 16 | 44 | 113 | 131 |
|---|---|---|---|---|---|
| | Total counts/min. | | | | |
| Acetoacetyl | 3 100 | 8 920 | 12 870 | 27 460 | 46 630 |
| β-Hydroxybutyryl | 1 080 | 3 240 | 4 240 | 7 250 | 13 280 |

presumably at the expense of re-reduction of DPN by some oxidizable substrate which, as is discussed below, we believe to be AMP.

A surprising and perhaps significant finding was that the re-reduction of the added DPN was not associated with the oxidation of fatty acids, as was expected, but with a very great increase in fatty acid synthesis. The increases in the amounts of β-hydroxybutyryl and acetoacetyl (cf. Table 8) were paralleled by a rise of butyryl from 2130 (0·02 μmole of acetate incorporated) to 3360 (0·032 μmole) counts/min. and of hexanoyl + higher fatty acyls from 990 (0·01 μmole) to 9060 (0·087 μmole) counts/min. This may be compared with the experiment of Fig. 5, where during the first 15 min. of synthesis in the presence of 0·4 μmole of DPNH, 0·04 μmole of acetate was incorporated into fatty acids; or with the first experiment described in this section, where the addition of 0·4 μmole of DPNH resulted in the synthesis of a further 0·035 μmole of saturated fatty acids. Evidently the reduction of DPN did not occur at the expense of fatty acyls, or of β-hydroxybutyryl, but at the expense of some other substrate, now thought to be AMP. This oxidation of AMP was associated with a marked increase in the synthesis of certain saturated fatty acids.

*Chain length of fatty acids synthesized by the FASF*

The paper chromatograms of the acylhydroxamates, as found in several experiments, indicated that our preparations must have synthesized butyric, hexanoic and octanoic acids at least. Since the solvent systems used for these chromatograms could not resolve acid hydroxamates with a chain length greater than $C_8$, the fatty acids extracted from the incubations were fractionated by column chromatography according to the reversed-phase technique of Howard & Martin (1950).

From an experiment similar in every respect to that shown in Fig. 5, the fatty acids were extracted after various intervals of incubation and resolved by reversed-phase partition chromatography. In Table 9 the results obtained on one sample, drawn from a bulk incubation 45 min. after addition of acetate, are shown. At this time (steady state) 0·181 μmole of DPNH had been utilized and 0·058 μmole of acetate incorporated into fatty acids. The FASF can effect the synthesis of fatty acids of all chain lengths up to $C_{18}$, but is most efficient for the short-chain acids.

Table 9. *Chain-length specificity of FASF as measured by incorporation of* [$^{14}$C]*acetate into various fatty acids*

Standard conditions of assay were employed with 0·4 μmole of DPNH and 10 mg. of undialysed FASF/3 ml. Incubation at room temperature for 45 min. For the extraction of labelled fatty acids, triglyceride fat obtained from mammary gland and containing 25 mg. of mixed acids was added to the sample before saponification.

| Fatty acid chain length | Specific activity (counts/min. at infinite thickness of 2 cm.$^2$ sample) | Fatty acid (%, w/w, of total) | Radioactivity of fatty acid (% of total) |
|---|---|---|---|
| $C_4 + C_6$ | 3731 | 7·9 | 46·50 |
| $C_8$ | 1111 | 25·8 | 45·20 |
| $C_{10}$ | 101 | 15·7 | 2·5 |
| $C_{12}$ | 178 | 7·3 | 2·05 |
| $C_{14}$* | 70 | 6·7 | 0·74 |
| $C_{16}$* | 31 | 27·5 | 1·35 |
| $C_{18}$ | 116 | 9·1 | 1·66 |

* The tetradecanoic acid sample contained also hexadec-9-enoic and the hexadecanoic some octadec-9-enoic acid.

*Relative roles of DPN and TPN in fatty acid synthesis by the FASF as revealed by chromatography*

The results in Table 4 gave no indication that TPN might play a specific role in fatty acid synthesis by the mammary-gland enzymes. Since, however, these conclusions were based on incorporation of acetate into total fatty acids, the possibility that TPN may cause a shift of synthesis from shorter to longer acids, without necessarily affecting the sum total of synthesis, had to be considered. Hydroxamates were prepared from all samples of the three experiments shown in Table 4 and chromatographed. The incubations in these experiments, however, were done at 38°, a condition which, as we found later, leads to significant deacylation of at least crotonyl–CoA and possibly of other acyl–CoA

derivatives also. The relative proportions of butyryl and crotonyl as shown by the paper chromatograms of the hydroxamates do not reflect, therefore, the total amounts of these acids formed in these experiments. The products split off from CoA by deacylases would accumulate in the incubation mixture and would not form hydroxamates, but would be extracted after the usual saponification into total fatty acids.

As the significance and extent of deacylase action in the FASF at 38° was appreciated, we realized that by the procedures used by us in extracting fatty acids and purifying these from contaminating [$^{14}$C]acetate by chromatography (cf. Popják & Tietz, 1954) our total fatty acid samples obtained from incubations made at 38° could contain a far higher proportion of crotonic acid than samples obtained from incubations at room temperature. By the usual methods of liquid–liquid partition chromatography it is doubtful if crotonic acid could be separated from other short-chain fatty acids; but we found that the vapour-phase chromatographic technique of James & Martin (1952) gave an excellent separation of crotonic acid from butyric and hexanoic acids provided that the development was made with a slow gas flow through the column (10–15 ml. of $N_2$/min. with a 4 ft. column at 137°).

An experiment was therefore carried out at 38° under standard conditions of fatty acid synthesis with DPNH, TPNH or their combination as hydrogen donors. At the end of incubation 20 mg. of a mixture of butyric, crotonic, hexanoic and octanoic acids was added to the flasks and the whole saponified. After extraction of fatty acids and usual purification from contaminating [$^{14}$C]acetate the four short-chain acids were resolved by vapour-phase chromatography; acids higher than octanoic were not analysed. Although the results shown in Table 10 revealed a surprisingly large amount of labelled crotonate when DPNH or DPNH + TPNH was employed, they gave no support to the contention that TPNH in any way enhanced the reduction of unsaturated to the saturated acid. In fact the amounts of the saturated acids formed when both nucleotides were present were less than with DPNH alone. The synthesis observed with TPNH alone can be accounted for by the slow formation of small amounts of DPNH from TPNH in the FASF. The crotonate:butyrate ratio with TPNH alone was very similar to that found in another experiment (not reported here) with DPNH alone at 6·5 min. after the addition of acetate.

Table 10. *Synthesis of short-chain fatty acids from* [$^{14}$C]*acetate by the FASF. Test for specificity of pyridine nucleotides*

Standard conditions of assay with 10 μmoles of potassium acetate, 0·8 μmole of DPNH or TPNH and 10 mg. of dialysed FASF in a final vol. of 3 ml. Incubation at 38° for 45 min. Fatty acid synthesis expressed in terms of acetate μmoles incorporated per flask.

| Fatty acid | Fatty acid synthesis in the presence of DPNH | TPNH* | DPNH + TPNH* |
|---|---|---|---|
| Crotonic | 81·00 | 13·80 | 125·5 |
| Butyric | 8·53 | 3·24 | 5·3 |
| Hexanoic | 8·06 | 2·90 | 7·6 |
| Octanoic | 4·80 | 3·55 | 3·7 |

* Generated from TPN with glucose 6-phosphate (2 μmoles).

*Experiments designed to elucidate further the relationship between DPNH and TPNH in fatty acid synthesis*

*TPNH in the spectrophotometric system.* In some experiments TPNH instead of DPNH was employed in the spectrophotometric assay system. Use was made of the presence of TPN-specific glucose 6-phosphate dehydrogenase in the FASF to generate the reduced coenzyme from added TPN and glucose 6-phosphate. The failure to demonstrate a specific effect of TPNH on fatty acid synthesis from [$^{14}$C]acetate (cf. Tables 4 and 10) was duplicated in a failure to demonstrate an oxidation of TPNH in the FASF on addition of acetate in the presence of CoA, cysteine, ATP and $Mg^{2+}$ ions, i.e. under conditions in which DPNH was readily utilized. A typical experiment is shown in Fig. 7, curve *A*, which records the instantaneous and stoicheiometric formation of 0·3 μmole of TPNH by the FASF on addition of 0·3 μmole of glucose 6-phosphate. The rate of the slow and apparently non-specific utilization of TPNH (0·01–0·03 μmole/hr./10 mg. of protein at 38°) in the absence of added substrate was only slightly increased by the introduction of acetate and could be accounted for by the formation of DPNH from TPNH (see later). The disappearance of not more than 0·016 μmole of TPNH could be attributed to acetate in 28 min. The addition of oxidized glutathione caused, however, a rapid oxidation of TPNH by the TPNH-specific glutathione reductase (Mapson & Goddard, 1951) also present in the FASF. This and other similar experiments indicated that TPNH, under the conditions of fatty acid synthesis, did not undergo significant destruction.

TPN, however, in the absence of ATP is destroyed by the FASF (particularly at 38°), and is only partially reduced by glucose 6-phosphate to a substance which on continued incubation ceases to react enzymically either as TPNH or DPNH (Fig. 7, curve *B*). At room temperature this destruction is much less marked (cf. Fig. 8).

*Transhydrogenase.* The reduction of TPN by DPNH is known to occur in mammalian tissues

(Kaplan *et al.* 1953). An attempt was made to demonstrate the presence of such a transhydrogenase in the FASF in experiments based on those of Kaplan *et al.* (1953). Three of the type shown in Fig. 8 were performed in which we made use of the TPN-specific glucose 6-phosphate dehydrogenase and TPNH–glutathione reductase present in the FASF. Two cuvettes contained the complete system with TPN and DPNH, whereas TPN was omitted from the third cell. After 20 min. GSSG was added to test for TPNH; this time was sufficient in the experiments of Kaplan *et al.* (1953) to allow for the reduction of about 0·35 $\mu$mole of TPN by DPNH. Fig. 8 shows that the rate of fall of optical density after the addition of GSSG was the same in the complete system containing TPN and DPNH as in the control without TPN. At the end of a second 20 min. period glucose 6-phosphate was added to one of the cells containing the complete system and to which GSSG had not previously been added. A rise in optical density immediately occurred, corresponding to the formation of 0·13 $\mu$mole of TPNH (Fig. 8, curve 3). When there was no further rise in optical density the addition of GSSG caused an immediate fall in optical density which was 8·5 times more rapid during the next 10 min. than the corresponding rate in the control cells. The rate fell off with time and finally became the same as that in the controls. This experiment demonstrated that there was not sufficient transhydrogenase present in the FASF to generate TPNH in detectable amounts (at least 0·02 $\mu$mole in our assay system).

*Destruction and interconversion of pyridine nucleotides.* In these studies DPN, DPNH, TPN and TPNH (0·2–0·8 $\mu$mole) were incubated with the FASF in the presence and absence of ATP and other cofactors of fatty acid synthesis and then tested for all four nucleotides. The destruction of TPN in the absence of ATP has already been referred to (cf. Fig. 7, curve *B*). A similar destruction also of DPN by the FASF occurred when ATP was omitted from the incubation mixture; nicotinamide, in the amounts used by Kaplan *et al.* (1953), did not protect DPN. Although ATP protected both nucleotides against destruction, no TPN was formed when DPN and ATP were incubated together. TPNH, prepared either by chemical reduction of TPN or generated at the beginning of the experiment from TPN by glucose 6-phosphate

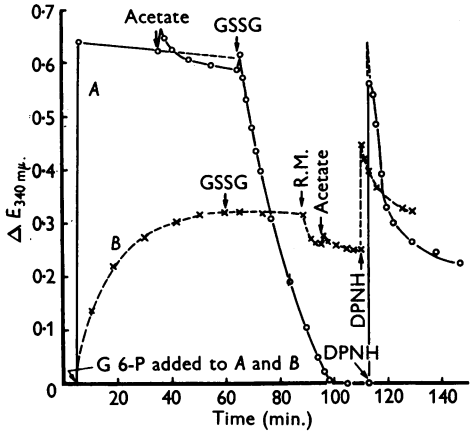


Fig. 7. Reduction of TPN by glucose 6-phosphate dehydrogenase in the FASF as measured by u.v. light absorption at 340 m$\mu$. Curve *A*, the cuvette contained initially: CoA, 2 $\mu$moles; cysteine, 30 $\mu$moles; ATP, 30 $\mu$moles; $Mg^{2+}$, 20 $\mu$moles; TPN, 0·38 $\mu$mole; tris buffer, pH 9, 100 $\mu$moles; protein (undialysed FASF) 10 mg.; total vol. 2·85 ml.; temp. 38°. Additions were made at arrows; glucose 6-phosphate (G 6-P), 0·3 $\mu$mole in 0·075 ml.; potassium acetate, 50 $\mu$moles in 0·1 ml.; oxidized glutathione (GSSG), 1·4 $\mu$moles in 0·05 ml.; DPNH, 0·3 $\mu$mole in 0·1 ml. Dotted straight line after 35 min. gives the extrapolated rate of TPNH disappearance which would have been observed if acetate had not been added; the dotted spike after 110 min. indicates the theoretical level of optical density due to addition of DPNH. Curve *B*, the cuvette contained in a vol. of 2·85 ml. initially only $Mg^{2+}$, TPN, buffer and protein in the same amounts as for curve *A*. Additions were: G 6-P, 0·6 $\mu$mole in 0·15 ml.: GSSG, 1·4 $\mu$moles; reaction mixture added at R.M. consisted of CoA, 2 $\mu$moles, cysteine, 30 $\mu$moles and ATP 30 $\mu$moles in 0·65 ml.; potassium acetate, 50 $\mu$moles; DPNH, 0·1 $\mu$mole in 0·03 ml. Temp. 38°.

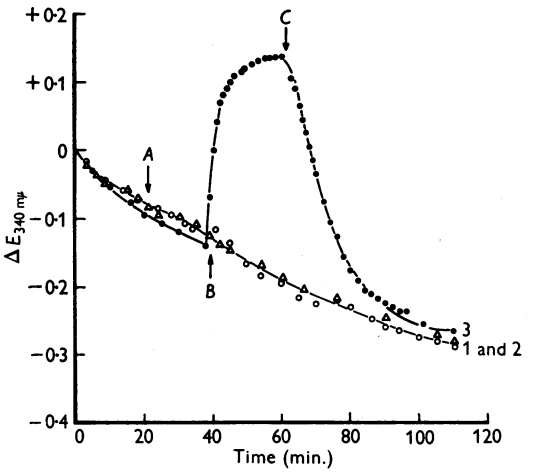


Fig. 8. Test for the transhydrogenase reaction in the FASF. The complete system contained 0·4 $\mu$mole of DPNH, 0·25 $\mu$mole of TPN, 8·3 mg. of FASF (dialysed), 230 $\mu$moles of potassium phosphate buffer, pH 7·5, and 100 $\mu$moles of nicotinamide, in a final vol. of 3 ml. Room temperature (1) ○, DPNH + TPN; (2) △, DPNH, no TPN; (3) ●, DPNH + TPN. At *A*, 5·6 $\mu$moles of GSSG were added to 1 and 2; at *B*, 0·18 $\mu$mole of glucose 6-phosphate was added to 3; at *C*, 5·6 $\mu$moles of GSSG were added to 3.

dehydrogenase, showed much greater stability than TPN on incubation with FASF. However, small quantities of DPNH were always formed (up to 0·05 μmole) from TPNH in the presence or absence of ATP.

*Dephosphorylation reactions.* In a number of experiments the liberation of orthophosphate from ATP, ADP, AMP, TPN, TPNH and from inorganic pyrophosphate in the FASF was measured under conditions similar to those used in the study of fatty acid synthesis. It was found that orthophosphate was liberated from the three adenine nucleotides at about the same rate (about 1 μmole/10 mg. of protein/30 min. at 38° from 30 μmoles of the nucleotides), and that this rate did not vary greatly from one FASF to another. The rate of liberation of orthophosphate was linear with time up to 120 min. Sodium pyrophosphate was hydrolysed 20–30 times as fast as were the adenine nucleotides. Such rapid liberation of orthophosphate was also observed when ATP was incubated with acetate, CoA and cysteine, and was presumably due to the hydrolysis of inorganic pyrophosphate generated from ATP during the activation of acetate. The amounts of orthophosphate appearing in such experiments were no greater than could be accounted for by the stoicheiometry of the acetate activation reaction plus the slow non-specific hydrolysis of ATP, AMP and of CoA. CoA, TPN and TPNH were also hydrolysed by the FASF, but at a rate not greater than that for adenine nucleotides

*Reduction of DPN by AMP.* Popják & Tietz (1955) reported that the fatty acid-synthesizing soluble enzyme preparations of mammary gland consumed oxygen in the presence of acetate and that this oxygen consumption depended on the amount of ATP added. There is now good evidence indicating that this oxygen consumption is due to an 'AMP-oxidase' that is probably linked with a xanthine oxidase system, both present in the FASF.

When DPN and AMP, in amounts equivalent to those arising in our system during the course of fatty acid synthesis, were incubated together with dialysed or undialysed FASF, reduction of DPN occurred (Fig. 9), the rate and extent of which depended upon the temperature at which the experiment was carried out. At room temperature reduction of DPN did not occur for about 1 hr. If the reaction mixture was first incubated at 38° for 5 min. with either the AMP or the FASF omitted, and the reaction then initiated by the addition of the missing component, the reduction of DPN commenced after about 15 min. The reduction was more rapid if the mixture was left at 38° than it was at room temperature, but in either case the 'lag period' was shorter than if the reaction mixture was left at room temperature throughout. Hypoxanthine reduced DPN more rapidly at room temperature without showing a lag period, ATP and ADP reduced DPN at one-eighth and one-half of the rate obtained with AMP.

It was thought possible that during the lag phase AMP was degraded to hypoxanthine and that this was oxidized by a xanthine oxidase-type enzyme. The presence of such an enzyme in the FASF has, in fact, been demonstrated in a number of experiments in which hypoxanthine or xanthine was used as substrate and potassium ferricyanide, 2:6-dichlorophenolindophenol or oxygen was used as electron acceptor.

The part played by the reduction of DPN by AMP in fatty acid synthesis was tested in the following experiments. The reduction of DPN by this reaction was inhibited by the xanthine oxidase inhibitors 2:5:6-triamino-4-styrylpyrimidine (CB 1019), phenylpurine and pteridine aldehyde (2-amino-6-formyl-4-hydroxypteridine) (Bergel, 1956). These substances caused considerable inhibition of the reduction of DPN by AMP during the first

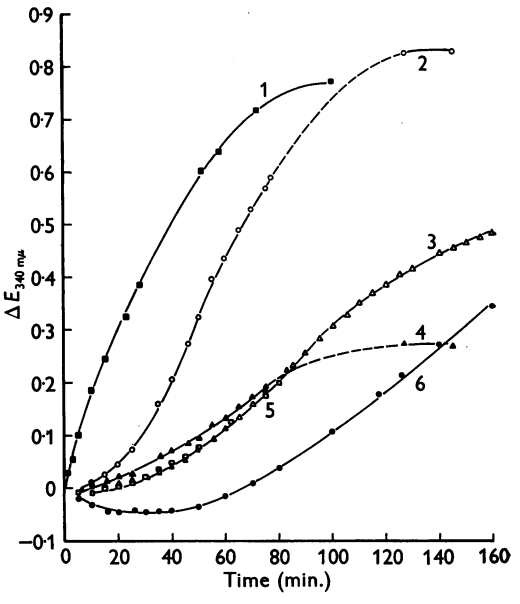


Fig. 9. Reduction of DPN by AMP. The test system contained 2 μmoles of AMP, 0·4 μmole of DPN, 100 μmoles of tris buffer, pH 9, 20 μmoles of $MgCl_2$ and 8·3 mg. of FASF (dialysed) in a final vol. of 3 ml. (1) ■, Hypoxanthine (2 μmoles), instead of AMP, at room temperature throughout; (2) ○, preliminary incubation at 38°, then read at 38° in an electrically heated cell housing; (3) △, preliminary incubation at 38°, then read at room temperature; (4) ▲, as (2) without AMP; (5) □, as (2) with $3{\cdot}4 \times 10^{-5}$ M CB 1019. Similar curves were obtained with $6{\cdot}8 \times 10^{-5}$ M pteridine aldehyde and with $6{\cdot}8 \times 10^{-5}$ M phenyl purine; (6) ●, read at room temperature throughout, no preliminary incubation at 38°.

Table 11. *Effects of xanthine oxidase inhibitors and of continuous reduction of DPN by alcohol dehydrogenase on fatty acid synthesis from* [$^{14}$C]*acetate*

Standard conditions of assay were used with 0·8 $\mu$mole of DPNH or TPN, 10 $\mu$moles (10 $\mu$c of $^{14}$C) of potassium acetate and 5 mg. of dialysed FASF in a final vol. of 3 ml. The additions were made at the beginning in final concentrations and amounts indicated. The fatty acid synthesis was started by introduction of acetate. Expts. 1 and 2 were carried out 3 weeks apart but with the same FASF preparation. Incubations were at 38° for 30 min. Results expressed as $\mu$mole of acetate incorporated into fatty acids per assay.

| Pyridine nucleotide used and other additions | Expt. 1 | Expt. 2 |
|---|---|---|
| Control: no DPNH or TPNH | 0·005 | 0·002 |
| DPNH | 0·136 | 0·147 |
| TPNH* | — | 0·008 |
| DPNH + TPNH* | — | 0·115 |
| DPNH + alcohol dehydrogenase (0·3 mg.) + ethanol (10 $\mu$moles) | — | 0·273 |
| DPNH + lipoic acid (2 $\mu$moles) | — | 0·138 |
| DPNH + phenyl purine ($6·8 \times 10^{-5}$ M) | 0·063 | — |
| DPNH + 2:5:6-triamino-4-styrylpyrimidine ($3·4 \times 10^{-5}$ M) | 0·159 | — |
| DPNH + pteridine aldehyde ($6·8 \times 10^{-5}$ M) | 0·136 | — |
| DPNH + hypoxanthine (2 $\mu$moles) | 0·214 | — |
| DPNH + hypoxanthine + 2:5:6-triamino-4-styrylpyrimidine | 0·134 | — |
| DPNH + hypoxanthine + pteridine aldehyde | 0·184 | — |

* TPN + glucose 6-phosphate (2 $\mu$moles).

30 min. of the reaction (cf. Fig. 9). Their effect upon the utilization of DPNH in the fatty acid-synthesizing system, at the concentrations given in the legend to Fig. 9, was variable. CB 1019 was without effect, pteridine aldehyde increased the utilization of DPNH by 30 %, whereas phenyl purine depressed the utilization to 25 % of the control value. These findings were reflected in an experiment in which the effect of the inhibitors upon [$^{14}$C]acetate incorporation into fatty acids was tested (Table 11). CB 1019 and pteridine aldehyde were without effect on incorporation of [$^{14}$C]acetate, whereas phenyl purine depressed it to one-half of the control value. Hypoxanthine increased incorporation by 55 %, but the addition of inhibitors with the hypoxanthine reduced the incorporation, in one instance to the control level. Alcohol dehydrogenase, although far more effective in keeping DPN fully reduced in our experiments than hypoxanthine, stimulated fatty acid synthesis only a little more than the purine.

## DISCUSSION

We think that the most significant part of our results is the demonstration for the first time of a fatty acid cycle (or spiral) in an organized enzyme system as postulated by Lynen & Ochoa (1953) and Lynen (1954). The same concept of synthesis of fatty acids by a stepwise lengthening of fatty acid chains by the addition of $C_2$ units to shorter chains has been expressed earlier (Popják, French, Hunter & Martin, 1951; Popják, 1952) on the basis of experiments on whole animals, although the nature of intermediates could not be defined. The mechanism of fatty acid synthesis according to these ideas is a reversal of the $\beta$-oxidation of fatty acids already achieved with purified enzyme preparations (cf. Lynen, 1954; Green, 1954). We may state now with some confidence that the steps involved in the synthesis of butyric acid involve the reduction of acetoacetyl–CoA to $\beta$-hydroxybutyryl–CoA, the dehydration of the latter to crotonyl–CoA, followed by a second step of reduction. The presumption is that a similar mechanism operates in the synthesis of higher acids also. In our enzyme preparations DPNH can act as the electron donor for both reductive steps. The first reductive reaction catalysed by the $\beta$-hydroxybutyryl dehydrogenase is a well-understood reaction (Wakil *et al.* 1954; Lynen *et al.* 1952), dependent on the participation of DPNH. However, the reduction of crotonyl–CoA with a natural electron donor has so far defied the efforts of those laboratories working with purified enzymes. Stansley & Beinert (1953) only succeeded in reducing crotonyl–CoA to butyryl–CoA with reduced benzyl viologen, which was itself kept reduced with xanthine oxidase. In their system no fatty acid higher than butyric was formed.

The report by Langdon (1955) that crude liver preparations will reduce crotonyl–CoA with TPNH made us concentrate our efforts particularly on this possibility, but all our attempts to demonstrate a specific role of TPNH in the synthesis of *n*-fatty acids containing an even number of carbons in their chain met with complete failure. In the absence of a demonstrable transhydrogenase reaction in the FASF, TPNH could not have been formed from DPNH, even if the FASF had contained catalytic

amounts of TPN. Our failure to demonstrate a significant effect of TPN or TPNH on fatty acid synthesis could not be attributed to the destruction of these coenzymes by the FASF as there was but a slight loss of TPN or TPNH in the FASF under conditions of fatty acid synthesis. The slight fatty acid synthesis observable when TPNH alone was used as electron donor could be attributed to small amounts of DPNH formed in our preparation. All these negative findings may be contrasted with the very positive effects of DPNH. The stoicheiometric relationship established between acetate-dependent DPNH utilization and acetate incorporation into fatty acids and their intermediates gave added support to our conclusion that DPNH acts as the primary electron donor in the reaction catalysed by both the β-hydroxyacyl dehydrogenase and fatty acyl dehydrogenase. Any attempt to establish a stoicheiometric relationship of this kind, in a complex series of reactions, with a partially purified enzyme preparation in which side reactions (e.g. the reduction of DPN by AMP or deacylation reactions) can take place, is certain to present difficulties; and if a correlation between DPNH utilization and [$^{14}$C]acetate incorporation can be shown to within 50 % something will have been achieved. In fact, most of the correlations obtained were considerably better than this (cf. Table 7). Such a satisfactory relationship could only be demonstrated in experiments carried out at room temperature, since at 38° interference from side reactions became so marked that the quantitative assessment of results became impossible.

At first we thought that the metabolism of AMP in the FASF, whereby DPN is reduced at its expense, does no more than provide a supplementary source of electrons for the reductive steps of synthesis. However, in experiments of long duration (over 50 min. of incubation), such as is partly shown in Fig. 6, and when the AMP-oxidase may be expected to come into full play, a degree of stimulation of fatty acid synthesis is observed far in excess of that attributable merely to generation of additional amounts of DPNH. This focused our attention on possible substances derived from AMP and which might exert a specific catalytic role in the second reductive step of fatty acid synthesis. Among these hypoxanthine was excluded by the experiments shown in Table 11, as it was found that this substance did not stimulate fatty acid synthesis any more than did alcohol dehydrogenase + ethanol. Preliminary experiments, however, strongly suggest that inosine monophosphate, derived from AMP by hydrolytic deamination, in the presence of DPNH catalyses, in an as yet undefined manner, the reduction of crotonyl–CoA to butyryl–CoA by the FASF. If further experiments confirm these results a good teleological reason may be provided for the splitting of the pyrophosphoryl from ATP during the acetate-activation reaction, since the metabolism of the resulting AMP would provide a catalyst for subsequent reactions.

## SUMMARY

1. A partially purified enzyme preparation has been obtained from lactating rabbit mammary gland that catalyses the synthesis of even-numbered-chain fatty acids from $C_4$ to $C_{18}$, with the shorter-chain fatty acids preponderating.

2. This synthesis is achieved from acetate, coenzyme A (CoA), adenosine triphosphate and stoicheiometric amounts of reduced diphosphopyridine nucleotide (DPNH). The intermediates involved have been studied by paper chromatography, as their hydroxamates. The findings strongly suggest that fatty acid synthesis in these enzyme preparations takes place by a stepwise condensation of $C_2$ units, through the reversal of the now well-established β-oxidation of even-numbered-chain fatty acids.

3. TPNH and lipoic acid in our system have no stimulatory effect upon the synthesis of even-numbered-chain fatty acids by the mechanism outlined above.

4. DPNH appears to act as a specific electron donor for both the reduction of β-ketoacyl–CoA to β-hydroxyacyl–CoA and of unsaturated fatty acyl–CoA to saturated fatty acyl–CoA. This latter reduction step appears to be stimulated in some manner, as yet undefined, by a metabolic derivative of adenosine 5-phosphate, which is not hypoxanthine, and which may possibly be inosine monophosphate.

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Mr R. Hancock participated in the development of certain of the chromatographic methods. It is a pleasure to acknowledge the skilled technical assistance of Miss Mary O'Donnell and Mr Barry Buckingham and the help of Miss J. Ashenden in the preparation of the diagrams. Fig. 5 is reproduced from Hele & Popják (1955) by permission of Elsevier Publishing Co.

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# The Amino Acid Composition of Fish Collagen and Gelatin

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The major protein constituent of the skin, bones, swim bladder and scales of fishes resembles in many ways the more widely studied collagen of mammals. Thus it contains hydroxyproline and hydroxylysine, shows striated fibrils with the electron microscope (Borasky, 1950; Schmitt, Gross & Highberger, 1955; Damodaran, Sivaraman & Dhavalikar, 1956), and has characteristic wide-angle and narrow-angle X-ray diffraction patterns (Bear, 1952).

Amino acid analyses of fish collagens have been reported by Beveridge & Lucas (1944), who used mainly gravimetric methods, for isinglass from the swim bladder of hake (*Urophycis*); by Block, Horwith & Bolling (1949) for scales of herring (*Clupea*) and by Neuman (1949), who used microbiological-assay techniques, for halibut skin and for gelatin prepared from the scales of an unspecified fish. The recent study on the composition of elastoidin from the fin rays of the shark (*Carcharinus melanopterus*) by Damodaran *et al.* (1956), who used resin chromatography, has confirmed the results of wide-angle X-ray diffraction work (Astbury, 1938) in placing this unusual protein within the collagen group.

Interest in fish collagens has recently been stimulated by the suggestion that their reduced structural stability compared with mammalian collagens (shown by a lower range of shrinkage temperatures), is related to a lower content of hydroxyproline (Gustavson, 1953). Interchain hydrogen bonding between hydroxyl groups of hydroxyproline and backbone carbonyl groups was, on this basis, suggested as an important stabilizing feature of the collagen structure (Gustavson, 1955). This hypothesis has since been applied, without further proof, in interpreting details of the structure of collagen at two levels of size, in connexion with X-ray diffraction (Ramachandran, 1956) and electron-microscope techniques (Reed, Wood & Keech, 1956) respectively. A summary of recent work on the connective tissues of fishes is given by Hamoir (1955).

The present investigation provides information about the composition of collagen from a few species representing a zoologically wide range of fish types, members of all three surviving classes, Elasmobranchii, Actinopterygii and Crossopterygii (Young, 1950; Trewavas, White, Marshall & Tucker, 1955) being included. The experimental methods were