SUMMARY

1. Estimations have been made of the ascorbic acid content of the particulate and non-particulate fractions of cytoplasm separated by high-speed centrifugation of sucrose dispersions of ox adrenal medulla and adrenal cortex, and of dog liver.

2. The ascorbic acid was found to be entirely or almost entirely in the non-particulate fraction.

3. These results are discussed and additional evidence is advanced for considering that the location of reduced silver particles does not necessarily indicate the localization of ascorbic acid within the cell.

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Biosynthesis of Fatty Acids by Slices and Cell-free Suspensions of Mammary Gland

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The experiments to be described were undertaken with the purpose of developing, if possible, cell-free enzyme preparations suitable for the study of the biosynthesis of fatty acids *in vitro*. The lack of even crude preparations of this kind until recently is probably responsible in a large measure for the paucity of detailed knowledge concerning the intermediary stages of fat metabolism.

While our work was in progress Gurin and his colleagues reported that 'homogenates' and some soluble enzyme preparations made from pigeon liver (Brady & Gurin, 1952a, b) and rat liver (Dituri & Gurin, 1953) synthesize long-chain fatty acids from [¹⁴C]acetate. In our experiments, we have used the mammary gland of lactating animals, which is known to be very active in synthesizing fat (cf. reviews by Folley, 1952; Popják, 1952a, b). The mammary gland of lactating rats was used mostly, but some experiments were also carried out with sheep udder tissue. The results described here have already been communicated in a preliminary form (Popják & Tietz, 1953a).

MATERIAL AND METHODS

Lactating rats, 7–14 days after parturition, were anaesthetized with ether and killed by dislocation of the neck. The abdominal pair of the mammary glands was removed

together with the overlying skin and packed in crushed ice. When the glands were thoroughly chilled, they were dissected and freed from connective tissue. One rat provided usually 5–6 g. of gland.

The sheep udder tissue, one portion packed in ice and another in solid CO₂, was transported from the slaughter house to the laboratory. The experiments with the cell-free suspensions prepared from the ice-cold tissue were set up 4 hr., and those with the slices 5 hr., after the killing of the sheep. The experiments with rat tissue were usually started 2–3 hr. after the death of the animals. (We are indebted to Dr S. J. Folley, F.R.S., for supplying the udder tissue from a lactating sheep.)

Preparation of tissue slices. Tissue slices were cut with a Stadie-Riggs (Stadie & Riggs, 1944) microtome and were kept in oxygenated ice-cold buffer until used. About 250 mg. of slices were incubated in 3 ml. of phosphate-saline (Krebs & Eggleston, 1940).

Preparation of suspensions. The tissues were minced finely with scissors and the mince was disintegrated for about 2 min. with 2 vol. of ice-cold buffer (0.154 M-KCl, 100 parts; 0.154 M-MgCl₂, 10 parts; 0.1 M-potassium phosphate buffer, pH 7.4, 35 parts) in a glass homogenizer tube provided with a loosely fitting pestle (difference between the diameter of the tube and that of the pestle, 0.40 mm.) which was driven by an electric motor. The suspension thus produced was filtered through four layers of gauze into another homogenizer tube with a tightly fitting pestle (difference between the diameter of the barrel and pestle, 0.10 mm.) and was ground further by hand. Large cell fragments and cell nuclei were removed by centrifugation at 400 g at 0° for 10 min. The fat which separated on the surface was removed and the supernatant fluid filtered through a small pad of cotton wool. This filtered supernatant, which contained 50 ± 5 mg. dry

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matter/ml. (corrected for salts but not for fat), was used in the experiments. Neither intact cells nor cell nuclei in the suspensions could be identified with the phase-contrast microscope; with the exception of some fat globules, most of the particles showed an intense Brownian movement when viewed with an oil-immersion lens. When suspensions from the sheep udder frozen in solid CO_2 were required, the frozen tissue was first broken up into a fine powder in a stainless-steel mortar chilled with solid CO_2 . The powder was then added to the buffer solution in the glass homogenizer tube; the rest of the procedure was as for the fresh tissues.

Incubations. All incubations (slices and suspensions) were made at 37.5° for 3 hr. in Warburg vessels, the centre well of which contained 0.2 ml. of 20% (w/v) KOH. The gas phase was O_2 or air. In the experiments with suspensions, the 'additions' were first measured into the flasks and the volume made up to 1 ml. with water; 2 ml. of suspension were then added and the incubations started immediately. In every experiment, except when the endogenous O_2 consumption of the preparations was being measured, acetate (containing $5 \mu\text{C}$ of [carboxy- ^{14}C]acetate, obtained from the Radiochemical Centre, Amersham, Bucks) was added to the incubation mixture in a final concentration of 0.02 M, equivalent to 60 μmoles of acetate/flask. Other substrates (denoted by 'Additions' in the Tables) were added in the following final concentrations: glucose, 0.3%; adenosine triphosphate (ATP), 0.01 M; pyruvate, oxaloacetate, citrate, α -oxoglutarate, succinate, malate and glutamate, 0.02 M.

Oxygen consumption. The values of $-Q_{\text{O}_2}$ ($\mu\text{l. O}_2/\text{mg. dry wt./hr.}$) reported are based on the total O_2 consumed during the first hour of incubation and do not represent just the initial rates of O_2 consumption.

Collection of CO_2 for measurement of oxidation of [^{14}C]acetate. The filter paper and alkali from the centre well of the flasks were added to 2 ml. of freshly boiled water in a centrifuge tube and the centre well was washed out five times with CO_2 -free water. One ml. of a 0.5% (w/v) solution of Na_2CO_3 was added to the centrifuge tube as 'carrier' and the contents of the tube were covered with a layer of high-boiling-point petroleum to prevent absorption of atmospheric CO_2 . After allowing several hours for the equilibration of the carbonate in the filter paper with the solution (it is extremely difficult to wash out all the alkali from the paper), the paper was withdrawn and washed with CO_2 -free water. The carbonate was precipitated as BaCO_3 by the addition of several drops of a saturated solution of BaCl_2 . The BaCO_3 was centrifuged and washed three times with freshly boiled water and twice with acetone and was plated for radioactive counting by filtration on to sample disks as described earlier (Popják, 1950).

Extraction of fatty acids and removal of [^{14}C]acetate from the fatty acids. The contents of the incubation flasks were saponified overnight with 1 ml. of 40% KOH on a boiling-water bath. In the experiments with slices the unsaponifiable matter was always extracted, but with the suspensions, in which the unsaponifiable matter amounted to only about 1% of the total fatty acids and which were found to contain only negligible amounts of radioactivity, this extraction was omitted. The hydrolysis mixture was acidified and extracted with 2 vol. of light petroleum (b.p. $40-60^\circ$).

In our earliest experiments the fatty acids were purified from contaminating [^{14}C]acetate by washing the light petroleum extract with 0.1 N acetic acid and then with

water. Although this technique was effective in removing all but traces of [^{14}C]acetate, it was extremely tedious and unsatisfactory because an appreciable amount of the short-chain fatty acids present in the mammary gland preparations was inevitably lost. A chromatographic technique was therefore resorted to. Dr A. J. P. Martin and Miss Joan Webb of this Institute have devised for the separation of *n*-butyric, propionic, acetic and formic acids a partition-chromatographic column (cf. Cornforth, Hunter & Popják, 1953), in which 0.5 N- H_2SO_4 supported by Hyflo Supercel forms the stationary phase and CHCl_3 (saturated with 0.5 N- H_2SO_4) the developing solvent. All fatty acids higher than propionic emerge from this column as a single narrow band. In practice, for the quantities of fatty acids obtained in a single experiment (25–50 mg.) a glass tube of 2 cm. diameter and fitted with a sintered-glass disk was packed with a CHCl_3 suspension of Hyflo Super-cel (25 g.) wetted with 0.5 N- H_2SO_4 (20 ml.). The light petroleum solution of the fatty acids (see above) was concentrated to about 1 ml., taken up in 5 ml. of CHCl_3 and transferred to the column. After the fatty acids were washed into the column with two further 5 ml. portions of CHCl_3 , 80 ml. of solvent were allowed to percolate through. The eluted fatty acids were titrated under N_2 with 0.01 N-ethanolic NaOH with bromthymol blue as indicator. The results were very well reproducible; in replicate analyses on the same cell-free suspension the titration values did not vary by more than 5%. When 5 μC of [^{14}C]acetate were added to 50 mg. of higher fatty acids and the mixture chromatographed the latter gave 1–3 counts/min. above background. The acetic acid could be eluted from the columns after passing through about 1 l. of 10% (v/v) *n*-butanol in CHCl_3 . Although these columns could be used repeatedly after removal of the [^{14}C]acetic acid, it was found more economical on account of the large volumes of solvent required to collect only the fatty acids higher than acetic and to discard the columns after using once.

Resolution to total fatty acids into individual acids by chromatography. The fatty acids were first fractionated by the reversed-phase partition chromatography technique of Howard & Martin (1950), the first solvent applied to the column being 45% (v/v) aqueous acetone. The first band emerging with this solvent contains all short-chain fatty acids up to and including octanoic; decanoic acid appears as the second band. On changing the solvent to 55% acetone, dodecanoic and tetradecanoic acids are eluted successively; 65% acetone is used to remove hexadecanoic (palmitic) acid and 75% acetone to remove octadecanoic (stearic) acid. Any monoethenoid acid will appear in this system with the saturated fatty acid having two carbon atoms fewer. Thus oleic (octadec-9-enoic) is eluted with palmitic acid and palmitoleic (hexadec-9-enoic) with myristic (tetradecanoic) acid. Of the unsaturated acids only oleic acid is present in significant amounts in our preparations and we separated it from palmitic acid by subjecting the mixture of the two (the band emerging with 65% acetone) to hydrogenation in ethyl acetate with 5% palladium on charcoal as catalyst at 20° for 24 hr. The fatty acids were then re-chromatographed and the stearic acid thus obtained represented the oleic acid in the original mixture. The hydrogenation was repeated on the palmitic acid fraction thus freed from oleic acid, but no stearic acid was formed during the second hydrogenation. The reversed-phase chromatographic columns were loaded with not more than 2 mg. total fatty acids/g. packing.

The short-chain fatty acids (45% acetone fraction) were further resolved on a column buffered with 2M- K_2HPO_4 :2M- KH_2PO_4 (6:1), pH 8.0. Caprylic, caproic, valeric and butyric acids can be separated on this column which is essentially the same as that already described (Cornforth *et al.* 1953) for the separation of valeric and butyric acids, except that the pH of the present column is higher.

Assay of ^{14}C . Whenever sufficient material was available, the radioactivity of the fatty acids was measured on the sodium salts in infinite thickness samples of 2 cm.² area. However, in the experiments with suspensions, this was seldom possible and the counts had to be taken on thin samples. For this purpose the sodium soaps, dissolved in 0.05–0.10 ml. water, were pipetted on to plastic disks lined with lens paper. An experimental self-absorption curve was prepared for [^{14}C]soaps plated with lens paper. The counts obtained on the thin samples were then corrected to infinite thickness with the help of this curve. The specific radioactivities of the carbon of the fatty acids (Table 10) were calculated as described earlier (Cornforth *et al.* 1953).

From the specific radioactivity and the amount of fatty acids found, the total amount of acetate converted into fatty acid was calculated.

RESULTS

The effect of substrates on fatty-acid synthesis and oxidation of [carboxy- ^{14}C]acetate by mammary gland slices

As a preliminary to the work with cell-free suspensions, the response of tissue slices to various substrates was tested in the hope that the information thus gained might prove useful in the later experiments. It has been shown by Folley & French (1950) and by Balmain, Folley & Glascock (1952) that rat mammary-gland slices do not utilize acetate for fatty-acid synthesis when acetate is the only substrate in the incubation medium; however, when glucose is also added, acetate is readily used. Slices of sheep udder on the other hand can synthesize fatty acids from acetate without any addition, although glucose causes a further stimulation in this tissue also.

These conclusions of Folley and his collaborators have been fully confirmed (Table 1) and it was found

Table 1. *Fatty-acid synthesis by rat and sheep mammary-gland slices from [carboxy- ^{14}C]acetate*

(Each flask contained 60 μ moles of acetate (5 μ C ^{14}C); 250 mg. of slices and 2.5 ml. of phosphate-saline, pH 7.4. Additions were as indicated (final concentrations in brackets). Final vol. 3 ml. Results are expressed as μ moles of acetate incorporated into fatty acids/100 mg. of initial dry weight of slices.)

Expt. no.	Species	Fatty-acid synthesis					
		None	Glucose (0.3%)	Pyruvate (0.02M)	Oxalo- acetate (0.02M)	α -Oxo- glutarate (0.02M)	Succinate (0.02M)
66	Rat	0.014	7.45	—	—	—	0.113
67	Rat	0.018	10.65	—	0.331	0.14	0.166
76	Rat	0.016	—	0.032	0.087	0.13	—
77	Rat	0.006	9.34	0.258	0.252	0.068	—
96	Sheep	0.293	3.67	1.26	1.18	1.62	2.54

Table 2. *Oxygen consumption and oxidation of acetate by rat and sheep mammary-gland slices in the presence of various substrates*

(Concentrations of substrates and other conditions as in Table 1.)

Expt. no.	Species	Additions	- Q_{O_2}	Acetate oxidized (μ moles/100 mg. initial dry wt.)
66	Rat	None	1.49	0.107
		Glucose	4.30	0.654
		Succinate	4.33	0.427
76	Rat	None	0.85	0.327
		Pyruvate	3.97	0.546
		Oxaloacetate	3.23	0.202
		α -Oxoglutarate	2.85	1.770
77	Rat	None	0.74	0.186
		Glucose	4.24	1.280
		Pyruvate	4.15	1.440
		Oxaloacetate	4.83	0.556
		α -Oxoglutarate	2.68	1.960
96	Sheep	None	1.46	4.730
		Glucose	1.56	4.680
		Pyruvate	2.10	4.550
		Oxaloacetate	3.02	1.730
		α -Oxoglutarate	2.14	1.570
		Succinate	5.92	5.910

in addition that oxidizable substrates other than glucose also stimulated fatty-acid synthesis from acetate. As can be seen from the results shown in Table 1 rat mammary-gland slices incorporated only traces of ^{14}C into fatty acids when [carboxy- ^{14}C]-acetate was the only substrate added to the medium. The addition of pyruvate, oxaloacetate, α -oxoglutarate or succinate, however, caused a significant utilization of acetate for fatty-acid synthesis although not as great as was found in the presence of glucose. In slices of sheep udder, pyruvate and the members of the citric acid cycle were nearly as effective stimulants as glucose and were far more active than in rat tissue.

The difference in behaviour of the mammary-gland slices of the two species was apparent also in their ability to oxidize acetate. As can be seen from the results shown in Table 2 rat mammary-gland slices oxidized only about one-fortieth of the amount oxidized by sheep-udder slices when acetate was the only substrate added. Even in Expt. 77 (Table 2) when the highest oxidation of acetate by rat mammary-gland slices was observed in the presence of glucose, pyruvate or α -oxoglutarate, the levels of oxidation were lower than that found with sheep tissue when incubated with acetate alone. α -Oxoglutarate in the rat tissue stimulated and in the sheep-udder slices inhibited oxidation of acetate. Oxaloacetate, which stimulated the oxygen consumption and fatty-acid synthesis in the slices from both species, had very little effect on acetate oxidation in the rat slices and was inhibitory in the ruminant tissue. Similar effects of oxaloacetate (stimulation of fatty-acid synthesis from acetate and inhibition of acetate oxidation) have been observed in ovarian tissues from the laying hen (Popják & Tietz, 1953b).

Fatty-acid synthesis from [carboxy- ^{14}C]acetate by cell-free suspensions of rat mammary gland

Suspensions, like slices, of rat mammary gland incorporated only traces of acetate into fatty acids when acetate, or acetate and ATP, were the only substrates added to the incubation medium. Glucose either alone or in combination with ATP did not promote fatty-acid synthesis in suspensions, in contrast to its behaviour in slices (Table 3).

The highest rate of incorporation of acetate into fatty acids by these suspensions has so far been observed in the presence of oxaloacetate and ATP (Table 4). Oxaloacetate alone showed a slight stimulation in most suspensions, but really significant fatty-acid synthesis occurred only when ATP was also added. The amount of ^{14}C incorporated into fatty acids by the most active suspensions under these conditions was about 50% of that used by slices in the presence of oxaloacetate. Heating for

Table 3. *Fatty-acid synthesis from [carboxy- ^{14}C]-acetate by suspensions of rat mammary gland in presence of glucose and ATP*

(The results are expressed as $\mu\text{moles} \times 10^{-3}$ of acetate incorporated into fatty acids/100 mg. dry weight of suspension.)

	Additions		
	None	ATP (0.01M)	Glucose (0.3%)
	3.79	2.72	4.76
	3.09	3.09	3.20
			Glucose + ATP
			5.43
			1.05

Table 4. *Effect of oxaloacetate and ATP on fatty-acid synthesis from [carboxy- ^{14}C]acetate by suspensions of rat mammary gland*

(The results are expressed as $\mu\text{moles} \times 10^{-3}$ of acetate used for fatty acid synthesis/100 mg. dry weight of suspension.)

	Additions		
	None	ATP (0.01M)	Oxaloacetate (0.02M)
	3.79	2.72	1.56
	3.09	3.09	5.54
	2.59	1.30	15.73
	2.63	2.25	10.30
	—	—	2.69
	2.56	2.56	7.20
			Oxaloacetate + ATP
			10.05
			12.10
			119.40
			69.50
			20.20
			69.70

5 min. in a boiling-water bath abolished completely the ability of the suspension to incorporate [^{14}C]-acetate into fatty acids.

The gas phase used in the incubation had a great effect on fatty-acid synthesis and on the oxygen consumption of the suspensions. As can be seen from the results shown in Table 5, the amount of acetate incorporated into fatty acids was more than doubled when the gas phase was changed from pure oxygen to air. At the same time the $-Q_{O_2}$ increased from about 1.7–1.9 to 2.2–2.7. Incubation under nitrogen on the other hand caused an 80–90% inhibition of fatty-acid synthesis (Table 5; Expts. no. 83, 89). In the two experiments carried out under nitrogen the gas was first bubbled through the suspensions (kept in ice) for 15 min. and then, after the addition of the suspensions to the incubation flasks, the contents of the latter were equilibrated with nitrogen in the water bath for 10 min. It was thought that the slight but definite fatty-acid synthesis observed in these two experiments might have been due to a lack of completely anaerobic conditions. The experiment was therefore repeated in such a way that the suspension, after saturation with nitrogen, was incubated first with oxaloacetate for 15 min. and the acetate and ATP were then tipped in from the side arm. The results (not shown in the Table) were the same, i.e. only 90% inhibition of synthesis was observed. Citrate alone or in

Table 5. *Effect of gas phase and citrate on fatty-acid synthesis and on oxygen consumption of suspensions of rat mammary gland*

(Fatty acid synthesis expressed as $\mu\text{moles} \times 10^{-3}$ of acetate incorporated into fatty acids/100 mg. dry weight of suspension. Final concentrations of additions were: oxaloacetate and citrate, 0.02 M; ATP, 0.01 M.)

Expt. no.	Additions	Gas phase				
		O ₂		Air		N ₂
		-Q _{O₂}	Fatty-acid synthesis	-Q _{O₂}	Fatty-acid synthesis	Fatty acid synthesis
83	(a) Oxaloacetate + ATP	1.67	36.7	2.22	91.5	11.2
	(b) Citrate	0.64	4.5	—	—	—
	(c) Citrate + ATP	1.97	5.3	—	—	—
89	Oxaloacetate + ATP	—	—	2.39	119.4	11.8
110	(a) Oxaloacetate + ATP	1.91	38.7	2.58	87.0	—
	(b) Oxaloacetate + ATP	1.86	43.5	2.49	97.0	—
	(c) Oxaloacetate + citrate + ATP	1.80	36.3	2.75	89.2	—
	(d) Oxaloacetate + citrate + ATP	1.93	31.7	2.50	84.6	—

combination with ATP was unable to stimulate fatty-acid synthesis (Table 5, Expt. no. 83); the amounts of ^{14}C incorporated into fatty acids by the suspensions in the presence of citrate were as low as were usually found when acetate or acetate and ATP were the only substrates added to the incubation medium. Furthermore, the addition of citrate to oxaloacetate and ATP did not affect the results obtained in the presence of the two last-named substances alone (Table 5, Expt. no. 110c, d). Experiment no. 110, which was carried out in duplicate, revealed also that the results with a particular suspension were reproducible to within about 10%.

α -Oxoglutarate, in contrast to oxaloacetate, stimulated fatty-acid synthesis to a significant degree even in the absence of added ATP; nevertheless ATP when added to α -oxoglutarate caused a further stimulation, particularly when the gas phase was air (Table 6). Pyruvate was also quite effective especially in combination with ATP, although the amount of acetate used for fatty-acid synthesis was not as great as in the presence of oxaloacetate and ATP (Table 7). As can be seen from the results shown in the same Table, succinate had no effect whatever on fatty-acid synthesis. Similarly negative results were obtained with malate and glutamate.

Since Balmain *et al.* (1952) have shown that glycerol stimulated fatty-acid synthesis from acetate in slices of rat mammary gland, glycerol was also tested in the suspensions, but either alone or in combination with other substances was found to have no effect on fatty-acid synthesis (Table 8).

Table 6. *Comparison of the effects of α -oxoglutarate and oxaloacetate on fatty-acid synthesis from [carboxy- ^{14}C]acetate by suspensions of rat mammary gland*

(Results are expressed as $\mu\text{moles} \times 10^{-3}$ of acetate used for fatty-acid synthesis/100 mg. dry weight of suspension. Final concentrations of additions were: oxaloacetate and α -oxoglutarate, 0.02 M; ATP, 0.01 M.)

Additions	Expt. no. 83 Gas phase, O ₂	Expt. no. 89 Gas phase, air
None	—	2.54
ATP	—	1.30
Oxaloacetate	—	15.73
Oxaloacetate + ATP	36.7	119.40
α -Oxoglutarate	30.5	31.90
α -Oxoglutarate + ATP	34.6	74.70

Table 7. *Comparison of the effects of pyruvate and succinate with that of oxaloacetate on fatty-acid synthesis from [carboxy- ^{14}C]acetate by suspensions of rat mammary gland*

(Results are expressed as $\mu\text{moles} \times 10^{-3}$ acetate used for fatty-acid synthesis/100 mg. dry weight of suspension. Final concentrations of additions: oxaloacetate, pyruvate and succinate, 0.02 M; ATP, 0.01 M.)

Additions	Expt. no. 90
None	2.63
ATP	2.25
Oxaloacetate	10.30
Oxaloacetate + ATP	69.50
Pyruvate	18.40
Pyruvate + ATP	34.60
Succinate	3.11
Succinate + ATP	2.05

Table 8. *Comparison of the effect of glycerol and oxaloacetate on fatty-acid synthesis from [carboxy- ^{14}C]acetate by suspensions of rat mammary gland*

(Fatty acid synthesis expressed as $\mu\text{moles} \times 10^{-3}$ of acetate used/100 mg. dry weight of suspension. Expt. no. 93. Final concentrations of additions: ATP, 0.01M; glycerol, 0.002M; oxaloacetate, 0.02M.)

Additions	Fatty acid synthesis
None	2.56
ATP	2.56
Glycerol	2.75
Glycerol + ATP	2.48
Oxaloacetate	7.20
Oxaloacetate + ATP	69.70
Oxaloacetate + glycerol + ATP	57.20

Table 9. *Fatty-acid synthesis from [carboxy- ^{14}C]acetate by suspensions of sheep udder*

(Results are expressed as $\mu\text{moles} \times 10^{-3}$ of acetate used for fatty-acid synthesis/100 mg. dry weight of suspension. In (a), the suspension was prepared from fresh udder tissue; in (b) the suspension was prepared from the same udder tissue as in (a) but the tissue was kept frozen in solid CO_2 for 5 days. Final concentration of substrates, 0.02M.)

Substrate added	Without ATP	With ATP (0.01M)
Acetate (a)	69.4	10.2
Acetate (b)	46.8	6.8
Acetate + oxaloacetate (a)	111.0	62.3

Fatty-acid synthesis from [carboxy- ^{14}C]acetate by suspensions of sheep udder

Suspensions of sheep udder, like slices of this tissue, can use acetate for fatty-acid synthesis very effectively without any further stimulant, although the addition of oxaloacetate to the medium nearly doubled the amount of acetate incorporated into fatty acids (Table 9). The amounts of acetate utilized by the sheep-udder suspension were very similar to those used by the suspensions of rat tissue in the presence of oxaloacetate and ATP. However, ATP in a concentration of 0.01M acted in the sheep-udder suspension as a strong inhibitor of fatty-acid synthesis and of respiration also (see below). The suspension prepared from the udder tissue kept frozen in solid carbon dioxide for 5 days was almost as active as that made from the fresh udder in respect of fatty-acid synthesis.

Oxygen consumption of cell-free suspensions of rat mammary gland and of sheep udder

In every experiment the oxygen consumption of the suspensions was measured for the whole incubation period (3 hr.). The oxygen uptakes could not under all experimental conditions be related directly to fatty-acid synthesis. For example,

usually the highest values of $-Q_{O_2}$ were observed when succinate was added to the suspensions, no doubt on account of the high succinic dehydrogenase activity of the preparations (cf. Moore & Nelson, 1952), yet no significant fatty-acid synthesis occurred. For this reason we wish to record only some of the results which illustrate the different responses of the suspensions made from the rat and sheep tissues to ATP (Figs. 1 and 2). In the suspensions of rat mammary gland the effect of ATP was to increase very significantly the oxygen uptake, whereas in the sheep-udder suspension ATP caused a dramatic inhibition of oxygen uptake. We do not think that the simultaneous inhibition of fatty-acid synthesis by ATP in sheep-udder suspension (Table 9) is necessarily connected with the lowered respiration of the preparation because, as can be seen from Fig. 2, oxaloacetate had an even greater inhibiting effect on the oxygen consumption than ATP yet it nearly doubled the amount of acetate incorporated into fatty acids (Table 9).

The addition of acetate (0.02M) in all suspensions, from either rat or sheep tissues, invariably depressed the oxygen consumption of the preparations. This phenomenon again does not reflect the ability or inability of the suspensions to oxidize acetate. Suspensions of rat mammary gland oxidized only traces of acetate, whereas the preparation from the fresh sheep udder oxidized 0.19 μmoles of acetate/100 mg. dry weight, but in the presence of ATP (0.01M) only 0.06 μmoles of acetate were oxidized/100 mg. dry weight. Oxaloacetate diminished the amount of acetate oxidized by the suspension of sheep udder to an almost negligible level, an effect qualitatively similar to that observed in slices.

Type of fatty acids synthesized by suspensions of rat mammary gland

The fatty acids extracted from four rat mammary suspensions after incubation with [carboxy- ^{14}C]acetate in the presence of oxaloacetate and ATP were pooled and resolved into individual acids by chromatography as described under Methods. The results are shown in Table 10. All the saturated *n*-fatty acids containing an even number of carbon atoms from 6 to 18 and also oleic acid were obtained. We suspect that the small amount of butyric acid, which we expected to find, was lost during the various manipulations owing to the solubility in water and volatility of this acid. Since all the acids were radioactive it may be concluded that the preparations synthesize both short- and long-chain fatty acids. The figures in the last column of Table 10 show that most of the ^{14}C found in the fatty acids (about 91%) could be accounted for by four fractions, namely, caproic, myristic, palmitic and oleic acids. It can be seen also from the data of Table 10

Fig. 1. The effect of substrates and ATP on O_2 consumption of rat mammary-gland suspensions. The additions were as indicated over the curves.

Table 10. *Type of fatty acids synthesized from [carboxy- ^{14}C]acetate by suspensions of rat mammary gland in the presence of oxaloacetate and ATP*

Fatty acid	Specific radioactivity (counts/min./C in infinite thickness)	Fatty-acid fraction as % of total	^{14}C re-covered in fraction as % of total
Hexanoic	6060	3.72	24.00
Octanoic	603	1.60	1.12
Decanoic	325	5.75	2.27
Dodecanoic	657	6.20	4.92
Tetradecanoic	925	11.60	13.62
Hexadecanoic	960	28.80	35.63
Octadecanoic	108	7.38	0.82
Octadec-9-enoic	382	35.00	17.62

that the fatty acids were not synthesized strictly in the proportions in which they occurred in the suspensions.

DISCUSSION

The work of Brady & Gurin (1952*a, b*), Dituri & Gurin (1953) and the results presented here demonstrate that the integrated structure of the cell is not required for fatty-acid synthesis. Whether or not a net increase occurred in the amount of fatty acids in our preparation, or in those of the American workers, cannot be decided at present. The measurement of the respiratory quotients of suspensions might have given this information (cf. Folley, 1952).

Fig. 2. The effects of acetate, oxaloacetate and ATP on O_2 consumption of a sheep-udder suspension. Curve marked (a) (b) (c) indicates the O_2 consumption under three experimental conditions, namely (a) ATP alone; (b) acetate + ATP; and (c) acetate + oxaloacetate + ATP.

Our experiments with the suspensions of rat mammary gland show that three conditions are required in this system for fatty-acid synthesis from acetate: (a) aerobic incubation; (b) the oxidation of either of the three keto acids, pyruvate, oxaloacetate or α -oxoglutarate and (c) the addition of ATP, although strictly the latter is not necessary with pyruvate or α -oxoglutarate. In suspensions of sheep udder neither the addition of the keto acids nor of ATP is required for fatty-acid synthesis from acetate, although oxaloacetate had a stimulating effect. The species differences in response to ATP are at present intriguing. Brady & Gurin (1952*b*) reported that in suspensions of pigeon liver and in a soluble enzyme preparation made from it ATP had either no effect on fatty-acid synthesis or in concentrations greater than $1 \times 10^{-3} M$ was inhibitory. There appear to be some significant differences between the mammary gland preparations and those made from pigeon liver. According to Brady & Gurin (1952*b*) the maximum stimulation of fatty-acid synthesis in the pigeon-liver preparation was observed in the presence of citrate, which in our system had no effect whatever; oxaloacetate, which showed the greatest stimulation in the mammary-

gland suspensions, had but a slight effect in the pigeon-liver suspensions. (It should, however, be pointed out that in their preliminary communication Brady & Gurin (1952*a*) reported that oxaloacetate had the greatest stimulating effect on fatty-acid synthesis in the pigeon-liver suspensions and that citrate was inhibitory.)

A further significant difference is that whereas in the suspensions of mammary gland incubation under nitrogen caused an 80–90% inhibition of fatty-acid synthesis, and therefore the energy required for the synthesis presumably has been derived from the oxidation of substrates, in the pigeon-liver preparations anaerobic conditions had only a slight inhibitory effect or none at all. Although these differences seem to be great, it is not likely that they are due to differences in the principal reactions of biosynthesis of fatty acids in the two systems; it seems more probable that they reflect qualitative and quantitative differences in the preparations in respect of the enzymes required for the activation of acetate.

The reason for the very striking effect of the gas phase, namely, the increased fatty-acid synthesis and higher oxygen consumption of the suspensions when the gas phase was changed from pure oxygen to air, is at present obscure. It is possible that, under high oxygen tension, oxidation of —SH groups occurred, resulting in an inactivation of enzymes and/or coenzymes.

The species differences in the metabolism of the mammary gland, which were elucidated by Folley and his colleagues (see Folley, 1952) in experiments with tissue slices, are apparent even in the suspensions. For example, while suspensions of rat mammary gland are virtually unable to oxidize acetate, preparations from sheep udder were fairly efficient in this respect. It seems probable that in the mammary gland of the rodent there are blocks in the reactions of the citric acid cycle, which do not exist in the tissues of the ruminant. Such an assumption might explain why the sheep-udder preparation does not require the addition of ATP. On the other hand we cannot yet offer a satisfactory explanation for the marked inhibition of fatty-acid synthesis, of acetate oxidation and of oxygen consumption caused by ATP in the sheep-udder suspensions.

The value of the mammary-gland preparations in the study of the biosynthesis of fatty acids seems to us to lie in their ability to synthesize fatty acids of all chain lengths. The fact that the fatty acids in the suspensions of rat mammary gland were not synthesized strictly in the proportions in which they were present suggests that different enzymes might be involved in the synthesis of groups of acids of varying chain lengths. It is of interest to mention here that Green & Mii (1953) reported that in the

oxidation of fatty acids of different chain lengths from C_4 to C_{18} at least three enzymes covering specific ranges of the acids are involved. The stepwise increase in the specific activity of the fatty acids from C_{10} to C_{18} (cf. Table 10) resembles the findings in the milk fatty acids of the lactating goat (Popják, French, Hunter & Martin, 1951) in which it was shown that such a stepwise increase in the specific activity of the acids after injection of [*carboxy*- ^{14}C]acetate was due to the stepwise elongation of a shorter acid to a longer one.

SUMMARY

1. The biosynthesis of fatty acids from acetate in tissue slices and cell-free suspensions of rat and sheep mammary gland have been studied.

2. In tissue slices, in addition to glucose, pyruvate and members of the citric acid cycle stimulated fatty-acid synthesis from acetate. These substances were particularly effective with slices of sheep udder.

3. Cell-free suspensions of the mammary gland of lactating rats and sheep can synthesize fatty acids from [*carboxy*- ^{14}C]acetate.

4. In suspensions of rat mammary gland three conditions are required for fatty-acid synthesis: (a) aerobic incubation; (b) the oxidation of either pyruvate, oxaloacetate or α -oxoglutarate; and (c) the addition of adenosine triphosphate to some extent. The best results were obtained in the presence of oxaloacetate and adenosine triphosphate and when the gas phase was air instead of pure oxygen.

5. All the saturated *n*-fatty acids containing an even number of carbon atoms from 6 to 18 and also oleic acid were synthesized in the rat mammary suspensions.

6. Suspensions of sheep udder used acetate for fatty-acid synthesis even without further addition, although oxaloacetate had a stimulating effect in this preparation also, but adenosine triphosphate in a concentration of 0.01M inhibited fatty-acid synthesis.

7. While the suspension of sheep udder oxidized appreciable amounts of acetate, suspensions of rat mammary gland were virtually inactive in this respect.

8. The implications of the results are discussed.

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Paper-chromatographic Analysis of Dakin's Samples of ' β -Hydroxyglutamic Acid'

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Dakin twice claimed the isolation from proteins of β -hydroxyglutamic acid. In his first paper (Dakin, 1918) he obtained a 10.5% yield but later (Dakin, 1919) he reported more variable yields of 2–10%. The compound had been obtained by acid and by tryptic digestion of casein and also by acid hydrolysis of gliadin and glutenin. Many workers have been unable to repeat this work and doubt is now generally cast on its validity (Nicolet & Shinn, 1942; Bailey, Chibnall, Rees & Williams, 1943; Beveridge & Lucas, 1944). It is of note that Dakin himself in a footnote to one of his papers (Dakin, 1941) commented on the fact that one reaction of his amino acid was inconsistent with its structure as a β -hydroxy- α -amino acid and that he was therefore exploring further the possibility that it was a γ -hydroxy compound. The amino acid still, however, continues to occupy space in the standard textbooks, since, using classical methods of analysis and isolation, it is difficult to be certain of the absence from a protein of such a substance. The only certain way to assess the earlier work is to re-examine the original samples in question. Dr H. B. Vickery has now kindly obtained for us two samples of β -hydroxyglutamic acid from Dakin's collection. These we have analysed by the simpler and far more specific methods now available.

MATERIALS

Two of Dakin's specimens were obtained from the Dakin Collection, Adelphi College. They were labelled: (1) 'Hydroxyglutamic acid *ex* casein and trypsin.' (2) 'Hydroxyglutamic acid, analysed.' (1) was a mixture of brown particles and powder, (2) comprised 1 mg. only of brown particles. They will be referred to here as Dakin's specimens nos. 1 and 2, respectively.

For comparison with Dakin's samples, synthetic specimens of DL- β -hydroxyglutamic and DL-*allo*- β -hydroxy-

glutamic acids were obtained by Dr Vickery from Dr Pfister. Both substances were white crystalline powders. The methods of synthesis and the convention of nomenclature have been described by Leanza & Pfister (1953).

METHODS

The analyses were performed on 0.1% (w/v) solutions of the amino acids in 10% (v/v) aqueous isopropanol. The two Dakin specimens dissolved immediately in the solvent, whereas the two synthetic compounds only dissolved slowly after much shaking. The amino acids used as markers were in the same solvent at a concentration of 0.01 M.

One-way paper chromatograms were run with phenol:water in the presence of NH_3 and HCN. Two-way chromatograms were run with this system first followed by collidine:lutidine:water in the presence of diethylamine vapour. The apparatus and working details were as described by Dent (1948). Both the synthetic isomers of β -hydroxyglutamic acid gave brownish purple spots on the chromatogram which could be readily distinguished from the adjacent spots given by other amino acids.

Periodate treatment of the amino acids was carried out by adding about 2 vol. of saturated aqueous KIO_4 and 0.5 vol. of saturated aqueous Na_2CO_3 to 1 vol. of amino acid solution. The mixture was allowed to stand for 0.5 hr. and was then electrolytically desalted in a micromodification (Dent, 1952) of the apparatus of Consden, Gordon & Martin (1947).

RESULTS

Chromatograms of the various samples compared with authentic amino acids were prepared as shown on Pl. 1. They were prepared with the intention of showing whether Dakin's samples contained any β -hydroxyglutamic acids, whether they contained any other β -hydroxyamino acids or any of the commonly occurring amino acids, and if so which. The conclusions drawn from the analyses are written into the description of the Plate (p. 55).