3. Adenosine 5′-phosphate, L-aspartate, L-asparagine, fumarate and glycerol each stimulated 'factor' activity but had no effect on the enzymic hydrolysis of carbamyl phosphate. This stimulation generally increased with time.

4. Some characteristics of the bacterial hydrolysis of carbamyl phosphate are described.

5. The results indicate that the bacterial 'factor' is not a carbamylphosphatase.

## REFERENCES

Berenblum, I. & Chain, E. (1938). *Biochem. J.* **32**, 295.

Eggleston, L. V. (1957). *Biochem. J.* **65**, 735.

Gomori, G. (1955). In *Methods in Enzymology*, vol. 1, p. 143. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.

Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.

Jones, M. E., Spector, L. & Lipmann, F. (1955*a*). *Conférences et Rapports, III$^e$ Congr. Int. Biochim., Bruxelles*, p. 278.

Jones, M. E., Spector, L. & Lipmann, F. (1955*b*). *J. Amer. chem. Soc.* **77**, 819.

Krebs, H. A., Eggleston, L. V. & Knivett, V. A. (1955). *Biochem. J.* **59**, 185.

Lowry, O. H. & Lopez, J. A. (1946). *J. biol. Chem.* **162**, 421.

Peel, J. L., Fox, M. & Elsden, S. R. (1955). *Biochem. J.* **60**, xxxiii.

Stafford, A., Watson, T. R. & Rand, M. J. (1955). *Biochim. biophys. Acta*, **18**, 319.

# The Formation of Acetoacetate in Homogenates of the Mammary Gland

By C. TERNER

*National Institute for Research in Dairying, University of Reading, England, and The Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts, U.S.A.**

(*Received* 10 *October* 1957)

Apart from the liver, which is generally considered the principal site of ketogenesis (see Weinhouse, 1952; Campbell & Best, 1956), the rumen is also recognized as a source of the ketone bodies circulating in the blood of ruminants (see Shaw, 1956).

Mammary tissue, which possesses high lipogenic activity (Balmain, Folley & Glascock, 1952) and which is able to oxidize acetoacetate (Terner, 1955), must synthesize acetoacetyl-coenzyme A as an intermediate; this may give rise to ketone bodies. As reported in previous papers (Terner, 1954, 1956*b*), the addition of fumarate in increasing amounts to respiring mammary homogenates metabolizing $^{14}$C-labelled acetate or pyruvate resulted in a progressive increase in the rate of fatty acid synthesis. In continuation of this work it was found that, when the rate of fatty acid formation was limited by decreasing the amount of added fumarate, acetoacetate was formed from acetate or pyruvate. In contrast with the powerful ketogenicity of pyruvate and acetate, glucose and lactate displayed only weak ketogenic activity. This analogous metabolic behaviour of glucose and lactate offers an explanation for the differences in ketogenic activity of the various substrates in terms of their ability to maintain an adequate level of reduced pyridine nucleotides in an actively oxidative environment and suggests a mechanism for the well-known antiketogenic properties of glucose. The results further indicate that the lactating mammary gland may deserve consideration as a site of ketone-body production in dairy animals suffering from ketosis. A preliminary account of part of this work has been given (Terner, 1956*c*, 1957).

* Present address.

## EXPERIMENTAL

The material, reaction mixtures, reagents and methods were the same as described in the earlier paper on lipogenesis in guinea-pig mammary homogenates (Terner, 1956*b*). Whole homogenates of mammary tissue were used; kidney-cortex homogenates were centrifuged at low speed for a few minutes to remove broken cells and nuclei.

*Determination of acetoacetate.* This was determined manometrically by the aniline method (Ostern, 1933; Edson, 1935) as modified by Krebs & Eggleston (1945). When radioactive substrates were employed, the 'bound $CO_2$' was first liberated from the medium by the addition of 1 ml. of a mixture of equal volumes of 50% citric acid and 3N-perchloric acid. The $CO_2$ was absorbed during the next 30 min. period in the NaOH in the centre wells of manometer flasks which had served to absorb the $CO_2$ during the measurements of $O_2$ consumption. The contents of the centre wells were transferred to a few millilitres of water in test tubes. The centre wells were washed with dil. HCl and charged with fresh portions (0·2 ml.) of 2N-NaOH and a strip of filter paper. The acetoacetate was then decarboxylated by adding 1 ml. of aniline citrate from the side arms of the vessels. After the reaction was complete, the contents of the centre wells were transferred to a second set of test tubes. Since aniline will also decarboxylate oxaloacetate, treatment with $Al_2(SO_4)_3$ (Krebs & Eggleston, 1945) was in some experiments interposed between the collection of the respiratory $CO_2$ and the addition of

aniline citrate. No radioactive $CO_2$ was liberated by the treatment with $Al_2(SO_4)_3$.

*Determination of radioactivity.* Carrier $K_2CO_3$ was added to all test tubes containing the NaOH from the centre wells and the carbonate precipitated with $BaCl_2$. The $BaCO_3$ was washed, dried, weighed and plated on planchets of 2·0 or 4·9 cm.$^2$ area. In earlier experiments 'infinitely thick' plates were prepared and counted with an end-window counter. The counting rates of thin samples prepared in later work were determined with a gas-flow counter and corrected for self-absorption. The specific activity of the radioactive substrates was determined by wet combustion of the material and counting as $BaCO_3$. The results were calculated in terms of amount of substrate appearing in the products. In calculating the amount of randomly labelled glucose oxidized to $CO_2$ and water, a correction was applied for the appearance in the respiratory $CO_2$ of C-3 and C-4 of that fraction of glucose which gave rise to $^{14}C$-labelled acetoacetate. Although the radioactivity of only the terminal carboxyl group of acetoacetate was determined, it was assumed that both halves of the molecule were equally labelled and the radioactive acetoacetate was calculated as being derived from two molecules of radioactive pyruvate, lactate or acetate, and from one molecule of randomly labelled glucose respectively.

All labelled compounds were obtained from the Radiochemical Centre, Amersham.

*Units.* Respiratory rates are expressed as $Q_{O_2}$ (μl./mg. dry wt./hr.). $Q_{ac.-ac.}$ denotes the rate of change in acetoacetate content of the reaction mixture as determined by the manometric method (1 μmole of acetoacetate = 22·4 μl.). Data derived from measurements of radioactivity are expressed as μm-moles/100 mg. dry wt./hr. The dry weights of the homogenates were determined by evaporation in a steam oven and were corrected for the salt content of the diluting fluid.

## RESULTS

*Acetoacetate formation and fatty acid synthesis in mammary homogenates.* Experiments reported in this paper reveal an inverse relationship between the rate of acetoacetate formation by mammary homogenates and fumarate concentration. As shown in Table 1, the amount of pyruvate carbon appearing in acetoacetate decreased progressively with the increase in fumarate concentration. The effect of fumarate on fatty acid and acetoacetate formation from acetate is shown in Table 2. In addition to the isotopic data, a small but definite net increase in the amount of acetoacetate in the absence of fumarate, determined by the manometric technique, indicated a net synthesis of the ketone body.

*Effect of inhibitors on acetoacetate formation.* Since, even in the absence of added fumarate, substrate carbon appeared in the respiratory $CO_2$, the Krebs cycle was functioning, if only to a limited extent. Previous studies of the effects of various inhibitors on aerobic phosphorylation and lipogenesis in mammary homogenates (Terner, 1956*a*, *b*) were therefore extended to include their effects on the formation of acetoacetate. The addition of

Table 1. *Effect of fumarate on formation of acetoacetate from pyruvate*

Mammary homogenate (dry wt. 42·5 mg.), basal medium, [2-$^{14}C$]pyruvate (0·01 M) and acetoacetate (0·01 M) in all flasks. Incubation period was 75 min.

| Additions | $-Q_{O_2}$ | Pyruvate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory $CO_2$ | Pyruvate (μm-moles/100 mg. dry wt./hr.) appearing in: Acetoacetate |
|---|---|---|---|
| None | 7·9 | 4800 | 4350 |
| Fumarate, 1 mM | 7·2 | 2160 | 1380 |
| Fumarate, 2 mM | 6·5 | 1060 | 1180 |
| Fumarate, 5 mM | 6·5 | 470 | 320 |
| Fumarate, 10 mM | 6·6 | 320 | 210 |

*p*-nitrophenol (0·2 mM) strongly depressed the incorporation of acetate carbon into acetoacetate, but retarded to a lesser extent its appearance in the respiratory $CO_2$ (Table 2). When [2-$^{14}C$]pyruvate was the substrate, the inhibitory action of *p*-nitrophenol on the rate of labelling of acetoacetate was relatively small, the radioactivity of the respiratory $CO_2$ being reduced somewhat more. The addition of fluoride (20 mM) also inhibited the appearance of pyruvate carbon in the respiratory $CO_2$ to a greater extent than its entry into acetoacetate. Arsenate (5–25 mM), especially when added in high concentration, diminished the formation of acetoacetate from pyruvate (Table 3).

*Effect of the presence of carrier acetoacetate on metabolism of mammary homogenates.* In order to determine the extent of dilution of [$^{14}C$]acetyl-coenzyme A, derived from the labelled substrates, by the acetyl-coenzyme A arising from the enzymic breakdown of initially unlabelled carrier acetoacetate, experiments were carried out in which all experimental conditions were duplicated, except that the carrier was either added or omitted. As shown in Table 4, the respiratory $CO_2$ contained more labelled carbon in the absence of the carrier than in its presence, as may be expected if, in the former case, some of the undiluted [$^{14}C$]acetoacetate had been broken down either enzymically during the incubation period, or spontaneously during the incubation period and during the subsequent period allowed for the absorption of the 'bound $CO_2$' (see Experimental). In the presence of fumarate, the radioactivity of the fatty acids and of the respiratory $CO_2$ was only slightly diminished by the presence of acetoacetate and only a small amount of the substrate carbon was trapped in the carrier.

*Effect of octanoate and glucose.* For the study of the effect on the present system of precursors of acetyl-coenzyme A of known ketogenic and anti-ketogenic properties, octanoate and glucose were

chosen. The addition of unlabelled octanoate to mammary homogenates metabolizing [*carboxy*-$^{14}$C]-acetate in the absence of fumarate resulted in a marked diminution, presumably by isotope dilution, of the radioactivity of the acetoacetate formed, accompanied by a much smaller decrease in the radioactivity of the respiratory $CO_2$ (Table 5). This finding is consistent with the ketogenic property of the fatty acid. The effect of glucose, however, was variable. In many preparations the addition of unlabelled glucose had almost no effect on the rate of appearance of [*carboxy*-$^{14}$C]-acetate carbon in acetoacetate, although the activity of the respiratory $CO_2$ was depressed.

Table 2. *Synthesis of fatty acids and acetoacetate from acetate*

Mammary homogenate (dry wt. 37·4 mg.), basal medium, [*carboxy*-$^{14}$C]acetate (5 mM) and acetoacetate (5 mM) in all flasks. Fumarate, 5 mM. Incubation period was 50 min.

| Additions | $-Q_{O_2}$ | $Q_{ac.-ac.}$ | Acetate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory $CO_2$ | Acetoacetate | Fatty acids |
|---|---|---|---|---|---|
| None | 7·3 | +0·2 | 6880 | 2930 | 58 |
| *p*-Nitrophenol, 0·2 mM | 5·5 | −0·4 | 4460 | 186 | 48 |
| Fumarate | 7·2 | −1·4 | 2210 | 250 | 1060 |
| Fumarate; *p*-nitrophenol, 0·2 mM | 5·7 | −0·5 | 1365 | 71 | 173 |
| Fumarate; *p*-nitrophenol, 0·4 mM | 3·8 | −0·3 | 404 | 45 | 0 |

Table 3. *Effect of inhibitors on formation of acetoacetate from pyruvate*

Mammary homogenate (dry wt. 62 mg.), basal medium, [2-$^{14}$C]pyruvate (7·5 mM) and acetoacetate (10 mM) in all flasks. Fluoride, 20 mM. Incubation period was 65 min.

| Additions | $-Q_{O_2}$ | Pyruvate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory $CO_2$ | Acetoacetate |
|---|---|---|---|
| None | 7·0 | 2940 | 3900 |
| *p*-Nitrophenol, 0·2 mM | 4·0 | 1280 | 2500 |
| Fluoride | 4·2 | 1030 | 3100 |
| Arsenate, 5 mM | 5·1 | 2280 | 2320 |
| Arsenate, 10 mM | 5·0 | 2060 | 2760 |
| Arsenate, 25 mM | 3·8 | 970 | 1800 |

Table 4. *Effect of carrier acetoacetate on metabolism of acetate*

Mammary homogenate (dry wt. 52·2 mg.), basal medium and [*carboxy*-$^{14}$C]acetate (5 mM) in all flasks. Additions: acetoacetate, 5 mM; fumarate, 5 mM. Incubation period was 55 min.

| Additions | $-Q_{O_2}$ | Acetate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory $CO_2$ | Acetoacetate | Fatty acids |
|---|---|---|---|---|
| None | 7·0 | 6600 | — | 60 |
| Acetoacetate | 7·6 | 4260 | 3200 | 20 |
| Fumarate | 9·0 | 1510 | — | 1840 |
| Acetoacetate, fumarate | 8·8 | 1090 | 150 | 1560 |

Table 5. *Fatty acid oxidation and acetoacetate formation*

Mammary homogenate (dry wt. 52 mg.), basal medium, [*carboxy*-$^{14}$C]acetate (7·5 mM) and acetoacetate (10 mM) in all flasks. Octanoate, 2·5 mM. Incubation period was 60 min.

| Additions | $-Q_{O_2}$ | Acetate (μm-moles/100 mg. dry wt./hr.) appearing in: Respiratory $CO_2$ | Acetoacetate |
|---|---|---|---|
| None | 6·7 | 5330 | 2280 |
| Octanoate | 6·7 | 4620 | 1090 |
| Fumarate, mM | 7·4 | 3930 | 1420 |
| Fumarate, mM; octanoate | 8·5 | 3590 | 534 |
| Fumarate, 2 mM | 8·2 | 2510 | 442 |
| Fumarate, 2 mM; octanoate | 9·0 | 2160 | 411 |
| Fumarate, 5 mM | 8·4 | 1245 | 250 |
| Fumarate, 5 mM; octanoate | 9·9 | 1230 | 169 |

When the rate of glucose breakdown was accelerated by the addition of hexokinase, the radioactivity of both the respiratory $CO_2$ and acetoacetate was diminished (Table 6), as might be expected to be the result of isotope dilution of the [$^{14}$C]acetyl-coenzyme A. In homogenates in which the rate of acetoacetate formation was slow in relation to the rate of $^{14}CO_2$ production, glucose increased the rate of incorporation of [*carboxy*-$^{14}$C]-acetate carbon into both the respiratory $CO_2$ and acetoacetate (Table 7). In the presence of glucose, the addition of *p*-nitrophenol inhibited the formation of acetoacetate from [*carboxy*-$^{14}$C]acetate to a much greater extent than it depressed the appearance of $^{14}$C in the respiratory $CO_2$ (Table 8).

*Glucose, pyruvate, lactate and acetate as precursors of acetoacetate.* A comparison of the rates of appearance in acetoacetate of $^{14}$C from labelled glucose, pyruvate and acetate showed that pyruvate and acetate were readily converted into acetoacetate. In many experiments acetate entered acetoacetate as rapidly as it appeared in the respiratory $CO_2$. Pyruvate appeared to show even stronger ketogenic tendencies, the amount of its carbon recovered from the ketone body often exceeding the amount oxidized to $CO_2$. The opposite was observed when [$^{14}$C]glucose (randomly labelled) was the only added substrate; only a small part of its carbon was found in acetoacetate, and by far the larger part appeared in the respiratory $CO_2$. It appears from Table 10 that glucose may undergo oxidation to $CO_2$ more readily than the other substrates examined. This may, however, be due to the contribution of C-3 and C-4 of that part of [$^{14}$C]glucose that may have been converted into fatty acids and for which no correction was applied. The low rate of conversion of glucose into acetoacetate was only slightly, if at all, increased

Table 6. *Effect of glucose and hexokinase on formation of acetoacetate from acetate*

Mammary homogenate (dry wt. 27·8 mg.), basal medium, [*carboxy*-$^{14}$C]acetate (4 mM) and acetoacetate (10 mM) in all flasks. Glucose, 20 mM. Incubation period was 60 min.

| Additions | $-Q_{O_2}$ | Acetate (μm-moles/100 mg. dry wt./hr.) appearing in | |
|---|---|---|---|
| | | Respiratory $CO_2$ | Acetoacetate |
| None | 8·4 | 4140 | 2470 |
| Glucose | 8·3 | 2700 | 2430 |
| Glucose; hexokinase, 4·5 units | 11·2 | 2720 | 2360 |
| Glucose; hexokinase, 9 units | 10·1 | 1990 | 1540 |
| Glucose; hexokinase, 18 units | 9·5 | 1070 | 830 |

Table 7. *Stimulation by glucose of acetoacetate formation from acetate*

Mammary homogenate (dry wt. 23·5 mg.), basal medium, [*carboxy*-$^{14}$C]acetate (5 mM) and acetoacetate (10 mM) in all flasks. Glucose, 20 mM. Incubation period was 85 min.

| Additions | $-Q_{O_2}$ | Acetate (μm-moles/100 mg. dry wt./hr.) appearing in | |
|---|---|---|---|
| | | Respiratory $CO_2$ | Acetoacetate |
| None | 4·0 | 2340 | 262 |
| Glucose | 8·0 | 3660 | 1520 |
| Fumarate, 1 mM | 7·5 | 3760 | 274 |
| Fumarate, 1 mM; glucose | 11·0 | 3660 | 1130 |
| Fumarate, 2 mM | 7·8 | 2140 | 156 |
| Fumarate, 2 mM; glucose | 9·2 | 1870 | 223 |

Table 8. *Effect of glucose and* p*-nitrophenol on formation of acetoacetate from acetate*

Mammary homogenate (dry wt. 59 mg.), basal medium, [*carboxy*-$^{14}$C]acetate (5 mM) and acetoacetate (10 mM) in all flasks. Glucose, 5 mM. Hexokinase, 18 units. Incubation period was 60 min.

| Additions | $-Q_{O_2}$ | Acetate (μm-moles/100 mg. dry wt./hr.) appearing in | |
|---|---|---|---|
| | | Respiratory $CO_2$ | Acetoacetate |
| None | 6·5 | 3650 | 1825 |
| Glucose | 7·3 | 2900 | 2670 |
| Glucose; *p*-nitrophenol, 0·2 mM | 6·0 | 1650 | 278 |
| Glucose; hexokinase | 7·6 | 2540 | 1615 |
| Glucose; hexokinase, *p*-nitrophenol, 0·2 mM | 7·4 | 1860 | 410 |
| Glucose; hexokinase, *p*-nitrophenol, 0·4 mM | 5·6 | 1590 | 186 |

Table 9. *Formation of acetoacetate from glucose and acetate*

Mammary homogenate (dry wt. 34 mg.), basal medium and acetoacetate (10 mM) in all flasks. Additions: [$^{14}$C]glucose (randomly labelled), 5 mM; unlabelled glucose, 5 mM; [*carboxy*-$^{14}$C]acetate, 5 mM; hexokinase, 18 units. Incubation period was 60 min.

| Additions | $-Q_{O_2}$ | Labelled substrate (acetyl-coenzyme A equivalents) (μm-moles/100 mg. dry wt./hr.) appearing in | |
|---|---|---|---|
| | | Respiratory $CO_2$ | Acetoacetate |
| [$^{14}$C]Glucose | 8·4 | 2135 | 585 |
| [$^{14}$C]Glucose, hexokinase | 10·3 | 4570 | 870 |
| [$^{14}$C]Acetate | 6·8 | 2040 | 2200 |
| [$^{14}$C]Acetate, glucose | 8·0 | 785 | 2220 |
| [$^{14}$C]Acetate, glucose, hexokinase | 9·4 | 756 | 1925 |

Table 10. *Formation of acetoacetate from pyruvate, lactate and glucose*

Mammary homogenate (dry wt. 15·8 mg.), basal medium and acetoacetate (10 mM) in all flasks. Additions: [2-$^{14}$C]-pyruvate, 5 mM; [2-$^{14}$C]DL-lactate, 5 mM; [$^{14}$C]glucose (randomly labelled), 5 mM; hexokinase, 18 units; *p*-nitrophenol, 0·2 mM. Incubation period was 80 min.

| Additions | $-Q_{O_2}$ | Labelled substrate (acetyl-coenzyme A equivalents) (μm-moles/100 mg. dry wt./hr.) appearing in | |
|---|---|---|---|
| | | Respiratory $CO_2$ | Acetoacetate |
| [2-$^{14}$C]Pyruvate | 8·3 | 3010 | 5320 |
| [2-$^{14}$C]Pyruvate, *p*-nitrophenol | 5·4 | 2190 | 4440 |
| [2-$^{14}$C]Lactate | 5·9 | 3580 | 812 |
| [2-$^{14}$C]Lactate, *p*-nitrophenol | 4·5 | 2380 | 332 |
| [$^{14}$C]Glucose | 8·8 | 4300 | 700 |
| [$^{14}$C]Glucose, *p*-nitrophenol | 8·1 | 5300 | 590 |
| [$^{14}$C]Glucose, hexokinase | 10·2 | 6000 | 780 |
| [$^{14}$C]Glucose, hexokinase, *p*-nitrophenol | 10·2 | 8200 | 710 |

Table 11. *Acetoacetate formation by kidney cortex*

Rabbit kidney-cortex homogenate (dry wt. 19·8 mg.), basal medium and acetoacetate (10 mM) in all flasks. Additions: [$^{14}$C]glucose (randomly labelled), 5 mM; [2-$^{14}$C]-pyruvate, 5 mM; [2-$^{14}$C]DL-lactate, 10 mM; [*carboxy*-$^{14}$C]-acetate, 5 mM. Incubation period was 50 min.

| Additions | $-Q_{O_2}$ | Labelled substrate (acetyl-coenzyme A equivalents) (μm-moles/100 mg. dry wt./hr.) appearing in | |
|---|---|---|---|
| | | Respiratory $CO_2$ | Acetoacetate |
| [$^{14}$C]Glucose | 20·0 | 13 280 | 2 090 |
| [2-$^{14}$C]Pyruvate | 15·9 | 4 770 | 11 160 |
| [2-$^{14}$C]Lactate | 17·0 | 7 920 | 1 430 |
| [1-$^{14}$C]Acetate | 17·6 | 8 620 | 9 680 |

by the addition of hexokinase, or of *p*-nitrophenol, or both, although these agents markedly accelerated the rate of $^{14}CO_2$ formation from glucose (Tables 9 and 10). In marked contrast with the powerful ketogenic properties of pyruvate and acetate, [2-$^{14}$C]lactate gave rise to only a small amount of acetoacetate, in a pattern strongly resembling that of glucose (Table 10). Similar observations were made when homogenates of rabbit-kidney cortex were substituted for mammary homogenates (Table 11).

## DISCUSSION

*Effect of fumarate on the pathways of acetyl-coenzyme A in mammary homogenates.* The appearance of radioactive-substrate carbon in the respiratory carbon dioxide shows that even in the absence of added fumarate the Krebs cycle was operative and supplied sufficient energy for the activation of acetate. Endogenous precursors of oxaloacetate were present in the preparation in only low concentration, as evidenced by the marked effect of fumarate when added in catalytic amounts (1 mM). Further increases in the concentration of added fumarate (up to 5–10 mM) resulted in the progressive stimulation of fatty acid synthesis (Terner, 1956*b*) and at the same time in the suppression of acetoacetate formation. The progressive decline in the radioactivity of the respiratory carbon dioxide caused by increasing amounts of fumarate has been discussed before as due to isotope dilution of the $C_4$ dicarboxylic acids derived from the radioactive substrate when passing

through the fumarate stage of the cycle (Terner, 1956*b*), and should therefore not be regarded as indicating an inhibition of the oxidative pathway.

*Exchange of acetyl-coenzyme A or synthesis of acetoacetate.* Since acetoacetate is broken down by mammary homogenates (see Table 2), the possibility must be considered that the appearance of labelled acetoacetate may have been due entirely or in part to an enzymic-exchange reaction between the labelled acetyl-coenzyme A derived from the substrate and the unlabelled acetoacetyl-coenzyme A produced by the activation of the carrier acetoacetate (see, for example, Beinert & Stansly, 1953). If that were the case, such an exchange reaction might be expected to occur also in the presence of fumarate, so that the addition of carrier acetoacetate should alter the distribution of isotope in the metabolic products studied; e.g. the dilution of [$^{14}$C]acetyl-coenzyme A by exchange with inactive acetoacetate should result in a marked depression of the radioactivity of the respiratory carbon dioxide and of the fatty acids. However, when fumarate was present, the addition of carrier acetoacetate resulted in only a small decrease of the activity of the fatty acids (Table 4); on the other hand, the labelling of the acetoacetate was suppressed by the addition of fumarate in amounts large enough to stimulate lipogenesis (Tables 1 and 2). In the presence of fumarate in low concentration, the appearance of acetate carbon in acetoacetate was inhibited by *p*-nitrophenol to a greater extent than its oxidation to carbon dioxide and water (Tables 2 and 8). Further, a measurable net increase in the amount of acetoacetate was detected by means of a non-isotopic method of analysis (Table 2). These observations make it appear unlikely that in the present experiments an exchange reaction played a major part in the labelling of acetoacetate. The amount of substrate carbon appearing in the carrier may therefore be assumed to provide a measure of the capacity of the tissue preparation to form acetoacetate from the various precursors studied.

*Factors influencing ketone-body formation and lipogenesis.* Lack of oxaloacetate or its precursors is known to result in the formation of ketone bodies *in vitro*. Previous workers chose the experimental conditions so as to prevent or severely restrict the functioning of the Krebs cycle. Lehninger (1946) and Recknagel & Potter (1951) studied the conversion of pyruvate into acetoacetate by washed rat-liver suspensions poisoned with malonate. Crandall & Gurin (1949) employed a washed liver homogenate in a study of the formation of acetoacetate from labelled pyruvate, acetate and fatty acids. Catalytic amounts of Krebs-cycle intermediates have been found to be effective in preventing the accumulation of acetoacetate; e.g. Weinhouse, Millington & Volk (1950) reported that fumarate in concentrations as low as 0·1–0·5 mM suppressed the formation of acetoacetate from [$^{14}$C]palmitate. In the present work much higher concentrations of fumarate, in excess of amounts needed to catalyse Krebs-cycle oxidations, were required to abolish ketone-body formation and, at the same time, to stimulate fatty acid synthesis. The two processes were mutually exclusive only under extreme conditions, i.e. when the concentration of fumarate was either very high or very low. By choosing a suitable intermediate concentration of fumarate, it was possible to allow both acetoacetate formation and lipogenesis to proceed at reduced but measurable rates.

As concluded in the earlier paper (Terner, 1956*b*), the necessity to add fumarate in quantities greatly exceeding the small amounts required to catalyse Krebs-cycle oxidations suggested an additional role of fumarate as a stimulant of lipogenesis by the generation of reduced pyridine nucleotide.

That the availability of reduced pyridine nucleotide is a requirement for lipogenesis in non-respiring extracts has been demonstrated by various workers. According to Langdon (1955, 1957) and Brady, Mamoon & Stadtman (1956), reduced triphosphopyridine nucleotide (TPN) is required for the synthesis of fatty acids by extracts of liver, whereas Hele, Popják & Lauryssens (1957) found that rabbit mammary-gland extracts required reduced diphosphopyridine nucleotide (DPN). Brady *et al.* (1956), using extracts of acetone-dried powders of pigeon liver, added citrate and fumarate in high concentration (0·01–0·1 M) to regenerate reduced TPN. In work with respiring mammary homogenates, the addition of fumarate in increasing amounts (1–10 mM) has been found to result in a progressive stimulation of fatty acid synthesis (Terner, 1954, 1956*b*) and, as shown in this paper, also in a progressive decrease in acetoacetate formation.

Since lipogenesis is a reductive process, the accumulation of acetoacetate in mammary homogenates metabolizing acetate or pyruvate in the presence of catalytic amounts of the oxaloacetate precursor appears to be a consequence of the low reductive capacity of the tissue preparation. In the studies of lipogenesis quoted above, in which cell-free extracts and fractions were employed, reducing conditions were more easily maintained, owing to the diminution to a low level, or to the complete absence, of oxidative reactions. The present series of studies, with respiring mammary and kidney homogenates, represents an attempt to study the balance of oxidative and reductive reactions in a vigorously oxidizing system, which, although cell-free, may approach more closely than a non-respiring extract the conditions in the living organ.

*Relative ketogenicity of various precursors of acetyl-coenzyme A.* The marked contrast in ketogenicity between pyruvate and acetate on the one hand, and glucose on the other, observed in mammary and kidney homogenates is in agreement with previous findings (see Weinhouse, 1952) and emphasizes the long-felt need for an explanation of the role of carbohydrate in ketogenesis. There is no reason to assume that acetyl-coenzyme A derived from glucose is less capable of forming acetoacetyl-coenzyme A than if derived from other precursors. As pointed out by Krebs (1950), pyruvate is an obligatory intermediate in the breakdown of carbohydrate and it cannot be doubted that carbohydrate can serve as a source of ketone bodies; its antiketogenic action therefore remains to be explained.

The low ketogenicity of lactate, analogous to that of glucose, observed in the present work offers a possible explanation of the low ketogenic power of carbohydrate, at least in the present system. Since the conversion of lactate into acetyl-coenzyme A differs from that of pyruvate in only one additional oxidative step, coupled with the reduction of DPN, it seems that the ability, shared by glucose and lactate, to generate reduced pyridine nucleotide is the fundamental property which determines their low ketogenic capacity, contrasting with the powerful ketogenicity of acetate and pyruvate. As shown previously (Terner, 1955), citrate accumulates in mammary homogenates incubated with pyruvate or acetate in the presence of large amounts of fumarate. Under these conditions, the malic dehydrogenase reaction may yield reduced pyridine nucleotide. The availability of the latter would facilitate the reduction of acetoacetyl-coenzyme A, thus starting the chain of reactions leading to the formation of fatty acids (see Lynen & Ochoa, 1953). The reductive step would be in competition with the enzymic deacylation of acetoacetyl-coenzyme A and, in the absence of reactions maintaining an adequate level of reduced pyridine nucleotides, the prevalence of the deacylation reaction should result in the formation of acetoacetate.

Since acetoacetyl-coenzyme A was undoubtedly formed from pyruvate, it must also have been formed at comparable rates from lactate as well as from glucose, but in the 'non-ketogenic substrates' it could then have been largely reduced to $\beta$-hydroxybutyryl-coenzyme A. This is supported by the observation that the 'non-ketogenic' substrates yielded small but measurable amounts of acetoacetate (Tables 10 and 11). Since oxidative reactions are known to occur mainly within the mitochondria, whereas the reductive synthesis of fatty acids has been demonstrated to take place in the soluble extra-mitochondrial fractions of mammary tissue (Hele *et al.* 1957), spatial separation may provide an explanation. The glycolytic system is also located in the soluble fractions, and glucose may be more effective in making reduced pyridine nucleotides available to the soluble enzyme systems than Krebs-cycle oxidations.

The recovery of most of the added carrier acetoacetate (Table 2) indicates that its reduction by reduced DPN occurred at a relatively insignificant rate and that acetoacetate in its non-activated form played but a minor part in the reactions studied.

*Effect of glucose on ketogenesis and lipogenesis.* The mechanism outlined above may explain not only the low ketogenicity of glucose when added as the only oxidizable substrate, but also its stimulating effect on the synthesis of fatty acids from acetate, originally observed by Folley & French (1950) and Balmain *et al.* (1952) in rat- and sheep-mammary slices, and later also found to occur in cell-free guinea-pig mammary homogenates metabolizing [*carboxy*-$^{14}$C]acetate in the presence of high concentrations of fumarate (Terner, 1956*b*). The effect of glucose in these systems may be attributable to two factors: the enhanced rate of activation of acetate by the extra energy generated by the breakdown of glucose, and the maintenance of a high level of reduced pyridine nucleotide, in an oxidative environment otherwise unfavourable for reductive synthesis.

The apparent lack of antiketogenic power of glucose in the presence of acetate, shown in Table 7, may have been due to the experimental conditions being such that the rate of formation of acetoacetyl-coenzyme A from acetyl-coenzyme A, originating from the combined sources, exceeded the rate of generation of reduced pyridine nucleotide coupled with the breakdown of glucose alone, to an extent that a large part of the acetoacetyl-coenzyme A escaped reduction and suffered deacylation.

*Ketogenesis in the mammary gland.* The well-known fact that ruminants are especially susceptible to ketosis (see Shaw, 1956) may be connected with their ability to utilize acetate and fatty acids as a major source of energy. The demonstration of the utilization of acetate for the synthesis of fatty acids in slices of mammary gland from ruminants is due to the original work of Folley & French (1950) and Balmain *et al.* (1952), who also showed that the concurrent metabolism of glucose facilitated the utilization of acetate by non-ruminant mammary tissue. The guinea-pig mammary-tissue preparation used in the earlier paper (Terner, 1956*b*) and in the present work, resembles ruminant-mammary tissue in its ability to utilize acetate.

An organ such as the lactating mammary gland, subjected to conditions of considerable strain and

utilizing relatively large amounts of acetate, must depend on a large and continuous supply of reduced pyridine nucleotide in order to promote lipogenesis and suppress ketogenesis. If the supply of glucose were reduced, or its breakdown retarded, the balance precariously maintained might easily be shifted in favour of ketogenesis. It thus seems not improbable that at least part of the acetoacetate found in the milk of ketotic cows (Robertson & Thin, 1953) originates in the udder itself, instead of being derived entirely from the liver and rumen. To those two important sites of ketogenesis the udder may possibly therefore be added as a third.

## SUMMARY

1. Conditions affecting the balance between ketogenesis and lipogenesis in actively respiring homogenates of the lactating mammary gland of the guinea pig have been studied.

2. [*carboxy*-$^{14}$C]Acetate and [2-$^{14}$C]pyruvate, when incubated with mammary homogenates in the absence of added fumarate, were oxidized to carbon dioxide and converted into acetoacetate at comparable rates. The addition of fumarate in increasing amounts (1–10 mM), which, as previously shown (Terner, 1956*b*), results in the progressive stimulation of fatty acid synthesis, caused a progressive inhibition of acetoacetate formation.

3. When unlabelled glucose was added to mammary homogenates metabolizing [*carboxy*-$^{14}$C]acetate in the absence of fumarate, it did not suppress and in some experiments promoted the incorporation of acetate carbon into acetoacetate. In the presence of both glucose and fumarate the formation of acetoacetate was abolished.

4. In marked contrast with the rapid rate of acetoacetate formation from acetate and from pyruvate, [$^{14}$C]glucose (randomly labelled) or [2-$^{14}$C]lactate gave rise to only small amounts of the ketone body. It is suggested that this difference resides in the ability of glucose and lactate to regenerate reduced pyridine nucleotide. The ability to maintain, in a vigorously oxidative system, conditions favourable for reductive reactions may be a common factor underlying not only the effects of fumarate on ketogenesis and lipogenesis in the present system, but also the well-known 'antiketogenic' properties of glucose and its stimulant action on lipogenesis.

## REFERENCES

Balmain, J. H., Folley, S. J. & Glascock, R. F. (1952). *Biochem. J.* **52**, 301.
Beinert, H. & Stansly, P. G. (1953). *J. biol. Chem.* **204**, 67.
Brady, R. O., Mamoon, A. E. & Stadtman, E. R. (1956). *J. biol. Chem.* **222**, 795.
Campbell, J. & Best, C. H. (1956). *Metabolism*, **5**, 95.
Crandall, D. I. & Gurin, S. (1949). *J. biol. Chem.* **181**, 829.
Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.
Hele, P., Popják, G. & Lauryssens, M. (1957). *Biochem. J.* **65**, 348.
Krebs, H. A. (1950). *Harvey Lect.*, 1948–49, series XLIV, 165.
Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
Langdon, R. G. (1955). *J. Amer. chem. Soc.* **77**, 5190.
Langdon, R. G. (1957). *J. biol. Chem.* **226**, 615.
Lehninger, A. L. (1946). *J. biol. Chem.* **164**, 291.
Lynen, F. & Ochoa, S. (1953). *Biochim. biophys. Acta*, **12**, 299.
Ostern, P. (1933). *Hoppe-Seyl. Z.* **218**, 160.
Recknagel, R. O. & Potter, V. R. (1951). *J. biol. Chem.* **191**, 263.
Robertson, A. & Thin, C. (1953). *Brit. J. Nutr.* **7**, 181.
Shaw, J. C. (1956). *J. Dairy Sci.* **39**, 402.
Terner, C. (1954). *Biochem. J.* **58**, xxxi.
Terner, C. (1955). *Biochem. J.* **60**, 88.
Terner, C. (1956*a*). *Biochem. J.* **64**, 523.
Terner, C. (1956*b*). *Biochem. J.* **64**, 532.
Terner, C. (1956*c*). *Fed. Proc.* **15**, 186.
Terner, C. (1957). *Proc. Soc. exp. Biol., N.Y.*, **96**, 801.
Weinhouse, S. (1952). *Brookhaven Symp. Biol.* **5**, 201.
Weinhouse, S., Millington, R. H. & Volk, M. (1950). *J. biol. Chem.* **185**, 191.

# Separation and Composition of the Phospholipids of Ox Heart

By G. M. Gray and Marjorie G. Macfarlane
*Lister Institute of Preventive Medicine, London, S.W. 1*

(*Received* 14 *March* 1958)

This work arose from a decision made in 1953 to prepare the acetal phospholipid described by Feulgen & Bersin (1939) and examine its susceptibility to various bacterial toxins. This acetalphosphatide, which at that time was regarded as a natural substance, is an acetal of a long-chain aliphatic aldehyde with glycerophosphorylethanolamine (I) from which aldehyde is liberated on treatment with acid or mercuric chloride. It was presumed that this compound, with only one fatty chain, could be readily separated from the classical ester phosphatide by chromatography. However, Schmidt, Ottenstein & Bessmann (1953) suggested from the results of hydrolysis of crude brain-lipid extracts that the natural 'plasmalogen' was a compound of