The Metabolism of Citric Acid in the Mammary Gland

1. THE EFFECT OF *p*-NITROPHENOL ON THE SYNTHESIS AND OXIDATION OF CITRIC ACID BY HOMOGENATES OF THE MAMMARY GLAND

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In a previous paper (Terner, 1951) it was reported that small amounts of citric acid accumulated in rat mammary slices metabolizing pyruvate in the presence of fumarate, and other evidence was presented suggesting that the citric acid cycle is operating in the mammary gland. Recently, Moore & Nelson (1952), using a cyclophorase preparation of lactating guinea pig mammary tissue, found that added citrate was oxidized slowly if at all, and concluded that 'not only does mammary tissue not oxidize citrate, but it contains an inhibitor to this oxidative step of the tricarboxylic acid cycle'. Since citric acid is an intermediate of the Krebs cycle, the inability of the mammary gland to oxidize citrate at adequate rates would rule out the cycle as a major metabolic pathway in this tissue.

In the present work homogenates of the lactating mammary gland of the guinea pig have been found capable of synthesizing citrate from pyruvate or acetate in the presence of fumarate and also of oxidizing added citrate at rapid rates. Under the experimental conditions employed the activity of the enzyme systems activating pyruvate or acetate and of the system condensing the active fragments with oxaloacetate exceeded the ability of the tissue to oxidize citric acid. These findings suggest a simple explanation for the formation of citric acid in the mammary gland and its secretion into milk. The observation that citric acid is metabolized at rates corresponding to those of other intermediates of the cycle provides additional and more conclusive evidence for the functioning of the Krebs cycle in the mammary gland. A preliminary account of part of this work has been given to the Biochemical Society (Terner, 1953).

EXPERIMENTAL

Material. The mammary glands of lactating guinea pigs were removed after the death of the animal and placed in ice-cold 0·154M-KCl. The tissue was passed through a Latapie mincer and washed three times in the cold KCl solution and strained on muslin. The minced tissue was then ground in an all-glass homogenizer (Potter & Elvejehm, 1936) in a medium of 0·154M-KCl containing 0·024M-$KHCO_3$ and 0·02M nicotinamide. The homogenate was further diluted with 0·154M-KCl to give a 1 in 5 homogenate (dry weight 40–50 mg./ml.).

The high fat content of lactating mammary tissue is shown by the following example. The pooled mammary glands of five lactating guinea pigs were found to contain: fat, 11·8%; fat-free dry matter, 8·7% and water, 79·5% (by difference). Thus 58% of the dry weight was fat. The washing of the minced tissue removed much of the milk and reduced the fat content of the tissue to 35% of its dry weight. This treatment also decreased the amounts of preformed oxidizable substrates present in the tissue. Portions of homogenates of 50 mg. dry wt. contained *ca.* 1 μmole each of citric and of lactic acids, corresponding to 80–100 mg. of citric acid and 30–40 mg. of lactic acid in 100 g. fresh weight of washed mammary tissue.

Reaction mixture. The basal medium contained in addition to 0·154M-KCl: $MgCl_2$, 0·01M; nicotinamide, 0·02M; cozymase, $1·25–2·5 \times 10^{-4}$M; adenosine triphosphate (ATP), 0·001M (or ATP, 2×10^{-4}M and adenylate, 2×10^{-4}M); phosphate buffer pH 7·4, 0·0025M and aminotrishydroxymethylmethane buffer pH 7·4, 0·02M. Further additions were made as stated in the text and in the tables. The gas phase was air. The homogenate (1 ml.) and hexokinase (when added) were placed in the side arm of the Warburg flask and were mixed with the medium in the main compartment immediately before the vessel was placed in the thermostat at 37°. The volume of the complete reaction mixture was 4·0 ml. Readings were begun after 10 min. equilibration. All manometric data have been corrected by extrapolation to include the equilibration period. After incubation the vessels were cooled in iced water and 3 ml. 1·5N perchloric acid (Neuberg, Strauss & Lipkin, 1944) were added. The contents of control flasks were deproteinized at the moment of the start of the incubation period. The protein-free filtrates were analysed after storage at −17°.

Analytical methods. Citric acid was estimated by Taylor's (1953) modification of the method of Weil-Malherbe & Bone (1949). Before the bromination the samples were boiled with sulphuric acid to remove 'pseudocitrates' (Pucher, Sherman & Vickery, 1936). A 4% thiourea solution, pH 9·2 (Natelson, Pincus & Lugovoy, 1948) was used in the final extraction of the pentabromoacetone from the light petroleum extract. Reagent blanks and complete sets of citric acid standards were prepared for each determination and were subjected to the same procedure as the samples to be analysed. Since *p*-nitrophenol (*p*NP) when present produced some yellow colour in the final alkaline thiourea extract, a number of blanks were set up containing *p*NP in amounts corresponding to those present in the portions of the protein-free filtrates under analysis and the colour of each sample was read against its appropriate blank. Pyruvate and α-oxoglutarate were determined by the specific methods of Friedemann & Haugen (1943); lactic acid was determined by the method of Barker & Summerson (1941)

and acetoacetate by the aniline method (Ostern, 1933; Edson, 1935) as modified by Krebs & Eggleston (1945). Inorganic phosphate was estimated by a modification of the method of Berenblum & Chain (1938) as described by Weil-Malherbe & Green (1951).

Reagents and preparations. The sodium salt of adenosine triphosphoric acid (ATP), supplied by Pabst Laboratories, Milwaukee, U.S.A. and the potassium salt prepared from the barium salt obtained from Boots Pure Drug Co., Nottingham, were used. Adenylic acid was obtained from Roche Products, Welwyn Garden City, cozymase (85% pure) from Schwarz Laboratories Inc., New York, U.S.A., and coenzyme II (TPN, 10% pure) from Sigma Chemical Company, St Louis, U.S.A. Hexokinase was prepared from baker's yeast according to Meyerhof (1927); extracts were prepared containing in 0·1 ml. about nine hexokinase units as defined by Berger, Slein, Colowick & Cori (1946). Crystalline potassium pyruvate was prepared according to Korkes, del Campillo, Gunsalus & Ochoa (1951). Commercial samples of sodium fumarate and *p*-nitrophenol were purified by repeated recrystallization. Oxaloacetic acid was a sample kindly given by Dr G. Popják, α-oxoglutaric acid (96% pure) by Dr A. L. Greenbaum.

Units. All metabolic quotients are expressed in the Q notation (μl./mg. dry wt./hr.). (Q_P = μl. H_3PO_4/mg. dry wt./hr., 1 μmole H_3PO_4 = 22·4 μl.) The dry weight of the homogenates was determined by evaporation in the steam oven and was corrected for the salt content of the diluting fluid, but not for the fat content of the tissue.

RESULTS

Respiration and oxidation of substrates in mammary gland homogenates. Since, as previously reported (Terner, 1954*a*), whole mammary homogenates incubated in the basal medium (see Methods) had a high endogenous respiration, it was not unexpected to find that the addition of a number of oxidizable substrates caused little or no stimulation of respiration, even when it could be shown by analytical methods that the added substances disappeared during incubation. In the presence of *p*NP, however, certain substrates were metabolized with a large increase in oxygen consumption. This reagent was therefore used throughout the present study in the concentration of 2×10^{-4} M which in a different system had been found to produce the greatest stimulation of respiration (Terner, 1954*a*).

Metabolism of pyruvate. When pyruvate was added to the reaction mixture it was found to disappear during incubation. The addition of *p*NP stimulated the respiration slightly or not at all, and only traces of citric acid were found in the absence or presence of the reagent. When fumarate and pyruvate were added together, appreciable amounts of citric acid accumulated. In this system the addition of *p*NP approximately doubled the oxygen consumption with only a small acceleration of the rates of pyruvate disappearance and of citric acid accumulation (Table 1).

In earlier experiments in which fumarate was added in smaller amounts, it was observed that the yield of citrate was diminished by prolonging the incubation period, while the rate of respiration remained linear. This suggested that, after the exhaustion of the added fumarate, the accumulated citrate was used to catalyse the oxidation of the remaining pyruvate. In all subsequent experiments fumarate was added in amounts equivalent to pyruvate or acetate in order to preserve the citric acid formed.

Oxidation of citric and α-oxoglutaric acids. When citrate was the only substrate added, the rate of respiration was not much higher than the endogenous respiration. Again, a large increase in oxygen consumption was produced by the addition of *p*NP. As shown in Tables 1 and 2 this was not accompanied by a corresponding increase in the rate of disappearance of citrate. Furthermore, citrate did not disappear as rapidly as pyruvate. In order to test whether the rate of oxidation of citric acid was limited by the lack of cofactors, TPN was added in some experiments and the rates of oxidation of citrate and α-oxoglutarate were compared. Tables 1 and 3 show that the two substances were metabolized by the same preparation at approximately the same rate and that the amount of citrate accumulating during the metabolism of pyruvate corresponded to the difference between the amounts of added pyruvate and of added citrate disappearing. The addition of TPN was without effect on the rate of disappearance of added citrate.

In the presence of α-oxoglutarate the stimulation of respiration by *p*NP was of the same order of

Table 1. *Oxidation and accumulation of citric acid in mammary homogenates*

Mammary homogenate, dry weight, 37·9 mg. per vessel. Basal medium. Additions: fumarate, 0·01 M; pyruvate, 0·01 M; citrate, 0·005 M; α-oxoglutarate, 0·005 M; *p*NP, 2×10^{-4} M. Incubation period, 45 min.

Additions	$-Q_{O_2}$	$-Q_{pyruvate}$	$-Q_{\alpha\text{-oxoglutarate}}$	$Q_{citrate}$
Fumarate, pyruvate	10·3	11·7	—	+5·5
Fumarate, pyruvate, *p*NP	24·0	15·0	—	+5·5
Citrate	10·7	—	—	−6·4
Citrate, *p*NP	19·6	—	—	−9·5
α-Oxoglutarate	9·8	—	6·2	—
α-Oxoglutarate, *p*NP	14·4	—	7·2	—

magnitude as in the presence of citrate or of pyruvate plus fumarate. However, while the rates of disappearance of citrate and of α-oxoglutarate were approximately equal, they were exceeded by the rate of disappearance of pyruvate.

Table 2. *Effect of* pNP *on metabolism of acetate and of pyruvate*

Mammary homogenate, dry weight 53 mg. per vessel. Basal medium. TPN ($6{\cdot}25 \times 10^{-5}$ M) in all vessels. Additions: fumarate, 0·005 M; pyruvate, 0·005 M; acetate, 0·005 M; citrate, 0·005 M; *p*NP, 2×10^{-4} M. Incubation period, 40 min.

Additions	$-Q_{O_2}$	$-Q_{pyruvate}$	$Q_{citrate}$
None	6·6	—	0
*p*NP	8·9	—	0
Fumarate, acetate	11·5	—	+3·4
Fumarate, acetate, *p*NP	14·9	—	+1·9
Fumarate, pyruvate	9·7	9·6	+2·6
Fumarate, pyruvate, *p*NP	19·3	10·6	+3·2
Citrate	9·7	—	−6·9
Citrate, *p*NP	18·2	—	−7·2

citric acid accumulated. Since *p*NP appears to retard the metabolism of acetate, time may be required for an effective concentration of citric acid to be built up in the reaction mixture before the stimulation of respiration by the nitrophenol can be observed.

Effect of fluoride. Liébecq & Peters (1949) have shown that the oxidation of pyruvate by brain suspensions of the pigeon is inhibited by fluoride. In the present experiments fluoride (0·01 M) inhibited both the rate of respiration and of substrate disappearance not only in the case of pyruvate, but also of citrate and α-oxoglutarate. This inhibition was reversed by the addition of *p*NP which despite the presence of fluoride produced its stimulating effects (Table 3).

Effect of pNP *and fluoride on the metabolism of succinate, fumarate and malate.* As shown in Tables 4 and 5 the effect of *p*NP on the respiration in the presence of succinate varied from a small inhibition to a small stimulation. Although succinate may be expected to be metabolized more rapidly than other intermediates of the Krebs cycle (see Krebs, 1943), its oxidation appeared to be in step with the oxidation of all other intermediates tested ($-Q_{O_2} = ca.$ 10). However, when more dilute suspensions of mammary tissue in phosphate buffer were incubated with succinate without the addition of cofactors, $-Q_{O_2}$ values exceeding 20 could be obtained.

Table 3. *Effect of fluoride and* pNP *on metabolism of mammary homogenates*

Mammary homogenate, dry weight, 46·5 mg. per vessel in Expt. 1; 53·3 mg. in Expt. 2. Basal medium. Additions: fumarate, 0·01 M; pyruvate, 0·01 M; citrate, 0·005 M; α-oxoglutarate, 0·01 M in Expt. 1; 0·005 M in Expt. 2; *p*NP, 2×10^{-4} M; fluoride, 0·01 M. Incubation period, 40 min.

Expt. no.	Additions	$-Q_{O_2}$	$-Q_{pyruvate}$	$-Q_{\alpha\text{-oxoglutarate}}$	$Q_{citrate}$
1	Fumarate, pyruvate	8·0	9·1	—	+3·3
	Fumarate, pyruvate, *p*NP	18·0	13·6	—	+4·9
	Fumarate, pyruvate, fluoride	5·2	6·6	—	+3·3
	Fumarate, pyruvate, fluoride, *p*NP	14·7	11·7	—	+4·2
	Citrate	9·6	—	—	−6·1
	Citrate, *p*NP	16·7	—	—	−7·5
	Citrate, fluoride	6·9	—	—	−4·8
	Citrate, fluoride, *p*NP	9·9	—	—	−5·1
	α-Oxoglutarate	9·0	—	6·5	—
	α-Oxoglutarate, *p*NP	16·3	—	10·6	—
	α-Oxoglutarate, fluoride	6·7	—	3·9	—
	α-Oxoglutarate, fluoride, *p*NP	8·1	—	6·7	—
2	Citrate	11·0	—	—	−5·2
	Citrate, *p*NP	18·9	—	—	−6·4
	Citrate, fluoride	5·1	—	—	−2·9
	Citrate, fluoride, *p*NP	15·8	—	—	−6·0
	α-Oxoglutarate	9·6	—	4·6	—
	α-Oxoglutarate, *p*NP	18·0	—	6·0	—
	α-Oxoglutarate, fluoride	5·9	—	2·9	—
	α-Oxoglutarate, fluoride, *p*NP	13·4	—	5·7	—

Metabolism of acetate. As in the case of pyruvate, citric acid accumulated during incubation of the homogenate with acetate plus fumarate. The metabolism of acetate differed from that of pyruvate in that *p*NP caused a marked inhibition of citric acid accumulation when acetate (plus fumarate) was its precursor (Table 2). Furthermore, the respiration was usually slightly inhibited by *p*NP although in some experiments an acceleration of respiration occurred after a lag period of 25 min. It seems probable that the delayed stimulation was the result of the action of *p*NP on the oxidation of the

The inhibitory effect of fluoride on the respiration in the presence of succinate, fumarate and L-malate

was not reversed by *p*NP (Table 4). Citrate did not accumulate in measurable amounts during the oxidation of those substrates whether in the absence or presence of *p*NP (Table 5).

Table 4. *Effect of* pNP *and fluoride on oxidation of succinate, fumarate and malate*

Mammary homogenates. Dry weight: 53 mg. per vessel in Expt. 1, 53·3 mg. per vessel in Expt. 2, 56 mg. per vessel in Expt. 3. Basal medium. Additions: fumarate, 0·005 M; *p*NP, 2×10^{-4} M; fluoride, 0·01 M; L-malate, 0·005 M; succinate, 0·01 M in Expt. 1, 0·02 M in Expt. 2. Incubation period 40 min.

Expt. no.	Additions	$-Q_{O_2}$
1	None	6·6
	*p*NP	8·9
	Fumarate	7·6
	Fumarate, *p*NP	10·2
	Succinate	11·3
	Succinate, *p*NP	12·0
2	None	7·5
	Fluoride	5·2
	Fumarate	9·1
	Fumarate, *p*NP	9·0
	Fumarate, fluoride	5·5
	Fumarate, fluoride, *p*NP	5·4
	Succinate	12·3
	Succinate, *p*NP	16·4
	Succinate, fluoride	9·2
	Succinate, fluoride, *p*NP	8·9
3	None	6·9
	L-Malate	8·4
	L-Malate, *p*NP	5·5
	L-Malate, pyruvate	8·6
	L-Malate, pyruvate, *p*NP	18·4

Table 5. *Effect of* pNP *on the oxidation of intermediates of the citric acid cycle*

Mammary homogenate, dry weight, 48·4 mg. per vessel. Basal medium; all added substrates 0·01 M; *p*NP, 2×10^{-4} M. Incubation period, 40 min.

Additions	$-Q_{O_2}$	$Q_{citrate}$
None	5·8	0
Succinate	10·3	0
Succinate, *p*NP	7·6	0
Fumarate	6·5	0
Fumarate, *p*NP	4·3	0
L-Malate	6·3	0
L-Malate, *p*NP	4·6	0
Oxaloacetate	6·9	3·2
Oxaloacetate, *p*NP	17·0	4·1
Fumarate, pyruvate	6·5	2·2
Fumarate, pyruvate, *p*NP	19·8	3·1

Metabolism of oxaloacetate. This substance was metabolized in the manner characteristic of fumarate plus pyruvate; large amounts of citric acid accumulated during its oxidation and the addition of *p*NP resulted in a large increase of oxygen consumption (Table 5).

Metabolism of acetoacetate. The observation that in some experiments *p*NP stimulated the respiration of mammary homogenates incubated with fumarate and acetoacetate, suggested that mammary tissue can attack the latter substance. However, this stimulatory effect of *p*NP could not always be observed and in other experiments *p*NP caused some inhibition. Examples of experiments showing stimulation or inhibition of acetoacetate oxidation by *p*NP are summarized in Tables 6 and 7.

Table 6. *Effect of fluoride and* pNP *on metabolism of acetoacetate*

Mammary homogenate, dry weight 46·7 mg. per vessel. Basal medium; all flasks contained fumarate (0·005 M) and acetoacetate (0·005 M), except where otherwise stated. Incubation period, 50 min.

Additions	$-Q_{O_2}$	$-Q_{ac.-ac.}$
No fumarate, no acetoacetate	7·4	—
No acetoacetate	8·2	—
None	8·2	2·7
*p*NP, 2×10^{-4} M	16·4	3·7
*p*NP, 4×10^{-4} M	5·6	2·1
Fluoride, 0·005 M	6·9	2·7
Fluoride, 0·005 M, *p*NP, 2×10^{-4} M	11·5	3·3
Fluoride, 0·005 M, *p*NP, 4×10^{-4} M	4·9	1·7
Fluoride, 0·01 M	5·9	2·6
Fluoride, 0·01 M, *p*NP, 2×10^{-4} M	9·3	3·3
Fluoride, 0·01 M, *p*NP, 4×10^{-4} M	4·6	2·0
Fluoride, 0·02 M	5·2	2·0
Fluoride, 0·02 M, *p*NP, 2×10^{-4} M	7·1	2·4
Fluoride, 0·02 M, *p*NP, 4×10^{-4} M	4·9	1·6

Table 7. *Metabolism of acetoacetate in mammary homogenates*

Mammary homogenate, dry weight 60 mg. per vessel. Basal medium. Fumarate (0·0025 M) and acetoacetate (0·0025 M) in all flasks. Additions: glucose, 0·005 M; *p*NP, 2×10^{-4} M. Incubation period, 40 min.

Additions	$-Q_{O_2}$	$-Q_{ac.-ac.}$
None	5·7	2·0
*p*NP	4·1	0·9
Glucose	5·8	2·0
Glucose, *p*NP	12·0	1·6

It seems that the concentration of *p*NP is critical, since while 2×10^{-4} M-*p*NP stimulated the rates of respiration and of disappearance of acetoacetate, 4×10^{-4} M-*p*NP tended to be inhibitory (Table 6). Fluoride (0·005–0·02 M) depressed the metabolism of acetoacetate, but this inhibition could be partly reversed by *p*NP, especially when the concentration of fluoride was low. The addition of glucose did not accelerate the breakdown of acetoacetate, but it seemed to afford some protection against the inhibitory action of *p*NP (Table 7); the stimulation of respiration caused by *p*NP in this experiment was due to the presence of glucose. Since the incubation

periods were short and the estimation of acetoacetate was carried out with the minimum delay, no correction was applied for any spontaneous decomposition of the ketone body that may have occurred. Only small amounts of citric acid accumulated during the metabolism of acetoacetate.

Oxidation of fatty acids. With octanoate as the only substrate added, there was a small but distinct increase in respiration which was further accelerated by the addition of fumarate. This increased rate of respiration was maintained over prolonged periods of incubation (Table 8), and is interpreted as showing oxidation of octanoate.

Table 8. *Oxidation of octanoate*

Mammary homogenate, dry weight 46·7 mg. Basal medium. Additions: fumarate, 0·005 M; octanoate, $1{\cdot}25 \times 10^{-3}$ M.

Additions	$-Q_{O_2}$	
Period (min.) ...	0–45	45–85
None	7·6	5·6
Fumarate	8·4	6·6
Octanoate	8·7	7·6
Fumarate, octanoate	10·5	8·7

Effect of phosphate acceptors. Various workers have found that the respiration of mitochondrial preparations can be accelerated either by 2:4-dinitrophenol or by added phosphate acceptors (Lardy & Wellman, 1952; Slater, 1953). It has been shown in a previous paper that mammary homogenates incubated with phosphate acceptors and glycolysable substrates, respond to the addition of *p*NP with increased respiration (Terner, 1954*a*). In the present experiments the addition of phosphate acceptors did not influence the rate of respiration of the preparation and the extent of stimulation by *p*NP remained the same in the presence of the acceptors as in their absence (Table 9).

DISCUSSION

Metabolism of citric acid in mammary tissue. An attempt to demonstrate the synthesis and oxidation of citric acid in isolated mammary tissue has been reported by Knodt & Peterson (1946). These authors found that the amount of citric acid originally present in cow-udder slices was increased on incubation, and that the accumulation of citric acid was accelerated by the addition of various substrates, e.g. glucose. On the other hand, when citric acid was added to the slices, a part was not recovered after incubation and was assumed to have been oxidized. It may be noted that the observed changes were small; viz. in six experiments the average increase in the amount of citric acid after 6 hr. incubation with glucose was 26·49 mg./100 g. of tissue, and the average decrease of added citric acid was 175·9 mg./100 g. of tissue. Expressed in the Q notation, these figures would correspond to $Q_{\text{citric acid}}$ values of $+0{\cdot}026$ and $-0{\cdot}17$ respectively.

In experiments with rat and rabbit mammary gland slices incubated with pyruvate in the presence of fumarate, citric acid was found to accumulate in small amounts, $Q_{\text{citric acid}}$ values of *ca.* $+1$ being observed (Terner, 1951). It is uncertain whether the full amount of citric acid formed was estimated in those experiments in view of the findings of Coxon (1953) that citrate formed in slices does not diffuse freely into the suspending medium. The high rates of citric acid metabolism in the present study demonstrate the advantage of the use of cell-free tissue preparations in which restrictions due to permeability barriers are reduced.

Relative rates of synthesis and oxidation of citric acid. It has been shown that in mammary homogenates capable of oxidizing added citrate at rapid rates, citric acid accumulated during the metabolism of pyruvate or acetate in the presence of fumarate. The preparation appeared to produce 'active acetate' more readily from acetate than from pyruvate, judging from the accumulation of

Table 9. *Effect of added phosphate acceptor on the metabolism of citric acid*

Mammary gland homogenates. Dry weight: Expt. 1, 53 mg.; Expt. 2, 49·1 mg. per vessel. Basal medium. Citrate (0·005 M) in all flasks. Additions: *p*NP, 2×10^{-4} M; fluoride, 0·01 M; glucose, 0·02 M; hexokinase, 18 units. Incubation period, 40 min.

Expt. no.	Additions	$-Q_{O_2}$	$-Q_{\text{citrate}}$	Q_P
1	None	9·7	6·9	—
	*p*NP	18·2	7·2	—
	Glucose, hexokinase	8·3	3·6	−13·4
	Glucose, hexokinase, *p*NP	18·1	3·9	+ 8·0
2	Fluoride	6·6	4·0	− 1·7
	Fluoride, *p*NP	14·6	8·4	− 0·6
	Fluoride, glucose, hexokinase	7·6	4·2	−14·0
	Fluoride, glucose, hexokinase, *p*NP	15·3	8·6	−12·8

larger amounts of citrate in the presence of the former. Analytical measurements revealed that the amounts of citrate accumulating during the oxidation of pyruvate corresponded closely to the difference, in molar equivalents, between the amounts of pyruvate and of citrate that the preparation was capable of metabolizing. The observation that α-oxoglutarate was metabolized at a rate not faster than citrate suggests that citrate was oxidized at a rate corresponding to the overall rate of the citric acid cycle and that the accumulation of citric acid during the metabolism of its precursors was not due to a deficiency in the cycle, but rather to an excessive rate of production of 'active acetate' and its condensation with oxaloacetate.

Origin of milk citric acid. Although citric acid has been reported to accumulate in suspensions of other tissues, the amounts of citric acid accumulating in mammary homogenates appear to be exceptionally large. Thus, while Coxon (1953) found that 4% of the pyruvate metabolized by pigeon brain brei appeared as citrate, the yield of citric acid in the present experiments amounted to 30–40% of the pyruvate disappearing, in preparations which at the same time oxidized added citrate at what appeared to be the full rate of the citric acid cycle. Coxon (1953) pointed out that 'the appearance of such intermediates at all in recognizable form is in itself a measure of the artificiality of the experimental conditions'. This must admittedly be borne in mind in interpreting the results of *in vitro* experiments, especially when the intermediate appearing under the experimental conditions employed is not known to accumulate *in vivo*. Milk, however, contains relatively large amounts of citric acid and, if conclusions regarding the function of intact tissue can be drawn from experiments carried out under *in vitro* conditions, the present findings would offer a simple explanation for the formation of this intermediate during the normal metabolic activity of mammary tissue.

p*NP as a reagent in metabolic studies.* The difficulty of the overlapping of the oxygen uptake due to the oxidation of an added substrate with the endogenous respiration of whole tissue homogenates is frequently encountered. It has been shown in the present paper that in the case of certain substrates the stimulation of respiration caused by *p*NP provided a convenient manometric method of testing the ability of the tissue preparation to oxidize the substrate. The observation that the amount of oxygen consumed under the influence of *p*NP corresponded to the amount theoretically required for the complete oxidation of the substrate disappearing, and not accounted for as citric acid, suggests that the oxidation of endogenous reserves present in the homogenate was suppressed during the oxidation of the added substrates.

Effect of p*NP on the metabolism of the Krebs cycle intermediates.* In a previous paper (Terner, 1954*a*) *p*NP has been shown to accelerate the oxygen consumption of mammary homogenates metabolizing glycolysable substrates. It appears from the present study that the stimulation of respiration by *p*NP is due to its action on certain intermediates of the Krebs cycle, namely oxaloacetate, pyruvate (in the presence of malate or fumarate), citrate and α-oxoglutarate. The oxidation of succinate, fumarate, malate and of acetate (plus fumarate) was either slightly inhibited or only slightly stimulated. The substances susceptible to the full stimulant action of *p*NP thus appeared to be either α-keto acids or their precursors. Malate presented an exception, but this substance is known to be slowly oxidized by enzyme preparations, unless its inhibitory oxidation product—oxaloacetate—is removed, for example, by transamination with glutamate (Potter, 1946). Using the latter technique, Moore & Nelson (1952) demonstrated that 'cyclophorase' preparations of the mammary gland of the guinea pig are capable of oxidizing malate. In the present experiments, *p*NP which did not accelerate the respiration of mammary homogenates metabolizing either pyruvate or fumarate (or malate) alone, caused a large increase in respiration in the presence of both. Under these conditions the metabolism of both pyruvate and malate must be equally affected by the reagent. It is notable that despite the large increase in oxygen uptake the rate of substrate utilization was accelerated, if at all, to a lesser extent.

The vigorous respiratory response to *p*NP of mammary homogenates metabolizing added citrate, combined with analytical measurements, showed that citric acid was metabolized at rates corresponding to those of the subsequent steps of the cycle. Thus, no evidence was found to support Moore & Nelson's (1952) suggestion of an inhibition of the oxidation of citric acid in mammary tissue.

Oxidation of acetoacetate in the mammary gland. Shaw (1942), using the arterio–venous technique, found that acetoacetate was not utilized by the udder of the cow. Knodt & Peterson (1946) showed that acetoacetate and acetone accumulated when cow udder slices were incubated with β-hydroxybutyrate. Peeters, Coussens & Sierens (1953) also observed that acetoacetate accumulated in the blood of the perfused isolated cow udder after addition of β-hydroxybutyrate to the system. After prolonged perfusion, however, the concentration of acetoacetate in the blood leaving the udder was found to be less than in the entering blood, suggesting the absorption and utilization of the ketone body by the gland. The present experiments clearly demonstrate the ability of mammary tissue to metabolize acetoacetate. The finding that the rate of its disappearance was influenced by metabolic

inhibitors points to the enzymic nature of the reactions concerned. The effect of *p*NP, although variable in different preparations, provides evidence for the oxidative removal of acetoacetate; e.g. in experiments in which *p*NP in suitable concentration accelerated the respiration, about 4 moles of oxygen were consumed for every mole of acetoacetate disappearing, a relationship expected for its complete oxidation via the Krebs cycle (Table 6).

Incomplete oxidation of pyruvate in mammary suspensions and effect of nitrophenols. While mitochondria or 'cyclophorase' preparations will, in the presence of dicarboxylic acids or bicarbonate, oxidize pyruvate to carbon dioxide and water (Green, Loomis & Auerbach, 1948; Judah, 1951; Bartley, 1953), the mammary homogenates employed in the present study oxidized pyruvate incompletely in the presence of fumarate. This was shown by the observation that the oxygen consumption of the preparation was too small to account for the complete oxidation of the pyruvate disappearing, even when a correction for the accumulated citric acid was applied. Judah (1951) found that 2:4-dinitrophenol inhibited the oxidation of pyruvate by mitochondrial preparations and that this inhibition could be reversed by the addition of dicarboxylic acids. In mammary homogenates, however, *p*NP did not inhibit the oxidation of pyruvate when this was the only added substrate and when fumarate was also added, the inhibitor caused a large increase in oxygen consumption. While Judah (1951) did not observe a stimulation of respiration by 2:4-dinitrophenol, Lardy & Wellman (1952) reported that the respiration of rat liver mitochondria metabolizing a number of substrates including pyruvate, citrate and α-oxoglutarate was stimulated by the addition of either 2:4-dinitrophenol or phosphate acceptors. In mammary homogenates, as shown in this and a previous communication (Terner, 1954*a*), added phosphate acceptors did not increase the rate of respiration and, when an acceleration of respiration was caused by *p*NP during the metabolism of a suitable substrate, the stimulating effect of the inhibitor could be demonstrated in the presence as well as in the absence of the phosphate acceptor system. The variety of results reported is doubtless due to the different preparations employed.

Whole tissue homogenates differ from tissue fractions in that, although disrupted, they contain all the components of the organized tissue. This may account for the close resemblance of the metabolism of pyruvate in mammary homogenates to its metabolism in mammary slices. The observation of incomplete oxidation of pyruvate, combined with the effect of nitrophenols in causing the complete oxidation of the substrate, has been taken to suggest the occurrence of synthetic reactions depending on energy derived from oxidative phosphorylation in mammary slices (Terner, 1951) and in bull spermatozoa (Melrose & Terner, 1953). The occurrence of similar phenomena in mammary homogenates suggest that they are capable of utilizing pyruvate in synthetic reactions. These have been investigated by means of tracer isotopes in an attempt to throw further light on the effect of *p*NP on oxidative and synthetic reactions (Terner, 1954*b*) and the results will be presented in a subsequent paper.

SUMMARY

1. Citric acid was oxidized by homogenates of the lactating mammary gland at approximately the same rate as α-oxoglutaric acid.

2. Citric acid accumulated in mammary homogenates metabolizing pyruvate or acetate in the presence of fumarate. The amount of citrate accumulating during the metabolism of pyruvate was equivalent to the difference between the amounts of pyruvate and of added citrate disappearing. It is concluded that the preparation metabolized citrate at a rate corresponding to the overall rate of the citric acid cycle, and that the accumulation of citric acid during the metabolism of its precursors was due to an excessive rate of production of 'active acetate' and its condensation with oxaloacetate. This observation suggests a simple explanation for the occurrence of citric acid in milk.

3. The addition of *p*-nitrophenol (2×10^{-4} M) greatly enhanced the respiration of mammary homogenates metabolizing pyruvate plus fumarate, oxaloacetate, citrate and α-oxoglutarate, but had much smaller or no stimulating effects in the presence of succinate, fumarate, malate and of acetate plus fumarate. The stimulation of respiration by *p*-nitrophenol was not accompanied by a corresponding acceleration of substrate disappearance. Phosphate acceptors did not accelerate the respiration unless *p*-nitrophenol was also added.

4. Mammary homogenates oxidized acetoacetate and octanoate.

5. Fluoride (0·01 M) inhibited the rates of respiration and of substrate disappearance. In the case of certain substrates this inhibition was reversed by the addition of *p*-nitrophenol.

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REFERENCES

Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.

Bartley, W. (1953). *Biochem. J.* **53**, 305.

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

Berger, L., Slein, M. W., Colowick, S. P. & Cori, C. F. (1946). *J. gen. Physiol.* **28**, 379.

Coxon, R. V. (1953). *Biochem. J.* **55**, 545.
Edson, N. L. (1935). *Biochem. J.* **29**, 2082.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Green, D. E., Loomis, W. F. & Auerbach, V. H. (1948). *J. biol. Chem.* **172**, 389.
Judah, J. D. (1951). *Biochem. J.* **49**, 271.
Knodt, C. B. & Peterson, W. E. (1946). *J. Dairy Sci.* **29**, 115.
Korkes, S., del Campillo, A., Gunsalus, I. C. & Ochoa, S. (1951). *J. biol. Chem.* **193**, 721.
Krebs, H. A. (1943). *Advanc. Enzymol.* **3**, 191.
Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
Lardy, H. A. & Wellman, H. (1952). *J. biol. Chem.* **195**, 215.
Liébecq, C. & Peters, R. A. (1949). *Biochim. biophys. Acta*, **3**, 215.
Melrose, D. R. & Terner, C. (1953). *Biochem. J.* **53**, 296.
Meyerhof, O. (1927). *Biochem. Z.* **183**, 176.
Moore, R. O. & Nelson, W. L. (1952). *Arch. Biochem. Biophys.* **36**, 178.
Natelson, S., Pincus, J. B. & Lugovoy, J. K. (1948). *J. biol. Chem.* **175**, 745.
Neuberg, C., Strauss, E. & Lipkin, L. E. (1944). *Arch. Biochem.* **4**, 101.
Ostern, P. (1933). *Hoppe-Seyl. Z.* **218**, 160.
Peeters, G., Coussens, R. & Sierens, G. (1953). *Arch. int. Pharmacodyn.* **95**, 153.
Potter, V. R. (1946). *J. biol. Chem.* **165**, 311.
Potter, V. R. & Elvejehm, C. A. (1936). *J. biol. Chem.* **114**, 495.
Pucher, G. W., Sherman, C. C. & Vickery, H. B. (1936). *J. biol. Chem.* **113**, 235.
Shaw, J. C. (1942). *J. biol. Chem.* **142**, 53.
Slater, E. C. (1953). *Nature, Lond.*, **172**, 975.
Taylor, T. G. (1953). *Biochem. J.* **54**, 48.
Terner, C. (1951). *Biochem. J.* **50**, 145.
Terner, C. (1953). *Biochem. J.* **55**, xxix.
Terner, C. (1954*a*). *Biochem. J.* **56**, 471.
Terner, C. (1954*b*). *Biochem. J.* **58**, xxxi.
Weil-Malberbe, H. & Bone, A. B. (1949). *Biochem. J.* **45**, 377.
Weil-Malberbe, H. & Green, R. H. (1951). *Biochem. J.* **49**, 286.

The Metabolism of Citric Acid in the Mammary Gland

2. THE EFFECT OF *p*-NITROPHENOL AND OF FLUORIDE ON THE SYNTHESIS OF CITRIC ACID IN FLUOROACETATE-BLOCKED HOMOGENATES

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It has been shown in the first paper of this series that relatively large amounts of citric acid accumulated in homogenates of the lactating mammary gland of the guinea pig incubated with pyruvate or acetate in the presence of fumarate, despite the ability of the preparation to oxidize added citrate at rates corresponding to the overall rate of the citric acid cycle. In homogenates metabolizing pyruvate, the addition of *p*-nitrophenol (*p*NP) greatly accelerated the oxygen consumption without more than a small increase in the amounts of pyruvate disappearing and citrate accumulating. When acetate was the substrate, however, *p*NP caused marked inhibition of citrate formation (Terner, 1955).

The effect of *p*NP and other agents has now been studied in mammary homogenates poisoned with fluoroacetate. This substance is known to block the citric cycle by its enzymic conversion into a fluorotricarboxylic acid which specifically inhibits aconitase (Peters, 1952*b*). In view of the absence of inhibitory effects of fluoroacetate on other reactions of the citric acid cycle (Liébecq & Peters, 1949) and the fact that the equilibrium of the condensing reaction is far in the direction of citrate synthesis (Ochoa, 1954), the rate of accumulation of citric acid may be expected to provide a measure of the rate of formation of 'active acetate'. In this system *p*NP caused a marked stimulation of respiration and citric acid synthesis, when pyruvate was the substrate, while it inhibited the formation of citrate from acetate. A preliminary account of part of this work has been given to the Biochemical Society (Terner, 1954).

EXPERIMENTAL

Material and methods. The mammary glands of lactating guinea pigs were passed through a Latapie mincer and the minced tissue was washed 3 times with cold 0·154M-KCl. The tissue was then ground in a Potter homogenizer in a medium of 0·154M-KCl containing 0·024M-$KHCO_3$ and 0·02M nicotinamide. The suspension was further diluted with 0·154M-KCl (isotonic) to give a 1 in 5 homogenate (dry wt. 50–60 mg./ml.).

Suspensions of kidney cortex (guinea pig or rabbit) were prepared essentially as described by Liébecq & Peters (1949). The kidneys were removed, cut in two and cooled in ice. Further operations were carried out in the cold room. The kidneys, after removal of the medulla, were passed through a Latapie mincer and then ground either in a mortar or in a Potter homogenizer. The medium was either 8·55% sucrose or the isotonic KCl containing $KHCO_3$ and