Pathways of Pyruvate Metabolism in the Mammary Gland*

By C. TERNER
National Institute for Research in Dairying, University of Reading

(*Received* 10 *March* 1951)

The utilization of glucose and acetate by lactating mammary tissue *in vitro* has been demonstrated by Folley & French (1949*a*, 1950). The observation that these substrates are metabolized with a high respiratory quotient is regarded as evidence for their utilization in milk-fat synthesis.

In the present paper an account is given of experiments on the metabolism of pyruvate in mammary tissue. The evidence obtained suggests that the Krebs citric acid cycle is operative in the mammary gland. However, only a fraction of the pyruvate metabolized is oxidized completely and a large part appears to be utilized in synthetic reactions, a conclusion supported by the use of 2:4-dinitrophenol (DNP), which is known to inhibit synthetic reactions by interfering with the transfer of phosphate-bond energy generated by the Krebs cycle (Lipmann, 1946; Loomis & Lipmann, 1948).

EXPERIMENTAL

Material. Mammary gland tissue of lactating rats, rabbits, or goats was removed immediately after the death of the animal. Sheep mammary tissue was obtained by biopsy under *cyclo*propane anaesthesia. Tissue slices, cut by the method of Stadie & Riggs (1944), were shaken in three changes of ice-cold saline to remove as much milk as possible; they were then blotted and 100–150 mg. portions weighed on a torsion balance and placed in the prepared media in Warburg flasks. These slices will be referred to as 'non-depleted' or 'untreated' slices.

'Depleted' tissue. In order to reduce their stores of preformed substrates, the slices were subjected in bulk to a preliminary incubation for 1 hr. at 37° in a 100 ml. measuring cylinder containing 50 ml. of saline through which a rapid stream of O_2 was passed. The tissue was then again shaken in ice-cold saline, blotted, and weighed portions transferred to Warburg flasks. 'Depleted' slices were used in all experiments described in this paper, unless otherwise stated.

Dry weights. At the end of the experimental period the cups were cooled in ice water and HCl added (final concn. 0·04 N); the tissue portions were removed from the media, washed in distilled water and dried in a steam oven overnight. All metabolic quotients were calculated from the final dry weight of the tissue. The ratio final dry weight/initial wet weight was 0·14–0·15 for lactating mammary gland slices, and 0·4–0·5 for pregnant mammary gland slices.

Saline media. Phosphate saline without calcium (Krebs & Eggleston, 1940) was the standard medium. The bicarbonate saline of Krebs & Henseleit (1932) was used in some experiments.

Analytical methods. Pyruvate was estimated by the method of Friedemann & Haugen (1943). The method involving extraction with benzene or toluene gave the same results as the direct method ('total hydrazones') and the latter was in a few determinations used for greater speed. The period of incubation of the sample with 2:4-dinitrophenylhydrazine was 5 min. in all cases.

Citric acid was determined by the method of Weil-Malherbe & Bone (1949), acetoacetic acid according to Krebs & Eggleston (1945), lactic acid by the method of Barker & Summerson (1941) and glucose by the method of Nelson (1944). For the estimation of 'total bound carbohydrate' the tissue in its medium (after withdrawal of a 0·5 ml. sample for pyruvate estimation) was hydrolysed with N-KOH at 100° for 30 min., followed by hydrolysis in N-H_2SO_4 at 100° for 180 min. Reducing sugar was then determined by the method of Nelson (1944) and calculated as glucose. For the determination of steam-volatile fatty acids, 1–2 g. portions of depleted mammary gland slices were incubated in large manometric flasks (Krebs & Eggleston, 1945) containing 20 ml. of saline and added substrates. At the end of the incubation period, 5 ml. of 2 N-H_2SO_4 were added and the tissue removed. The media were deproteinized with tungstic acid and steam distilled in the apparatus described by Markham (1942); this was followed by redistillation from HgO according to Friedemann (1938). The distillates were analysed by liquid-gas partition chromatography (James & Martin, 1951). These analyses were kindly carried out by Dr A. J. P. Martin.

R.Q. was determined by the method of Dickens & Šimer (1931), using Dickens & Greville (1933) flasks.

A diluted solution of pyruvic acid was kept as a stock solution and portions were neutralized before each experiment as recommended by Lipschitz, Potter & Elvehjem (1938).

RESULTS

Endogenous respiration and effect of fumarate. The endogenous respiration of lactating mammary tissue is high, and the increase of O_2 uptake on addition of substrates can often be relatively small. Preliminary incubation of the tissue slices without added substrates (see Experimental section) reduced the endogenous Q_{O_2} of fresh rat tissue from -4 to -8 (cf. Folley & French, 1949*a*) to -2 to -3. The depleted slices respired with high Q_{O_2} in the presence of glucose or pyruvate, but in many

* Preliminary communication, Terner (1950).

Table 1. *Effect of prolonged incubation on metabolism of lactating mammary tissue*

(Rat mammary gland slices incubated in Warburg flasks at 37° after (*a*) 60 min. aerobic incubation at 37°, (*b*) 60 min. storage in ice-cold saline. Phosphate saline without calcium (Krebs & Eggleston, 1940); gas, 100% O_2; glucose, pyruvate, 0·01 M.)

Exp. no.	Tissue portion	Preliminary treatment	Substrates added	$-Q_{O_2}$ during period 30–60 min.	$-Q_{O_2}$ during period 60–120 min.
1	1		None	2·9	2·2
	2	Incubated at 37°	Glucose at 60 min.	3·1	4·1
	3		Pyruvate at 0 min.	8·3	8·5
	4	Stored at 0°	Glucose at 60 min.	6·5	8·4
				10–30 min.	30–90 min.
2	1		None	1·1	1·0
	2	Incubated at 37°	Glucose at 30 min.	1·0	1·7
	3		Pyruvate at 30 min.	0·9	1·7
	4		Pyruvate at 0 min.	10·7	10·6
	5		None	7·4	4·2
	6	Stored at 0°	Pyruvate at 0 min.	8·9	8·1
	7		Pyruvate at 30 min.	5·2	6·5

experiments the increase of respiration was small or absent, when the addition of the substrate had been delayed for 30–60 min. from the start of the incubation period in the Warburg apparatus (Table 1). The addition of fumarate (0·002–0·01 M) to the medium in the Warburg flasks restored to the depleted tissue its ability to metabolize glucose or pyruvate, even when these substrates were added after long periods of incubation (up to 2 hr.). Fumarate alone did not increase the O_2 consumption above the endogenous level (Table 2).

In order to test whether prolonged incubation without added substrates damaged the respiratory mechanism of the tissue, slices from the same gland were divided into two portions *a* and *b*. Slices *a* were incubated at 37° for 60 min., while slices *b* were stored in ice-cold saline. Subsequently the depleted slices *a* respired in the presence of fumarate and either glucose or pyruvate at approximately the same high rates as the untreated slices *b* ($Q_{O_2} - 6$ to -11; cf. Folley & French, 1949*a*), while the endogenous respiration of slices *a* had been reduced to one-third or one-half of that of slices *b* (Table 1). The depletion process, therefore, does not impair the ability of the tissue to metabolize added substrates, and increases the magnitude of substrate respiration in relation to the endogenous respiration. Oxaloacetate or a precursor is an essential metabolite which is lost from the system during prolonged preliminary incubation. Fluoride (0·005–0·01 M) was in some experiments added to untreated tissue in order to inhibit the utilization of endogenous substrates.

Effect of fumarate and malonate on metabolism of pyruvate. When depleted tissue was incubated with malonate (0·0025–0·01 M) the increase of Q_{O_2} on addition of pyruvate was inhibited. In the presence of fumarate, the inhibition due to malonate was to some extent reversed. In mammary gland slices which had not been depleted, pyruvate was oxidized in the presence of malonate at rates of the same order of magnitude as those observed in depleted tissue incubated with fumarate, malonate and pyruvate (Tables 2 and 3).

Formation of citric acid. Knodt & Petersen (1946) reported the *in vitro* formation by mammary tissue of citric acid from endogenous substrates. Added substrates increased the amount of citric acid formed, but the authors could not exclude the possibility that these substances 'acted indirectly to increase citric acid by stimulating other metabolic processes which in turn increase citric acid formation'. In the present experiments, citric acid was not found in measurable quantities after the incubation of depleted mammary gland slices without added substrates. On incubation with fumarate and pyruvate, however, small amounts of citric acid accumulated (Table 4).

The role of fumarate in the metabolism of pyruvate, the inhibitory effect of malonate, its reversal by fumarate, and the formation of citric acid during the metabolism of pyruvate are evidence suggesting the operation of the citric acid cycle in the mammary gland (see Krebs & Eggleston, 1940).

Effect of DNP on metabolism of pyruvate. In the course of a study of the effect of inhibitors on the metabolism of pyruvate in lactating mammary gland slices of the rat, rabbit and sheep, the addition of 2×10^{-4} M-DNP was found to cause a large increase of the O_2 uptake in the presence of pyruvate plus fumarate (Fig. 1). When added in the absence of pyruvate, DNP caused inhibition of the endogenous respiration (cf. Dodds & Greville, 1934). Estimation of the amount of pyruvate disappearing showed that in the absence of DNP less oxygen was taken up by the tissue slices than corresponded to the complete oxidation of the pyruvate metabolized, the ratio extra O_2 taken up/pyruvate disappearing being less than 1. On addition of DNP, the rate at which

Table 2. *Effect of fumarate and malonate on respiration of lactating mammary tissue*

(Rat mammary gland slices incubated in Warburg flasks at 37° after (*a*) 60 min. aerobic incubation at 37°, (*b*) 60 min. storage in ice-cold saline. Phosphate saline; gas, 100% O_2; pyruvate, 0·01 M; malonate, 0·01 M.)

Exp. no.	Tissue portion	Preliminary treatment	Substrates added	$-Q_{O_2}$ during period (min.) 30–60	60–120
1	1		None	3·6	3·0
	2	Incubated at 37°	Fumarate (0·005 M) at 0 min.	4·0	3·6
	3		Fumarate (0·005 M) at 0 min.; pyruvate at 60 min.	5·0	7·1
2	1		None	2·9	2·4
	2		Pyruvate at 60 min.	2·3	4·4
	3		Fumarate (0·005 M) at 0 min.; pyruvate at 60 min.	3·0	6·4
	4	Incubated at 37°	Malonate at 0 min.; pyruvate at 60 min.	2·4	3·0
	5		Fumarate (0·01 M), malonate at 0 min.; pyruvate at 60 min.	3·6	5·6
	6		None	4·3	2·9
	7	Stored at 0°	Pyruvate at 60 min.	4·3	7·5
	8		Malonate at 0 min.; pyruvate at 60 min.	3·2	5·7

Table 3. *Effect of fumarate and malonate on metabolism of pyruvate in lactating mammary tissue*

(Rat mammary gland slices incubated at 37° in phosphate saline at 37°; gas, 100% O_2. Depleted slices, except in Exp. 4. Pyruvate, 0·005 M.)

Exp. no.	Incubation period (min.)	Tissue portion	Additions	$-Q_{O_2}$	$Q_{pyruvate}$
1	100	1	None	3·5	—
		2	Fumarate (0·005 M), pyruvate	5·9	−4·3
		3	Malonate (0·005 M), pyruvate	4·8	−2·9
2	85	1	None	2·0	—
		2	Fumarate (0·002 M), pyruvate	5·3	−4·3
		3	Malonate (0·0025 M), pyruvate	2·7	−3·0
3	170	1	None	1·4	—
		2	Fumarate (0·002 M), pyruvate	4·5	−3·2
		3	Malonate (0·01 M), pyruvate	1·3	−1·1
		4	Fumarate (0·002 M), malonate (0·01 M), pyruvate	2·3	−1·8
4	110	1	Malonate (0·01 M)	1·6	—
		2	Malonate (0·01 M), fumarate (0·005 M)	1·8	—
		3	Fumarate (0·005 M), pyruvate	5·4	−3·7
		4	Malonate (0·01 M), pyruvate	4·1	−2·9
		5	Malonate (0·01 M), fumarate (0·005 M), pyruvate	4·9	−3·4

Table 4. *Formation of citric acid in mammary tissue*

(Mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2; fumarate (0·002 M) in all cups; pyruvate 0·005 M; 2:4-dinitrophenol, 2×10^{-4} M. Exp. 1, rat; Exps. 2 and 3, rabbit. Incubation period, 60 min.)

Exp. no.	Tissue portion	Additions	$-Q_{O_2}$	$Q_{pyruvate}$	$Q_{citric\ acid}$
1	1	None	2·2	—	<0·1
	2	Pyruvate	4·3	−4·1	+1·2
	3	Pyruvate, DNP	6·2	−4·5	<0·1
	4	Pyruvate, fluoroacetate (0·005 M)	5·2	−3·5	+1·1
2	1	None	3·7	—	<0·1
	2	Pyruvate	9·5	−5·8	+0·4
	3	Pyruvate	8·9	−4·9	+0·3
	4	Pyruvate, DNP	13·4	−6·0	<0·1
	5	Pyruvate, DNP	13·3	−6·1	<0·1
3	1	None	2·9	—	<0·1
	2	Pyruvate	8·4	−5·4	+1·1
	3	Pyruvate, DNP	16·0	−6·8	<0·1

pyruvate was metabolized ($Q_{pyruvate}$) was not considerably, if at all, increased, although the O_2 consumption increased by 50–100 %. The stimulation of O_2 uptake by DNP was much greater in those experiments in which the addition of pyruvate had resulted in only a small stimulation of respiration above the endogenous level. When the increase due to addition of pyruvate had been large, the effect of DNP was relatively small. The ratio extra oxygen consumed/pyruvate disappearing was increased by DNP to a value of 1·5–2, indicating that pyruvate is more completely oxidized in the presence of DNP than in its absence (Table 5). No citric acid measurable by the experimental technique employed was found after incubation in the presence of DNP. In most experiments the increase of O_2 uptake due to DNP greatly exceeded the extra O_2 consumption that might have been expected from the complete oxidation of the citric acid accumulated in the absence of DNP (Table 4).

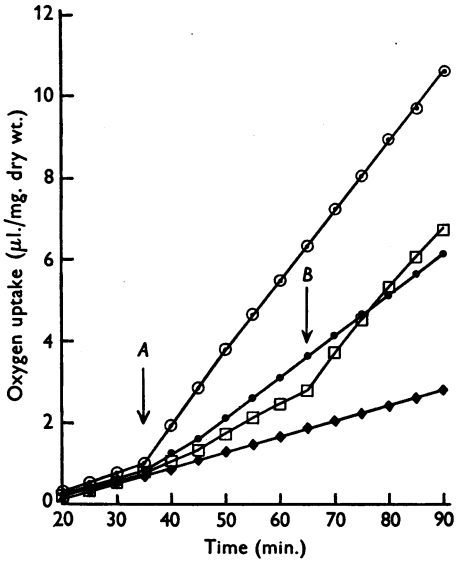

Fig. 1. Effect of 2:4-dinitrophenol on pyruvate metabolism in 'depleted' mammary tissue. Rat mammary gland slices incubated in phosphate saline at 37°; gas, 100 % O_2; 0·002 M-fumarate in all cups. ◆—◆, no addition; ●—●, pyruvate (0·01 M) added at *A*; □—□, pyruvate added at *A*, DNP (2×10^{-4} M) at *B*; ○—○, pyruvate and DNP at *A*.

Pyruvate metabolism of non-lactating mammary tissue. The largest and most regular increases in oxygen consumption on addition of DNP to mammary tissue metabolizing pyruvate were observed at the height of lactation. Mammary gland slices from pregnant rats (20th day of pregnancy), despite their low rate of respiration (Q_{O_2} about -1, cf. Folley & French, 1949*b*), showed a distinct increase of oxygen consumption on addition of pyruvate and 2×10^{-4} M-DNP (Fig. 2).

Formation of acetic acid. In view of the *in vitro* utilization of acetate by the mammary gland (Folley & French, 1950), accumulation of acetate derived from the oxidative decarboxylation of pyruvate is not to be expected. Acetate was found in small amounts in the steam distillates of the media recovered from large-scale experiments (see Experimental section), and was increased when pyruvate was present. Traces of propionic, butyric and *iso*-butyric acid found in some samples may have been

Table 5. *Effect of inhibitors on metabolism of mammary tissue*

(Rabbit mammary gland slices incubated at 37° in phosphate saline; gas, 100 % O_2; fumarate (0·002 M) in all cups; pyruvate, 0·005 M; glucose, 0·01 M; acetate, 0·01 M; fluoroacetate, 0·005 M; fluoride, 0·005 M.)

Tissue portion	Additions	$-Q_{O_2}$ during period (min.) 10–40	40–70	70–100	$-Q_{pyruvate}$ during period 40–100 min.	$-\Delta Q_{O_2}$ during period 40–100 min.	Ratio $\Delta Q_{O_2}/Q_{pyruvate}$
1	None	3·0	2·7	3·1	—	—	—
2	Pyruvate at 40 min.	3·2	6·9	10·0	5·4	5·3	1·0
3	Pyruvate at 40 min.	2·9	5·7	7·8	3·4	3·9	1·1
4	Pyruvate; DNP at 40 min.	3·0	14·7	17·3	6·9	13·0	1·9
5	Pyruvate; DNP at 40 min.	3·2	14·3	16·2	7·0	12·1	1·7
6	Glucose, acetate at 40 min.; DNP at 70 min.	3·1	10·2	16·9	—	—	—
7	Glucose, acetate at 40 min.; DNP at 70 min.	3·1	12·0	17·5	—	—	—
8	Fluoride at 0 min.; glucose, acetate at 40 min.; DNP at 70 min.	1·5	3·5	2·5	—	—	—
9	Fluoroacetate at 0 min.; pyruvate at 40 min.	1·0	1·0	1·0	1·1	—	—
10	Fluoroacetate at 0 min.; pyruvate, DNP at 40 min.	1·4	2·0	1·4	0·4	—	—
11	Pyruvate at 40 min.; fluoroacetate at 70 min.	2·8	7·0	4·4	3·6	—	—
12	Pyruvate at 40 min.; fluoroacetate at 70 min.	2·5	6·0	4·1	3·1	—	—

derived from tissue debris. The extra acetic acid accumulated in the presence of pyruvate was equivalent to 5–10 % of the pyruvate metabolized (Table 6).

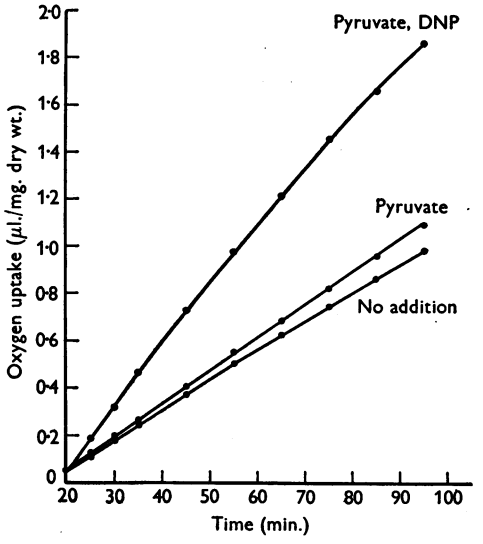

Fig. 2. Effect of 2:4-dinitrophenol on pyruvate metabolism in non-lactating mammary tissue. Rat mammary gland slices (20th day of pregnancy) (not depleted) incubated in phosphate saline at 37°. Fumarate (0·002 M) in all cups; additions: pyruvate (0·005 M), DNP (2×10^{-4} M).

Formation of lactic acid. Only small amounts of lactic acid were formed from pyruvic acid in the presence or absence of DNP (Table 7).

Formation of acetoacetic acid. Acetoacetic acid was not found in measurable amounts after incubation of mammary gland slices with fumarate and pyruvate, in the presence or absence of DNP.

Changes in tissue carbohydrate. Rat mammary gland slices washed in several changes of saline contained 0·25–0·3 % of 'total bound carbohydrate' (see Methods section). On incubation without added substrates, the carbohydrate content decreased slightly, but was approximately maintained in the presence of pyruvate. Table 8 shows that the changes in carbohydrate content were small in relation to the amount of pyruvate metabolized, and that less than 10 % of the pyruvate disappearing was utilized in the maintenance of the carbohydrate level. DNP appeared to inhibit the synthesis of carbohydrate from pyruvate.

Table 6. *Formation of acetic acid in mammary tissue*

(Mammary gland slices (approx. 1·5 g.) incubated at 37° in phosphate saline in large manometric flasks (Krebs & Eggleston, 1945); gas, 100 % O_2; tissue of Exps. 1 and 2 not depleted; tissue of Exp. 3 depleted by preliminary incubation for 60 min.; fumarate (0·005 M) in all cups; fluoride, 0·01 M; pyruvate, 0·002 M; DNP, 5×10^{-5} M; media of Exp. 3 were saponified before steam distillation; Exps. 1 and 3, rat; Exp. 2, rabbit. Incubation period 120 min.)

Exp. no.	Additions	$-Q_{O_2}$	$-Q_{pyruvate}$	$Q_{acetic\ acid}$
1	Fluoride	1·7	—	0·2
	Fluoride, pyruvate	2·8	2·1	0·3
2	Fluoride	1·2	—	0·1
	Fluoride, pyruvate	3·0	2·8	0·1
3	None	2·1	—	0·1
	Pyruvate	3·6	3·0	0·3
	Pyruvate, DNP	5·8	3·7	0·5

Table 7. *Formation of lactic acid in mammary tissue*

(Rabbit mammary gland slices incubated at 37° in phosphate saline; gas, 100 % O_2; fumarate (0·002 M) in all cups. Incubation period, 90 min.)

Exp. no.	Additions	$-Q_{O_2}$	$-Q_{pyruvate}$	$Q_{lactic\ acid}$
1	None	2·3	—	0·6
	Pyruvate (0·005 M)	4·0	5·1	0·9
	Pyruvate (0·005 M), DNP (2×10^{-4} M)	11·8	6·1	1·2
2	None	2·1	—	0·5
	Pyruvate (0·0025 M)	3·7	2·6	1·2
	Pyruvate (0·0025 M), DNP (5×10^{-5} M)	7·1	3·7	0·8

Table 8. *Changes in carbohydrate content of mammary gland slices*

(Rat mammary gland slices incubated at 37° in phosphate saline; gas, 100 % O_2. Incubation period, 120 min. Pyruvate, 0·01 M, fumarate, 0·02 M, 2:4-dinitrophenol, 2×10^{-4} M. Preliminary treatment: slices washed by shaking in ice-cold saline; no preliminary incubation.)

Tissue portion	Wet wt. (mg.)	Additions	O_2 uptake (μl.)	Pyruvate disappearing (μl.)	Total bound carbohydrate as glucose (μg.)	(μl.)
1	196·5	Not incubated	—	—	585	73
2	195·0	None	127	—	504	63
3	196·5	None	99	—	450	56
4	195·5	Fumarate, pyruvate	382	354	604	75
5	197·0	Fumarate, pyruvate	305	273	571	71
6	196·0	Fumarate, pyruvate, DNP	875	452	518	65

Effect of DNP on glucose metabolism. In contrast to its stimulating effect on the oxygen uptake of mammary tissue metabolizing pyruvate, 2×10^{-4}M-DNP did not increase, and in most experiments inhibited, respiration when glucose was the substrate (Table 9). Lower concentrations of DNP (2×10^{-5}M) stimulated respiration in the presence of glucose (Table 10). This may be partly due to the stimulation by DNP of aerobic glycolysis (see Dodds & Greville, 1934). 5×10^{-5}M-DNP was variable in its effect on glucose metabolism, causing definite stimulation in some experiments, and definite inhibition in others. It therefore appears that DNP affects carbohydrate metabolism in at least two ways: (1) by influencing the glycolytic breakdown of glucose to pyruvate; (2) by influencing the pathways of pyruvate metabolism, causing the larger part of pyruvate to be oxidized through the Krebs cycle.

Table 9. *Effect of* 2:4-*dinitrophenol on metabolism of mammary tissue*

(Rat mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2; fumarate (0·002M) in all cups; pyruvate, 0·05M; glucose, 0·005M; DL-lactate, 0·005M.)

Tissue portion	Additions	$-Q_{O_2}$ during period (min.) 30–60	60–120
1	None	3·3	2·8
2	Pyruvate at 30 min.	5·6	4·8
3	Pyruvate, 2×10^{-5}M-DNP at 30 min.	5·3	4·6
4	Pyruvate, 5×10^{-5}M-DNP at 30 min.	8·7	9·0
5	Pyruvate at 30 min., 10^{-4}M-DNP at 60 min.	5·0	7·9
6	Pyruvate at 30 min., 2×10^{-4}M-DNP at 60 min.	5·7	13·5
7	Glucose at 30 min.	4·5	3·9
8	Glucose at 30 min., 2×10^{-4}M-DNP at 60 min.	4·4	2·2
9	Lactate at 30 min.	4·1	2·3
10	Lactate at 30 min., 10^{-4}M-DNP at 60 min.	4·2	3·1
11	Lactate at 30 min., 2×10^{-4}M-DNP at 60 min.	4·2	2·2

It follows that in the presence of DNP in high concentration the formation of pyruvate from glucose will be inhibited and the stimulating effect of DNP on the metabolism of pyruvate will not be apparent. It is of interest, in this connexion, that in some experiments in which acetate was present in addition to glucose, 2×10^{-4}M-DNP caused some stimulation of O_2 consumption by mammary gland slices of the rabbit (Table 5), although in most experiments Q_{O_2} was inhibited by DNP in the presence of both glucose and glucose plus acetate. It seems plausible that 2-carbon fragments derived from acetate can under suitable conditions supplement the supply of 2-carbon fragments, thus enabling the Krebs cycle to proceed at an adequate rate.

Table 10. *Effect of* 2:4-*dinitrophenol on metabolism of glucose in mammary tissue*

(Rat mammary gland slices incubated at 37° in phosphate saline; gas, 100% O_2; fumarate (0·002M) in all cups; glucose, 0·005M; incubation period 90 min.)

Tissue portion	Additions	$-Q_{O_2}$
1	None	2·6
2	Glucose	6·8
3	Glucose, 10^{-5}M-DNP	6·4
4	Glucose, 2×10^{-5}M-DNP	10·5
5	Glucose, 5×10^{-5}M-DNP	2·7
6	Glucose, 2×10^{-4}M-DNP	2·3

Effect of fluoroacetate. Bartlett & Barron (1947) found that fluoroacetate inhibits the O_2 uptake and pyruvate utilization of tissue slices (kidney, heart, liver) and of minced muscle. Similar results were obtained with mammary gland slices (Table 5). As in yeast (Kalnitsky & Barron, 1947; Black & Hutchens, 1948), inhibition of respiration and pyruvate utilization were most nearly complete when fluoroacetate (0·005–0·05M) was present before pyruvate was added. Delayed addition of fluoroacetate often resulted in 30–50% inhibition of pyruvate utilization and respiration. In other experiments, fluoroacetate (0·01–0·025M) did not inhibit the oxygen uptake of mammary tissue metabolizing pyruvate. According to Bartlett & Barron

Table 11. *Effect of fluoroacetate on pyruvate metabolism in mammary tissue*

(Rat mammary gland slices incubated in phosphate saline at 37°; gas, 100% O_2; fumarate (0·002M) in all cups except no. 1; pyruvate (0·005M) and DNP (2×10^{-4}M) added after 15 min. equilibration; fluoroacetate (0·025M) added at time indicated.)

Tissue portion	Additions	$-Q_{O_2}$ during period (min.) 20–50	50–80	80–105	105–130
1	None	2·0	1·5	1·0	1·0
2	Pyruvate	5·6	5·6	5·2	3·4
3	Pyruvate, DNP	7·5	5·8	4·9	4·4
4	Pyruvate; fluoroacetate at 15 min.	5·8	5·4	4·7	4·5
5	Pyruvate; fluoroacetate at 50 min.	5·4	5·2	4·3	3·6
6	Pyruvate, DNP; fluoroacetate at 15 min.	4·5	3·6	2·5	1·7
7	Pyruvate, DNP; fluoroacetate at 50 min.	7·1	3·2	1·7	1·2

(1947) fluoroacetate inhibits synthetic reactions and causes accumulation of acetate. This may result in a low O_2/pyruvate ratio of the same order as in the absence of the inhibitor, so that conditions may obtain in which the respiration is not appreciably altered by the addition of fluoroacetate. When added to tissue slices metabolizing pyruvate in the presence of DNP, fluoroacetate caused progressive inhibition of O_2 consumption (Table 11).

Experiments in bicarbonate saline. In bicarbonate saline in equilibrium with 5% CO_2 and 95% O_2, DNP sometimes caused a marked acceleration of the disappearance of pyruvate (Table 12), while in experiments in phosphate saline in which CO_2 was absorbed by alkali, $Q_{pyruvate}$ was not or not appreciably increased by DNP. In bicarbonate saline a large decrease of pressure was observed in the manometers containing pyruvate and DNP. This was due to the retention of CO_2, which could be recovered from the medium by addition of acid at the end of the incubation period. A smaller uptake of gas was observed when pyruvate was metabolized in the absence of DNP, while an evolution of gas occurred when glucose was the substrate. When 1 mol. of pyruvate disappears by oxidation to CO_2 and water, the liberation of one equivalent of base results in the retention of 1 mol. of CO_2 as bicarbonate. The disappearance of pyruvate by complete oxidation may therefore be formulated thus:

$$CH_3.CO.COO^- + 2{\cdot}5\ O_2 \rightarrow 2CO_2 + HCO_3^- + H_2O. \quad (1)$$

Accordingly, for every mol. of pyruvate completely oxidized, the change of pressure in the manometer should be equivalent to the uptake of 0·5 mol. of gas. In the case of incomplete oxidation of pyruvate resulting in the formation of other acidic products less CO_2 will be retained; e.g. in the reaction

$$CH_3.CO.COO^- + \tfrac{1}{2}O_2 \rightarrow CH_3COO^- + CO_2. \quad (2)$$

0·5 mol. of gas should be evolved per mol. of pyruvate disappearing.

During the complete oxidation of glucose the uptake of O_2 and the evolution of CO_2 will balance. If, however, the pyruvate formed from glucose were incompletely oxidized, an increase of pressure should be observed; e.g. in the case of the formation of acetate, 4 mol. of CO_2 would be evolved and 2 mol. of O_2 taken up, resulting in the evolution of 2 mol. of gas per mol. of glucose disappearing.

$$C_6H_{12}O_6 + O_2 + 2HCO_3^- = 2CH_3CO.COO^- + 2CO_2 + 4H_2O, \quad (3)$$

$$2CH_3.CO.COO^- + O_2 \rightarrow 2CH_3COO^- + 2CO_2, \quad (4)$$

the overall reaction being

$$C_6H_{12}O_6 + 2O_2 + 2HCO_3^- \rightarrow 2CH_3COO^- + 4CO_2 + 4H_2O. \quad (5)$$

This is illustrated in Table 12, which shows manometric pressure changes of the relative magnitude and in the direction expected from the above considerations. No attempt is made to calculate 'Δ lactic acid', i.e. to differentiate between lactic acid formed from endogenous and from added substrates, since the oxidative removal of lactic acid during incubation without added substrates would result in high 'Δ lactic acid' values. The amounts of lactic acid determined were of the same order of magnitude in the different tissue portions of each experiment, except in tissue portion 6 of Exp. 2, in which lactic acid was increased. In this case the correction for the increased CO_2 evolution due to the extra lactic acid formed reduces the observed manometric pressure change to the small value expected for the complete oxidation of glucose in the presence of DNP.

It should be pointed out that any observed pressure change (Table 12) is the resultant of the pressure changes due to the two types of reactions discussed, and may be to some degree influenced by minor side reactions. The results, nevertheless, suggest that in bicarbonate saline also, DNP causes

Table 12. *Effect of* 2:4-*dinitrophenol on gaseous exchanges of mammary tissue*

(Rat mammary gland slices incubated in bicarbonate saline (Krebs & Henseleit, 1932); gas, 5% CO_2 + 95% O_2; fumarate (0·002 M) in all cups; pyruvate, 0·005 M; glucose, 0·0025 M. Exp. 1: incubation period 100 min.; additions after 15 min. equilibration; Exp. 2: incubation period 120 min.; additions after 20 min. equilibration. Manometric pressure changes calculated as μl. CO_2.)

Exp. no.	Tissue portion	Additions	CO_2 (μl.)	Pyruvate (μl.)	Glucose (μl.)	Lactic acid (μl.)	Dry wt. (mg.)
1	1	None	−16	—	—	<10	22·8
	2	Pyruvate	−28	−86	—	22	25·1
	3	Pyruvate, 2×10^{-4} M-DNP	−103	−150	—	20	24·8
	4	Glucose	+35	—	—	27	25·3
	5	Glucose, 2×10^{-4} M-DNP	+1	—	—	37	25·5
2	1	None	−16	—	—	<40	25·7
	2	Pyruvate	−43	−215	—	100	28·1
	3	Pyruvate, 2×10^{-4} M-DNP	−146	−289	—	104	26·2
	4	Glucose	+113	—	−98	80	27·8
	5	Glucose, 2×10^{-5} M-DNP	+67	—	−114	100	25·8
	6	Glucose, 5×10^{-5} M-DNP	+73	—	−88	148	28·0

more complete oxidation of pyruvate. Further, since acetate does not accumulate in appreciable amounts during the metabolism of pyruvate

Table 13. *Respiratory quotient of mammary gland slices metabolizing pyruvate*

(Rat mammary gland slices incubated at 37° in bicarbonate saline (Krebs & Henseleit, 1932); gas, 5% CO_2 + 95% O_2; fumarate (0·005 M) and fluoride (0·005 M) in all cups; pyruvate, 0·01 M; DNP, 2×10^{-4} M; incubation period 2 hr. Method of Dickens & Šimer (1931). All added pyruvate disappeared during incubation.)

Tissue portion	Additions	$-Q_{O_2}$	Q_{CO_2}	R.Q.
1	None	2·9	3·2	1·1
2	Pyruvate	7·3	11·5	1·6
3	Pyruvate	5·6	8·4	1·5
4	Pyruvate, DNP	9·3	12·5	1·3
5	Pyruvate, DNP	8·8	11·7	1·3

(Table 6), other acidic products, e.g. fatty acids, may be formed from pyruvate in mammary tissue.

Measurements of the respiratory exchanges by the method of Dickens & Šimer (1931) showed that DNP increases the O_2 consumption of mammary gland slices metabolizing pyruvate in bicarbonate saline and at the same time decreases the R.Q. (Table 13).

DISCUSSION

Endogenous and substrate respiration of mammary tissue. In comparing the oxygen consumption and substrate utilization of a tissue which has a high endogenous respiration, the problem arises whether the increase of oxygen consumption is due to the oxidation of the added substrate, and whether and to what extent the oxidation of endogenous substrates has been reduced (cf. Potter, 1944). According to Krebs & Eggleston (1940), added pyruvate competes for oxygen with the endogenous substrates of pigeon-breast muscle and suppresses their oxidation. Since the depleted and untreated mammary gland slices show a marked difference in their endogenous Q_{O_2}, but respire at similar rates in the presence of added substrate, it can be concluded that the oxidation of added substrates depresses the high endogenous respiration of untreated mammary tissue, at least to the level observed in depleted slices. It is more difficult to assess the effect of added substrates on the low endogenous respiration of depleted tissue. This, in contrast to the endogenous respiration of untreated tissue, is not appreciably inhibited by fluoride and may to a large extent be incapable of further reduction except by disorganizing the normal metabolic reactions of the tissue. Therefore, it may be preferable to use ΔQ_{O_2}, the increase of Q_{O_2} above the endogenous level, in comparing the rates of respiration and substrate utilization of depleted tissue. At any rate the error will be small when the endogenous respiration is low and the oxygen consumption in the presence of the added substrate is high.

Krebs cycle in the mammary gland. The relationship between fumarate and malonate in the metabolism of pyruvate in mammary gland slices is similar to that originally demonstrated by Krebs & Eggleston (1940) in minced pigeon-breast muscle. Moreover, in depleted mammary tissue, small amounts of citric acid were found to accumulate during the aerobic metabolism of pyruvate. It may therefore be concluded that the citric acid cycle operates in the mammary gland. In contrast to minced pigeon-breast muscle, mammary gland slices oxidize pyruvate incompletely; the low ratio oxygen consumed/pyruvate metabolized suggests that a part of the pyruvate disappearing is not oxidized to carbon dioxide and water, but is metabolized by pathways other than the Krebs cycle.

Effect of DNP on pyruvate metabolism. The acceleration of the respiration of mammalian tissue slices by dinitrophenols was first described by Dodds & Greville (1933). As shown in the present paper, the increase in respiration caused by DNP is not accompanied by a corresponding increase in pyruvate utilization. The low ratio $\Delta Q_{O_2}/Q_{pyruvate}$, about 1 in the presence of pyruvate and fumarate, was increased to 1·5 to 2 on addition of DNP. This inhibitor has been shown to inhibit synthetic reactions in bacteria without interfering with oxidation (Clifton, 1946) and to uncouple phosphorylation from oxidation in homogenates (Loomis & Lipmann, 1948). The experimental results described are consistent with the view that the acceleration of the respiration of mammary tissue by DNP is due to the inhibition of pathways of pyruvate metabolism depending on phosphate-bond energy, i.e. synthetic reactions, with the result that a larger part of the pyruvate is oxidized via the Krebs cycle.

Pathways of pyruvate metabolism. The first evidence for the existence of two pathways of pyruvate metabolism in brain tissue, complete oxidation, and incomplete oxidation resulting in the formation of acetate, was offered by Long (1938) and Long & Peters (1939). Recently, Coxon, Liébecq & Peters (1949), working with brain homogenates, concluded that the first step in the oxidation of pyruvate is an oxidative decarboxylation, the resulting 2-carbon fragments being either stabilized as acetate, or, if oxaloacetate is available, being incorporated in the Krebs cycle. That the ratio $\Delta Q_{O_2}/Q_{pyruvate}$ is less than 1 in most experiments with mammary gland slices suggests that in this tissue complete and incomplete oxidation of pyruvate occur side by side in the presence of oxaloacetate, the observed ratio being the resultant of the ratios of the two reactions. Since acetate does not accumulate in mammary

tissue it may be presumed that the 2-carbon fragments resulting from oxidative decarboxylation of pyruvate are utilized in the synthesis of other metabolic products. If 1 mol. of pyruvate were oxidized completely and 2 mol. were utilized in the synthesis of fatty acids, e.g. of butyric acid, the overall reaction

$$3CH_3CO.COOH + 2{\cdot}5O_2 = CH_3CH_2CH_2COOH + 5CO_2 + 2H_2O \quad (6)$$

would result in an oxygen/pyruvate ratio of 0·8. This ratio would be depressed to about 0·6 if longer chain saturated fatty acid (up to C_{18}) were formed from 2-carbon units derived from pyruvate. Thus the experimental results described in this paper suggest that 1 mol. of pyruvate is oxidized for every 2 mol. of pyruvate utilized in synthetic reactions. This relationship corresponds to a Meyerhof oxidation quotient of three. Meyerhof (1925, 1942) found values of three to six for the ratio substrate disappearing/substrate oxidized for lactic acid in muscle and sugar in yeast. In the presence of DNP, however, the ratio $\Delta Q_{O_2}/Q_{\text{pyruvate}}$ approaches 2·5, and complete oxidation becomes the predominant pathway of pyruvate metabolism.

It is, therefore, most probable that in the mammary gland 2-carbon fragments derived from pyruvate are utilized in synthetic reactions which are energized by phosphate-bond energy generated during the oxidation of part of the pyruvate via the Krebs cycle (see Lipmann, 1946). Pyruvate which has long been assumed to be a key intermediate between carbohydrate breakdown and fat synthesis (see Smedley-MacLean, 1936; Bloch, 1948), has recently been shown, in isotope experiments, to be a source of fatty acid carbon *in vivo* (Anker, 1948) and in rat-liver slices *in vitro* (Brady & Gurin, 1950). Fat synthesis by mammary tissue *in vitro* from glucose or acetate has been demonstrated by Folley & French (1950) by means of R.Q. determinations. This has been confirmed by Popják, French & Folley (1951), who observed the incorporation of carbon from ^{14}C-labelled acetate into milk fatty acids of the lactating goat. In view of the importance of fat synthesis in the mammary gland (see Folley, 1949), it may be assumed that pyruvate plays a part in the synthesis of fatty acids in this tissue both as a source of metabolic energy and of fatty acid carbon.

SUMMARY

1. In order to correlate the utilization of added substrates with their effect on respiration, lactating mammary gland slices (rat, rabbit, sheep) were depleted of preformed substrates by aerobic incubation without added substrates, prior to incubation in the Warburg apparatus. By this treatment the high endogenous respiration was reduced by 50–70 %; the ability of the depleted tissue to metabolize glucose or pyruvate was often impaired or lost, but was completely restored by the addition of fumarate in low concentrations.

2. Small amounts of citric acid accumulated when depleted mammary gland slices were incubated with fumarate and pyruvate. Fumarate reversed the inhibition of respiration caused by malonate. The above observations suggest that the citric acid cycle is operative in the mammary gland.

3. In phosphate saline the oxygen consumption of mammary tissue metabolizing pyruvate was increased by 50–100 % by 2×10^{-4}M-2:4-dinitrophenol (DNP), without a corresponding stimulation of pyruvate utilization. DNP thus increases the ratio oxygen consumed/pyruvate metabolized. Pyruvate is metabolized with high R.Q. which is reduced to 1·2–1·3 by DNP. These findings, and manometric measurements of gaseous exchanges in bicarbonate saline, indicate that pyruvate is oxidized more completely in the presence of DNP than in its absence.

4. Although 2×10^{-4}M-DNP accelerated respiration in the presence of pyruvate, it was inhibitory in the presence of glucose. Lower concentrations of DNP (2×10^{-5} to 5×10^{-5}M) stimulated respiration when glucose was metabolized. DNP therefore appears to affect carbohydrate metabolism in two ways: (*a*) by influencing the glycolytic breakdown of glucose to pyruvate; (*b*) by influencing the metabolic fate of pyruvate.

5. It is concluded that pyruvate is metabolized by mammary tissue by two pathways, (*a*) oxidation via the Krebs cycle, and (*b*) synthetic reactions. About 2 mol. of pyruvate appear to be utilized in synthetic reactions for every mol. of pyruvate oxidized. Inhibition of synthetic reactions by DNP increases the fraction of pyruvate oxidized and thus increases the overall oxygen consumption and the oxygen/pyruvate ratio.

After this paper had been submitted for publication, a short note appeared describing the oxidation of tricarboxylic acid intermediates in lactating mammary gland cyclophorase (Moore & Nelson, 1951). This confirms the evidence presented in the present paper suggesting the operation of the Krebs cycle in the mammary gland.

I wish to thank Dr S. J. Folley, F.R.S., for his interest in this work, Dr A. T. Cowie for biopsies on sheep, Dr A. J. P. Martin, F.R.S., for chromatographic analyses, and Prof. R. A. Peters, F.R.S., for the gift of a sample of sodium fluoroacetate.

REFERENCES

Anker, H. S. (1948). *J. biol. Chem.* **176**, 1337.
Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
Bartlett, G. R. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 67.
Black, S. & Hutchens, J. O. (1948). *Arch. Biochem.* **17**, 211.
Bloch, K. (1948). *Cold Spr. Harb. Sym. quant. Biol.* **13**, 29.
Brady, R. O. & Gurin, S. (1950). *Fed. Proc.* **9**, 155.
Clifton, C. E. (1946). *Advanc. Enzymol.* **6**, 269.
Coxon, R. V., Liébecq, C. & Peters, R. A. (1949). *Biochem. J.* **45**, 320.
Dickens, F. & Greville, G. D. (1933). *Biochem. J.* **27**, 1479.
Dickens, F. & Šimer, F. (1931). *Biochem. J.* **25**, 973.
Dodds, E. C. & Greville, G. D. (1933). *Nature, Lond.*, **132**, 966.
Dodds, E. C. & Greville, G. D. (1934). *Lancet*, **1**, 398.
Folley, S. J. (1949). *Biol. Rev.* **24**, 316.
Folley, S. J. & French, T. H. (1949*a*). *Biochem. J.* **45**, 117.
Folley, S. J. & French, T. H. (1949*b*). *Biochem. J.* **45**, 270.
Folley, S. J. & French, T. H. (1950). *Biochem. J.* **46**, 465.
Friedemann, T. E. (1938). *J. biol. Chem.* **123**, 161.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
James, A. T. & Martin, A. J. P. (1951). *Biochem. J.* **48**, vii.
Kalnitsky, G. & Barron, E. S. G. (1947). *J. biol. Chem.* **170**, 83.
Knodt, C. B. & Petersen, W. E. (1946). *J. Dairy Sci.* **29**, 115.
Krebs, H. A. & Eggleston, L. V. (1940). *Biochem. J.* **34**, 442.
Krebs, H. A. & Eggleston, L. V. (1945). *Biochem. J.* **39**, 408.
Krebs, H. A. & Henseleit, K. (1932). *Hoppe-Seyl. Z.* **210**, 33.
Lipmann, F. (1946). *Advanc. Enzymol.* **6**, 231.
Lipschitz, M. A., Potter, V. R. & Elvehjem, C. A. (1938). *J. biol. Chem.* **123**, 267.
Long, C. (1938). *Biochem. J.* **32**, 1711.
Long, C. & Peters, R. A. (1939). *Biochem. J.* **33**, 759.
Loomis, W. F. & Lipmann, F. (1948). *J. biol. Chem.* **173**, 807.
Markham, R. (1942). *Biochem. J.* **36**, 790.
Meyerhof, O. (1925). *Biochem. Z.* **162**, 43.
Meyerhof, O. (1942). In *A Symposium on Respiratory Enzymes*. Madison: University of Wisconsin Press.
Moore, R. O. & Nelson, W. L. (1951). *Fed. Proc.* **10**, 226.
Nelson, N. (1944). *J. biol. Chem.* **153**, 375.
Popják, G., French, T. H. & Folley, S. J. (1951). *Biochem. J.* **48**, 411.
Potter, V. R. (1944). *Advanc. Enzymol.* **4**, 201.
Smedley-MacLean, I. (1936). *Ergebn. Enzymforsch.* **5**, 285.
Stadie, W. C. & Riggs, B. C. (1944). *J. biol. Chem.* **16**, 187.
Terner, C. (1950). *Biochem. J.* **47**, xlix.
Weil-Malherbe, H. & Bone, A. B. (1949). *Biochem. J.* **45**, 377.

Hippuric Acid Synthesis in the Rat

By H. R. V. ARNSTEIN and A. NEUBERGER
National Institute for Medical Research, Mill Hill, London, N.W. 7

(*Received* 16 *February* 1951)

The conjugation of benzoic acid with glycine in the mammalian body which was discovered by Wöhler more than 120 years ago, has been the subject of a large number of publications. Many workers have tried to establish the source of the glycine which is made available for this conjugation without obtaining a really satisfactory answer. Lehmann (1851) suggested that the 'nitrogenous constituent of hippuric acid which we may regard as fumaramide or as glycine, is undoubtedly derived from the animal albuminous substances'. This idea was further elaborated by Wiechowski (1906), who believed that glycine or glycine precursors are formed in large amounts during the normal metabolism of proteins. Since administration of big doses of benzoate may lead to the excretion of considerable quantities of hippuric acid and a corresponding diminution of urea output, Wiechowski (1906) concluded that at least a large part of the normal catabolism of protein to urea takes place through glycine. An alternative possibility, namely that administration of benzoate stimulates the breakdown of tissue proteins and thus makes glycine available, was first put forward by Ringer (1911) and further discussed by Delprat & Whipple (1921). The observation made by various workers that the total nitrogen of the urine increased somewhat after giving large doses of benzoate was quoted as evidence for this assumption. However, other workers calculated that the glycine content of tissue proteins was not large enough to account for the amounts of hippuric acid excreted in experiments in which benzoate was fed over long periods. It was therefore suggested that the glycine was derived from other amino-acids (Magnus-Levy, 1907) or synthesized from ammonia and acetic acid (Cohn, 1893, 1905) or glyoxylic acid (Friedmann & Tachau, 1911) respectively. It was shown later that addition to the diet of glycine or of proteins rich in glycine accelerates the rate of excretion of hippuric acid in the rabbit (Griffith & Lewis, 1923) and increases markedly the output of hippuric acid in the pig (Abderhalden & Strauss, 1914; Csonka, 1924) and to a lesser extent in the dog (Quick, 1926). A similar