Anaerobic and Aerobic Glycolysis in Lactating Mammary Gland and in Nervous Tissue

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The stimulating effect of dinitrophenols on respiration and aerobic glycolysis of tumour slices was described by Dodds & Greville (1933, 1934) and has since been confirmed by many workers (for reviews see Peiss & Field, 1948; Tyler, 1950).

In a study of the effect of 2:4-dinitrophenol (DNP) on the metabolism of lactating mammary gland slices (Terner, 1951*c*), it was observed that the addition of 2×10^{-4} M-DNP caused marked stimulation of respiration when pyruvate was metabolized, but caused inhibition when glucose was the substrate.

In the present paper the results of a study of anaerobic and aerobic glycolysis and of the effect of DNP on the aerobic metabolism of glucose in lactating mammary gland slices and in nervous tissue are reported. It was found that under certain conditions DNP does not influence the breakdown of glucose and the formation of lactic acid in a parallel manner.

Preliminary accounts of part of this work have been given to the Biochemical Society (Terner, 1951*a*, *b*).

EXPERIMENTAL

Material. Mammary gland tissue of lactating rats and rabbits was removed immediately after the death of the animal and placed in ice-cold saline. Slices cut by the method of Stadie & Riggs (1944) were shaken in ice-cold saline to remove as much milk as possible, blotted and portions of approximately equal wet weight (150–200 mg.) were placed in the prepared media in conical Warburg flasks. In some experiments the slices were subjected to a preliminary incubation in saline without added substrates for 1 hr. at 37° in a 100 ml. measuring cylinder aerated by a rapid stream of O_2, and then treated as above. They are referred to as 'depleted' tissue (Terner, 1951*c*). Slices of the grey matter of guinea pig brain were cut by the technique of Deutsch (1936). Ox retina was prepared as described by Terner, Eggleston & Krebs (1950); portions of about 100 mg. wet weight were weighed and placed in the media in Warburg flasks. At the end of the incubation period the flasks were cooled in ice water and 1 ml. of 2N-HCl was added to the medium (4 ml.). The mammary gland slices and portions of retina were removed, washed rapidly in distilled water, dried in the steam oven and weighed. Portions of the acidified medium were taken for chemical analysis. The brain slices were not removed from the medium; the contents of the flask were poured into a small mortar and ground to a smooth suspension. Portions of this suspension were taken for analysis. The dry weight of the brain slices was taken as 20% of their initial wet weight.

Homogenates. Mammary tissue was homogenized in the homogenizer of Folley & Watson (1948), brain in the homogenizer of Potter & Elvehjem (1936). For dry weight, determination portions of the homogenates were evaporated and dried in the steam oven.

Saline media. Phosphate saline without calcium (Krebs & Eggleston, 1940) or bicarbonate saline (Krebs & Henseleit, 1932) were used in tissue slice experiments. A reaction mixture similar to that described by Potter & Le Page (1949) was used for glycolysis in homogenates.

In most aerobic experiments, substrates and inhibitors were placed in the side arm of the Warburg flask and added to the medium after an equilibration period of 10–15 min. In those cases, the incubation periods given in the tables do not include the equilibration period before the addition of the substrates. In anaerobic experiments strictly anaerobic conditions were maintained by a stick of yellow phosphorus in the centre well.

Analytical methods. Pyruvate was estimated by the method of Friedemann & Haugen (1943) after deproteinization with metaphosphoric acid; lactic acid by the method of Barker & Summerson (1941) after deproteinization with trichloroacetic acid; and glucose according to Nelson (1944). Cozymase was estimated spectrophotometrically after reduction with ethanol and alcohol dehydrogenase according to Kornberg (1950). Fluorimetric estimations of the nicotinamide-ribose fraction of cozymase by the method of Huff & Perlzweig (1947) were kindly carried out by Mr D. E. Hughes.

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

Cozymase preparations used in early experiments were samples of about 30% purity given by Mr D. E. Hughes and by Dr H. McIlwain. Later, a commercial product of 85% purity was obtained from Schwarz Laboratories Inc. Crystalline alcohol dehydrogenase (Racker, 1950) was a gift from Dr E. Racker.

Hexokinase was prepared from baker's yeast according to Meyerhof (1927). Before each experiment 20 mg. of the powder were ground in 2 ml. of distilled water in a glass homogenizer (Potter & Elvehjem, 1936) and the insoluble matter centrifuged off (see Needham, Siminovitch & Rapkine, 1951). The activity of the resulting extract was determined by the manometric method of Colowick & Kalckar (1943); 0·1 ml. of the solution contained 6–8 hexokinase units as defined by Berger, Slein, Colowick, & Cori (1946). The potassium salts of adenosinetriphosphate

Table 1. *Effect of pyruvate and nicotinamide on anaerobic glycolysis of rat mammary tissue*

(Rat mammary gland slices incubated in bicarbonate saline at 37°; gas, 5% CO_2 + 95% N_2; glucose, 0·01 M; pyruvate 0·005 M; nicotinamide, 0·01 M.)

Exp. no.	Tissue portion no.	Additions	$Q^{N_2}_{CO_2}$ (µl./mg. dry wt./hr.) during period (min.) 10–40	40–70	70–100	100–130	10–130
1	1	None	2·7	1·7	1·3	1·2	1·7
	2	Pyruvate	3·4	2·5	1·9	1·8	2·4
	3	Pyruvate, nicotinamide	3·4	2·5	1·8	1·7	2·4
	4	Glucose	4·3	2·8	2·5	2·3	3·0
	5	Glucose, nicotinamide	7·4	5·3	4·2	3·5	5·2
	6	Glucose, pyruvate	6·2	4·8	4·0	3·8	4·7
	7	Glucose, pyruvate	4·9	3·8	3·5	3·2	3·8
	8	Glucose, pyruvate, nicotinamide	8·9	7·5	7·0	6·0	7·3
	9	Glucose, pyruvate, nicotinamide	8·0	7·3	6·0	5·6	6·7

Exp. no.	Tissue portion no.	Additions	$Q^{N_2}_{CO_2}$ (µl./mg. dry wt./hr.) during period (min.) 10–40	40–70	70–100	100–150	10–150
2	1	None	2·9	1·6	1·5	1·1	1·6
	2	Nicotinamide	2·8	2·2	1·8	1·2	1·8
	3	Glucose, nicotinamide	4·8	4·1	2·7	2·4	3·1
	4	Glucose, pyruvate, nicotinamide	8·5	8·2	6·6	5·5	7·3
	5	Glucose, pyruvate, nicotinamide	10·6	10·5	9·1	8·4	9·4

(ATP) and fructose-1:6-diphosphate were prepared from commercial barium salts supplied by Schwarz Laboratories Inc.

Units. All metabolic quotients are expressed in the Q notation (µl./mg. dry wt./hr.). $Q_{\text{lactic acid}}$ denotes the rate of lactic acid production as determined by chemical estimation, while $Q^{N_2}_{CO_2}$ applies to acid formation calculated from changes in manometric pressure.

RESULTS

Anaerobic glycolysis in mammary gland slices. Lactating mammary gland slices of the rat or rabbit, incubated with glucose under anaerobic conditions, produced lactic acid at slow rates ($Q^{N_2}_{CO_2}$ 2–3). In rat tissue the rate of glycolysis was accelerated by the addition of nicotinamide (0·01–0·02 M) and was further increased and stabilized by the addition of pyruvate (0·001–0·005 M). Maximal rates of glycolysis were observed when all factors were present in the medium before incubation started. Delayed addition of glucose, e.g. after 10 min. equilibrium in the bath at 37°, or later, failed to promote rapid glycolysis even when pyruvate and nicotinamide were present from the start.

The instability of anaerobic glycolysis in rat mammary gland slices is also shown in the results of Folley & French (1949), who observed that with glucose as the only substrate, the initially high rate of glycolysis decreased rapidly after short (approx. 30 min.) periods of incubation. In the present experiments, high rates of glycolysis ($Q^{N_2}_{CO_2}$ up to 10) could be maintained for prolonged periods (up to 3 hr.) when glucose, pyruvate and nicotinamide were added (Table 1). Cozymase (10^{-4} to $2{\cdot}5 \times 10^{-4}$ M), in addition to nicotinamide, was no more effective than nicotinamide alone.

In rabbit mammary tissue, however, pyruvate, nicotinamide and cozymase were unable to support a rate of glycolysis ($Q^{N_2}_{CO_2}$) greater than about 3.

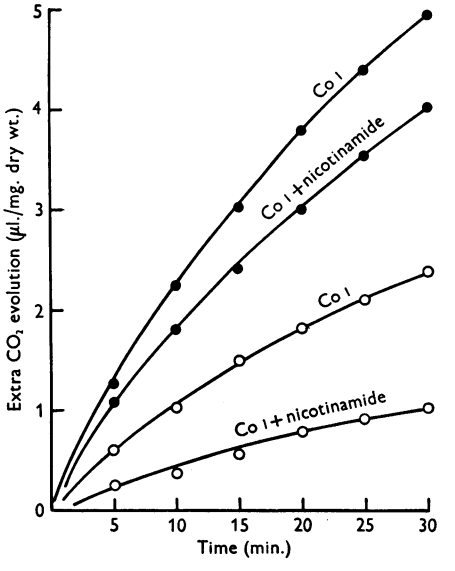

Fig. 1. Effect of nicotinamide on acid production from cozymase. Mammary gland homogenate incubated in bicarbonate saline at 37°; gas, 5% CO_2 + 95% N_2. Cozymase, 1·8 µmoles; nicotinamide, 0·02 M. ●—●, rabbit tissue (10 mg. dry wt.); ○—○, rat tissue (17 mg. dry wt.).

Inactivation of cozymase in mammary tissue. In order to compare the effect of nicotinamide on the breakdown of cozymase in rat and in rabbit mammary tissue, homogenates were incubated in

bicarbonate saline under anaerobic conditions and the extra acid production due to the decomposition of cozymase was measured manometrically as described by McIlwain & Rodnight (1949) (Fig. 1). Comparison of the manometric data with the results of direct estimation of cozymase showed a close relationship between the rates of acid formation and breakdown of cozymase (Table 2).

metrically by the method of Huff & Perlzweig (1947). Analysis of the reaction mixtures by the methods employed by Kornberg & Lindberg (1948) suggests that the pyrophosphatase is also active in the mammary gland of the rabbit (Table 3).

Other factors controlling glycolysis. If the enzymic inactivation of cozymase were the only factor restricting the breakdown of glucose in rabbit

Table 2. *Breakdown of cozymase by mammary tissue*

(Cozymase, 1·95 μmoles incubated with 1·0 ml. of homogenate (1 g. tissue + 7 ml. of water) in bicarbonate saline at 37°; gas, 5% CO_2 + 95% N_2; nicotinamide, 0·04 M.)

Source of tissue	Dry wt./ml. homogenate (mg.)	Flask no.	Inhibitor	Incubation period (min.)	Cozymase broken down (μmoles)	Inhibition (%)	Extra CO_2 evolved (μmoles)	Inhibition (%)
Rat	20·9	1	None	25	1·53	—	1·85	—
		2	Nicotinamide	25	0·62	59	0·76	59
		3	None	50	1·88	—	2·95	—
		4	Nicotinamide	50	0·99	47	1·50	56
Rabbit	17·2	1	None	25	1·53	—	2·45	—
		2	Nicotinamide	25	1·26	18	2·01	18
		3	None	50	1·88	—	3·48	—
		4	Nicotinamide	50	1·88	0	3·20	8

Table 3. *Breakdown of cozymase by rabbit mammary tissue*

(Experimental conditions as in Table 2. Cozymase, 2 μmoles; nicotinamide, 0·02 M. Fluorescence of nicotinamide-ribose fraction of cozymase determined according to Huff & Perlzweig (1947).)

Exp. no.	Flask no.	Incubation period (min.)	Inhibitor	Cozymase recovered (%)	Fluorescence (%)
1	1	0	None	100	100
	2	20	None	48	80
	3	20	Nicotinamide	53	88
	4	40	None	20	58
	5	40	Nicotinamide	26	78
2	1	0	None	100	100
	2	40	None	6	68
	3	40	Nicotinamide	6	82

In rat mammary tissue, the amounts of acid produced were approximately equivalent to the amounts of cozymase broken down, as found by McIlwain & Rodnight (1949) for nervous tissue. In rabbit mammary tissue, however, 1·5–2 moles of acid appeared to be produced for every mole of cozymase broken down. Nicotinamide (0·01–0·04 M) caused 50–80% inhibition of cozymase breakdown in rat tissue, but less than 20% inhibition in rabbit mammary tissue. The results thus suggest that in the rat the inactivation of cozymase is mainly due to a nucleotidase which is inhibited by nicotinamide (Mann & Quastel, 1941; Handler & Klein, 1942), while in the mammary gland of the rabbit cozymase appears to be broken down by another enzyme. Kornberg & Lindberg (1948) have demonstrated the presence in rabbit kidney of an enzyme which hydrolyses the pyrophosphate linkage of cozymase leaving intact the nicotinamide-ribose moiety which can be determined fluori-

mammary slices, it should be possible, by adding large amounts of cozymase, to increase the rate of lactic acid formation during short periods of incubation. This, however, was not found to be the case. Addition of adenosinetriphosphate (0·001 M) was also without effect. Fructose-1:6-diphosphate, however, was broken down to lactic acid more readily than glucose, but, as in the case of glucose, the addition of pyruvate was necessary. Relatively high rates of lactic acid formation were observed in the presence of glucose, fructose-1:6-diphosphate, pyruvate and cozymase (Table 4). Yeast hexokinase appeared to have some effect in accelerating glycolysis when added to the complete system. Its effect was more clearly shown in glycolysing homogenates of mammary tissue in which the rate of lactic acid formation could be increased by hexokinase by more than 100%. Hexokinase not only accelerated the initial rate of glycolysis but, in some experiments, also checked its decrease with time (Table 5).

Table 4. *Anaerobic glycolysis in rabbit mammary tissue*

(Rabbit mammary gland slices incubated in bicarbonate saline at 37°; gas, 5% CO_2 + 95% N_2. Glucose, 0·005M; pyruvate, 0·005M; fructose-1:6-diphosphate (HDP), 0·005M; cozymase (CoI), $1·5 \times 10^{-4}$M; fluoride (0·05M in Exp. 1; 0·025M in Exp. 2); ATP, 0·001M; hexokinase 0·2 ml.; nicotinamide (0·02M) in all cups. Incubation period: 40 min. in Exps. 1 and 2, 60 min. in Exp. 3.)

Exp. no.	Tissue portion no.	Additions	$Q_{\text{lactic acid}}$ (μl./mg. dry wt./hr.)
1	1	Glucose, pyruvate, CoI	1·4
	2	Glucose, pyruvate, CoI, hexokinase	1·9
	3	Glucose, pyruvate, CoI, hexokinase, ATP	2·2
	4	Glucose, pyruvate, CoI, hexokinase, ATP, NaF	2·1
	5	Pyruvate, HDP, CoI	3·0
	6	Glucose, pyruvate, HDP	3·4
	7	Glucose, HDP, CoI	2·6
	8	Glucose, pyruvate, HDP, CoI	4·3
	9	Glucose, pyruvate, HDP, CoI	4·3
	10	Glucose, pyruvate, HDP, CoI, hexokinase	5·4
2	1	Glucose, pyruvate, ATP, NaF	1·3
	2	Glucose, pyruvate, CoI, NaF	2·0
	3	Glucose, pyruvate, CoI, ATP, NaF	3·0
	4	Glucose, pyruvate, CoI, ATP	3·2
	5	HDP, pyruvate, CoI	7·0
	6	HDP, pyruvate, CoI, NaF	7·3
3	1	Glucose, pyruvate, CoI	2·0
	2	Glucose, pyruvate, CoI	2·3
	3	Glucose, HDP, CoI, hexokinase	1·5
	4	Glucose, HDP, CoI, hexokinase	2·0
	5	Glucose, pyruvate, CoI, hexokinase	2·1
	6	Glucose, pyruvate, CoI, hexokinase	2·6
	7	Glucose, pyruvate, HDP, CoI	4·6
	8	Glucose, pyruvate, HDP, CoI	5·2
	9	Glucose, pyruvate, HDP, CoI, hexokinase	6·5
	10	Glucose, pyruvate, HDP, CoI, hexokinase	6·6

Table 5. *Effect of hexokinase on glycolysis in mammary gland and brain homogenates of the lactating rabbit*

(The reaction mixtures (4 ml.) contained: $KHCO_3$, 0·025M: potassium phosphate pH 7·4, 0·0025M; $MgCl_2$, 0·005M; nicotinamide, 0·02M; cozymase (CoI), $2·5 \times 10^{-4}$M; ATP, $2·5 \times 10^{-4}$M; glucose, 0·005M; pyruvate, 0·005M; fructose-1:6-diphosphate, 0·001M; 1 ml. homogenate (1 g. tissue + 8 ml. water). Temperature, 37°; gas, 5% CO_2 + 95% N_2. In Exp. 1 the mammary gland and brain tissue are from the same animal.)

Exp. no.	Tissue	Flask no.	Amount of hexokinase added (ml.)	$Q^{N_2}_{CO_2}$ (μl./mg. dry wt./hr.) during period (min.) 15–25	25–35	35–50	50–65	15–65
1	Mammary gland	1	None	14·9	8·5	6·0	4·2	8·0
		2	0·05	17·9	10·8	7·6	6·0	9·3
		3	0·1	21·7	15·4	11·6	9·6	13·2
		4	0·2	28·1	23·1	22·0	21·2	22·9
	Brain	5	None	60·0	58·0	36·9	18·5	40·5
		6	0·05	58·5	55·0	28·6	18·2	36·7
		7	0·1	62·0	52·0	24·2	18·1	36·7
		8	0·2	70·6	36·7	26·0	18·7	36·9
		9	0·3	65·6	29·4	24·7	18·1	32·6
				$Q^{N_2}_{CO_2}$ (μl./mg. dry wt./hr.) during period (min.) 10–20	20–30	30–45	45–60	10–60
2	Mammary gland	1	None	26·7	12·3	9·1	7·0	12·6
		2	0·05	32·0	14·9	12·0	9·0	15·7
		3	0·1	35·1	19·6	14·6	12·2	18·6
		4	0·15	37·7	22·2	18·2	15·9	22·1
		5	0·2	40·0	24·9	20·9	16·9	24·2
		6	0·30	42·1	26·9	21·0	17·3	25·1

The addition of insulin (2·5 mg./100 ml.) and of DNP (10^{-4}M) had no effect on the rate of glycolysis in the absence or presence of hexokinase. On the other hand, in brain homogenates of lactating and non-lactating rats and rabbits, no comparable acceleration of glycolysis was observed on addition of hexokinase; in some brain homogenates inhibition of glycolysis was probably the result of the disturbing effect of large amounts of added hexokinase on the balance of enzymes within the glycolytic system (see Meyerhof & Wilson, 1949).

respiration of mammary gland slices metabolizing glucose, although it greatly increases the respiration in the presence of pyruvate. To investigate this further, the effect of DNP on the metabolism of glucose and pyruvate was studied by chemical analysis of the principal metabolites involved. An experiment in which depleted mammary tissue of the rabbit was used is shown in Table 6. DNP (2×10^{-4}M) almost completely inhibited glucose breakdown and reduced the respiration to the endogenous level (cup 5). The addition of pyruvate

Table 6. *Effect of cozymase and 2:4-dinitrophenol on metabolism of lactating mammary tissue*

(Depleted rabbit mammary gland slices, incubated at 37° in phosphate saline without Ca (Krebs & Eggleston, 1940). Gas, 100% O_2; fumarate (0·002M) in all cups. Additions: pyruvate 0·005M; glucose, 0·005M; nicotinamide, 0·01M; cozymase (CoI), $2{\cdot}5 \times 10^{-4}$M; DNP, 2×10^{-4}M. Incubation period: 80 min. $Q_{\text{lactic acid}}$ corrected for lactic acid formed in absence of added substrates.)

Tissue portion no.	Additions	Q_{O_2} (μl./mg. dry wt./hr.)	Q_{pyruvate} (μl./mg. dry wt./hr.)	Q_{glucose} (μl./mg. dry wt./hr.)	$Q_{\text{lactic acid}}$ (μl./mg. dry wt./hr.)
1	None	−2·3	—	—	—
2	Pyruvate	−4·0	−5·8	—	0·3
3	Pyruvate, DNP	−11·8	−6·9	—	0·6
4	Glucose	−5·2	—	−2·0	0·3
5	Glucose, DNP	−2·8	—	−0·3	0·7
6	Glucose, nicotinamide, CoI	−6·2	—	−1·8	0·4
7	Glucose, nicotinamide, CoI, DNP	−3·2	—	−0·1	0·3
8	Glucose, pyruvate	−6·3	−5·8	−1·5	1·0
9	Glucose, pyruvate, nicotinamide, CoI	−6·7	−6·1	−1·3	1·6
10	Glucose, pyruvate, nicotinamide, DNP	−8·8	−4·7	−0·8	1·4
11	Glucose, pyruvate, nicotinamide, CoI, DNP	−10·1	−5·7	−1·4	3·3

Table 7. *Effect of 2:4-dinitrophenol on glycolysis in lactating mammary gland slices*

(Rat mammary gland slices incubated at 37° in bicarbonate saline. Fumarate, 0·02M, in all aerobic cups. Glucose, 0·005M; pyruvate, 0·005M; nicotinamide, 0·02M; DNP, 5×10^{-5}M. Incubation period: 70 min.)

Tissue portion no.	Additions	Experimental conditions	Q_{glucose} (μl./mg. dry wt./hr.)	Q_{pyruvate} (μl./mg. dry wt./hr.)	$Q_{\text{lactic acid}}$ (μl./mg. dry wt./hr.)
1	None	Aerobic	—	—	0·7
2	Glucose	Aerobic	−4·3	—	0·7
3	Glucose, nicotinamide	Aerobic	−4·2	—	0·6
4	Glucose, DNP	Aerobic	−2·6	—	2·4
5	Glucose, nicotinamide, DNP	Aerobic	−2·8	—	2·8
6	Glucose, pyruvate, nicotinamide	Aerobic	−2·9	−5·8	1·6
7	Glucose, pyruvate, nicotinamide, DNP	Aerobic	−1·6	−6·4	2·4
8	Glucose, nicotinamide	Anaerobic	−3·4	—	6·1
9	Glucose, pyruvate, nicotinamide	Anaerobic	−4·7	−2·2	10·7

The results of chemical determination of lactic acid produced by homogenates agreed closely with the manometric data. In experiments with tissue slices, however, in which the rate of lactic acid production was relatively slow, the additional evolution of gas resulting from the decomposition of added unstable substances and from side reactions introduced large errors which made the manometric measurement of glycolysis impossible and lactic acid was in those cases determined chemically.

Effect of DNP on aerobic glycolysis in mammary gland slices. As previously reported (Terner, 1951c), DNP in high concentration (2×10^{-4}M) inhibits the

partly restored the breakdown of glucose and also caused accumulation of lactic acid (cup 10) which was greatly accelerated by the further addition of cozymase (cup 11). High rates of lactic acid formation were thus dependent on the presence of pyruvate, cozymase and DNP, and could not be maintained when one of those factors was omitted.

Although under those conditions lactic acid formation could be greatly increased, the rate of glucose breakdown was not accelerated. The results suggested that aerobic lactic acid formation could, under certain conditions, be increased without a corresponding increase and even with some inhibi-

tion of glucose breakdown. Owing to the relatively low rates of glucose utilization in rabbit tissue, the experiments were repeated under conditions in which larger changes could be observed in the amounts of glucose metabolized. Rat mammary gland slices were incubated in bicarbonate saline without a preceding depletion period (Table 7). A large increase in aerobic lactic acid formation was observed on addition of DNP (5×10^{-5} M) while the breakdown of glucose was inhibited. In fresh rat mammary slices, in contrast to depleted rabbit tissue, the addition of pyruvate, cozymase or nicotinamide was not required under aerobic conditions. DNP had no effect on glycolysis under anaerobic conditions.

Table 8. *Effect of* 2:4-*dinitrophenol on aerobic glycolysis in retina*

(Ox retina incubated in bicarbonate saline at 37°; gas, 5% CO_2 + 95% O_2. All cups contained glucose (0·005 M) and L-glutamate (0·001 M). Incubation period: 80 min.)

Tissue portion no.	Concn. of DNP (M)	$Q_{glucose}$ (μl./mg. dry wt./hr.)	$Q_{lactic\ acid}$ (μl./mg. dry wt./hr.)
1	0	−11	17
2	2×10^{-5}	−14	22
3	5×10^{-5}	−21	32
4	2×10^{-4}	−26	43

Experiments on other tissues. No inhibitory effect of DNP in the range of concentrations used (2×10^{-5} to 2×10^{-4} M) was observed during a study of aerobic glycolysis in retina, brain or in bull spermatozoa (see Melrose & Terner, 1951). In retina increasing concentrations of DNP accelerated both glucose breakdown and lactic acid formation by approximately equivalent increments (Table 8). In brain tissue the highest rates of glucose breakdown and lactic acid formation were observed when glutamate, nicotinamide, cozymase and DNP were added at the same time. Omission of glutamate, cozymase or of DNP resulted in a relatively low rate of aerobic glycolysis (Tables 9 and 10).

The known effects of glutamate in increasing aerobic lactic acid formation and of pyruvate in reversing the inhibition by glutamate of anaerobic glycolysis in brain slices (Weil-Malherbe, 1938) were also shown in these experiments. The prevention by nicotinamide of the enzymic destruction of cozymase in brain tissue has been shown to increase aerobic glycolysis (McIlwain, 1950). In the present experiments the addition of DNP, glutamate and cozymase could more than double the highest rates of aerobic lactic acid formation observed in the presence of glutamate and cozymase alone. In brain slices in the presence of glutamate, cozymase and DNP, the rate of glucose breakdown ($Q_{glucose}$) under aerobic conditions reached the level observed under anaerobic conditions, i.e. the Pasteur effect was completely inhibited (Table 10).

Table 9. *Effect of cozymase and* 2:4-*dinitrophenol on aerobic lactic acid formation in brain tissue*

(Guinea pig brain cortex slices incubated in bicarbonate saline; gas, 5% CO_2 + 95% O_2. Complete system: pyruvate, 0·005 M; glucose, 0·005 M; L-glutamate, 0·01 M; nicotinamide, 0·02 M; cozymase (CoI), $1{\cdot}25 \times 10^{-4}$ M; DNP, 10^{-4} M. Incubation period: 80 min.)

Tissue portion no.	Experimental conditions	$Q_{lactic\ acid}$ (μl./mg. dry wt./hr.)
1	Complete system	15·7
2	No DNP	8·9
3	No nicotinamide, no CoI	9·9
4	No glutamate	6·3
5	No glutamate, no DNP	5·5
6	No glutamate, no nicotinamide, no CoI	4·9

Origin of lactic acid formed under the influence of DNP. In view of the inhibitory effect of DNP on aerobic glucose breakdown in mammary tissue the question arises of the mechanism by which the increased amounts of lactic acid are formed.

It is conceivable that the extra lactic acid might be formed by the reduction of pyruvic acid, as a result of the inhibition by DNP of its oxidative removal. This is, however, not the case in mammary tissue, where DNP does not decrease $Q_{pyruvate}$ and does not increase lactic acid formation from pyruvate (see Terner, 1951*c*). Similarly, in brain

Table 10. *Effect of* 2:4-*dinitrophenol on glycolysis in brain tissue*

(Guinea pig brain cortex slices incubated in bicarbonate saline at 37°. Glucose, 0·005 M; pyruvate, 0·005 M; L-glutamate, 0·005 M; DNP, 2×10^{-4} M; nicotinamide, 0·02 M added with cozymase (CoI), 5×10^{-5} M. Incubation period: 60 min.)

Tissue portion no.	Additions	Experimental conditions	$Q_{glucose}$ (μl./mg. dry wt./hr.)	$Q_{pyruvate}$ (μl./mg. dry wt./hr.)	$Q_{lactic\ acid}$ (μl./mg. dry wt./hr.)
1	Glucose, glutamate	Aerobic	−3·5	—	5·4
2	Glucose, glutamate, DNP	Aerobic	−6·5	—	9·2
3	Glucose, glutamate, CoI, DNP	Aerobic	−8·3	—	12·2
4	Glucose, glutamate, pyruvate, CoI	Aerobic	−3·8	−4·9	5·2
5	Glucose, glutamate, pyruvate, CoI, DNP	Aerobic	−7·2	−4·0	12·5
6	Glucose, pyruvate, CoI	Anaerobic	−7·8	−1·1	16·2
7	Glucose, glutamate, pyruvate, CoI	Anaerobic	−8·4	−3·7	16·0

tissue, under aerobic conditions DNP does not stimulate the reduction of pyruvate to lactate (Table 11). The increased disappearance of pyruvate in the presence of glutamate shown in Table 11 is probably due to transamination and can also be observed under anaerobic conditions (Table 10).

Table 11. *Effect of* 2:4-*dinitrophenol on metabolism of pyruvate in brain tissue*

(Guinea pig brain cortex slices incubated in bicarbonate saline at 37°. Gas, 5% CO_2 + 95% O_2. Pyruvate, 0·005 M; L-glutamate, 0·01 M; DNP, 10^{-4} M. Incubation period: 65 min.)

Tissue portion no.	Additions	$Q_{pyruvate}$ (μl./mg. dry wt./hr.)	$Q_{lactic\ acid}$ (μl./mg. dry wt./hr.)
1	None	—	<0·5
2	Pyruvate	−5·2	2·1
3	Pyruvate, DNP	−5·9	1·6
4	Pyruvate, glutamate	−7·5	2·4
5	Pyruvate, glutamate, DNP	−7·9	2·4

DISCUSSION

Factors controlling glycolysis in mammary tissue

Breakdown of cozymase. Nicotinamide, which in rat mammary tissue inhibits the breakdown of cozymase, is almost ineffective in mammary tissue of the rabbit. Diphosphopyridine nucleotidase, which is specifically inhibited by nicotinamide (Handler & Klein, 1942), therefore appears to be the main cozymase-splitting enzyme in rat mammary tissue. Evidence has been presented suggesting that in the mammary gland of the rabbit the destruction of cozymase is catalysed mainly by the diphosphopyridine pyrophosphatase of Kornberg & Lindberg (1948). This difference in the enzymic composition of the mammary glands of the two species studied may at least partly explain the inability of rabbit mammary tissue to break down glucose, even in the presence of added cozymase, at rates comparable with those attained by rat mammary tissue. In rat mammary gland slices high rates of anaerobic glycolysis can be maintained by nicotinamide without added cozymase, and under aerobic conditions the addition of nicotinamide is unnecessary (Table 7).

The hexokinase reaction. Higher rates of lactic acid formation from hexosediphosphate than from glucose have been observed in cell-free extracts and homogenates (Ochoa, 1941; Utter, Wood & Reiner, 1945). It has been pointed out by Utter *et al.* (1945) that under those conditions 'phosphatase activity may be greater than the hexokinase activity so that little phosphorylation of glucose could occur'. Meyerhof & Wilson (1949) have shown that the rate of glycolysis in tumour homogenates and extracts depends on the balance between the activities of hexokinase and adenosinetriphosphatase. Tissue slices, however, represent a relatively balanced system and anaerobic glycolysis can usually be maintained at high rates with glucose as the only added substrate. The observations that in mammary gland slices hexosediphosphate could give rise to higher rates of lactic acid formation than could glucose, and that added hexokinase could further accelerate glycolysis in slices and in homogenates of mammary tissue, suggest that the rate of glycolysis in this tissue may be limited by a relatively slow hexokinase reaction. Meyerhof (1927) found that the formation of lactic acid from glucose in muscle extracts was facilitated by the addition of yeast hexokinase and attributed this to the lability of muscle hexokinase. Brain hexokinase, on the other hand, was found to be relatively stable (Meyerhof & Wilson, 1949). The present experiments (Table 5) accordingly point to an unstable or deficient hexokinase in mammary tissue. This may be expected, since the lactating mammary gland is under the influence of anterior pituitary and adrenal cortical hormones (see Folley, 1950), which have been shown to inhibit the hexokinase reaction (Cori, 1950). However, added hexokinase did not stimulate glycolysis in brain homogenates of lactating and non-lactating rabbits and rats. No conclusions are drawn from the present results regarding a hormonal inhibition of the hexokinase reaction in mammary tissue, a question which remains a subject for further study.

Effect of pyruvate on glycolysis. Pyruvate has long been known to have a stimulating effect on anaerobic glycolysis (Mendel, Bauch & Strelitz, 1931; Elliott & Henry, 1946). According to Meyerhof & Wilson (1949) pyruvate produces a stimulating effect on lactic acid formation by facilitating the dismutation between pyruvate and triosephosphate in cases in which the formation of pyruvate from glucose is inhibited owing to damage of the enzymes which transform 3-phosphoglyceric acid to pyruvic acid. In the present experiments pyruvate is shown to accelerate and stabilize anaerobic glycolysis in rat and rabbit mammary gland slices (Tables 1 and 3). In depleted mammary gland slices incubated with glucose, pyruvate also increases aerobic lactic acid formation, especially in the presence of DNP and cozymase (Table 6).

Aerobic glycolysis and Pasteur effect

Accumulation of lactic acid under aerobic conditions. The balance sheets presented in this paper show that, as is to be expected, the rate of aerobic lactic acid formation is the resultant of the relative rates of glucose breakdown and pyruvate oxidation. In mammary tissue pyruvate is oxidized as fast as it can be formed from glucose, and therefore lactic acid does not accumulate (Tables 6 and 7). In nervous tissue (Tables 9 and 10) and in spermatozoa

(Melrose & Terner, 1951) in which the glycolysing power of the tissue exceeds its capacity to oxidize the breakdown products of glycolysis, lactic acid is formed by reduction of the excess pyruvate. Thus, the predominance of the glycolytic over the oxidative capacity of a tissue will tend to produce the conditions associated with tissues which have a high aerobic glycolysis, i.e. high rates of glucose breakdown and accumulation of lactic acid. In tissues such as the mammary gland, in which the oxidative capacity is high in relation to their glycolytic power, the rapid aerobic re-oxidation of reduced cozymase at the triosephosphate level will prevent the functioning of the oxidation-reduction step and pyruvate, even if added in excess, will not be reduced to lactic acid (Tables 6 and 7).

The above remarks are based on a scheme which explains the Pasteur effect in terms of a competition between oxygen and pyruvate for the hydrogen of reduced cozymase (Ball, 1939). This scheme, modified by the above considerations relating to the variations in the balance of the glycolytic and oxidative powers of different tissues (see also Warburg, Posener & Negelein, 1924), appears to be in agreement with most experimental observations reported in this paper. It is therefore used as a working hypothesis regarding the mechanism of the Pasteur effect under the present experimental conditions.

Inhibition of the Pasteur effect by DNP. In tissues with a high aerobic glycolysis, e.g. cerebral cortex (in presence of glutamate) and retina, which show a relatively small Pasteur effect for the reasons outlined above, DNP further increases glucose breakdown and lactic acid formation. In mammary tissue, however, DNP in concentrations which produce stimulation of glycolysis and lactic acid formation in other tissues (Tables 6 and 7), causes inhibition of glucose breakdown. Nevertheless, lactic acid formation can be greatly increased by concentrations of DNP inhibitory to glucose breakdown, even in depleted mammary tissue with low glycolytic activity, provided cozymase and pyruvate is added. No explanation is offered for the inhibitory effect of DNP on the breakdown of glucose in mammary tissue in contrast to its accelerating effect in other tissues. However, it would appear that the increased accumulation of lactic acid caused by DNP is not due to its simply accelerating the rate of carbohydrate breakdown.

Since the Pasteur effect is defined as the action of oxygen in diminishing carbohydrate breakdown and in accelerating lactic acid accumulation (see Dixon, 1937) it may be argued that, although DNP inhibits the Pasteur reaction in nervous tissue, it does not do so in mammary tissue in which it does not increase glucose breakdown concomitantly with lactic acid formation. In fact, since under the experimental conditions of Table 7 the rates of glucose breakdown under anaerobic and aerobic conditions in the absence of DNP are of the same order of magnitude (cups 2, 3 and 9), there is, from the point of view of carbohydrate breakdown, no Pasteur effect in mammary tissue. On the other hand, it has generally been considered sufficient, in studies of the Pasteur reaction, to compare the rates of aerobic and anaerobic lactic acid formation (see Burk, 1939). Accordingly, had the present investigations been based on measurements of lactic acid production alone, it might have been inferred that mammary tissue exhibits a considerable Pasteur effect (e.g. Folley & French, 1949), and the increased accumulation of lactic acid under the influence of DNP might have led to the conclusion that the breakdown of glucose was also accelerated, i.e. that the Pasteur effect was inhibited. Various authors have calculated the rates of aerobic carbohydrate breakdown from manometric data of respiration and acid production (see Dixon, 1937; Dickens, 1951). In those calculations the complete oxidation of the substrate was assumed. Although that method may be applicable to many tissues, it would produce erroneous results in others; e.g. in mammary tissue (Terner, 1951*c*), and in spermatozoa (Melrose & Terner, 1951), where ratios of oxygen/substrate were found to be low and to be altered by DNP. The direct determination of the metabolites involved is therefore to be preferred in such studies.

SUMMARY

1. Anaerobic and aerobic glycolysis in mammary tissue has been studied and compared with glycolysis in nervous tissue. The rates of phosphorylation of glucose and of pyruvate formation, and the enzymic inactivation of cozymase were found to limit the rate of anaerobic lactic acid formation in mammary tissue.

2. In the presence of glucose, pyruvate and nicotinamide, high rates of anaerobic glycolysis ($Q_{\text{lactic acid}}$ approx. 10 μl./mg. dry wt./hr.) could be maintained in rat mammary gland slices for periods up to 3 hr.; when pyruvate or nicotinamide were omitted low rates of glycolysis were observed ($Q_{\text{lactic acid}}$ 2–3). Mammary gland slices of the rabbit, in the presence of glucose, pyruvate, nicotinamide and cozymase produced lactic acid at rates $>$ $Q_{\text{lactic acid}}$ approx. 3.

3. The enzymic decomposition of cozymase in homogenates of rat mammary tissue was strongly inhibited by nicotinamide (0·01–0·04 M). In mammary tissue of the rabbit, however, nicotinamide was almost ineffective in preventing the destruction of cozymase. In rabbit mammary tissue, in contrast to rat tissue, the breakdown of cozymase appears to be catalysed by the diphosphopyridine pyrophosphatase shown by Kornberg & Lindberg (1948) to occur in rabbit kidney.

4. In rabbit mammary gland slices, fructose-1:6-diphosphate in the presence of pyruvate and cozymase gave rise to higher rates of anaerobic lactic acid formation than glucose. The addition to this system of glucose and of yeast hexokinase could further increase the rate of glycolysis. In glycolysing homogenates the formation of lactic acid was accelerated by more than 100 % on addition of hexokinase. The possibility of hormonal inhibition of the hexokinase reaction in lactating mammary tissue is considered.

5. Under aerobic conditions, 2:4-dinitrophenol (DNP) increased glucose breakdown and lactic acid formation in brain cortex slices and in retina in a parallel manner. In brain slices, in the presence of glucose, glutamate and cozymase, DNP increased the aerobic rate of glucose breakdown to its anaerobic level, thus completely inhibiting the Pasteur effect. In mammary gland slices, however, lactic acid formation could be increased without a corresponding acceleration of glucose breakdown and even with some inhibition. In 'depleted' mammary tissue, DNP caused increased accumulation of lactic acid when glucose, pyruvate and cozymase were present. In the absence of added pyruvate or cozymase, this increase was much smaller or absent.

6. The effect of DNP on the Pasteur reaction is discussed.

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