The Effect of *p*-Nitrophenol on the Pasteur Reaction and on Aerobic Phosphorylation in Suspensions of the Mammary Gland

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It is well known that certain inhibitors of the 'Pasteur effect', in particular the nitrophenols, are also inhibitors of oxidative phosphorylation. The Pasteur effect and oxidative phosphorylation have been studied extensively in suspensions of intact yeast cell (Seits & Engelhardt, 1949; Lynen & Koenigsberger, 1951). Recently, Meyerhof & Fiala (1950) demonstrated the two phenomena and their simultaneous inhibition by *p*-nitrophenol (*p*NP) in a dried-yeast preparation, and concluded that the Pasteur effect results from a phosphorylating step.

In work with animal tissues, the Pasteur effect and aerobic phosphorylation have so far been studied in different types of preparation, the former in tissue slices, the latter in broken-cell preparations, and conclusions regarding the common effect of inhibitors on the two phenomena have been made by analogy, a procedure which is open to criticism (see Judah & Williams-Ashman, 1951).

The difficulty of obtaining broken-cell preparations which exhibit a Pasteur effect has led to the view that this phenomenon is dependent to some degree on the structural integrity of the tissue (see Dickens, 1951). In the present work a marked Pasteur effect and vigorous aerobic phosphorylation are shown to occur in cell-free suspensions of the lactating mammary gland of the guinea pig. Both phenomena are sensitive to the action of *p*NP which supports the widely held view that they are closely connected. The results presented extend the findings of Meyerhof & Fiala (1950) to animal tissues. A preliminary account of part of this work has been given to the Biochemical Society (Terner, 1953*a*).

EXPERIMENTAL

Material. The mammary glands of lactating rats or guinea pigs were removed after the death of the animals and placed in ice-cold isotonic KCl. The tissue was passed through a Latapie mincer and washed three times in cold KCl and strained on muslin. The minced tissue was then ground in an all-glass homogenizer (Potter & Elvejehm, 1936) in a medium of isotonic KCl containing 0·024 M-$KHCO_3$ and 0·02 M nicotinamide. To prepare brain suspensions, the brains of two guinea pigs were trimmed to remove as much white matter as possible and were ground in a glass homogenizer in the solution described above, without preliminary washing. The suspensions were further diluted with isotonic KCl to give in most cases a 1 in 5 suspension (dry wt. 40–50 mg./ml.).

Although the mammary suspensions contained shreds of connective tissue, they were, on microscopic examination, found to contain nuclei but to be free from unbroken cells. Brain suspensions, however, contained relatively large numbers of whole cells.

Reaction mixture. The basal medium contained in addition to isotonic KCl: $MgCl_2$, 0·005–0·01 M; nicotinamide, 0·02 M; cozymase, $1·25–2·5 \times 10^{-4}$ M; adenosine triphosphate (ATP), 0·001 M; phosphate buffer, pH 7·4, 0·0025 M; and either glycylglycine buffer, pH 7·4, 0·02 M (all aerobic and some anaerobic experiments), or $KHCO_3$, 0·024 M (anaerobic experiments). Further additions were made as stated in the text and in the tables. In aerobic experiments the gas was air. Cytochrome *c* was not a factor limiting the rate of respiration of mammary suspensions under the experimental conditions described and was only added where stated. When anaerobic conditions were required, a stick of yellow phosphorus was placed in the centre-well and the manometer was gassed with 5% CO_2 + 95% N_2, or with 100% N_2.

The suspension (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 and were continued at intervals of 5 min. 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. 20% trichloroacetic acid or 1·5 N perchloric acid (Neuberg, Strauss & Lipkin, 1944) were added. Several zero-time control flasks, containing all the constituents in the various combinations used, were deproteinized at the moment of the start of the incubation period. The protein-free filtrates were either analysed immediately or after neutralization to pH 6·5–6·6 and storage at −17°.

Analytical methods. Inorganic phosphate was estimated by a modification of the method of Berenblum & Chain (1938) described by Weil-Malherbe & Green (1951); lactic acid by the method of Barker & Summerson (1941); fructose 1:6-diphosphate (HDP) by the determination of inorganic phosphate liberated after 3 min. hydrolysis in 0·2 N alkali at 100° (Kiessling, 1938) and also by the estimation of fructose according to Roe (1934).

Reagents and preparations. The potassium salts of HDP and of ATP were prepared from commercial barium salts. Cozymase (85% pure) was a product of Schwarz Laboratories Inc., New York, U.S.A. Cytochrome *c* was obtained commercially as a dried preparation.

Hexokinase was prepared from baker's yeast according to Meyerhof (1927). Before each experiment 20 mg. of the powder were ground in 2 ml. distilled water and the insoluble matter was centrifuged off; 0·1 ml. of the extract contained about 9 hexokinase units as defined by Berger, Slein, Colowick & Cori (1946). An extract of rabbit-muscle acetone powder was prepared as described by Green, Needham & Dewan (1937). Crystalline potassium pyruvate was prepared according to Korkes, del Campillo, Gunsalus & Ochoa (1951). A sample of *p*NP was purified by repeated crystallization from hot water. Sodium fluoroacetate was kindly given by Sir Rudolph Peters. Insulin free from glycogenolytic factor was a gift to Dr Folley from Dr K. Hallas Møller of Novo Terapeutisk Laboratorium, Copenhagen.

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 formation as determined by chemical estimation, while $Q^{N_2}_{CO_2}$ applies to acid production calculated from changes in manometric pressure. The rate of change in the inorganic phosphate content of the reaction mixture is given by Q_P (µl. H_3PO_4/mg. dry wt./hr., 1 µg. atom P = 22·4 µl.). Q_{HDP} similarly denotes the change in its HDP content (1 µmol. HDP = 22·4 µl.). The dry weight of the suspension was determined by evaporation in the steam oven and was corrected for the salt content of the diluting fluid.

RESULTS

Anaerobic glycolysis and phosphorylation in mammary-gland suspensions

In earlier experiments suspensions of the mammary glands of rats and rabbits were prepared. While these preparations showed high glycolytic activity when incubated with reaction mixtures reinforced with yeast hexokinase (see Terner, 1952), their respiratory activity was poor. Under anaerobic conditions, in the presence of glucose, HDP, fluoride and pyruvate, a system employed by LePage (1948) in the study of tumour suspensions, a rapid esterification of inorganic phosphate accompanied the formation of lactic acid. When pyruvate was omitted from the fluoride-blocked system, vigorous carbon dioxide production and phosphorylation occurred without the formation of lactic acid (Table 1). The latter results point to the presence in the mammary gland of a very active enzyme system capable of producing phosphoglyceric acid and α-glycerophosphate by the dismutation of triose phosphate (see Green *et al.* 1937). Although the rate of carbon dioxide evolution due to the dismutation reaction of triose phosphate declined after an initial period of high activity, it was maintained for about 30 min. at a rate almost equal to that of the oxido-reduction between triose phosphate and pyruvate. Table 2 shows that during short periods of incubation fluoride did not inhibit the rate of carbon dioxide production from HDP. The addition of glucose and hexokinase as a phosphate-acceptor system approximately doubled the rate of acid formation whether in the absence or presence of fluoride. Insulin (free from glycogenolytic factor: 2·5 mg./100 ml.) was without effect.

Under aerobic conditions rat-mammary suspensions produced large amounts of lactic acid, and the rate of uptake of inorganic phosphate did not exceed the rate of anaerobic phosphorylation. 2:4-Dinitrophenol had no effect on lactic acid formation or phosphorylation (Table 1). Since considerable difficulty had been experienced during the preparation of these suspensions because of their tough connective tissue, it was concluded that the respiratory mechanism had been damaged. The mammary gland of the guinea pig, on the other hand, was found to be of softer texture and no difficulty was encountered in preparing actively respiring suspensions.

Table 1. *Phosphorylation and glycolysis in mammary suspensions of low respiratory activity*

(Rat-mammary suspension, dry wt. 19 mg. per vessel. Basal medium. All vessels also contained: glucose, 0·005 M; fructose 1:6-diphosphate (HDP), 0·0025 M; hexokinase, 18 units; ADP, 5×10^{-4} M; fluoride, 0·02 M; cytochrome *c*, 1×10^{-5} M. Additions: pyruvate, 0·005 M; fumarate, 0·005 M; 2:4-dinitrophenol (DNP), 2×10^{-4} M; iodoacetate, 0·002 M. Incubation period, 40 min.)

Vessel no.	Additions	Q_{O_2}	$Q^{O_2}_P$	$Q^{O_2}_{\text{lactic acid}}$
1	None	−1·8	−11·5	—
2	DNP	−3·1	−12·1	—
3	Pyruvate, fumarate	−2·2	−17·5	+24·8
4	Pyruvate, fumarate, DNP	−1·9	−17·9	+24·8
5	Iodoacetate	0	+2·7	0
		$Q^{N_2}_{CO_2}$	$Q^{N_2}_P$	$Q^{N_2}_{\text{lactic acid}}$
6	None	+20·5	−16·0	0
7	Pyruvate	+31·5	−22·0	+32·0
8	Iodoacetate	+3·5	+5·0	0

Table 2. *Anaerobic metabolism of fructose 1:6-diphosphate in mammary suspensions*

(Rat-mammary suspension, 31·7 mg. dry wt. per vessel. Basal medium; HDP (0·004 M) in all flasks. Additions: glucose, 0·02 M; fluoride, 0·01 M; hexokinase, 18 units; insulin (Novo), 2·5 mg./100 ml. Incubation period, 25 min.)

Vessel no.	Additions	$Q^{N_2}_{CO_2}$
1	None	24·2
2	Insulin	23·7
3	Fluoride	24·7
4	Fluoride, insulin	24·1
5	Glucose, hexokinase	54·4
6	Glucose, hexokinase, insulin	52·5
7	Fluoride, glucose, hexokinase	43·8
8	Fluoride, glucose, hexokinase, insulin	42·8

Aerobic phosphorylation and Pasteur effect. As shown in Tables 3–5, guinea pig mammary-gland suspensions are capable of vigorous aerobic phosphorylation in the absence of fluoride. Only small amounts of lactic acid were found to accumulate under aerobic conditions, as compared with the large amounts formed by anaerobic glycolysis; thus, mammary suspensions show a marked Pasteur effect. When the balance of respiration and glycolysis in the suspension was disturbed by the addition of an extract of rabbit-muscle powder, the respiration was unaffected and despite the greatly accelerated rates of aerobic and anaerobic lactic acid formation a Pasteur effect was found to persist (Table 3).

Effect of p*-nitrophenol.* The addition of *p*NP (1×10^{-4}–4×10^{-4} M) caused inhibition of aerobic phosphorylation and increased the accumulation of lactic acid, but was without effect in anaerobic experiments (Table 4). In the concentration range used, *p*NP did not inhibit and usually stimulated the respiration. The greatest stimulation of respiration, accompanied by effective inhibition of phosphorylation and increased lactic acid formation,

Table 3. *Aerobic and anaerobic glycolysis and phosphorylation in mammary suspensions*

(Guinea pig mammary suspension. Dry wt., 28·4 mg. per vessel. Basal medium; glucose, 0·01 M; HDP, 0·0025 M; hexokinase, 18 units. Rabbit-muscle acetone powder extract, 0·1 ml. Incubation period, 30 min. Initial inorganic P, 580 µg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	$Q_{CO_2}^{N_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	Aerobic	18·3	—	−26·2	2·8
2	Muscle extract	Aerobic	18·2	—	−24·7	16·1
3	None	Anaerobic	—	24·7	−3·5	24·7
4	Muscle extract	Anaerobic	—	49·0	−21·4	48·6
5	Iodoacetate, 0·001 M	Anaerobic	—	0	+13·1	1·5

Table 4. *Effect of inhibitors on phosphorylation and lactic acid accumulation in mammary suspensions*

(Guinea pig mammary suspension, dry wt. 44·8 mg. per vessel. Basal medium; glucose (0·01 M), HDP (0·0025 M) and hexokinase (18 units) in all flasks. Additions: pyruvate, 0·01 M; iodoacetate, 0·001 M; arsenite, 0·0025 M. Incubation period, 35 min. Initial inorganic P, 914 µg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	Aerobic	11·1	−12·6	2·1
2	*p*NP, 2×10^{-4} M	Aerobic	16·1	−5·9	7·9
3	*p*NP, 4×10^{-4} M	Aerobic	16·5	+6·1	10·5
4	Pyruvate	Aerobic	11·0	−12·6	4·5
5	Pyruvate; *p*NP, 2×10^{-4} M	Aerobic	11·9	+1·1	10·5
6	Pyruvate; *p*NP, 4×10^{-4} M	Aerobic	12·1	+7·8	10·7
7	Iodoacetate	Aerobic	5·6	−3·1	0·7
8	Pyruvate; iodoacetate	Aerobic	5·0	−2·3	1·6
9	Arsenite	Aerobic	7·9	+2·9	7·9
10	None	Anaerobic	—	−1·5	19·9
11	*p*NP, 4×10^{-4} M	Anaerobic	—	+1·9	20·4
12	Iodoacetate	Anaerobic	—	+5·1	1·6

Table 5. *Effect of pyruvate and fluoride on aerobic phosphorylation and lactic acid formation in mammary suspensions*

(Guinea pig mammary suspension, dry wt. 38 mg. per vessel. Basal medium; glucose (0·01 M), HDP (0·0025 M) and hexokinase (18 units) in all flasks. Additions: pyruvate, 0·01 M; fluoride, 0·01 M. Incubation period, 40 min. Initial inorganic P, 735 µg.)

Vessel no.	Additions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	13·4	−15·2	5·2
2	*p*NP, 1×10^{-4} M	17·0	−11·4	7·8
3	*p*NP, 2×10^{-4} M	18·0	−5·4	10·9
4	*p*NP, 4×10^{-4} M	16·3	+4·8	11·9
5	Pyruvate	10·8	−11·9	8·5
6	Pyruvate; *p*NP, 1×10^{-4} M	12·4	−7·2	10·3
7	Pyruvate; *p*NP, 2×10^{-4} M	12·6	+0·3	11·0
8	Pyruvate; *p*NP, 4×10^{-4} M	11·8	+2·3	12·4
9	Pyruvate; fluoride	8·6	−17·7	6·9
10	Pyruvate; fluoride; *p*NP, 1×10^{-4} M	7·5	−12·0	8·1
11	Pyruvate; fluoride; *p*NP, 2×10^{-4} M	7·5	−8·1	9·1
12	Pyruvate; fluoride; *p*NP, 4×10^{-4} M	7·3	−7·4	9·0

Table 6. *Effect of fluoride on metabolism of mammary suspensions*

(Guinea pig mammary suspension, dry wt. 46·8 mg. per vessel. Basal medium; glucose (0·01 M), hexokinase 18 units, HDP (0·005 M), pyruvate (0·01 M) in all flasks. Additions: iodoacetate, 0·001 M; arsenite, 0·0025 M; *p*-nitrophenol (*p*NP), 4×10^{-4} M. Incubation period, 40 min. Initial inorganic P, 750 μg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	Aerobic	9·0	−5·8	7·5
2	*p*NP	Aerobic	10·0	+7·3	7·7
3	Fluoride, 0·005 M	Aerobic	7·2	−13·0	3·7
4	Fluoride, 0·005 M; *p*NP	Aerobic	7·0	−1·8	5·5
5	Fluoride, 0·01 M	Aerobic	6·7	−16·3	3·3
6	Fluoride, 0·01 M; *p*NP	Aerobic	6·7	−3·6	5·4
7	Fluoride, 0·02 M	Aerobic	5·6	−15·5	4·1
8	Fluoride, 0·02 M; *p*NP	Aerobic	5·8	−4·9	5·2
9	Iodoacetate	Aerobic	5·4	+3·8	2·0
10	Arsenite	Aerobic	4·7	+8·4	6·4
11	Arsenite; iodoacetate	Aerobic	2·4	+7·0	1·9
12	None	Anaerobic	—	+7·2	11·6
13	Fluoride, 0·005 M	Anaerobic	—	+1·7	11·6
14	Fluoride, 0·01 M	Anaerobic	—	−3·2	8·0
15	Fluoride, 0·02 M	Anaerobic	—	−2·5	7·2

Table 7. *Effect of fluoride on dephosphorylation and* P/O *ratios*

(Calculated from data of experiment shown in Table 6. Q_{-P} denotes the rate of dephosphorylation, calculated as the difference in the corresponding anaerobic vessels between the theoretical and observed Q_P; corrected Q_P = observed aerobic Q_P minus Q_{-P}.)

Vessel no.	Concn. of fluoride (M)	*p*NP	Q_{-P}	Corr. Q_P	$-Q_{O_2} \times 2$	P/O ratio
1	None	−	18·8	−24·6	18·0	1·4
2	None	+	18·8	−11·5	20·0	0·6
3	0·005	−	13·3	−26·3	14·2	1·8
4	0·005	+	13·3	−11·5	14·0	0·8
5	0·01	−	4·8	−21·1	13·4	1·5
6	0·01	+	4·8	−8·4	13·4	0·6
7	0·02	−	4·7	−20·2	11·2	1·8
8	0·02	+	4·7	−9·6	11·6	0·8

Table 8. *Effect of inhibitors on aerobic metabolism*

(Guinea pig mammary suspensions. Dry wt.: 43·5 mg. per vessel in Expt. 1; 44 mg. per vessel in Expt. 2. Vessels 1–5 of Expt. 1 and vessels 1 and 2 of Expt. 2 contained the basal medium without added substrates. All other vessels contained the complete reaction mixture which includes: glucose, 0·02 M; HDP, 0·0025 M; hexokinase, 18 units. Additions: *p*NP, 4×10^{-4} M; fluoride, 0·005 M; fluoroacetate, 0·01 M; malonate, 0·01 M; arsenite, 0·0025 M. Incubation period, 30 min. in Expt. 1; 40 min. in Expt. 2. Initial inorganic P, 750 μg.)

Expt. no.	Vessel no.	Reaction mixture	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	1	Basal	None	Aerobic	5·7	—	—
	2	Basal	Fluoride	Aerobic	5·0	—	—
	3	Basal	Fluoroacetate	Aerobic	3·2	—	—
	4	Basal	Malonate	Aerobic	2·2	—	—
	5	Basal	Arsenite	Aerobic	1·1	—	—
	6	Complete	None	Aerobic	10·4	−16·9	+6·7
	7	Complete	*p*NP	Aerobic	14·0	+2·2	+14·4
	8	Complete	Malonate	Aerobic	5·8	−2·5	+7·4
	9	Complete	Malonate, *p*NP	Aerobic	5·8	+1·5	+9·3
	10	Complete	None	Anaerobic	—	−5·4	+20·6
2	1	Basal	None	Aerobic	9·8	+4·7	−0·4
	2	Basal	*p*NP	Aerobic	8·1	+10·8	−0·1
	3	Complete	None	Aerobic	12·7	−13·9	+4·3
	4	Complete	*p*NP	Aerobic	18·0	+6·6	+12·4
	5	Complete	Malonate	Aerobic	6·0	+2·6	+3·3
	6	Complete	Malonate, *p*NP	Aerobic	5·0	+3·8	+3·4
	7	Complete	Fluoroacetate	Aerobic	8·7	−4·5	+9·3
	8	Complete	Fluoroacetate, *p*NP	Aerobic	9·8	+6·5	+9·8
	9	Complete	None	Anaerobic	—	+3·1	+16·6

was produced by 2×10^{-4} M-pNP. Lower concentrations (1×10^{-4} M) only partly inhibited phosphorylation and the Pasteur effect (Table 5).

Effect of other inhibitors. Iodoacetate (0·001–0·002 M) which abolished glycolysis caused the liberation of large amounts of inorganic phosphate. Arsenite which is known to inhibit the oxidation of pyruvate (see Stocken & Thompson, 1949) has also been reported to be a powerful inhibitor of aerobic phosphorylation (Lehninger, 1949). In the present experimental conditions, arsenite (0·0025 M) inhibited phosphorylation and also increased the accumulation of lactic acid. Like iodoacetate, arsenite inhibited the respiration of the suspension, but left a considerable residual respiration which was reduced to a low level only by the combined action of the two inhibitors (Table 6). Fluoroacetate (0·01 M), an inhibitor of the Krebs cycle (see Peters, 1952), inhibited the Pasteur effect almost as effectively as pNP, but only partly inhibited the uptake of inorganic phosphate, the remaining phosphorylation being inhibited by pNP. Malonate (0·01 M), however, although it inhibited respiration and phosphorylation, did not increase lactic acid formation (Table 8).

Effect of inhibitors on the endogenous respiration. In the absence of added substrates the respiration of the suspensions in the basal medium was relatively high ($-Q_{O_2}$, 5–10). Fluoride and pNP had no effect on the endogenous respiration, while fluoroacetate, malonate and arsenite were strongly inhibitory. Suspensions respiring in the absence of added substrates and phosphate acceptors did not maintain the initial level of organic phosphate; its breakdown was further accelerated by pNP (Table 8).

Effect of pyruvate and fluoride. The addition of pyruvate to a glycolysing suspension did not increase the respiration nor the phosphorylation. The amount of lactic acid accumulating under aerobic conditions was, however, increased, often to the extent that pNP had no further effect. Pyruvate also suppressed the stimulation of respiration by pNP (Tables 4 and 5). Fluoride in the presence of pyruvate inhibited the respiration, higher concentrations (0·02 M) causing an inhibition of the same order of magnitude as arsenite or iodoacetate, while generally increasing the net uptake of inorganic phosphate (Table 6). The increased uptake of inorganic phosphate was often not very pronounced (Table 5), but in the presence of pNP or under anaerobic conditions fluoride appeared to retard the breakdown of organic phosphate.

In some guinea pig mammary suspensions fluoride in higher concentrations had an adverse effect on anaerobic glycolysis which partly offset its protective action against phosphatases with a resulting low net uptake of inorganic phosphate in relation to the amount of lactic acid produced (Table 6). In rat-mammary suspensions, however, the net uptake of inorganic phosphate in the presence of 0·02 M fluoride could be as high as 70–80% of the equivalent of the amount of acid produced both in the presence or absence of added pyruvate (Table 1).

Metabolism of fructose 1:6-diphosphate. Although in the experiments described above the addition of HDP was not essential for the demonstration of glycolysis and phosphorylation, the rates of glycolysis were somewhat higher in its presence than in its absence. When the phosphate-acceptor system was omitted and HDP was the only added substrate, the initial level of inorganic phosphate was approximately maintained in respiring suspensions and only small amounts of lactic acid were formed, while on addition of pNP or under anaerobic conditions the increased rate of lactic acid formation was accompanied by the liberation of inorganic phosphate. A marked stimulation of respiration by pNP could also be observed in the absence of the phosphate-acceptor system (Table 9).

Table 9. *Metabolism of fructose 1:6-diphosphate*

(Guinea pig mammary suspensions, dry wt. 46 mg. per vessel in Expt. 1; 42·8 mg. per vessel in Expt. 2. Basal medium; HDP, 0·005 M in Expt. 1; 0·0025 M in Expt. 2. Additions: pNP, 4×10^{-4} M; pyruvate, 0·01 M; fluoride, 0·005 M. Incubation period: 40 min. in Expt. 1; 30 min. in Expt. 2. Initial inorganic P: 900 μg. in Expt. 1; 760 μg. in Expt. 2.)

Expt. no.	Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$-Q_{HDP}$	$Q_{lactic\ acid}$
1	1	None	Aerobic	7·4	−1·3	3·9	0·8
	2	pNP	Aerobic	9·7	+6·3	4·6	4·0
	3	Pyruvate, fluoride	Aerobic	5·6	−4·3	3·4	2·9
	4	Pyruvate, fluoride, pNP	Aerobic	8·0	+3·4	4·9	5·4
	5	None	Anaerobic	—	+10·9	6·2	12·1
	6	Pyruvate, fluoride	Anaerobic	—	+3·7	6·1	11·0
2	1	None	Aerobic	7·8	−0·3	4·1	1·3
	2	pNP	Aerobic	14·0	+12·9	6·0	6·4
	3	Pyruvate	Aerobic	7·1	−0·5	3·9	4·2
	4	Pyruvate, pNP	Aerobic	14·5	+14·9	6·2	7·8
	5	None	Anaerobic	—	+8·9	6·3	11·3

When arsenate replaced the phosphate in the reaction mixture, the rate of aerobic glycolysis was increased, and the addition of *p*NP had no further effect. A Pasteur effect, however, remained since the rates of both HDP-breakdown and lactic acid accumulation did not reach anaerobic levels (Table 10). The effect of arsenate on aerobic glycolysis resembled the effect of *p*NP in the presence of phosphate in accelerating the rate of lactic acid formation more than the equivalent rate of HDP-breakdown so that the amount of substrate available for oxidation was decreased without a corresponding inhibition of respiration. Thus, two effects common to arsenate and *p*NP were to inhibit the Pasteur effect and to increase the ratio of oxygen consumed/substrate oxidized to a value which approached the theoretical ratio for the complete oxidation of substrate (see Terner, 1951*c*).

Pasteur effect and phosphorylation in brain suspensions. While the rate of anaerobic glycolysis in mammary-gland suspensions supplemented with yeast hexokinase was maintained at almost linear rates for at least 60 min., brain suspensions were less stable and showed a rapid decline of the initially high rate of glycolysis which could not be prevented by the addition of hexokinase (Terner, 1952). Since at the same time the rate of respiration decreased less rapidly, it was necessary, in order to compare aerobic and anaerobic metabolic rates, to limit the duration of incubation to a short period during which anaerobic glycolysis was maintained (30 min. or less). Table 11 shows that with these precautions it was possible to observe in brain suspensions essentially the same phenomena as in mammary-gland suspensions, namely a marked Pasteur effect sensitive to *p*NP and arsenite, stimulation of respiration by 2×10^{-4} M-*p*NP and its inhibition by fluoride. However, no appreciable net uptake of inorganic phosphate occurred in the absence of fluoride and even in its presence the uptake was relatively small. Nevertheless, phosphorylation occurred as shown by the liberation of large amounts of inorganic phosphate in the presence of inhibitors of oxidative phosphorylation. Thus, the results extend to mammary-gland and brain suspensions the view of Meyerhof & Fiala (1950), based on experiments with disintegrated yeast preparations, that the Pasteur effect and aerobic phosphorylation are closely connected.

When brain suspensions were incubated with HDP in the absence of added phosphate acceptors, anaerobic carbon dioxide production ceased after a short period of relatively high activity, while the respiration continued. In one experiment $-Q_{O_2}$ calculated for the period of 20 min. was: without HDP, 6·4; with HDP, 8·7; with HDP + *p*NP (2×10^{-4} M) 12·1. During the same interval $Q^{N_2}_{CO_2}$ was 18·0. In contrast to the stimulation of respiration

Table 10. *Effect of arsenate on metabolism of fructose* 1:6-*diphosphate*

(Guinea pig mammary suspension, dry wt., 46·7 mg. per vessel. Usual basal medium except that phosphate buffer was omitted. All vessels contained: HDP, 0·0045 M; Additions: phosphate, 0·0038 M; arsenate, 0·0038 M; *p*NP, 4×10^{-4} M. Incubation period, 50 min.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	$-Q_{HDP}$	$Q_{\text{lactic acid}}$
1	Phosphate	Aerobic	8·8	3·8	1·0
2	Phosphate; *p*NP	Aerobic	12·7	6·4	8·4
3	Phosphate	Anaerobic	—	7·3	13·6
4	Arsenate	Aerobic	8·3	5·4	6·9
5	Arsenate; *p*NP	Aerobic	10·0	5·4	7·1
6	Arsenate	Anaerobic	—	6·7	13·4

Table 11. *Effect of inhibitors on phosphorylation and lactic acid accumulation in brain suspension*

(Guinea pig brain suspension, dry wt. 37·6 mg. per vessel. Basal medium; glucose (0·01 M), HDP (0·005 M) in all flasks; cytochrome *c* (1×10^{-5} M) in all aerobic flasks. Additions: pyruvate, 0·01 M; fluoride, 0·01 M; arsenite, 0·0025 M; iodoacetate, 0·001 M. Incubation period, 30 min. Initial inorganic P, 750 μg.)

Vessel no.	Additions	Experimental conditions	$-Q_{O_2}$	Q_P	$Q_{\text{lactic acid}}$
1	None	Aerobic	15·3	−2·1	5·5
2	*p*NP, 2×10^{-4} M	Aerobic	18·2	+16·1	10·6
3	*p*NP, 4×10^{-4} M	Aerobic	15·3	+20·0	11·9
4	Pyruvate, fluoride	Aerobic	8·2	−6·6	5·7
5	Pyruvate, fluoride, *p*NP, 2×10^{-4} M	Aerobic	12·8	+1·6	7·4
6	Pyruvate, fluoride, *p*NP, 4×10^{-4} M	Aerobic	11·3	+1·6	8·7
7	Arsenite	Aerobic	4·8	+23·9	10·1
8	Iodoacetate	Aerobic	5·7	+10·1	1·2
9	Arsenite, iodoacetate	Aerobic	3·1	+18·0	1·2
10	None	Anaerobic	—	+18·4	21·7
11	Pyruvate, fluoride	Anaerobic	—	−3·6	20·1
12	Iodoacetate	Anaerobic	—	+18·4	1·0

by *p*NP shown in this experiment, Tyler (1950) observed no augmentation, but only inhibition of respiration on adding 2:4-dinitrophenol to brain suspensions in the presence of HDP.

Phosphorus/oxygen ratios. Measurements of P/O ratios are usually made in highly purified systems in which phosphatase activity is low or suppressed by the addition of fluoride (see Krebs, Ruffo, Johnson, Eggleston & Hems, 1953). In the suspensions used in the present work, phosphatase action considerably reduced the uptake of inorganic phosphate, although under aerobic conditions, the rate of phosphorylation exceeded the rate of dephosphorylation. An estimate of the rate of destruction of organic phosphate was obtained from the differences between the amount of inorganic phosphate expected to be esterified during anaerobic glycolysis and the amount of inorganic phosphate actually taken up. Since the formation of 1 mole of lactic acid should result in the esterification of 1 mole of inorganic phosphate, the difference between the amount of phosphate equivalent to the amount of lactic acid formed and the amount of phosphate actually disappearing may be taken as a measure of the dephosphorylating capacity of the system. The quantity thus obtained, when added to the observed net uptake of phosphate, gives the amount of phosphate which would have been taken up if dephosphorylation could have been completely prevented. The P/O ratios calculated in this manner for mammary-gland and brain suspensions were of the order of 1·5. Lynen & Koenigsberger (1951), who determined the rate of dephosphorylation in respiring yeast cells by adding cyanide to inhibit respiration and glycolysis and measuring the ensuing liberation of inorganic phosphate, obtained P/O ratios of the order of 1·0. The corrected rates of aerobic phosphorylation for the experiment illustrated in Table 6 are given in Table 7. It can be seen that lower concentrations of fluoride (up to 0·005 M) afforded little protection while higher concentrations (0·01–0·02 M) caused about 75 % inhibition of dephosphorylation. As is to be expected, the P/O ratios, calculated as described above, were of the same order of magnitude in the presence and absence of fluoride.

DISCUSSION

Pasteur effect and aerobic phosphorylation. Until recently it has been found difficult to study oxidative phosphorylation in broken-cell preparations which also exhibit a Pasteur effect. Meyerhof & Fiala (1950) were the first to demonstrate both phenomena in the same preparation of disintegrated yeast cells. The phosphate uptake was small but distinct, and was inhibited by *p*NP. A disadvantage noted by the authors was that 'in such cases, where the yeast is nearly completely killed and the phosphate can be washed out, fermentation prevails too much over respiration and fermentative phosphorylation too much over oxidative phosphorylation'. In the present experiments oxidative phosphorylation and a Pasteur effect have been demonstrated in suspensions of mammary tissue which were practically free from intact cells. Rapid rates of oxidative phosphorylation could be observed, which greatly exceeded the rates of glycolytic phosphorylation. Mammary suspensions thus provide a system which is better balanced and more suitable for the study of the phenomena concerned. In these preparations the 'Pasteur mechanism' was so powerful that on addition of a muscle extract which doubled the rate of anaerobic glycolysis, the amount of lactic acid accumulating under aerobic conditions did not exceed one-third of the amount produced by anaerobic glycolysis.

Birmingham & Elliott (1951) described the suppression of lactic acid formation in brain suspensions glycolysing under aerobic conditions; no measurements of phosphorylation were made. Their preparations, on microscopic examination 'showed the presence of numbers of nerve and other cells', an observation also made with brain suspensions used in the present work. Although subjected to the same experimental conditions, brain suspensions differed from mammary suspensions by their inability to promote a rapid net esterification of inorganic phosphate. This no doubt resulted from an excessive rate of dephosphorylation due to the high adenosine triphosphatase (ATP-ase) content of the brain preparations, which was found by Meyerhof & Geliazkowa (1947) to be the cause of the rapid decline of anaerobic glycolysis. In mammary suspensions, however, the ATP-ase activity appeared to be sufficiently low to permit the net esterification of appreciable amounts of inorganic phosphate without the necessity of adding fluoride.

In the absence of fluoride, a full Pasteur effect was observed in both mammary and brain suspensions. In the presence of fluoride, which made it necessary also to add pyruvate, the aerobic lactic acid level was raised and was in most cases not further increased to an appreciable extent by *p*NP. It has been shown that this masking of the Pasteur effect was due to the presence of an excess of pyruvate. Since fluoride and pyruvate are as a rule added to cell-free preparations in order to check ATP-ase activity (see Potter & Siekevitz, 1952), it is not surprising that Clowes & Keltch (1951), in experiments with glycolysing suspensions, did not observe any effect on the aerobic formation of lactic acid by 4:6-dinitro-*o*-cresol added in concentrations which inhibited phosphorylation.

Metabolic rates under aerobic conditions and effect of nitrophenols. It has been assumed by various

workers that a rate-determining step of the glycolytic chain of reactions is retarded under aerobic conditions. Thus, in order to explain the decrease of the rate of glucose disapperance in yeast cells, Lynen & Koenigsberger (1951) suggested that the hexokinase reaction might be inhibited in the presence of air. However, in the present experiments the rate of HDP-breakdown was found to be influenced by the presence and absence of oxygen in the same way as the breakdown of glucose. Further, when the esterification of inorganic phosphate was measured in a system supplemented with glucose and yeast hexokinase, the hexokinase reaction did not appear to be impaired in the presence of air. Factors other than the hexokinase reaction must therefore be involved. Meyerhof & Fiala (1950) found that the activity of aldolase in fermenting yeast was not reduced by oxygen, and therefore thought it improbable that aldolase regulates the speed of fermentation.

According to Johnson (1941) and Lynen (1941) the rate of oxidation of triose phosphate is retarded by high ATP/ADP ratios and low levels of inorganic phosphate resulting from aerobic phosphorylation. Accordingly, it is now accepted that phosphate acceptors and phosphatases play an important part in the regulation of rates of substrate oxidation by controlling the availability of ADP and of inorganic phosphate (see Potter & Siekevitz, 1952). Recently, Siekevitz & Potter (1953) and Lardy & Wellman (1953) reported that the oxidation of various substrates can be accelerated by the addition of either phosphate acceptors or of 2:4-dinitrophenol; the latter was found to enhance the latent 'ATP-ase' activity of the system. This concept would explain satisfactorily the present observations that in mammary suspensions, metabolizing HDP in the absence of added phosphate acceptors, *p*NP accelerated the breakdown of HDP and at the same time stimulated the respiration and liberation of inorganic phosphate. However, while Lardy & Wellman (1952) found that in rat-liver mitochondria the accelerating effect of phosphate acceptors on the respiration could be duplicated by 2:4-dinitrophenol, the present experiments show that in whole suspensions incubated with substrates in the presence of phosphate acceptors the respiration can be considerably augmented by the further addition of a nitrophenol. This may be partly explained by the observations reported previously that the increased respiration resulting from the addition of nitrophenols to mammary-gland slices (Terner, 1951*c*), bull spermatozoa (Melrose & Terner, 1953) and mammary suspensions (Terner, 1953*b*), metabolizing various substrates, was not accompanied by a corresponding increase in the amount of substrate oxidized, with the result that the ratio of oxygen consumed/substrate oxidized was increased, and approached the theoretical ratio corresponding to the complete oxidation of the substrate. Apart from the accumulation of intermediates, the incomplete oxidation of a substrate may be due to its partial utilization in synthetic reactions which, if dependent on energy derived from oxidative phosphorylation, will be inhibited by nitrophenols. Synthetic reactions may also be expected to compete with the added phosphate-acceptor system and reduce the P/O ratio. The accepted average P/O ratio for the oxidation of pyruvate by the Krebs cycle is 3·0 (Ochoa & Stern, 1952). The P/O ratios which were calculated indirectly and may be assumed to represent the maximal value attainable under present experimental conditions were of the order of 1·5 (see p. 477).

The suggestion that the Pasteur effect is due to the retardation of the oxidation of triose phosphate owing to the lack of inorganic phosphate within the cell has been followed up by Lynen and his associates in experiments with intact yeast cells (Holzer & Lynen, 1950; Lynen & Koenigsberger, 1951; Holzer & Holzer, 1953). In view of the demonstration of a Pasteur effect sensitive to nitrophenols in broken-cell preparations of yeast and of mammary tissue, this may require further consideration. In broken-cell preparations, in contrast to suspensions of yeast cells, the glycolytic enzymes are for the most part in solution. In most of the present experiments the level of inorganic phosphate at the end of the incubation period was relatively high, and although a concentration gradient in respect of inorganic phosphate has been shown to exist between the mitochondria and the surrounding solution (Bartley & Davies, 1952), the glycolytic enzyme system was unlikely to suffer from a lack of inorganic phosphate even during vigorous aerobic phosphorylation. Moreover, when HDP was metabolized in the absence of an added phosphate acceptor, the initial level of inorganic phosphate was not diminished, but a full Pasteur effect could nevertheless be demonstrated. Thus it seems improbable that under the present experimental conditions the rate of breakdown of HDP was retarded by a lack of inorganic phosphate.

Arsenate is known to accelerate glycolysis by making it independent of phosphorylation (Warburg & Christian, 1939*a*, *b*; Meyerhof & Junowicz-Kocholaty, 1942) and the inhibition of the Pasteur effect in its presence may therefore be expected. The observation in mammary suspensions that nitrophenols and arsenate accelerate the accumulation of lactic acid more than the breakdown of HDP remains unexplained. Similar observations have been reported previously, when it was found that 2:4-dinitrophenol could cause increased aerobic lactic acid formation in mammary slices while at the same time inhibiting the disappearance of glucose,

which was the only added substrate (Terner, 1952). It seems possible that the oxidation-reduction step of glycolysis also plays a part and this appears to be supported by the observation that added pyruvate increased the amount of lactic acid formed under aerobic conditions. Since only part of the added pyruvate was reduced to lactate, the rate of oxidation of triose phosphate may be a limiting factor. According to older views concerning the Pasteur effect, the aerobic reoxidation of reduced cozymase, formed during the oxidation of glyceraldehyde phosphate, may prevent the reduction of pyruvate to lactic acid (see Ball, 1939). To explain the inhibition of the Pasteur reaction by 2:4-dinitrophenol the suggestion was made in preliminary notes (Terner, 1951*a*, 1951*b*) that the nitrophenol might interfere with the aerobic reoxidation of reduced cozymase, thus favouring its reoxidation by the coupled reduction of pyruvate to lactate. However, attempts in unpublished experiments to obtain evidence of an inhibition by nitrophenols of the aerobic oxidation of reduced cozymase have been unsuccessful and this hypothesis now appears to be unlikely.

SUMMARY

1. A Pasteur effect coupled with aerobic phosphorylation has been demonstrated in cell-free suspensions of mammary gland incubated with fructose 1:6-diphosphate (HDP) in the presence of glucose and yeast hexokinase as the phosphate-acceptor system.

2. Both phenomena were inhibited by *p*-nitrophenol (*p*NP, $2\text{–}4 \times 10^{-4}$ M) which at the same time could cause up to 50% stimulation of respiration; *p*NP had no effect on the respiration of the suspensions in the absence of added substrates.

3. Similar results were obtained with brain suspensions, which, however, contained considerable numbers of unbroken cells.

4. A Pasteur effect was also demonstrated in mammary suspensions incubated with HDP, in the absence of added phosphate acceptors. In these experiments the initial level of inorganic phosphate was approximately maintained under aerobic conditions; the addition of *p*NP resulted in stimulation of respiration and an increase in the amount of inorganic phosphate in the medium.

5. In the absence of added phosphate acceptors, *p*NP and also arsenate accelerated the aerobic breakdown of HDP; the increase in the amount of lactic acid accumulating exceeded the increase expected from the acceleration of the breakdown of HDP. No effect of *p*NP was observed under anaerobic conditions.

6. The addition of pyruvate, or pyruvate plus fluoride (0·005–0·02 M), resulted in increased aerobic formation of lactic acid; in their presence, *p*NP produced little or no further inhibition of the Pasteur reaction and its stimulating effect on respiration was suppressed.

7. Phosphorus/oxygen ratios for the overall reaction were determined by an indirect method and were found to be of the order of 1·5 under conditions supporting aerobic phosphorylation.

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Sedimentation and Diffusion of Human Albumins

4. LOW CONCENTRATION BEHAVIOUR OF SERUM ALBUMINS IN CASES OF LIVER DISEASE

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It has long been realized that the liver, even if not directly responsible for the synthesis of serum albumin, is at least intimately involved in its metabolism. The possible role of the liver in albumin formation was discussed by Madden & Whipple (1940), who concluded that it is highly likely that the liver constitutes a site of production. Further evidence in favour of this view is forthcoming from the work of Roberts & White (1949) and the radioactive studies by Peters & Anfinsen (1950).

The very close connexion between the liver and albumin formation suggests the possibility of alterations in the protein when that organ is diseased. Quantitative changes in the serum proteins have been reported by a number of authors who used the electrophoretic technique. Thus cases of cirrhosis have been studied by Gray & Barron (1943), Thorn, Armstrong & Davenport (1946), Martin (1949*a*), Rafsky, Weingarten, Krieger, Stern & Newman (1950), and Franklin *et al.* (1951). Sera from patients with infective hepatitis formed the basis of work by Gray & Barron (1943), Martin (1946), Cohen & Thompson (1947), Rafsky *et al.* (1950) & Franklin *et al.* (1951). A common feature of the patterns is the reduction in the relative concentration of albumin, although this decrease is by no means specific to diseases of the liver (Gutman, 1948).

Qualitative alterations in the serum albumin are indicated by two types of evidence. First, Luetscher (1939) and Hoch-Ligeti & Hoch (1948) found alterations in the electrophoretic characteristics of the albumin peaks when examined under special conditions. Secondly, albumin separated by electrophoresis of sera from patients with liver disease was shown to behave abnormally in its ability to inhibit the cephalin cholesterol flocculation of γ-globulin, and in its effect on the thymol flocculation test, etc. (Kabat, Hanger, Moore & Landow, 1943; Moore, Pierson, Hanger & Moore, 1945; Hanger, 1947; Martin, 1949*b*). Unfortunately, the conditions used to isolate the albumin were not designed to remove all the α-globulin, so that the conclusions which can be drawn are not quite so definite as might be desired.

The abnormal molecular weights found by sedimentation and diffusion measurements for serum and urinary albumins in nephrosis (Charlwood 1952*b*), and the above considerations, have prompted a similar investigation in cases of liver disease, with the object of obtaining evidence of any abnormalities in albumin synthesis.

The main complication likely to be caused by the conditions prevailing in liver diseases lay in the generally raised bilirubin content of the sera. This could affect the actual molecular weight of the albumin, to which bilirubin is largely bound (Martin, 1949*c*). The colour, too, might affect optical conditions, more especially in the diffusiometer, and give rise to anomalous results. Both these points are discussed later.

EXPERIMENTAL

Preparation of materials. Blood was collected from patients selected on the basis of a preliminary diagnosis of primary liver disease. It was processed as previously described (Charlwood, 1952*a*), the buffer in all cases being veronal (pH 8·6, $I=0{\cdot}1$). Separations were controlled by the photoelectric arrangement described by Warren & Charlwood (1953), as in other similar work (Charlwood, 1954). Samples were diluted and redialysed, prior to sedimentation and diffusion, against a solution containing 0·05 I-NaCl + 0·02 I phosphate buffer, pH 8·0.

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