minimum levels of inorganic phosphate in yeast have been measured.

5. An attempt is made to relate these effects to the effect of the same substances on glucose catabolism and polysaccharide synthesis. Various discrepancies are pointed out.

6. The hypothesis of Lynen (1941) and of Lynen & Koenigsberger (1951) that the Pasteur effect is related to the lower inorganic phosphate level obtaining aerobically appears to be untenable, for propionitrile, which inhibits the Pasteur effect, has no effect on phosphorylation phenomena.

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The Effects of Phosphate Acceptors, *p*-Nitrophenol and Arsenate on Respiration, Phosphorylation and Pasteur Effect in Cell-Free Suspensions

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The first evidence that phosphorylation reactions play a part in the oxidation of pyruvic acid in animal tissues was provided by Banga, Ochoa & Peters (1939) who showed that inorganic phosphate and adenine nucleotide reactivate the pyruvic oxidase system of dialysed dispersions of pigeon brain and of rabbit kidney. Later workers have attributed the restoration of the respiratory activity of deficient systems by 2:4-dinitrophenol to its ability to 'replace' either inorganic phosphate (Loomis & Lipmann, 1948; Teply, 1949) or adenine nucleotide (Judah & Williams-Ashman, 1951). In washed kidney dispersions (cyclophorase), dinitrophenol has been found to abolish phosphorylation without inhibition or with slight stimulation of respiration (Loomis & Lipmann, 1948; Cross, Taggart, Covo & Green, 1949). Lardy & Wellman (1952) observed that dinitrophenol accelerated the respiration of liver mitochondria oxidizing various substrates and that this stimulant action could be reproduced by the addition of phosphate acceptors such as adenylate or glucose plus hexokinase, the effects of the nitrophenol and the phosphate acceptors not being additive.

Since it had been found (Terner, 1954*b*, 1955*a*) that *p*-nitrophenol increased the respiration of mammary-gland homogenates metabolizing fumarate and pyruvate, with and without glucose plus hexokinase, further studies have been made to compare the effects of *p*-nitrophenol and of phosphate acceptors on the oxygen consumption of mitochondrial preparations metabolizing fumarate and pyruvate. The effects of *p*-nitrophenol and of arsenate on aerobic phosphorylation and citric acid formation were studied in suspensions blocked with fluoracetate.

In extension of previous studies of the Pasteur effect in cell-free suspensions (Terner, 1954*a*), it could be shown that the addition of large amounts of adenine nucleotide resulted in a marked increase in the amount of lactic acid accumulating under aerobic conditions, i.e. that the inhibition of the Pasteur effect by *p*-nitrophenol could be reproduced by the addition of adenine nucleotide.

EXPERIMENTAL

Material. Homogenates in 0·154 M-KCl or in 8·55% sucrose were prepared as previously described (Terner, 1955*b*) and used either without further treatment, or after centrifuging twice for short periods at low speed to remove cell debris and nuclei. The resultant mixture of mitochondria and soluble tissue constituents is designated 'suspension'. Washed suspensions were prepared by further centrifuging at 7000 *g* for 30 min. and resuspending the sediment in KCl. After two or three washings the suspension contained almost pure mitochondria, with few if any nuclei, and will be referred to as 'mitochondria'.

The 'supernatant fraction' was the clear solution recovered after the first centrifuging at high speed and was free from nuclei and mitochondria. Nuclei were isolated as described by Hogeboom, Schneider & Striebich (1952).

Unless otherwise stated, the experimental animals were guinea pigs.

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Table 1. *Effect of phosphate acceptors and* p-*nitrophenol on respiration of kidney-cortex mitochondria*

Kidney-cortex mitochondria, dry wt., 13·1 mg./vessel. Basal medium, ATP (0·0005 M), fumarate (0·0025 M) and pyruvate (0·001 M) in all flasks. Additions: glucose, 0·02 M; hexokinase, 18 units. *p*-Nitrophenol (*p*NP) (2×10^{-4} M) was tipped in after 30 min. incubation. Q_{O_2} was calculated from the O_2 uptake during 15 min. periods before and after adding *p*NP. Further additions of adenine nucleotide were made as AMP.

Total adenine nucleotide (M)	$-Q_{O_2}$ —		$-Q_{O_2}$ Glucose + hexokinase		$-Q_{O_2}$ Supernatant	
	—	*p*NP	—	*p*NP	—	*p*NP
0·0005	18·8	44·3	31·0	45·6	21·6	75·0
0·001	23·8	52·0	40·6	53·0	28·0	68·4
0·002	31·0	52·8	45·8	43·4	34·8	71·0
0·004	37·7	47·0	44·3	33·3	45·5	65·5

Reaction mixture. The basal medium contained: KCl, 0·154 M; $MgCl_2$, 0·01 M; nicotinamide, 0·02 M; cozymase, $1·25 \times 10^{-4}$ M; aminotrishydroxymethylmethane buffer, pH 7·4, 0·02 M and phosphate buffer, pH 7·4, 0·005–0·01 M. Further additions were made as stated in the text and in the tables. The gas phase was air. In experiments in which the uptake of inorganic phosphate was measured, the homogenate or suspension (1 ml.) and hexokinase were pipetted into 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. All manometric data have been corrected by extrapolation to include the equilibration period (10 min.). After incubation the vessels were cooled in ice water and 3 ml. of 1·5 N perchloric acid was added to each. The contents of control flasks, some of which contained arsenate in the same amounts as used in experimental vessels, were deproteinized at the time the incubation was started. The protein-free filtrates were analysed after storage at −17°, the phosphate analyses being completed within 24 hr.

Analytical methods. Citric acid was estimated by Taylor's (1953) modification of the method of Weil-Malherbe & Bone (1949) with some small changes and precautions outlined previously (Terner, 1955*a*); lactic acid by the method of Barker & Summerson (1941). Inorganic phosphate was estimated by Weil-Malherbe & Green's (1951) modification of the method of Berenblum & Chain (1938). When this method was used, arsenate, although it reacts more slowly with the molybdate reagent than inorganic phosphate, gave rise to some colour in the final extract. The development of arsenomolybdate can be prevented by the addition of tartaric acid (Feigl, 1937). Since the reagent containing tartaric acid described by Feigl, when used in the extraction method, also delayed the formation of phosphomolybdic acid, the amount of tartaric acid was decreased to a level which caused only slight inhibition of the formation of phosphomolybdate and minimized the interference due to arsenate. Portions of protein-free filtrates containing 0·3–2·5 μmole of inorganic phosphorus and up to 12 μmoles of arsenate were pipetted into glass-stoppered tubes containing 6 ml. of an *iso*butanol–benzene (1:1) mixture (Martin & Doty, 1949), followed by 2 ml. of the molybdate–tartaric acid reagent (2·5 g. of ammonium molybdate and 1·5 g. of tartaric acid in 100 ml. of 0·5 N-H_2SO_4). The tubes were shaken for 30 sec., the aqueous layer was removed from each and the solvent layer dried over anhydrous Na_2SO_4. A measured portion was transferred to a test tube containing 5 ml. of acid ethanol, 0·2 ml. of dil. $SnCl_2$ was added and the absorption measured in a Spekker absorptiometer with a red filter (Ilford 608), as outlined in the procedure of Weil-Malherbe & Green (1951). The extraction method has the advantage over the method of Pett (1933) that prolonged warming of the sample in acid reagents can be avoided, the ice-cold sample being extracted with practically no delay.

Table 2. *Effect of phosphate acceptors and* p-*nitrophenol on respiration of mammary mitochondria*

Mammary mitochondria, dry wt., 18 mg./ml. Basal medium, ATP (0·0005 M), AMP (0·0005 M), fumarate (0·0025 M) and pyruvate (0·02 M), in all vessels. Additions: glucose, 0·02 M; hexokinase, 18 units; AMP, 0·003 M. *p*-Nitrophenol (*p*NP) (2×10^{-4} M) was added from side arms of the flasks after 30 min. incubation. Q_{O_2} was calculated from the O_2 uptake before and after mixing.

Acceptors added	$-Q_{O_2}$ 0·5 ml. of suspension/vessel		$-Q_{O_2}$ 1·0 ml. of suspension/vessel	
	—	*p*NP	—	*p*NP
None	16·0	41·2	11·4	43·8
Glucose, hexokinase	32·7	43·0	22·9	44·0
AMP	31·8	23·5	36·0	21·1
Glucose, hexokinase, AMP	43·5	31·0	44·7	22·2

In experiments in which ^{14}C-labelled substrates were used, the respiratory CO_2 was collected and its radioactivity measured as described by Popják & Tietz (1954).

Reagents and preparations. These were the same as previously employed (see Terner, 1955*a*). Samples of fluoroacetate were kindly given by Sir Rudolph Peters, α-oxoglutarate (96% pure) by Dr A. L. Greenbaum. [2-^{14}C]Pyruvate was obtained from the Radiochemical Centre, Amersham.

Units. Except when quantities are given in μmoles, metabolic quotients are used and expressed in the *Q* notation (μl./mg. dry wt./hr.). (Q_P = μl. of H_3PO_4/mg. dry wt./hr., 1 μmole of H_3PO_4 = 22·4 μl.) Dry weights were determined by evaporation in the steam oven of portions of the homogenates and suspensions, and were corrected for the salt content of the diluting fluids.

RESULTS

Stimulation of respiration by phosphate acceptors. As shown in Tables 1 and 2, the O_2 consumption of mitochondrial suspensions of kidney cortex

and of mammary gland metabolizing fumarate plus pyruvate was accelerated by the addition of *p*-nitrophenol, glucose plus hexokinase, or adenosine 5-phosphate (AMP). Although the present observations are in general agreement with those of Lardy & Wellman (1952) made on liver mitochondria, they further reveal a relationship between the Q_{O_2} and the amount of adenine nucleotide present in the system. Not only was the rate of respiration accelerated progressively as the concentration of AMP was increased, but the elevated rate in the presence of the glucose–hexokinase system was further enhanced by the addition of AMP. The highest rates of respiration were observed in the presence of *p*-nitrophenol (2×10^{-4} M), which increased the Q_{O_2} of kidney and mammary mitochondria at low levels of adenine nucleotide. When the respiratory rate was elevated by the presence of AMP in high concentration, the addition of *p*-nitrophenol resulted in further stimulation in kidney mitochondria, but caused inhibition in mammary mitochondria. When glucose and hexokinase were included in the system, *p*-nitrophenol increased the O_2 consumption of mammary and kidney mitochondria at low levels of adenine nucleotide, but was inhibitory or without effect in the presence of larger amounts of AMP. Kidney mitochondria, however, when incubated in the presence of the corresponding supernatant fraction (see below), responded to *p*-nitrophenol with increased respiration even at the highest levels of AMP tested.

The stimulation of respiration by AMP and by glucose plus hexokinase could also be observed in whole homogenates (Table 3). In mammary homogenates the addition of glucose alone was without effect, as may be expected from the previously reported observation that the hexokinase activity of mammary homogenates is low and variable, probably because of the instability of the enzyme (Terner, 1952; Kittinger & Reithel, 1953).

In homogenates and suspensions containing fluoride and fluoroacetate, the stimulant action of *p*-nitrophenol was smaller and, especially when glucose and hexokinase were present, of short duration, as will be described in the study of phosphorylation below. The respiratory response of mammary homogenates blocked by fluoroacetate AMP added in increasing concentration is shown in Table 4.

Effect of supernatant fraction. Although the addition of the isolated nuclear fraction was without effect on the respiration of the mitochondria, the addition of the supernatant fraction of the original kidney homogenate greatly enhanced the stimulating action of *p*-nitrophenol, but had much less effect on the oxygen consumption in the absence of the nitrophenol (Table 1). The corresponding effect of the supernatant fraction of mammary tissue was much smaller.

The supernatant fraction of kidney cortex was found to contain an enzyme which liberated inorganic phosphate from adenosine triphosphate

Table 3. *Effect of phosphate acceptors and* p-*nitrophenol on respiration of mammary homogenates*

Mammary homogenate, dry wt., 45·3 mg./vessel. Basal medium, ATP (0·0005 M), fumarate (0·0025 M) and pyruvate (0·01 M), in all flasks. Additions: glucose, 0·02 M; hexokinase, 18 units; *p*-nitrophenol (*p*NP), 2×10^{-4} M (added from the side arms of the flasks after 30 min. incubation). Q_{O_2} was calculated from the O_2 uptake during the 15 min. periods before and after adding *p*NP. Further additions of adenine nucleotide were made as AMP.

Total adenine nucleotide (M)	$-Q_{O_2}$			
	—		Glucose + hexokinase	
	—	*p*NP	—	*p*NP
0·0005	6·6	17·3	8·8	18·1
0·001	7·3	17·3	11·5	15·6
0·002	8·4	17·8	11·5	13·9
0·004	9·3	14·6	11·0	12·7

Table 4. *Effect of phosphate acceptors and* p-*nitrophenol on respiration of mammary homogenates in the presence of fluoroacetate*

Mammary homogenate, dry wt., 46·3 mg./vessel. Basal medium, fumarate (0·01 M), pyruvate (0·01 M), fluoride (0·01 M) and fluoroacetate (0·0025 M), in all flasks. Additions: glucose, 0·02 M; hexokinase, 18 units; *p*-nitrophenol (*p*NP), 2×10^{-4} M. Q_{O_2} values are given for the periods 10–25 min. and, in parentheses, for 25–40 min.

AMP added (M)	$-Q_{O_2}$			
	—		Glucose + hexokinase	
	—	*p*NP	—	*p*NP
0	3·3 (2·2)	3·7 (2·4)	2·9 (2·4)	3·1 (2·5)
0·0005	6·0 (4·7)	11·0 (7·9)	8·1 (7·1)	11·5 (8·5)
0·001	6·0 (4·9)	10·9 (7·5)	11·1 (7·0)	11·3 (7·6)
0·002	8·7 (4·8)	10·0 (6·1)	12·3 (7·2)	10·7 (6·5)
0·005	11·5 (3·2)	10·0 (5·1)	13·0 (5·9)	9·8 (2·8)

(ATP) at pH 7·4, in the presence of Mg^{2+} ions. *p*-Nitrophenol did not enhance the activity of the enzyme in the supernatant or in a fraction prepared by precipitation with ammonium sulphate (30–60 % saturation).

Since the addition of the supernatant to the mitochondrial fraction resulted in the appearance of an 'endogenous' respiration, the possibility was considered that under these conditions the effects of *p*-nitrophenol might be due to its action on the metabolism of endogenous substrates. However, measurement of the radioactivity of the respiratory CO_2 produced by kidney mitochondria metabolizing [2-^{14}C]pyruvate in the presence of fumarate showed that the supernatant fraction greatly increased the production of $^{14}CO_2$ in the presence of *p*-nitrophenol, but less so in its absence. No such enhancement of the action of *p*-nitrophenol by the supernatant fraction of mammary time was observed in mammary mitochondria. However, in both kidney and mammary mitochondria, the addition of AMP stimulated the production of $^{14}CO_2$ concomitantly with the stimulation of respiration already described (Table 5).

Table 2 also shows that when the concentration of adenine nucleotide was low, the Q_{O_2} values decreased as the amount of tissue added was increased. In the presence of high concentrations of AMP or in the presence of *p*-nitrophenol the Q_{O_2} values were independent of the enzyme concentration within the limits of the experimental conditions employed.

Since in the presence of high concentrations of AMP the enhanced rates of respiration were of relatively short duration and the effect of *p*-nitrophenol tended to be inhibitory, the total adenine nucleotide concentration chosen for use in the subsequent experiments in which phosphorylation was measured was 0·001 M.

Myokinase and adenylic deaminase in the mammary gland. Since the phosphorylation of adenylate is brought about by the action of myokinase (Barkulis & Lehninger, 1951), the presence of this enzyme in the mammary gland seems likely. Popják & Tietz (1955) observed that adenosine diphosphate (ADP) but not AMP could replace ATP in promoting lipogenesis in their mammary preparation, and concluded that this suggested the presence of either powerful myokinase activity, or of some other reaction leading to the formation of ATP from ADP. Further evidence was obtained in manometric experiments illustrated in Fig. 1, which showed that extracts of acetone-dried powders of mammary gland, prepared according to Kaplan & Lipmann (1948), in combination with yeast hexokinase caused the phosphorylation of glucose by ATP to go further than yeast hexokinase alone. Since the extracts prepared from guinea pig, rat, rabbit and sheep mammary tissue were almost devoid of hexokinase activity, their activity in the presence of added hexokinase must be attributed to myokinase. In these experiments a slow but steady uptake of gas following the rapid evolution of CO_2 (see Weil-Malherbe & Bone, 1951) and a decrease in

Table 5. *Effect of adenylate and of supernatant fraction on the metabolism of kidney mitochondria*

Kidney-cortex mitochondria, dry wt., 15·7 mg./vessel. Basal medium, ATP (0·0005 M), fumarate (0·0025 M) and [2-^{14}C]pyruvate (0·005 M), in all flasks. Additions: *p*-nitrophenol (*p*NP), 2×10^{-4} M; a portion of the supernatant fraction of original homogenate equivalent to one-half of the mitochondrial fraction (metabolic data obtained in the presence of added supernatant fraction are in parentheses). Incubation period, 50 min.

Additions	O_2 uptake (μmoles)	[^{14}C]Pyruvate (μm-moles) appearing in respiratory CO_2
None	13·1 (16·7)	510 (572)
*p*NP	25·6 (35·7)	3790 (5220)
AMP, 0·0015 M	19·1 (25·2)	2100 (2230)
AMP, 0·0015 M; *p*NP	22·8 (37·2)	3680 (5550)
AMP, 0·0035 M	25·2 (29·5)	2880 (3420)
AMP, 0·0035 M; *p*NP	23·6 (36·1)	3490 (4700)

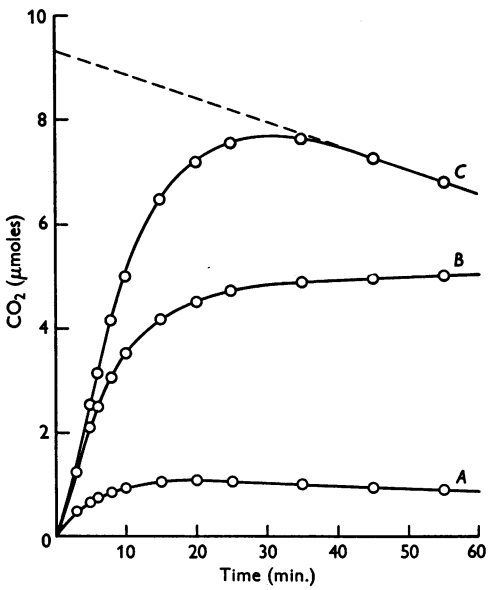

Fig. 1. Myokinase and adenylate deaminase activity of mammary tissue. The reaction mixture contained $MgCl_2$, 0·01 M; glucose, 0·02 M; fluoride, 0·005 M; iodoacetate, 0·001 M; $KHCO_3$, 0·0024 M; ATP, 13·2 μmoles of labile P ($P_{10} - P_0$). Volume, 2·0 ml. Temp., 30°. Gas: $CO_2 + N_2$ (5:95). *A*, Guinea-pig mammary acetone-dried powder extract, 0·5 ml.; *B*, yeast hexokinase, 9 units; *C*, mammary extract + yeast hexokinase.

optical extinction at 265 mμ. of samples deproteinized before and after incubation (see Kalckar, 1947) were indicative of the presence of adenylic deaminase.

Inhibition of the Pasteur effect by adenine nucleotide. As previously reported (Terner, 1954*a*), a Pasteur effect inhibited by *p*-nitrophenol can be demonstrated in cell-free homogenates of mammary gland. Further experiments now described show that the addition of adenine nucleotide in relatively large amount to glycolysing cell-free suspensions also resulted in increased accumulation of lactic acid under aerobic conditions. The cell-free suspensions used in these experiments were prepared by centrifuging mammary or kidney-cortex homogenates at slow speed to remove broken cells and nuclei. The resultant supernatant fraction, which contained the mitochondria and the soluble glycolytic enzymes, was incubated in the medium similar to that previously employed for glycolysis (Terner, 1954*a*), except that the concentration of inorganic phosphate was increased to 0·01 M. Both kidney and mammary suspensions showed a marked Pasteur effect. The addition of *p*-nitrophenol, AMP or ATP increased the amount of lactic acid accumulating under aerobic conditions (Table 6); 0·007 M AMP had a greater effect than 0·003 M.

Phosphorylation in mammary homogenates and kidney suspensions blocked by fluoroacetate. In previous papers (Terner, 1955*a*, *b*) the effect of *p*-nitrophenol on oxidation and citric acid formation in homogenates blocked by fluoroacetate has been reported. Phosphorylation has now been studied in the same system (Tables 7 and 8). A small uptake of

Table 6. *Inhibition of Pasteur effect by* p-*nitrophenol and adenine nucleotide*

Mammary suspension (freed from nuclei and cell debris), dry wt., 32 mg./vessel. Basal medium, ATP (0·001 M), glucose (0·04 M), fructose 1:6-diphosphate ($1{\cdot}25 \times 10^{-3}$ M) and hexokinase (18 units), in all flasks. *p*-Nitrophenol (*p*NP), 2×10^{-4} M. Incubation period, 35 min.

Additions	Experimental conditions	$-Q_{O_2}$	$Q_{\text{Lactic acid}}$
None	Aerobic	13·4	5·8
AMP, 0·003 M	Aerobic	15·1	10·8
AMP, 0·007 M	Aerobic	14·7	17·2
ATP, 0·007 M	Aerobic	15·1	11·0
*p*NP	Aerobic	17·8	17·1
AMP, 0·003 M; *p*NP	Aerobic	15·3	18·5
AMP, 0·007 M; *p*NP	Aerobic	14·4	17·8
ATP, 0·007 M; *p*NP	Aerobic	13·4	11·5
None	Anaerobic	—	27·4
AMP, 0·003 M	Anaerobic	—	26·6
AMP, 0·007 M	Anaerobic	—	23·5

Table 7. *Effect of* p-*nitrophenol and of arsenate on citric acid synthesis and aerobic phosphorylation*

Mammary homogenate, dry wt., 60·9 mg./vessel. Basal medium, ATP (0·001 M), glucose (0·02 M), hexokinase (18 units), fluoride (0·01 M) and fluoroacetate (0·005 M), in all flasks. Additions: fumarate, 0·01 M; pyruvate, 0·01 M. Incubation period, 40 min. Initial inorganic P, 972 μg.

Additions	$-Q_{O_2}$	$-Q_P$	Q_{Citrate}
None	3·5	7·2	0·2
*p*NP, 2×10^{-4} M	3·5	0·2	0·1
Fumarate	4·0	11·0	0·4
Fumarate; *p*NP, 2×10^{-4} M	3·2	2·4	0·6
Fumarate; arsenate, 0·01 M	3·8	1·5	0·9
Fumarate; pyruvate	7·2	16·7	3·4
Fumarate; pyruvate; *p*NP, 2×10^{-4} M	12·2	15·5	7·2
Fumarate; pyruvate; *p*NP, 4×10^{-4} M	12·3	2·5	7·2
Fumarate; pyruvate; arsenate, 0·005 M	6·9	14·2	3·3
Fumarate; pyruvate; arsenate, 0·005 M; *p*NP, 2×10^{-4} M	8·4	6·4	4·6
Fumarate; pyruvate; arsenate, 0·01 M	5·8	11·0	3·3
Fumarate; pyruvate; arsenate, 0·02 M	5·5	6·2	2·4
Fumarate; pyruvate; arsenate, 0·04 M	4·3	3·2	1·8

Table 8. *Effect of* p-*nitrophenol on citric acid synthesis and aerobic phosphorylation*

Kidney-cortex suspension (centrifuged once), dry wt., 28 mg./vessel. Basal medium, ATP (0·001 M), fluoride (0·01 M), fluoroacetate (0·005 M), glucose (0·02 M) and hexokinase (18 units), in all flasks. Additions: fumarate, 0·01 M; pyruvate, 0·01 M. Incubation period, 35 min. Initial inorganic P, 1455 μg.

Additions	$-Q_{O_2}$	$-Q_P$	Q_{Citrate}
None	6·3	14·5	0
*p*NP, 2×10^{-4} M	6·7	3·7	0
Fumarate	13·4	45·8	4·6
Fumarate; *p*NP, 2×10^{-4} M	11·3	5·9	4·4
Fumarate; pyruvate	20·4	61·6	8·9
Fumarate; pyruvate; *p*NP, 2×10^{-4} M	26·7	29·6	8·6
Fumarate; pyruvate; *p*NP, 4×10^{-4} M	29·0	4·8	11·4

inorganic phosphate, sensitive to inhibition by *p*-nitrophenol, was observed in the absence of added fumarate. This might have been due to the oxidation of endogenous substrates present in the crude mammary homogenates; washed kidney suspensions (mitochondria), however, were virtually devoid of endogenous oxidizable material as shown by the absence of respiration on incubation without added substrates.

Fumarate, when added as the only oxidizable substrate, caused a larger uptake of inorganic phosphate, which was further increased by the addition of pyruvate. In kidney suspensions, fumarate alone gave rise to appreciable amounts of citric acid (Liébecq & Peters, 1949), and in mammary homogenates only small amounts of citrate were formed unless both fumarate and pyruvate were present.

In many experiments the rate of respiration declined with time, but remained linear in the presence of *p*-nitrophenol. As only the overall rates are reported in the tables, the apparently higher rates of respiration in the presence of *p*-nitrophenol were largely due to the maintenance of the initial rate. Nevertheless, in this system the initial respiratory rate was usually enhanced by *p*-nitrophenol in mammary homogenates, but less so in kidney suspensions.

Citrate synthesis and inhibition of phosphorylation. In mammary homogenates metabolizing fumarate plus pyruvate in the presence of fluoroacetate, the synthesis of citric acid was accelerated by 2×10^{-4} M *p*-nitrophenol, which only partially inhibited phosphorylation; no further acceleration was observed when phosphorylation was abolished by *p*-nitrophenol in higher concentration (4×10^{-4} M) (Tables 7 and 9). Thus the higher overall respiratory rates observed in the presence of *p*-nitrophenol were paralleled by increased accumulation of citric acid but much less so by the degree of inhibition of phosphorylation. In washed kidney suspensions metabolizing fumarate plus pyruvate, the effect of *p*-nitrophenol on the rate of citric acid formation was small or absent (Tables 8 and 10).

Effect of arsenate on phosphorylation and citric acid synthesis. In both mammary and kidney preparations, arsenate was found to be a much less effective inhibitor of phosphorylation than *p*-nitrophenol. Although a measurable depression of phosphorylation was produced by relatively low concentrations (0·005 M), increasing amounts caused only small increments in the degree of inhibition. At the same time, arsenate caused inhibition of both respiration and citric acid formation (Tables 7, 9 and 10). Crane & Lipmann (1953) found that the inhibition of respiration by arsenate increased with time and

Table 9. *Effect of* p-*nitrophenol and of arsenate on phosphorylation and citric acid synthesis*

Mammary homogenate, dry wt., 55·7 mg./vessel. Basal medium, ATP (0·001 M), fumarate (0·01 M), pyruvate (0·01 M), fluoroacetate (0·005 M), glucose (0·02 M), hexokinase (18 units) and fluoride (0·01 M), in all flasks. Incubation period, 35 min. Initial inorganic P, 967 μg.

Additions	$-Q_{O_2}$	$-Q_P$	$Q_{Citrate}$
None	8·9	19·8	4·9
*p*NP, 2×10^{-4} M	14·9	17·9	10·7
*p*NP, 4×10^{-4} M	14·8	1·5	11·5
Arsenate, 0·005 M	8·2	16·0	4·1
Arsenate, 0·005 M; *p*NP, 2×10^{-4} M	10·1	5·1	7·5
Arsenate, 0·02 M	8·6	7·8	3·1
Arsenate, 0·02 M; *p*NP, 2×10^{-4} M	11·2	1·3	5·4

Table 10. *Effect of* p-*nitrophenol and arsenate on phosphorylation and citric acid synthesis*

Rabbit-kidney cortex suspension (centrifuged twice), dry wt., 15·2 mg./vessel. Basal medium, ATP (0·001 M), fluoroacetate (0·005 M), fluoride (0·01 M), glucose (0·01 M) and hexokinase (18 units), in all flasks. Additions: fumarate, 0·01 M; pyruvate, 0·01 M. Incubation period, 50 min. Initial inorganic P, 930 μg.

Additions	$-Q_{O_2}$	Q_P	$Q_{Citrate}$
None	1·6	+10·4	0
Pyruvate	6·1	+4·7	0
Fumarate	11·2	−23·0	3·9
Fumarate; *p*NP, 1×10^{-4} M	10·4	+3·7	3·9
Fumarate; pyruvate	14·5	−46·0	7·2
Fumarate; pyruvate; *p*NP, 1×10^{-4} M	14·4	−11·2	7·4
Fumarate; pyruvate; *p*NP, 2×10^{-4} M	14·4	+2·9	7·4
Fumarate; pyruvate; *p*NP, 4×10^{-4} M	15·1	+4·4	9·2
Fumarate; pyruvate; arsenate, $1{\cdot}25 \times 10^{-3}$ M	14·8	−40·2	5·7
Fumarate; pyruvate; arsenate, 0·0025 M	13·2	−32·8	5·5
Fumarate; pyruvate; arsenate, 0·005 M	13·2	−26·2	4·1

Table 11. *Effect of* p-*nitrophenol and arsenate on phosphorylation*

Kidney-cortex suspension (centrifuged twice), dry wt., 18·8 mg./vessel. Basal medium, ATP (0·001 M), fumarate (0·01 M), pyruvate (0·01 M), fluoroacetate (0·005 M), fluoride (0·01 M), glucose (0·02 M) and hexokinase (18 units), in all flasks. Incubation period, 50 min. Initial inorganic P, 1000 μg. All figures denote phosphate uptakes calculated as Q_P.

Concn. of arsenate (M)	Concn. of *p*NP			
	—	10^{-4} M	2×10^{-4} M	4×10^{-4} M
—	−44·6	−36·3	−17·0	+7·2
0·0025	−33·2	−17·2	−1·5	—
0·005	−27·8	−9·8	−1·5	—
0·01	−24·6	—	—	—

Table 12. *Effect of* p-*nitrophenol and arsenate on aerobic phosphorylation*

Mammary homogenate, dry wt., 42·9 mg./vessel. Basal medium, ATP (0·001 M), glucose (0·02 M), hexokinase (18 units), fluoride (0·01 M) and fluoroacetate (0·005 M), in all flasks. Additions: fumarate, 0·01 M; pyruvate, 0·01 M; α-oxoglutarate, 0·01 M; arsenate, 0·02 M. Incubation period, 40 min. Initial inorganic P, 1430 μg.

Additions	$-Q_{O_2}$	Q_P
None	3·2	−2·5
*p*NP, 2×10^{-4} M	2·1	−1·0
Fumarate	3·5	−4·5
Fumarate; *p*NP, 2×10^{-4} M	2·7	+2·1
Fumarate; pyruvate	11·4	−35·8
Fumarate; pyruvate; *p*NP, 2×10^{-4} M	13·4	−23·0
Fumarate; pyruvate; *p*NP, 4×10^{-4} M	11·5	−1·6
Fumarate; pyruvate; arsenate	5·5	−6·3
Fumarate; pyruvate; arsenate; *p*NP, 2×10^{-4} M	7·3	+1·2
α-Oxoglutarate	10·8	−32·2
α-Oxoglutarate; *p*NP, 2×10^{-4} M	7·7	−5·5
α-Oxoglutarate; *p*NP, 4×10^{-4} M	7·4	+0·4
α-Oxoglutarate; arsenate	7·7	−9·7

attributed it to the reductive formation of arsenite. This complication did not arise in the present work; in all experiments the respiratory rates in the presence of arsenate were either linear, or, where they declined, the rate of decline did not exceed that of the tissue incubated without arsenate. Under the present experimental conditions in which the initial concentration of inorganic phosphate was about 0·01 M, arsenate in a concentration at least as high as 0·04 M had to be added to produce an inhibition of phosphorylation comparable to that caused by *p*-nitrophenol (Table 7). This is in accord with the findings of Crane & Lipmann (1953), who found that an arsenate/phosphate ratio of 5:1 was required for phosphorylation to be inhibited as shown by the depression of the ^{32}P turnover of ATP.

Low concentrations of arsenate, although relatively ineffective, made the phosphorylating system more susceptible to the action of low concentrations of *p*-nitrophenol. The presence of arsenate reduced, but did not abolish, the stimulation of respiration and citric acid synthesis by *p*-nitrophenol (Table 9). The mutual potentiation of the effects of arsenate and *p*-nitrophenol on phosphorylation is illustrated in Table 11.

The phosphorylations supported by the oxidation of fumarate or α-oxoglutarate appeared to be more susceptible to inhibition by low concentrations of *p*-nitrophenol or of arsenate than the phosphorylations associated with the oxidation of fumarate plus pyruvate (Tables 7 and 12).

Time-course of phosphorylation and phosphorus/O_2 ratios. Only low phosphorus/O_2 ratios can be calculated from the preceding tables. However, in many experiments the inorganic phosphate initially present was esterified so rapidly, especially during the oxidation of fumarate plus pyruvate, that at the end of the usual incubation period of 40 min. it was nearly exhausted. By shortening the incubation period, rather than by increasing the amount of inorganic phosphate in the medium, higher ratios were obtained. It was found that the uptake of inorganic phosphate was highest during the first 10 min., which coincided with the equilibration period. On the assumption that the O_2 consumption during the first and second 10 min. periods was the same, phosphorus/O_2 ratios approaching 3·0 could be calculated for the equilibration period (Tables 13 and 14), a value which corresponds to the ratio expected for the conversion of fumarate and pyruvate into citrate (see Ochoa & Stern, 1952).

Even during short incubation periods, which precluded the danger of exhaustion of inorganic phosphate, the phenomena previously described could be observed; notably, citrate formation was increased by low concentrations of *p*-nitrophenol which caused only slight inhibition of phosphorylation (Table 13).

DISCUSSION

Phosphate turnover and metabolic rates. According to recent views, the rates of oxidations in phosphorylating systems are controlled by the balance between the rates of synthesis and breakdown of ATP (Potter & Recknagel, 1951; Lardy & Wellman, 1952, 1953). In mitochondrial preparations of low adenosine triphosphatase activity and containing adenine nucleotide in catalytic amounts the addition of an acceptor such as the glucose–hexokinase system may be expected to facilitate the turnover of phosphate by accelerating the regeneration of ADP. In the present experiments, however, the presence of adenine nucleotide in relatively large amount was necessary in addition to the glucose–hexokinase system to increase the respiratory rate to the maximum level attained under the influence of *p*-nitrophenol. High rates of respiration were also

Table 13. *Time-course of phosphorylation and citric acid synthesis*

Mammary homogenate, dry wt., 34·6 mg./vessel. Basal medium, ATP (0·001 M), fumarate (0·005 M), pyruvate (0·005 M), glucose (0·01 M), hexokinase (18 units), fluoride (0·01 M) and fluoroacetate (0·005 M), in all vessels. Initial inorganic P, 806 μg. Oxygen uptake during first 10 min. was obtained by linear extrapolation.

		Incubation period (min.)							
		0–10				0–20			
Series	Additions	O (μatoms)	P (μatoms)	Citrate (μmoles)	P/O ratio	O (μatoms)	P (μatoms)	Citrate (μmoles)	P/O ratio
1	None	4·2	−11·4	1·4	2·7	10·5	−24·0	2·5	2·3
2	*p*NP, 1×10^{-4} M	4·6	−10·2	2·1	2·2	11·4	−18·6	3·8	1·6
3	*p*NP, 2×10^{-4} M	6·4	−7·0	2·9	1·1	15·9	−9·9	5·1	0·6
4	*p*NP, 4×10^{-4} M	6·2	−2·0	3·1	0·3	15·4	−1·6	5·4	0·1
5	Arsenate, 0·0025 M	4·2	−8·4	1·4	2·0	10·5	−18·2	2·7	1·7
6	Arsenate, 0·0025 M; *p*NP, 2×10^{-4} M	5·7	−2·6	2·3	0·5	14·2	−6·3	4·7	0·4
7	Arsenate, 0·005 M	3·9	−6·6	1·0	1·7	9·8	−14·9	2·6	1·5
8	Arsenate, 0·005 M; *p*NP, 1×10^{-4} M	4·8	−2·6	2·0	0·5	11·9	−10·2	3·7	0·9
9	Arsenate, 0·005 M; *p*NP, 2×10^{-4} M	6·1	+0·2	2·5	—	15·3	−4·1	4·6	0·3

Table 14. *Time-course of phosphorylation and citric acid synthesis*

Mammary homogenate, dry wt., 43 mg./vessel. Basal medium, ATP (0·001 M), fumarate (0·01 M), pyruvate (0·01 M), glucose (0·02 M), hexokinase (18 units), fluoride (0·005 M) and fluoroacetate (0·005 M), in all vessels. Initial inorganic P, 770 μg. Oxygen uptake during first 10 min. was obtained by linear extrapolation.

		Incubation period (min.)							
		0–10				0–20			
Series	Additions	O (μatoms)	P (μatoms)	Citrate (μmoles)	P/O ratio	O (μatoms)	P (μatoms)	Citrate (μmoles)	P/O ratio
1	None	5·5	−16·5	2·2	3·0	11·0	−23·7	3·4	2·1
2	*p*NP, 2×10^{-4} M	8·4	−7·7	3·3	0·9	16·7	−11·0	5·8	0·7
3	*p*NP, 4×10^{-4} M	8·6	−6·4	3·4	0·6	17·2	−4·9	7·6	0·1
4	Arsenate, 0·005 M	5·2	−7·4	1·3	1·4	10·4	−12·8	2·2	1·2
5	Arsenate, 0·005 M, *p*NP, 2×10^{-4} M	6·7	−2·1	2·6	0·4	13·3	−2·1	4·5	0·1
6	Arsenate, 0·025 M	4·5	−4·6	0·9	0·2	9·0	−6·5	1·2	0·7

elicited by the addition of large amounts of AMP alone; furthermore, a progressive acceleration of respiration could be shown to result from a gradual increase in the amount of AMP added. Since the elevated rate of respiration in the presence of *p*-nitrophenol was of the same order of magnitude whether at lower or higher levels of adenine nucleotide, the apparent increase of respiration due to *p*-nitrophenol diminished in extent as the amount of AMP was increased. As shown in Table 2, the optimum amount of adenine nucleotide may vary according to the tissue concentration. Since in work of this nature a standard concentration of ATP, usually 0·001 M, irrespective of the activities of the tissue preparations is generally employed, the diversity of the effects of nitrophenols on respiratory rates reported in the literature may be at least in part explained.

Effects of p-*nitrophenol.* In experiments in which the phosphorylation accompanying the oxidation of fumarate plus pyruvate was only partially inhibited, the uptake of phosphate accompanying the oxidation of fumarate alone was practically abolished by *p*-nitrophenol. Thus it appears that the inhibitory action of the nitrophenol could be antagonized by a vigorous rate of phosphorylation.

Nitrophenols are believed to act at two points in the chain of events leading to the esterification of inorganic phosphate. They are assumed to cause the hydrolysis of a hypothetical intermediary high-energy compound at a stage preceding the formation of ATP (Slater, 1953; Boyer, Falcone & Harrison, 1954; Lehninger, 1955), and have been shown to activate the latent 'adenosine triphosphatase' activity of mitochondria (Siekevitz & Potter, 1953; Lardy & Wellman, 1953). Conditions seem to have been found in the present experiments such that the hydrolytic actions of *p*-nitrophenol, present in a critical concentration, could be allowed either to take effect or be overcome.

Effect of arsenate on phosphate metabolism. Arsenate, which as shown by Crane & Lipmann (1953) inhibits competitively the incorporation of inorganic phosphate into ATP, may be expected to interfere with the formation of the high-energy phosphate intermediate. When present in amounts which inhibited phosphorylation, arsenate also retarded the metabolism of pyruvate, depressing the rates of respiration and of citric acid formation. The different mode of action of the two inhibitors is also shown by the observation that although their inhibitory effects on the uptake of phosphate were cumulative, some stimulation of respiration and citric acid formation could be observed when *p*-nitrophenol was added to a system partially inhibited by arsenate. The effect of arsenate on the Pasteur reaction has been discussed previously (Terner, 1954*a*).

Effect of adenine nucleotide on the Pasteur reaction. Johnson (1941) and Lynen (1941) proposed that the exhaustion of intracellular inorganic phosphate by aerobic phosphorylation is an important factor in retarding the rate of aerobic glycolysis. It was further suggested by Johnson (1941) that a high ATP/ADP ratio, maintained by aerobic phosphorylation, may be expected to retard aerobic glycolysis. In a review of these concepts, Krebs (1954) pointed out that either ADP or inorganic phosphate or both may be the limiting factor controlling the rates of respiration and of aerobic glycolysis, but that it was uncertain which of the three alternatives applied. It has already been stated (Terner, 1954*a*) that the demonstration of a Pasteur effect in cell-free preparations of yeast and of mammary gland does not bear out the hypothesis that the level of inorganic phosphate is an important factor in regulating the rate of aerobic glycolysis. In previous and in the present experiments, high initial phosphate levels and short incubation periods were employed to avoid undue depletion of phosphate in reaction mixtures in which the glycolytic enzymes were mainly in solution. In the present experiments, the addition of either AMP or ATP resulted in increased accumulation of lactic acid in respiring cell-free suspensions of kidney or mammary gland. Under the influence of the myokinase and hexokinase reactions and of phosphatases both nucleotides give rise to ADP, the presence of which is required for glycolysis to proceed.

It may be noted that, in the experiments in which aerobic glycolysis was studied, the stimulating action of *p*-nitrophenol or of adenine nucleotide on the respiration of the cell-free system was small or absent. In these experiments fumarate had been omitted, to avoid excessive phosphorylation resulting from a fully functioning Krebs cycle, which might suppress the formation of lactic acid. When fumarate was present, both adenine nucleotide and *p*-nitrophenol accelerated the rate of respiration. Thus the effects of adenine nucleotide as well as those of nitrophenols of accelerating the respiration and inhibiting the Pasteur effect appear to be due to an acceleration of rates of reactions limited by the availability of the AMP system.

SUMMARY

1. The effects of *p*-nitrophenol and of added phosphate acceptors on respiration and aerobic glycolysis in cell-free suspensions of mammary gland and kidney cortex have been studied.

2. In homogenates or mitochondrial suspensions metabolizing fumarate plus pyruvate the addition of *p*-nitrophenol (2×10^{-4} M) resulted in maximal stimulation of respiration. The addition of adenosine 5′-phosphate (AMP) in amounts increasing from

0·0005 to 0·004 M resulted in progressive acceleration of respiration. Glucose plus hexokinase accelerated respiration, which was further increased by AMP.

3. The addition to the mitochondrial fraction of the particle-free supernatant of kidney cortex greatly enhanced the stimulating action of *p*-nitrophenol on the respiration. The supernatant fraction of mammary tissue did not show a corresponding activity.

4. In cell-free suspensions showing glycolysis under aerobic conditions, the addition of AMP or adenosine triphosphate in large amount resulted in the accumulation of lactic acid, thus inhibiting the Pasteur effect.

5. In mammary homogenates metabolizing fumarate plus pyruvate in the presence of fluoride and fluoroacetate, phosphorus/oxygen ratios approaching 3·0 were observed. Acceleration of respiration and citric acid formation resulted from the addition of 2×10^{-4} M *p*-nitrophenol, which caused only partial inhibition of phosphorylation. No further acceleration of respiration and citric acid formation was observed when the concentration of *p*-nitrophenol was raised to 4×10^{-4} M, at which it abolished phosphorylation. Arsenate, when present in effective concentration, inhibited respiration, citric acid formation and phosphorylation.

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The Effect of Fumarate and of *p*-Nitrophenol on the Synthesis of Fatty Acids in Homogenates of the Mammary Gland

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In preceding papers the effects of *p*-nitrophenol on the oxidative metabolism of pyruvate and acetate and on oxidative phosphorylation in mammary homogenates have been described (Terner, 1954*a*, 1955*a*, *b*, 1956). These studies have now been extended to the well-known ability of mammary tissue to synthesize fatty acids, demonstrated in tissue slices by Balmain, Folley & Glascock (1952) and in cell-free preparations of mammary gland by Popják & Tietz (1954, 1955). The latter authors found that in their preparation fatty acid synthesis was not inhibited by 2:4-dinitrophenol by more than

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