The author thanks Mr B. H. Bendall, Conservator of Forests, of the Wood and Forests Department, Adelaide, Australia, Mr A. C. Harris, Conservator of Forests, of the Government of Western Australia Forests Department, Perth, Australia, Mr W. E. Hillis, of the Division of Forest Products, C.S.I.R.O., Australia, Mr C. L. Hofmann, of the Forestry Commission of New South Wales, Division of Wood Technology, Australia, Dr F. N. Howes, of the Royal Botanic Gardens, Kew, and Mr W. Wilks, of the Department of Forestry, Brisbane, Australia, for specimens of eucalypt heartwoods.

REFERENCES

- Bate-Smith, E. C. (1956). *Sci. Proc. R. Dublin Soc.* **27**, 165.
 Bate-Smith, E. C. (1958). *Proc. Linn. Soc. Lond.* **169**, 198.
 Blakely, W. F. (1955). *A Key to the Eucalypts*, 2nd edition. Canberra: Forestry and Timber Bureau.
 Carr, D. J. & Carr, S. G. M. (1959). *Nature, Lond.*, **184**, 1549.
 Erdtman, H. (1958). In *Proc. IVth int. Congr. Biochemistry, Vienna*, **2**. *Symp. II. Biochemistry of Wood*, p. 1. Ed. by Kratzl, K. & Billek, G. London: Pergamon Press.
 Hathway, D. E. (1958). *Biochem. J.* **70**, 34.
 Hathway, D. E. (1960). In *Chromatographic and Electrophoretic Techniques*, vol. 1, *Chromatography*, p. 308. Ed. by Smith, I. London: William Heinemann Medical Books Ltd.
 Hathway, D. E. & Seakins, J. W. T. (1959a). *Biochem. J.* **72**, 369.
 Hathway, D. E. & Seakins, J. W. T. (1959b). *Biochem. J.* **72**, 37p.
 Koch, J. E. & Krieg, W. (1938). *Chem.-tech. Z.* **62**, 140.
 Linstedt, G. (1950). *Acta chem. scand.* **4**, 448.
 Mueller, F. von (1879). In *Eucalyptographia*. Melbourne: Government Printer.
 Roberts, E. A. H., Wight, W. & Wood, D. J. (1958). *New Phytol.* **57**, 211.
 Williams, A. H. (1955). *Chem. & Ind.* p. 120.

Biochem. J. (1962) **83**, 84

The Rate of Changes in Inorganic Phosphate Content of Ehrlich Ascites-Tumour Cells

By J. O. LAWS AND L. H. STICKLAND

Department of Experimental Pathology and Cancer Research, School of Medicine, Leeds 2

(Received 9 August 1961)

A good deal is known about the content of inorganic phosphate in the cells of the Ehrlich ascites tumour under different conditions (e.g. Chance & Hess, 1959; Ibsen, Coe & McKee, 1958; Kvamme, 1958; Braun, Erdelyi & Udvardy, 1960), but the changes in inorganic phosphate when the conditions are varied are very rapid, and little is known about the rate at which they occur. This paper gives evidence of the rates, in varying circumstances, at which the inorganic phosphate in these cells is esterified.

Four difficulties had to be dealt with before reliable and reproducible measurements could be made: (1) the inorganic phosphate content of a freshly prepared washed suspension of the cells is variable; (2) at 37° the inorganic phosphate of the cells rapidly leaks out into the medium (Kvamme, 1958); (3) at 37° the rate of the changes is too great to be measured conveniently; (4) phosphate buffer cannot be used for such measurements, and most other buffers inhibit the glycolysis of the cells at 37°.

The first point was dealt with by incubating the cells in nitrogen before beginning the measurements, a procedure which increased the inorganic phosphate content to a fairly constant high value, from

which subsequent reduction under different conditions could be observed. Leakage from the cells was minimized by keeping them at a low temperature throughout, and the rate of the changes in inorganic phosphate was decreased by carrying out the experiments at 12° instead of at 37°. Only one of the common buffers alternative to phosphate did not inhibit the glycolysis of these cells at 37°, namely bicarbonate buffer, but at 12° the inhibitory effect of tris was negligible, and in some experiments at 12° this buffer was used.

EXPERIMENTAL

Abbreviations. P_i, Inorganic phosphate.

Ascites-tumour cells. The Ehrlich ascites tumour [a strain originated by Klein (1951) from a solid Ehrlich carcinoma] was maintained in mice of the BalbC or C3H strain, and the ascitic fluid was removed from mice showing a sufficient accumulation of ascites, usually 7–10 days after inoculation. The combined fluid from anything up to 20 mice was filtered through glass wool, and the cells were washed, without preliminary treatment with water to lyse red blood cells, by centrifuging at 0° for 1–1.5 min. at 500g. Washing was with ice-cold calcium-free Krebs–Ringer solution, and from four to six washes with 5–10 vol. of that solution sufficed to remove ascitic fluid and all but very

slight traces of red blood cells. The cells were finally packed tightly by centrifuging for 10 min. at the same speed.

Buffers. A standard 0.1M-phosphate (Na_2HPO_4 - NaH_2PO_4) buffer, pH 7.5, was prepared from the Tables given by Lillie (1954), and its pH was checked by the glass electrode. The other buffers, except bicarbonate, were brought to the same pH colorimetrically (phenol red) by the addition of NaOH or HCl as required, and made up to 0.1M. The concentration of bicarbonate required, at the various temperatures used, to give the pH aimed at when in equilibrium with 5% of CO_2 in the gas phase, was calculated from the Table given by Umbreit (1957).

Determinations. Respiration was measured in air by the usual Warburg method, protein by the method of Robinson & Hogden (1940), glucose by the method of Hanes (1929), lactic acid by the method of Barker & Summerson (1941), P_i by the method of Fiske & Subbarow (1925).

Experimental procedure. When the time-course of changes was to be followed, the experiments were conducted in 150 ml. Erlenmeyer flasks shaken in a water bath at the requisite temperature. Samples (2 ml.) were withdrawn at the times required, and pipetted into 2 ml. of either 10% (w/v) trichloroacetic acid or M-perchloric acid. With buffers other than bicarbonate, anaerobic conditions were maintained by passing a rapid stream of N_2 , purified by passage over heated copper, through the flask, and aerobic experiments were conducted in air. With bicarbonate buffer, N_2 (O_2 -free) + CO_2 (95:5) was used for anaerobic experiments, and O_2 + CO_2 (95:5) for aerobic experiments.

It was impossible to bubble the gas through the cell suspensions, because of uncontrollable frothing, so for anaerobic experiments all the components of the reaction mixtures were freed from dissolved O_2 , either by boiling or by prolonged exposure to a vacuum, with frequent shaking. Bicarbonate solutions were brought to equilibrium with N_2 + CO_2 (95:5) before use.

For experiments on glucose utilization, the reaction mixtures were equilibrated in the bath, with gas passing, where necessary, for 10 min.; then the glucose solution was added, and, after further shaking for 1 min. to ensure complete mixing, the zero-time sample was removed, and further samples were taken as required. For experiments on changes in P_i concentration the initial samples were taken immediately before the addition of the glucose, and the moment of addition of the glucose was taken at zero time. In experiments in which glucose was not present, the equilibration was carried out in N_2 + CO_2 (95:5), and at zero time, after the removal of the initial samples, the gas mixture was changed to O_2 + CO_2 (95:5). In all experiments in which glucose was present its concentration was 0.2%.

Experiments on the leakage of P_i and protein from the cells were carried out in a similar manner, duplicate samples being treated at once with an equal volume of 10% (w/v) trichloroacetic acid, and further duplicates were cooled at once in ice and centrifuged rapidly at 0° as soon as possible. The cell deposit and the supernatant were each made up to 2 ml. and treated with 2 ml. of 10% (w/v) trichloroacetic acid. The protein precipitated in the latter fraction was estimated, and P_i determinations were done on both fractions, as well as on the samples of the whole suspension. Such experiments carried out during phases of rapid change of P_i gave fallacious results, as the changes in P_i continued during the cooling and centrifuging operations.

In the experiments which follow dealing with changes in P_i , trichloroacetic acid was added to the whole suspension, and the results include both intra- and extra-cellular P_i . Results are throughout expressed as $\mu\text{moles of } \text{P}_i/\text{g. dry wt. of cells}$, and rates of change in the form of q_P ($\mu\text{moles of } \text{P}_i/\text{mg. dry wt./hr.}$).

Samples of all cell suspensions were dried to constant weight and a correction was made for the salt content of the medium. The cell concentration in the reaction mixtures was, unless otherwise stated, in the region of 20 mg. dry wt./2 ml. sample.

RESULTS

Leakage of P_i

Kvamme (1958) and Wu (1959) have demonstrated the high permeability of the Ehrlich ascites-tumour cell to P_i and to proteins (including enzymes). We have done a number of experiments with the object of finding conditions to minimize this leakage. Our results have been rather variable, and those quoted below are representative.

In a freshly prepared suspension of cells, 9% of the P_i was found to be in the supernatant; after 1 hr. at 37° with no buffer this had increased to 35%, in 20 mM-tris to 63%, in 46 mM-bicarbonate to 62% and in 20 mM-imidazole to 80%. The extra-cellular protein (taking the total protein as roughly 80% of the dry weight, as determined by the biuret method on cell suspensions) was initially 5%, rising under the same conditions respectively to 7, 10, 12 and 16%, and in 20 mM-phosphate to 9%. It is not known whether the harmful effect of the buffers is due to a specific effect of the ions, or merely to the change of pH (the buffers were each at pH 7.5, and the pH of an unbuffered cell suspension is in the range pH 6.0-6.5).

Variation of the salt composition of the basic medium (NaCl , KCl , CaCl_2 and MgSO_4 were each varied independently from 0 to twice iso-osmotic concentration) did not decrease the rate of leakage. For instance, in one such experiment, in unbuffered medium, the extracellular P_i rose in 1 hr. at 37° from 8% to 35% in normal Krebs-Ringer solution, and to 35-40% in the modified solutions. The extracellular protein rose in all equally from 2 to 5%. The addition of Dextraven (Benger Laboratories Ltd.; an inert viscous polysaccharide plasma substitute) also caused no improvement.

The leakage of P_i was susceptible to decrease of temperature. At 20° extracellular P_i increased, in unbuffered Krebs-Ringer solution, in 1 hr. from 8 to 20%, with no increase in extracellular protein; at 12° , in 46 mM-bicarbonate buffer, in 20 min. extracellular P_i increased from 8.5 to 13.5%, and extracellular protein from 2 to 3.5%.

For the purposes of the experiments to be described, the cells had to be kept anaerobic for some time to allow the concentration of P_i to rise

to a constant figure. In a suspension made from cells kept, while still packed tight in centrifuge tubes, under nitrogen for 2 hr. at 20°, the initial extracellular P_i was 20%, rising after incubation for 20 min. in 46 mM-bicarbonate buffer at 12° to 30%. This rate of leakage of P_i , though still considerable, would not seriously interfere with measurements of the rate of change of concentration of P_i in the cells during the first 10 min. at 12°.

Choice of buffer

In phosphate buffer (20 mM, pH 7.5) the respiration of Ehrlich ascites-tumour cells is linear for at least 2 hr. (at 37° q_{O_2} approx. -0.5), whereas glucose utilization progressively decreases in rate from the beginning (at 37° q_{Glc}^N approx. -1.0 initially). When measurements of intracellular P_i , and its rapid changes, are to be made, phosphate buffer is inadmissible, and some other suitable buffer has to be found. The obvious choice, tris, was found to be inhibitory of the glycolysis of these cells at 37°, and so were all the other usual buffers at pH 7.5, with the exception of bicarbonate (Table 1). No buffer, other than veronal, had any significant inhibitory effect on the respiration. It was later found that tris has only a slight inhibitory action on glucose utilization at 12° (Table 2).

Changes in the concentration of P_i in Ehrlich ascites-tumour cells

At 37°. Freshly prepared suspensions of Ehrlich ascites-tumour cells contain a variable amount of P_i (from 30 to 80 μ moles/g. dry wt.). When such suspensions were incubated in nitrogen at 37° in the absence of added substrate the P_i rose, fairly rapidly at first, until an almost steady concentration was reached after about 1 hr. (average about 110 μ moles/g.; see Fig. 1, left-hand side). When glucose was added, in nitrogen at 37°, there was a rapid fall in P_i , complete within 1 min., to about 40 μ moles/g., independent of the starting concentration, and exactly the same change was observed with glucose in oxygen. The curves obtained with glucose present, whether in nitrogen or in oxygen, showed a kink at the first minute in which the P_i value fell below the steady value reached after 4 min. This was regularly observed in experiments at 37°, though not at 12° (see below); no explanation can be offered. In oxygen alone, without glucose, the change in P_i was variable, depending on the initial value; if the latter were high, then the value fell somewhat, though it was never as low as in the presence of glucose, and if the initial value were low it remained stationary or rose a little.

Table 1. *Effect of various buffers on the anaerobic rate of utilization of glucose by Ehrlich ascites-tumour cells at 37°*

'Control' values give the rate of utilization in 20 mM-phosphate, pH 7.5.

Buffer	Concn. (mM)	pH	Duration of expt. (min.)	$-q_{Glc}^N$		Inhibition (%)
				Control	Expt.	
Bicarbonate	15.4	7.4	30	0.93	0.71	24
Bicarbonate	30.8	7.7	30	0.93	0.90	3
Bicarbonate	61.6	8.0	30	0.93	1.03	-16
Bicarbonate	30.8	7.7	30	0.83	0.78	7
Citrate	20	7.5	15	1.00	0.68	32
Glycylglycine	20	7.5	60	0.48	0.23	51
Imidazole	20	7.5	15	0.81	0.33	60
Imidazole	20	8.0	15	0.81	0.37	54
Maleate	20	7.5	30	0.87	0.62	28
Maleate	20	8.0	15	1.07	0.84	22
Phosphate	20	8.0	30	0.93	1.09	-17
Tris	20	7.5	60	0.59	0.28	52
Veronal	20	7.5	15	1.00	0.55	45
EDTA	20	7.5	15	1.00	0.25	75

Table 2. *Effect of some buffers on the rate of anaerobic utilization of glucose by Ehrlich ascites-tumour cells at 12°*

'Control' values give the rate of utilization in 20 mM-phosphate, pH 7.5.

Buffer	Concn. (mM)	pH	Duration of expt. (min.)	$-10^3 q_{Glc}^N$		Inhibition (%)
				Control	Expt.	
Bicarbonate	22.6	7.4	15	15	17	-13
Bicarbonate	22.6	7.4	10	17	15	12
Tris	20	7.5	10	17	16	6
Triethanolamine	20	7.5	10	17	7.5	56

Cell suspensions subjected to a preliminary incubation in nitrogen (sometimes unbuffered, sometimes buffered with bicarbonate at pH 7.7) for 1 hr. at 37°, in which the P_i content had risen to 100 μ moles/g. or more, were now used for the study of the further changes in P_i under four sets of conditions: (a) further incubation in nitrogen; (b) incubation in oxygen; (c) incubation in nitrogen with addition of 0.2% of glucose; (d) incubation in oxygen with addition of 0.2% of glucose. The results of such a set of experiments are shown in Fig. 1 (right-hand side). In nitrogen the slow increase in P_i continued; in oxygen there was a slow fall to a value of between 80 and 90 μ moles/g.; in the presence of glucose, whether in nitrogen or oxygen, the fall was extremely rapid (complete in less than 1 min.) to a value between 50 and 60. The initial fall in 1 min. to a figure below that reached in the steady state was invariably observed here also.

The final concentration of P_i in these experiments with cells preincubated in nitrogen never reached the same low value as in the same suspension used fresh, but usually fell by about the same amount, even though starting at a higher concentration. It was at this point that we became acquainted with the work of Kvamme (1958), and realized that in the course of the preincubation period a large part of the P_i had left the cells, and was removed from participation in the esterification processes.

Cells incubated in nitrogen for 1 hr. at 37°, in spite of their loss of intracellular P_i , still respired and glycolysed actively. q_{O_2} and $q_{Glucose}^{N_2}$ in incubated cells were never less than 90% of their values in the fresh cells.

At 20°. In a cell suspension incubated in nitrogen for 1 hr. at 37° in bicarbonate buffer, up to 65% of the P_i was found to be extracellular, i.e. about

70 μ moles/g. in the medium and 40 μ moles/g. in the cells. If, instead, the cells were kept under nitrogen at room temperature (about 20°) in the minimum volume of medium and in the absence of buffer (i.e. packed tight in centrifuge tubes filled with nitrogen) for 2–3 hr., their P_i content rose to about the same value (90–110), of which about 80% was still intracellular. Suspensions made from cells treated in this manner were used to carry out experiments of the kind described above, the experiments also being done at room temperature. Fig. 2 illustrates such an experiment, showing the changes in P_i in nitrogen, oxygen, nitrogen + glucose, and oxygen + glucose, both in the fresh suspension and in that made from cells kept for 3 hr. under nitrogen at 20°. The changes were of the same kind as at 37°, but slower. The rates were still too fast for accurate measurement, so the temperature for further experiments was reduced to 12°.

At 12°. The results of a typical experiment at 12° are shown in Fig. 3. The cells as before had been kept tightly packed in the centrifuge tubes under nitrogen for 3 hr. at room temperature, and then were suspended in the requisite volume of ice-cold Krebs–Ringer solution and kept at 0° until used. Their P_i content had on this occasion risen to 117 μ moles/g. On incubation at 12° in nitrogen the P_i rose very slowly (to 118 in 40 min.); in oxygen it fell rather faster, approximately linearly to 104 in 20 min., and then more slowly to 88 in 60 min.; in the presence of glucose it fell, rapidly and approximately linearly, to a value of 75 in 10 min., and to a final steady value of about 60 after 20 min., the same rate of change being observed in nitrogen as in oxygen. The steady residual P_i concentration in the presence of glucose varied in different experiments

Fig. 1. Course of the changes in P_i in Ehrlich ascites-tumour cells at 37°; left, in a fresh suspension; right, in the same suspension kept at 37° in N_2 for 1 hr. □, in N_2 ; ×, in O_2 ; Δ, in N_2 + glucose (0.2%); ▽, in O_2 + glucose (0.2%).

Fig. 2. Course of the changes in P_i in Ehrlich ascites-tumour cells at 20°; left, in a fresh suspension; right, in the same batch of cells kept at 20° under N_2 for 3 hr. □, in N_2 ; ×, in O_2 ; Δ, in N_2 + glucose (0.2%); ▽, in O_2 + glucose (0.2%).

from 50 to 70 $\mu\text{moles/g.}$; in most experiments the steady concentration in oxygen + glucose was slightly higher than that in nitrogen + glucose (as illustrated in Fig. 3), but in some they were equal.

From the linear parts of these curves the rate of change of P_i was calculated, and expressed in terms of q_P . The results of eight such experiments are given in Table 3, together with the averages of several experiments at 37° and at 20°. In nitrogen (or in nitrogen + carbon dioxide, 95:5) there was no change, in air (or in oxygen + carbon dioxide, 95:5) a fall in P_i at a rather variable rate (q_P ranged from -0.007 to -0.040, average -0.020), and in the presence of glucose q_P was between -0.16 and

-0.32 (average -0.26) and was almost invariably the same whether oxygen were present or not.

Effect of iodoacetate. The results so far indicated that the fall in P_i observed in Ehrlich ascites-tumour cells is chiefly the result of glucose breakdown, and that respiration causes only a very slow and much smaller decrease in P_i . We therefore studied the effect of inhibiting the glycolysis by iodoacetate. Preliminary experiments showed that at 12°, mM-iodoacetate is required for complete suppression of glycolysis (0.1 mM, 41%; 0.2 mM, 58%; 0.5 mM, 85%; mM, 100% inhibition). Iodoacetate was made up to 0.1 M solution, which was diluted with Krebs-Ringer solution for addition to the flasks at a final concentration of mM.

The effect of mM-iodoacetate on the rate of change of P_i in Ehrlich ascites-tumour cells is shown in Table 4. The slow rate observed in air (or in oxygen + carbon dioxide, 95:5) was unaffected (average q_P -0.02); the anaerobic rate in the presence of glucose was, as expected, reduced almost to zero, whereas aerobically in the presence of glucose there was a residual decrease in P_i of variable magnitude (average q_P -0.065). It will be noted that the rate in the presence of oxygen, glucose and iodoacetate runs roughly parallel to that in oxygen alone, but is invariably about two or three times as high.

Metabolic rates of Ehrlich ascites-tumour cells at 12°. Since the rates of change of P_i could not be measured at temperatures higher than 12°, it is important to establish what effect this lowering of the temperature has on the rates of respiration and glycolysis of the cells. The usual methods were employed, but owing to the slowness of the re-

Fig. 3. Course of the changes in P_i in Ehrlich ascites-tumour cells at 12°; the cells had been kept at 20° under N_2 for 3 hr. $\square$, in N_2 ; $\times$, in O_2 ; Δ , in N_2 + glucose (0.2%); ∇ , in O_2 + glucose (0.2%).

Table 3. Rate of change of P_i in Ehrlich ascites-tumour cells in the presence and in the absence of oxygen, and in the presence and in the absence of glucose

Temp.	Buffer	Gas phase ...	$-10^3 q_P$			
			N_2	Air	With glucose (0.2%)	
					N_2	Air
12°	Tris		0.5	2.7	29	29
12	Tris		—	1.0	25	25
12	Tris		—	1.3	27	27
12	Tris		-0.5	0.7	16	16
		Gas phase ...	With glucose (0.2%)			
			$N_2 + CO_2$	$O_2 + CO_2$	$N_2 + CO_2$	
					$N_2 + CO_2$	$O_2 + CO_2$
12	Bicarbonate	-0.5		4.0	32	32
12	Bicarbonate	—		3.8	26	27
12	Bicarbonate	—		1.6	25	25
12	Bicarbonate	—		0.7	30	26
	Average ...	0		2.0	26	26
20	Various (average of three expts.)	0		9	75	70
37	Various (average of five expts.)	-3		50	> 250	> 250

Table 4. *Effect of iodoacetate (mM) on the rate of change of P_i in Ehrlich ascites-tumour cells*
Temperature, 12°. Buffers: A, bicarbonate; B, tris.

- 10 ³ q _P								
Anaerobic		Aerobic		Anaerobic with glucose (0.2 %)		Aerobic with glucose (0.2 %)		
Control	Iodo- acetate	Control	Iodo- acetate	Control	Iodo- acetate	Control	Iodo- acetate	
A {	-0.5	0	4.0	4.0	32	0.5	32	11
	—	—	3.8	3.8	26	0	27	8
	—	—	1.6	1.6	25	—	25	5
	—	—	0.7	0.7	30	0	26	7
B {	0.5	0	2.7	2.7	29	2	29	8.5
	—	—	1.1	1.6	25	0	25	3
	—	—	1.3	1.3	26	—	26	6
	-0.5	-0.5	0.7	0.7	16	0	16	3
Average	0	0	2	2	26	0.5	26	6.5

actions the highest possible concentrations of cells had to be used, and this led to technical difficulties. The viscosity of a dense cell suspension is very high; in respiration measurements this was overcome by increasing the volume of fluid in the manometer vessels to 4 ml., but in experiments on glucose utilization sampling was difficult, and the results are less accurate than usual. A further difficulty was that in control experiments (e.g. anaerobically in the presence of glucose and iodoacetate) there was an increase in the total reducing power of the reaction mixture. Greater accuracy could have been obtained by lowering the initial glucose concentration, but this was undesirable as 0.2% is approaching the lowest concentration at which the Crabtree effect (the diminution of Q_{O_2} in the presence of glucose; Crabtree, 1929) is observed.

For all these experiments cells which had been kept under nitrogen at 20° for 2–3 hr. were used, so that they were in the same condition as those used for the measurement of changes in P_i.

Table 5 gives the results of measurements of respiratory rate at 12°, and shows the absence of any significant inhibition by mM-iodoacetate. In these experiments the Crabtree effect appears to be somewhat diminished by iodoacetate (Crabtree quotient increased from 0.65 to 0.78).

Table 6 shows the rate of glucose utilization at 12° in the presence and the absence of oxygen, and the effect of iodoacetate on them. Glucose utilization was slowed down by the lower temperature to a smaller degree than was the respiration;

$q_{O_2}^{12^\circ}/q_{O_2}^{37^\circ} = 0.10$, whereas $q_{Glucose}^{12^\circ}/q_{Glucose}^{37^\circ} = 0.18$.

Iodoacetate (mM) completely suppressed the disappearance of glucose anaerobically, but not aerobically. The iodoacetate-resistant glucose utilization in the presence of oxygen was variable in quantity, amounting to between 10 and 25% of the

Table 5. *Respiration and the Crabtree effect in Ehrlich ascites-tumour cells at 12° and the effect of iodoacetate (mM)*

The bottom row of figures represents averages of the values given in the last four rows.

-10 ³ q _{O₂}			
Endogenous		With iodoacetate	
		Endogenous	With glucose (0.2%)
4.2	3.8	—	—
4.0	3.6	—	—
4.2	3.1	—	—
4.6	2.1	—	—
3.6	—	3.7	—
2.6	1.9	2.5	2.0
3.2	2.1	3.1	2.6
2.7	1.5	2.6	1.9
4.4	2.9	4.1	3.2
3.2	2.1	3.1	2.4

uninhibited anaerobic rate. The range of variation was similar to that seen in the rate of decrease of P_i under the same conditions (see Table 4).

These experiments on the effect of iodoacetate on the glucose metabolism of Ehrlich ascites-tumour cells at 12° gave results qualitatively similar to those of Wu & Racker (1959) at 30°.

DISCUSSION

The experiments described here were undertaken as a preliminary to a study of the rates of change in Ehrlich ascites-tumour cells of the concentrations of the adenine nucleotides under similar conditions. No such results are yet available, except that during the preliminary incubation in nitrogen, in which the P_i content of the cells reaches a high value, their ATP content is greatly depleted. How-

Table 6. *Rate of utilization of glucose by Ehrlich ascites-tumour cells at 12° and the effect of iodoacetate (mM)*

Glucose concentration was 0.2%. Cell concentration was between 50 and 70 mg. dry wt./2 ml. sample.

Buffer and pH	Duration of expt. (min.)	Gas phase	$-10^3 q_{O_2}$			
			Without iodoacetate		With iodoacetate	
			N ₂ or N ₂ + CO ₂	Air or O ₂ + CO ₂	N ₂ or N ₂ + CO ₂	Air or O ₂ + CO ₂
Bicarbonate, 7.7	10		16	10	—	—
Bicarbonate, 7.7	10		15	8	—	—
Phosphate, 7.5	15		21	15	—	—
Bicarbonate, 7.7	15		19	10	0	6
Bicarbonate, 7.7	10		14	13	0	2
Tris, 7.5	10		14	12	0	2
Bicarbonate, 7.7	10		17	15	0	4
Phosphate, 7.5	10		18	17	0	2
Bicarbonate, 7.7	10		14	13	0	7
Average		...	17	13	0	4

ever, in yeast it has been found (Stickland, 1956*a*; Laws & Stickland, 1958, 1959) that the rates of increase in ATP, when glucose is added, run parallel to the rates of decrease in P_i . The comparison with yeast is, however, probably unprofitable, and attempts have been made to observe similar changes in a normal mammalian tissue, namely, guinea-pig-kidney cortex, whose metabolism approaches quantitatively most nearly to that of Ehrlich ascites cells, but so far without success.

The most curious aspect of the results given above is the identity of the values of q_P anaerobically and aerobically in the presence of glucose (Table 3). The rate of glucose breakdown at 12° is diminished by some 25% in the presence of oxygen, and it might be expected that the rate of disappearance of P_i would be similarly diminished. However, in oxygen some oxidation of glucose appears to take place [Bloch-Frankenthal & Weinhouse (1957) estimated that 10% of the glucose disappearing aerobically was oxidized] in addition to the glycolysis, as is shown by the experiments in the presence of iodoacetate (Table 6), and q_P in oxygen may be made up of two components, associated respectively with the glycolysis and the oxidation. The slow rates of change of P_i associated with oxidative metabolism remain noteworthy; in yeast $q_P^{O_2+Glc}$ is some seven times as great as $q_P^{N_2+Glc}$, q_P for the endogenous respiration is of a similar magnitude to that in the presence of glucose and oxygen (about one-third as great; Stickland, 1956*b*).

The Pasteur effect, determined from the rates of either glucose utilization or lactic acid production in the presence and the absence of oxygen, was less at 12° than at 37° (the quotient $q_{Glc}^{O_2}/q_{Glc}^{N_2}$ was 0.6 at 37° and 0.75 at 12°). This is presumably related to the greater diminution of the respiration compared with the glycolysis at the lower temperature.

The lowered rate of utilization of glucose at 37° in the presence of tris and absence of phosphate has been observed by Ibsen, Coe & McKee (1960), who showed that the rate could be restored by adding phosphate in addition to the tris, i.e. the phenomenon is interpreted as a result of the lack of phosphate, not of an inhibitory effect of tris. However, the cells utilize glucose in bicarbonate buffer, in the absence of added phosphate, as fast as they do in phosphate buffer itself, so absence of phosphate is not alone enough to explain the lowered rate in tris buffer.

SUMMARY

1. Ehrlich ascites-tumour cells have a variable content of inorganic phosphate, but after incubation in nitrogen at 37° for 1 hr. or at 20° for 3 hr. this rises to a uniform high value, from which changes can be observed when the cells are subsequently incubated under various conditions.

2. The rate of such changes is too great to be measured at 37°, but is linear for 10 min. or more at 12°.

3. Values are given for the rates of change of inorganic phosphate in the presence and the absence of oxygen and in the presence and the absence of glucose, and the effect on these rates is recorded of iodoacetate at a concentration (mM) sufficient to suppress glycolysis.

4. The rate of fall of inorganic phosphate is slow in the presence of oxygen alone, but rapid in the presence of glucose.

5. Presence or absence of oxygen does not affect the rate of fall of inorganic phosphate when glucose is present.

We wish to express our thanks to the Trustees of the Will Trust, set up by the late Mrs M. O. Gordon, for the support they have given to this work.

REFERENCES

- Barker, S. B. & Summerson, W. H. (1941). *J. biol. Chem.* **138**, 535.
- Bloch-Frankenthal, L. & Weinhouse, S. (1957). *Cancer Res.* **17**, 1082.
- Braun, S., Erdelyi, M. & Udvardy, A. (1960). *Nature, Lond.*, **188**, 857.
- Chance, B. & Hess, B. (1959). *J. biol. Chem.* **234**, 2404.
- Crabtree, H. G. (1929). *Biochem. J.* **23**, 536.
- Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 387.
- Hanes, C. S. (1929). *Biochem. J.* **23**, 99.
- Ibsen, K. H., Coe, E. L. & McKee, R. W. (1958). *Biochim. biophys. Acta*, **30**, 384.
- Ibsen, K. H., Coe, E. L. & McKee, R. W. (1960). *Cancer Res.* **20**, 1399.
- Klein, G. (1951). *Exp. Cell Res.* **2**, 518.
- Kvamme, E. (1958). *Acta physiol. scand.* **42**, 204.
- Laws, J. O. & Stickland, L. H. (1958). *Arch. Biochem. Biophys.* **75**, 333.
- Laws, J. O. & Stickland, L. H. (1959). *Nature, Lond.*, **184**, 1246.
- Lillie, R. D. (1954). *Histopathological, Technical and Practical Histochemistry*, p. 451. New York: The Blakiston Company Inc.
- Robinson, H. W. & Hogden, C. G. (1940). *J. biol. Chem.* **135**, 727.
- Stickland, L. H. (1956a). *Biochem. J.* **64**, 515.
- Stickland, L. H. (1956b). *Biochem. J.* **64**, 498.
- Umbreit, W. W. (1957). In *Manometric Techniques*, 3rd ed., p. 23. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publishing Co.
- Wu, R. (1959). *Cancer Res.* **19**, 1217.
- Wu, R. & Racker, E. (1959). *J. biol. Chem.* **234**, 1036.

Biochem. J. (1962) **83**, 91

Treatment of Corticotrophin with Periodate and Borohydride

By H. B. F. DIXON

Department of Biochemistry, University of Cambridge

(Received 10 July 1961)

Periodate reacts with the terminal serine residue of corticotrophin (Geschwind & Li, 1954). Dixon (1956a) showed that it destroys the corticotropic action of the hormone and claimed that the melanocyte-stimulating activity was unchanged. In later unpublished assays he and A. B. Lerner and M. Ruth Wright found that the melanocyte-stimulating action of corticotrophin was increased by periodate treatment. Geschwind & Li (1958) demonstrated that treatment of corticotrophin with periodate destroyed not only its property of evoking depletion of adrenal ascorbic acid, which Dixon (1956a) had used in his study, but also its property of maintaining adrenal weight.

M. S. Raben in preliminary unpublished experiments (personal communication) found that periodate-treated samples of corticotrophin still retained some fat-mobilizing activity in the mouse. In view of this, when Engel & Engel (1958) reported that the ketogenic action of corticotrophin, which is mediated by an extra-adrenal mechanism, resembled its corticotropic action in that it could be reversibly abolished by the same oxidation-reduction procedure, it became interesting to prepare, characterize and test periodate-treated corticotrophin. The present paper concerns the preparation and characterization of such a material, and the accompanying paper (Boright, Engel, Lebovitz, Kostyo & White, 1962) describes some of its biological properties.

METHODS

Treatment of corticotrophin with periodate and borohydride. Pig corticotrophin A_1 (20.7 mg.), prepared by the method of Dixon & Stack-Dunne (1955) with the minor modifications cited by Waller & Dixon (1960), was dissolved in 8.6 ml. of water. Ammonium hydrogen carbonate (395 mg.) was added. Into this solution there was rapidly introduced 1 ml. of 25 mM- $NaIO_4$ (final concentration 2.5 mM), followed 30 sec. later by 54 mg. of KBH_4 , which was quickly stirred in. After 2 min. 2-mercaptoethanol (0.1 ml.) was added; the solution was acidified with acetic acid (3 ml.) and diluted to about 100 ml.

A reducing agent such as mercaptoethanol was added because, once the borohydride was destroyed by acidification, the iodide that had been produced was otherwise oxidized by air to iodine which might then have attacked the product. Mercaptoethanol is preferred to thioglycollic acid, which was used in much of the work, because it avoids the danger of acylation by thio esters which are generated during storage of thioglycollic acid (cf. White, 1960).

The product was desalted by adsorbing it on a carboxylic resin (Amberlite CG-50, type II), washing away the salts and un-ionized substances with dilute acetic acid and displacing the product with 50% acetic acid as described by Dixon (1959). The solution in acetic acid was dried while frozen and the product (14.9 mg.) submitted to chromatography.

Ion-exchange chromatography on Amberlite IRC-50 (Amberlite CG-50, 200-mesh). Chromatography was carried out according to the methods of Hirs, Moore & Stein (1953) and Dixon & Stack-Dunne (1955). The stock solution of