The Effect of Washing Procedures on the Phosphate Metabolism of Ehrlich Ascites-Tumour Cells

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Laws & Stickland (1962) showed that when washed Ehrlich ascites-tumour cells were kept in nitrogen, either at 37° or at 20°, their inorganic phosphate content rose in a matter of a few hours to a constant value of about 100 μ moles/g. dry wt., and that when glucose was subsequently added this concentration fell rapidly again. The rate of this fall, measured at 12°, in the presence of oxygen was equal to that in the absence of oxygen; in the absence of glucose but presence of oxygen there was an extremely slow fall in the concentration of inorganic phosphate.

In later experiments it was found, as expected, that the concentration of ATP was high in the freshly-prepared washed cells, and that it fell to a low value when the cells were incubated in nitrogen, the time-scale of this fall being similar to that of the increase in the concentration of inorganic phosphate. When such cells were further incubated with glucose, either with or without oxygen present, there was no (or only small and irregular) resynthesis of ATP. It seemed possible that this failure was to be attributed to the washing process, and in further experiments with unwashed cells the concentration of ATP returned almost to that found in the fresh cells. The present paper deals with some of the changes brought about in Ehrlich ascites-tumour cells by washing.

Washing of the cells involves (a) cooling to 0°, (b) the action of an abnormal medium (Krebs-Ringer solution in place of ascitic fluid) and (c) mechanical disturbance during resuspension. The effect of each of these factors was studied, by using as test reactions three aspects of the phosphate metabolism of the cells: (a) the rate of resynthesis of ATP, (b) the rate of disappearance of inorganic phosphate during endogenous respiration and (c) the rates of disappearance of inorganic phosphate in the presence of glucose both with and without oxygen.

EXPERIMENTAL

Abbreviations. P_i , Inorganic phosphate; q_{P_i} , rate of change of the concentration of P_i (μ moles/mg. dry wt./hr.); q_{-P} , rate of change of the concentration of the total 'energy-rich' phosphate, calculated from the changes in the

concentration of (ADP plus 2ATP) (μ moles/mg. dry wt./hr.). The use of this expression is desirable, rather than q_{ATP} , since in the early stages of ATP resynthesis there are also significant changes in the ADP concentration.

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 strain, and grown for use either in mice of this strain or in C3H mice. The ascitic fluid was removed from mice showing a sufficient accumulation, usually 7–10 days after inoculation, and any ascites showing more than a trace of blood was rejected. Cell counts on the suspensions, after filtration through glass wool, showed that less than 1% of the total cell volume consisted of erythrocytes. The standard washing procedure, departures from which are noted below in the appropriate places, was by centrifuging at 300g for 4 min. at 0°. The cells were resuspended in ice-cold Ca^{2+} ion-free Krebs-Ringer solution, and four washes were given. In the work by Laws & Stickland (1962) the cells were resuspended after each centrifuging by shaking with just sufficient violence to produce a uniform suspension; in the present study cells treated in this way were compared with others in which the gentlest possible stirring with a glass rod was substituted for the shaking, and with unwashed cells. For experiments with unwashed cells the filtered ascites was used, diluted appropriately with Krebs-Ringer solution, or with cell-free ascitic fluid obtained by centrifuging another portion of ascites at 1000g for 10 min.

Buffers. For experiments on the rate of change of the concentration of P_i or of ATP, at 12°, tris buffer (0.01 M-tris-HCl, pH 7.5) was used. For measurements of glucose utilization at 37° phosphate buffer (0.02 M- NaH_2PO_4 -NaOH, pH 7.5) was used.

Determinations. Respiration was measured in air by Warburg manometry, glucose by the method of Hanes (1929), and P_i by the method of Fiske & Subbarow (1925).

Determination of ADP and ATP was essentially by the method of Cohn & Carter (1951). For each determination a sample of ascites cells containing about 300 mg. dry wt. of cells in 10 ml. was added to 1 ml. of 50% (w/v) trichloroacetic acid, and after 1 hr. at room temperature was centrifuged. The cell residue was washed twice with 5% (w/v) trichloroacetic acid, and the extract and washings, after neutralization with ammonia to pH 8.2, were diluted to 25 ml. A 1 ml. sample was removed for the determination of P_i . To the remainder 2 ml. of 50% (w/v) barium acetate solution was added. After this solution had stood at 0° for 2 hr., the precipitated barium salts were collected by centrifuging. Barium was removed by the addition of Amberlite resin CG-120 (H^+ form), and the resin was filtered off and thoroughly washed with water. The combined filtrate and washings were neutralized with am-

monia to pH 8.2 and added to a column of Amberlite resin CG-400 (Cl⁻ form), about 0.2 cm.² in cross-section and about 3 cm. long. This quantity of resin was adequate for the amounts of nucleotide and P_i added, and gave better separation than the larger columns used by Laws & Stickland (1958). The rate of flow, with a head of pressure of about 40 cm. water, was between 100 and 150 ml./hr. The samples were washed on the column with 50 ml. of 3 mM-HCl; ADP was eluted with 200 ml. of Cohn & Carter's (1951) 'solution C' (0.02M-NaCl plus 0.01N-HCl), and ATP with 200 ml. of 'solution D' (0.2M-NaCl plus 0.01N-HCl), the eluates being collected in 50 ml. fractions. The extinction coefficient at 260 mμ at pH 2 was measured on each fraction, and the concentrations of ADP and ATP were calculated as by Laws & Stickland (1958). Determinations of dry weight were made on all cell suspensions, the suspensions in ascitic fluid being washed with Krebs-Ringer solution before being dried. Drying was carried out overnight in an oven with circulating air at 60°.

Experimental procedure. Unless otherwise stated, the cells were cooled to 0° and kept at 0° until the experiment was started. Most experiments involved keeping the cells in nitrogen at 20° for various periods to allow the P_i content to rise and the ATP content to fall. With unwashed cells the procedure was to flush the vessel with oxygen-free nitrogen, cork it, and immerse it in a water bath at 20°. For the washed cells, the centrifuge tubes containing the tightly packed cells after the last wash were similarly filled with nitrogen and incubated at 20°, and the cells were resuspended evenly to their original volume with the Krebs-Ringer solution, with the minimum of mechanical disturbance, immediately before use.

For measurements of changes in the concentrations of ADP and ATP, the undiluted suspensions (i.e. at the same concentration as was present in the original ascites, about 30 mg. dry wt./ml.) were transferred to 250 ml. Erlenmeyer flasks that were already in the bath at 12° and contained sufficient tris buffer, pH 7.5, to give a final concentration of 0.01M. The zero-time sample of 10 ml. was removed immediately, shaking in air was begun and further 10 ml. samples were taken as required.

For measurements of changes in the concentration of P_i in the presence of glucose, aerobically and anaerobically, there were prepared in the bath at 12° two Erlenmeyer flasks, each containing measured volumes of 0.1M-tris buffer, pH 7.5, and Krebs-Ringer solution such that when 3-4 ml. of cell suspension was added the cell concentration was about 7-10 mg. dry wt./ml. and the tris concentration 0.01M. The solutions in the flask designed for the anaerobic experiment had been boiled and cooled in a stream of nitrogen, and a rapid stream of nitrogen was passing through this flask before the addition of the cells. After about 1 min. of shaking to obtain complete mixing, duplicate 2 ml. samples were transferred into 2 ml. of 10% (w/v) trichloroacetic acid, then immediately at zero-time glucose was added (a 4% solution, free from oxygen, in quantity to give a final concentration of 0.2%), and further 2 ml. samples were withdrawn as required. All the samples were left at room temperature for about 1 hr. before being centrifuged, and P_i was determined in 2 ml. portions of the supernatants.

In experiments in which glucose disappearance was followed, 1 min. of shaking was allowed after the addition of the glucose solution, to obtain thorough mixing, before the

initial sample (2 ml. containing about 20 mg. dry wt. of cells) was taken. For respiration measurements about 50 mg. dry wt. of cells was present in each manometer vessel, in a total volume of 4 ml.

RESULTS

Changes in the concentrations of inorganic phosphate, adenosine diphosphate and adenosine triphosphate during incubation of cells in nitrogen at 20°

The rapid increase in the concentration of P_i from a value of 30-50 μmoles/g. dry wt. to about 100 μmoles/g. dry wt. was described by Laws & Stickland (1962). Fig. 1 illustrates the parallel decrease in the concentration of ATP in washed cells from about 10-15 μmoles/g. dry wt. to about 2 μmoles/g. dry wt. under the same circumstances. The fall was almost complete within 1 hr. Associated with the rapid fall in the concentration of ATP there was always observed a temporary rise in that of ADP, which, when the concentration of ATP had reached its minimum, returned to about its original value. Fig. 2 shows that a similar course was followed in unwashed ascites cells.

This treatment in nitrogen at 20° was used in our experiments to bring the concentration of ATP to a low level from which subsequent resynthesis could be observed. It was important to know what was the optimum period of incubation in nitrogen, and Fig. 2 shows the result of returning to air samples of unwashed cells that had been incubated in nitrogen at 20° for various periods. The initial sample maintained its concentration of ATP almost unchanged in air at 12°; after 60 min. in nitrogen at 20° there was a rapid resynthesis of

Fig. 1. Changes in the concentrations of P_i (x), ADP (Δ) and ATP (□) in washed Ehrlich ascites-tumour cells on incubation in nitrogen at 20°.

ATP in air at 12° so that its concentration rose to about 85% of the original level; after 90 min. the resynthesis was slower and less complete; and after 120 min. there was a lag in ATP synthesis which made the evaluation of the rate difficult. In later experiments a 60 min. period of preincubation in nitrogen at 20° was used as a standard procedure, even though in some samples of cells the concentration of ATP had not quite reached its minimum value, and that of P_i had definitely not reached its maximum.

Table 1 gives the average concentrations of P_i , ADP and ATP in Ehrlich ascites-tumour cells before and after incubation in nitrogen at 20°. Washing caused a small decrease in the concentration of ATP; this was not apparently due to loss by

leakage into the medium, as neither the ascitic fluid nor the supernatant from a washed suspension contained appreciable amounts of ATP. Incubation in nitrogen at 20° for 1 hr. brought the concentration of ATP down to the same low level in both unwashed and washed cells.

Factors involved in the washing procedure

Temperature change. Samples of cells cooled at once in ice and kept at 0° were compared with other samples kept at 20° and at 37° for the same period (30–45 min.). In all samples ATP resynthesis was normal, and their other properties were those of normal cells (see below).

Change of medium. Samples of cells were put through a 'mock' washing procedure, in which they were centrifuged and resuspended by shaking in the same ascitic fluid, and were compared with samples to which exactly the same treatment was given, except that the ascitic fluid was replaced by Krebs-Ringer solution. Both samples showed the behaviour characteristic of washed cells (see below). Further, when a suspension of unwashed cells was diluted up to fivefold, it made no difference whether the diluent was ascitic fluid or Krebs-Ringer solution.

Effects of shaking during resuspension. Several experiments were carried out to compare the rate of resynthesis of cells unwashed, washed with the gentlest possible stirring, and washed with shaking (e.g. Fig. 3). With unwashed cells, ATP synthesis was rapid and linear, and the concentration of ATP reached about 87% of the original value. The

Fig. 2. Changes in the concentration of ATP in unwashed Ehrlich ascites-tumour cells. Curve A shows the fall in the concentration of ATP during incubation in nitrogen at 20°; curves B, C, D and E show the changes in the concentration of ATP in air at 12°, after incubation in nitrogen at 20° for 0, 60, 90 and 120 min. respectively.

Table 1. Concentrations of inorganic phosphate, adenosine diphosphate and adenosine triphosphate in Ehrlich ascites-tumour cells before and after washing, and before and after incubation in nitrogen at 20° for 60–90 min.

Experimental details are given in the text. 'Fresh' indicates immediately after the filtration of the cell suspension for unwashed cells, and immediately after the final wash for washed cells. The numbers of observations are given in parentheses.

Condition of cells	Concn. (μmoles/g. dry wt.)		
	P_i	ADP	ATP
Unwashed			
Fresh	35 (6)	6.8 (6)	15.3 (6)
After N_2 at 20°	79 (14)	4.3 (14)	1.9 (14)
Washed			
Fresh	34 (10)	4.6 (12)	12.0 (12)
After N_2 at 20°	77 (17)	3.8 (22)	2.0 (22)

Fig. 3. Effect of washing on the resynthesis of ATP by Ehrlich ascites-tumour cells: unwashed ($\square$), washed gently ($\triangle$), or washed with shaking ($\times$). Each suspension had been incubated in nitrogen at 20° for 1 hr.; the concentrations of ATP before this treatment are indicated by the lines in the top left corner with the appropriate marks. The ATP resynthesis was in air at 12°.

Fig. 4. Changes in the concentration of ADP during re-synthesis of ATP (in air at 12°) by unwashed and washed Ehrlich ascites-tumour cells (the same experiment as in Fig. 3): unwashed (□), washed gently (Δ), or washed with shaking (×).

Table 2. Effect of washing and shaking on the rate of synthesis of 'energy-rich' phosphate in Ehrlich ascites-tumour cells

Experimental details are given in the text. q_{P} is the rate of change, at 12°, of the concentration of (ADP + 2ATP). The numbers of observations are given in parentheses.

Condition of cells	$10^2 q_{\text{P}}$ (μmoles/mg. dry wt./hr.)	
	Average	Range
Unwashed	10.1 (5)	6.3–16.5
Washed gently	4.1 (5)	2.5–4.5
Washed with shaking	2.0 (5)	1.5–2.9

Table 3. Effect of washing on the endogenous rate of disappearance of inorganic phosphate, in air, in Ehrlich ascites-tumour cells

Experimental details are given in the text. q_{P_i} is the rate of change, at 12°, of the concentration of P_i . The numbers of observations are given in parentheses.

Condition of cells	$-10^2 q_{\text{P}_i}$ (μmoles/mg. dry wt./hr.)	
	Average	Range
Unwashed	7.8 (10)	4–13
Washed gently	3.0 (5)	0–7
Washed with shaking	0.9 (7)	0–2

Table 4. Effect of washing on the rate of disappearance of inorganic phosphate, in air or in nitrogen, in Ehrlich ascites-tumour cells in the presence of glucose

Experimental details are given in the text. $q_{\text{P}_i}^{\text{Glc}}$ is the rate of change, at 12°, of the concentration of P_i in the presence of glucose. The numbers of observations are given in parentheses.

Condition of cells	$-10^2 q_{\text{P}_1}^{\text{Glc}}$ ($\mu\text{moles/mg. dry wt./hr.}$)				$\frac{q_{\text{P}_1}^{\text{Glc}} \text{ in air}}{q_{\text{P}_1}^{\text{Glc}} \text{ in N}_2}$
	In air		In N ₂		
	Average	Range	Average	Range	
Unwashed	49 (10)	32-61	23 (10)	17-35	2.17
Washed gently	36 (8)	26-42	26 (8)	19-33	1.40
Washed with shaking	28 (10)	23-39	27 (10)	20-38	1.03

gently washed cells showed a slower, though still initially linear, resynthesis, and the concentration of ATP reached a final value of 74 % of the original. The shaken cells showed a considerable lag period, and a slower resynthesis rate, and the concentration of ATP reached only 55 % of the original value. The concentration of ADP in each case showed (Fig. 4) a temporary increase, reaching a maximum during the phase of rapid ATP synthesis and falling again to a steady level.

Table 2 gives the results of five such experiments, showing the values of q_{P} derived from the rates of change of the concentration of (ADP plus 2ATP). Even the most careful washing procedure decreases q_{P} to less than one-half of its value in the unwashed cells, and shaking of the cells decreased it to less than one-fifth.

Laws & Stickland (1962) showed that the rate of disappearance of P_i in washed cells respiring without added substrate was unexpectedly small (the average q_{P_i} was 0.02). Unwashed cells (Table 3) gave a much higher value (0.08), which was decreased to 0.03 by the gentlest possible washing, and in the present series of experiments to 0.01 by washing accompanied by shaking. As Table 3 shows, this rate seems to be more variable than that of most metabolic processes in Ehrlich ascites-tumour cells.

Laws & Stickland (1962) found a rapid decrease in the concentration of P_i in Ehrlich ascites-tumour cells at 12° in the presence of glucose; surprisingly, the same rate was obtained in air and in nitrogen. This equality of rate has now been shown to be a result of the washing procedure, and chiefly of the shaking employed in the earlier experiments. Table 4 shows that in unwashed cells the $q_{\text{P}_i}^{\text{Glc}}$ was on average rather more than twice as great in air as in nitrogen; the ratio never exceeded 3 nor fell below 1.5. Washing of the cells left the anaerobic rate almost unaltered, but decreased the aerobic rate. Even the mildest washing brought the average ratio down to 1.4, and if the cells were shaken the ratio, as in our earlier experiments, approached unity. Many experiments showed an immediate fall in the concentration of P_i at a con-

stant rate (Fig. 5); in others there was a lag of 2–4 min. before the linear part of the curve began. In unwashed cells (Fig. 5A) there was always a great divergence in rate between the aerobic and anaerobic experiments; in shaken cells (Fig. 5D) the rates were always almost identical. The behaviour of the carefully washed cells was variable (Figs. 5B and 5C), showing sometimes a fairly wide divergence in rates, sometimes very little.

Fig. 5. Effect of the washing of Ehrlich ascites-tumour cells on the rate of disappearance of P_i in the presence of glucose: in air ($\square$) and in nitrogen ($\times$). Curves A, unwashed cells; curves B and C, cells washed gently; curves D, cells washed with shaking. Curves A, B and D were obtained with the same batch of cells, curves C with another batch.

Table 5. Change in cell volume associated with the washing and shaking of Ehrlich ascites-tumour cells

Experimental details are given in the text. The cell volumes are expressed relative to that of unwashed cells, which is taken to be 100. The numbers of observations are given in parentheses.

Condition of cells	Relative cell volume	
	Average	Range
Washed gently	103 (9)	91–111
Washed with shaking	128 (22)	114–139

Table 6. Rate of respiration of Ehrlich ascites-tumour cells, with and without washing

Experimental details are given in the text. The rate of respiration was measured at 12°. The numbers of observations are given in parentheses.

Condition of cells	$-10^3 q_{O_2}$ ($\mu\text{moles/mg. dry wt./hr.}$)	
	Average	Range
Unwashed	6.1 (6)	5.3–6.8
Washed twice gently	5.1 (4)	4.2–5.9
Washed four times gently	4.8 (9)	3.8–5.5
Washed four times with shaking	4.1 (9)	3.1–5.0

During washing the cells occupied a progressively greater volume in the centrifuge tube. This effect was not solely due to the change of medium from the protein-rich ascitic fluid to a simple salt solution, since the swelling was specifically associated with a shaking of the suspension (Table 5).

Microscopical examination of these cells in the fresh state showed that the majority of the washed cells were in fact swollen and more translucent than normal cells. The swelling appeared to affect the surface region of the cells, starting on one side as a translucent bleb which spread by extension, or coalesced with other blebs, until the whole cell surface was affected, the cytoplasm around the nucleus remaining granular.

Respiration and glucose utilization of unwashed and washed cells

The gross catabolic processes of washed Ehrlich ascites-tumour cells had in the experiments of Laws & Stickland (1962) proceeded at rates similar to those widely reported for such cells. The disturbances in various aspects of phosphate and ATP metabolism caused by washing, etc., appeared not to have affected the rates of the catabolic processes. It was confirmed that unwashed cells differed little in their rates of respiration and glucose catabolism from washed cells.

Respiration. Table 6 shows the q_{O_2} values of cells washed and shaken compared with that of unwashed cells. The respiration of the unwashed cells was probably augmented by the presence of substrates in the ascitic fluid. The removal of ascitic fluid by two gentle washes decreased the q_{O_2} from 0.06 to 0.05; further gentle washing had little more effect, and even the shaking procedure decreased it only to 0.04.

Glucose utilization. As explained by Laws & Stickland (1962), measurement of glucose disappearance at 12° is inaccurate, so these comparisons were made at 37°. Table 7 shows that washing decreased the rate of glucose utilization only by some 25%, and that there was little difference between cells that had been shaken and those that had not.

Pasteur effect. The last column of Table 7 (the Pasteur quotient, i.e. $q_{Glc}^{Air}/q_{Glc}^{N_2}$) shows that washing did not appreciably affect the efficiency of the Pasteur effect.

DISCUSSION

There are as many different ways of preparing ascites-tumour cells for biochemical experiments as there are research workers. Very few (e.g. Dewey & Green, 1959; Harbers & Heidelberger, 1959) have used unwashed cells. Most workers cool the cells in ice, a procedure that we find harmless

Table 7. *Effect of washing on the rate of utilization of glucose by Ehrlich ascites-tumour cells at 37° in nitrogen and in air*

Experimental details are given in the text. The Pasteur quotient is $q_{\text{Glc}}^{\text{Air}}/q_{\text{Glc}}^{\text{N}_2}$. The numbers of observations are given in parentheses.

Condition of cells	$-q_{\text{Glc}}^{\text{N}_2}$ ($\mu\text{moles/mg. dry wt./hr.}$)		$-q_{\text{Glc}}^{\text{Air}}$ ($\mu\text{moles/mg. dry wt./hr.}$)		Pasteur quotient
	Average	Range	Average	Range	
Unwashed	1.27 (3)	0.99–1.45	0.71 (3)	0.55–0.82	0.56
Washed gently	1.03 (3)	0.92–1.12	0.53 (3)	0.49–0.58	0.52
Washed with shaking	0.93 (3)	0.84–1.01	0.53 (3)	0.49–0.57	0.57

with respect to the reactions that we have measured, though Chance & Hess (1959) in some experiments avoided this because of disturbances in electrolyte metabolism described by Hempling (1958). The washing procedures used vary from a single wash (e.g. Hess & Chance, 1961) to washing repeatedly an unspecified number of times to remove erythrocytes, usually at 0° with Krebs–Ringer or Krebs–Ringer phosphate solution. In no case is any indication given of the method used to resuspend the cells after centrifuging; the degree of disturbance required would vary with the centrifugal force used to deposit the cells, which varies from 100*g* for 3 min. to 1500*g* for 10 min. Some workers (e.g. Papaconstantinou & Colowick, 1961) have used a loosely fitting TenBroeck homogenizer to ensure an even suspension of cells after the washing was completed.

Our results show that, although washed cells (even those subjected four times to a minimal amount of shaking during their preparation) still respire and utilize glucose at an almost unimpaired rate, and show a normal Pasteur effect, they have suffered severely in several aspects of their phosphate metabolism. In particular, in unwashed cells the rate of disappearance of P_i on the addition of glucose is up to three times as great in air as in nitrogen, though in the shaken cells the rates are equal. This suggests some degree of ‘uncoupling’ of the aerobic phosphorylation, which is borne out by the greatly decreased rate of ATP synthesis in air in the absence of glucose. An alternative explanation of the reduced yield of ATP, though not of the more greatly decreased rate of its appearance, in the shaken cells might be found in a loss of AMP from such cells during the period of incubation in nitrogen at 20°. There is no indication of a loss of nucleotides by diffusion out of the cells, but the possibility of deamination of AMP to IMP remains.

These changes are also present to a large degree in cells that have been washed with the greatest possible precautions to avoid mechanical disturbance. It can only be concluded that washed cells

are abnormal and should be used only when circumstances render it necessary, and with these considerations in mind.

SUMMARY

1. Washed Ehrlich ascites-tumour cells, depleted of ATP by incubation in nitrogen, show a small and erratic resynthesis of ATP when incubated in air.

2. Unwashed cells show a rapid resynthesis of ATP in air, raising the concentration of ATP to about 90 % of the original value.

3. The damage is caused by the mechanical disturbance involved in the washing process, and is exacerbated by shaking.

4. The rate of decrease in the concentration of inorganic phosphate in washed cells on the addition of glucose in the presence of air is the same as that in the absence of air. In unwashed cells the rate is more than twice as great in air as in nitrogen.

5. These changes in the behaviour of the cells on washing are present to a large extent even when the gentlest possible treatment of the cells is used to resuspend them after centrifuging.

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