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THE MECHANISM OF INSULIN ACTION: THE EFFECT OF INSULIN ON PHOSPHATE TURNOVER IN RED-CELL SYSTEMS

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The findings reported in the preceding paper (Dormandy & Zárday, 1965) suggested that insulin has an immediate electrochemical effect on unwashed red cells suspended in a variety of simple inorganic saline solutions. The term 'electrochemical' was used to emphasize that experimental conditions and procedures were designed to show up immediate physical rather than sustained metabolic changes. The insulin effects on ionic partitions, E_h and pH, were consistent with the hypothesis that the hormone induces a shift in the trans-membrane redox-potential gradient; and this raised the question whether, under more physiological conditions, such a primary action might alter or reset the pattern of cell metabolism. The consensus of opinion among workers in the insulin field seems to be that red-cell metabolism is not sensitive to insulin; but it was noted that, while the immediate anion shifts and pH changes could no longer be observed in cell suspensions incubated at 37° in glucose-rich media, the addition of insulin to such preparations caused an immediate though transient rise in the extracellular inorganic phosphate concentration. It was therefore decided to reinvestigate the effects of the hormone on red-cell metabolism centred on phosphorus (P) turnover rather than on such 'classical' insulin parameters as glucose uptake or CO_2 evolution. The findings are reported in the present paper.

METHODS

An account of the preparations of unwashed red-cell suspensions, of two ways of bicarbonate-CO_2 buffering, and of a number of techniques of estimation have been given in the previous paper. Only additional material and procedures are here described.

Material

Glucose–phosphate–bicarbonate buffer (*GPB*). The composition in m-mole/l. was: NaCl, 140; KCl, 3; $CaCl_2$, 0·2; $MgCl_2$, 0·1; Na_2HPO_4, 1·1; KH_2PO_4, 0·4; $NaHCO_3$, 26 and glucose to 300 mg/100 ml.; equilibrated with nitrogen 95 %–CO_2 5 %.

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Radioactive phosphorus. ^{32}P was added as $Na_2H^{32}PO_4$ to give final concentrations of the order of 0·001 m-mole/l., depending on the activity of the material.

Experimental methods

Radioactive counting. ^{32}P activity in the extracellular fluid was counted on dried 0·02 ml. samples in a thin-window Geiger–Müller counter. Radioactivity in cut-out chromatographic spots was counted directly, using unstained paper of the same area as background. Counting rates were 200–300 counts/min over a sufficient length of time to give a statistical error of less than 2·5 % (in most cases less than 2 %).

Chromatography. The technique of separation of cellular phosphorus (P) fractions was based, with minor modifications, on the two-way chromatographic method described by Hanes & Isherwood (1949) and Caldwell (1953). 10 % trichloroacetic-acid (TCA) extracts were prepared from the spun cell deposits (Prankerd & Altman, 1955): a haemoglobin-free stromal fraction was not analysed separately. The total activity in the TCA-insoluble precipitate was counted in all cases. The chromatograms were run on Whatman No. 4 filter paper, pretreated with 1 % oxalic acid (Moses & Edwards, 1960). Samples (0·2 ml., representing approximately 20 μl. packed-cell volume), were applied over areas of less than 1 cm in diameter and dried in a cold-air current. The pad technique was used with over-running. Markers for the various P fractions were obtained commercially (Biochemica Boehringer, Mannheim, Germany). While hexose diphosphate esters as a group readily separate as two fairly well-defined spots, there appears to be a variable admixture of glucose to fructose diphosphate. Mainly for this reason the two esters (or groups of esters) were treated as one and are tabulated as hexose-diphosphate (HEX-DP). The other TCA-soluble cellular P fractions separated were 2,3-di-phosphoglycerate (2,3-DPG), adenosine triphosphate (ATP), adenosine diphosphate (ADP), and inorganic phosphate (IP). Of the total radioactivity applied to the paper 83–96 % was recoverable from these five spots. When expressed as percentage fractional activities of the TCA extract the activity in these five fractions was taken as 100 %: i.e. the lost residue was divided proportionately between them. All chromatograms were run in duplicate or triplicate and the tabulated results are means.

Chemical P estimation. The paper spots containing P fractions were ashed and oxidized and the orthophosphate was estimated photocolorimetrically (Berenblum & Chain, 1938; Caldwell, 1953; Prankerd & Altman, 1955).

Glucose. 'True glucose' was estimated by a glucose-oxidase method based on that of Hugget & Nixon (1956). 'True sugar' was estimated by the copper-reducing method of Haslewood & Strookman (1939) as modified by Nelson (1944). The limitations of both methods are discussed below.

Procedures

Basic design. This was similar in all experiments. Unwashed red cells were resuspended in glucose–phosphate–bicarbonate buffer, deoxygenated and divided into prospective insulin and control suspensions. Samples of these were treated in parallel throughout, including centrifugation and chromatography. Both insulin and control series were incubated at 37° C in a water-bath equipped with a slow rocking device. Open buffering was used in Expts. *A–C*, *E* and *H*: i.e. the cell suspensions were at equilibrium with a virtually infinite gas space of nitrogen 95 %–CO_2 5 %. Closed buffering (as defined by Dormandy & Zárday, 1965) was used in Expts. *D*, *F* and *G*. In all experiments a 20–30 min period was allowed for pre-equilibration at 37° C. Micro-samples were withdrawn for haematocrit determinations. ^{32}P was added at zero time. Insulin and control solutions were added at the times indicated. These solutions were identical in volume and HCl content: their total volume never exceeded 5 % (v/v) of the whole suspension.

The incubation flasks had side arms with rubber caps. Samples for analysis were withdrawn by an air-tight technique into closed syringes. The syringes were spun. For investigations relating to the extracellular fluid only centrifugation was for 3–4 min at room tem-

perature to obtain cell-free supernatants. For analysis of cellular material the closed syringes were spun at 30–37° C for 15 min at 2000–2600 *g*. The supernatants were then aspirated into another set of syringes. They were replaced in the first set by ice-cold mM acetate buffer (pH 4·2) to which a trace of saponin had been added. The haemolysates were evacuated and immediately frozen. Small samples were used for haemoglobin estimation. For acid extraction and chromatography TCA of appropriate strength and volume was added to the thawed haemolysates to give a 10 % (weight/original red-cell volume) solution.

For experiments requiring incubation for more than 3 hr all glassware was autoclaved. As in previous experiments, all glassware was thoroughly siliconed.

Insulin concentrations. A number of experiments were carried out to establish both the minimal insulin concentrations which had a discernible effect and the minimal insulin concentrations which gave the maximal insulin effect obtainable. Under most experimental conditions this 'effective range' was of the order of 1–1000 times the minimal effective concentration. One series of such experiments is described below (Expt. *C*). In all other experiments the insulin concentrations were about 5–10 times higher than the upper limit of the effective range: i.e. in none was the insulin concentration limiting. As indicated in the tables and figures, these excess insulin concentrations were of the order of 800–1000 μ-units/ml. extracellular fluid, i.e. about 10 times higher than the physiological plasma-insulin range as estimated by immunoassay (Yallow & Berson, 1960).

Expt. A. The effect of insulin on cell/extracellular ^{32}P exchange (*Fig.* 1). This was studied in parallel with changes in extracellular Cl^-, IP, and glucose concentrations. The insulin and control systems were divided into three suspensions each. These were treated as parallel triplicates. Insulin and control solutions and ^{32}P were added at zero time. To counteract a possible chemical or enzymic break-down of insulin, repeat control and insulin solutions were added to the control and insulin suspensions respectively at 4 and 8 hr. Samples were withdrawn every hour. The supernatants were analysed chemically and counted for radioactivity.

Expt. B. The influence of the cell/extracellular-volume ratio (*Table* 1). In three essentially similar experiments three blood-cell suspensions of different cell/extracellular-volume ratios were investigated. Extracellular-fluid samples were collected at 3, 6 and 10 hr only.

Expt. C. The influence of insulin concentrations (*Fig.* 2). Duplicate series of cell suspensions were prepared as before, each series consisting of four suspensions. ^{32}P was added to all suspensions at zero time. Between 0 and 5 min one of three insulin solutions was added to one and a control solution was added to the fourth suspension in each series. Samples were withdrawn immediately before and after the addition of the insulin or control solutions, every 15 min for the first hour, and at 1½ and 2 hr.

Expt. D. The influence of pH (*Table* 2). In a similar series of experiments the pH of the suspending fluids was varied by altering the concentration of $NaHCO_3$. Incubation was in closed syringes.

Expt. E. The effect of insulin after pre-incubation with ^{32}P (*Fig.* 3). Three blood-cell suspensions were incubated in parallel. *a*. To the insulin suspensions insulin solution and ^{32}P were added at zero time. *b*. To the control suspensions control solution and ^{32}P were added at zero time. *c*. To the delayed insulin suspensions control solution and ^{32}P were added at zero time and insulin solution was added at the times indicated in Fig. 3. At the same times another volume of control solution was added to *a* and *b*.

Expt. F. The effect of insulin of ^{32}P fractional activities (*Tables* 3 *and* 4). An insulin and a control series, consisting of 6 cell suspensions each, were incubated in closed syringes. At the times indicated in Tables 3 and 4 one suspension from each series was spun. TCA extracts were prepared from the cells and the extracted P fractions were separated by chromatography. ^{32}P activity in the extracellular fluid, in the TCA-insoluble precipitate and in the chromatographic spots (fractional activities) were expressed as percentages of the total count in the suspension.

Expt. G. The insulin on ^{32}P relative specific activities (*Table* 5). Cell suspensions were incubated in closed syringes and treated as in Expt. *F*. Both radioactivity and the P content of the separated chromatographic spots were determined. Relative specific activities were calculated and expressed as counts/min/μg P.

Expt. H. The effect of insulin on extracellular glucose E_h and pH (*Fig.* 4). Glucose uptake was estimated both by a glucose-oxidase and by a copper reducing method and extracellular E_h and pH changes were monitored as described by Dormandy & Zárday (1965). Insulin was added to the insulin systems at 0 and at 8 hr.

RESULTS

The insulin effects on P turnover are shown in Tables 1–5 and Figs. 1–3.

TABLE 1. The effect of insulin on extracellular ^{32}P activity in three blood-cell suspensions (Expt. *B*). The different cell/extracellular-fluid-volume ratios are indicated (Hcr). Insulin concentrations 2 μg/100 ml. *Ins* = insulin systems. *Co* = control systems

	Time (hr)	Expt. 1 Hcr 42 % *Ins*	Expt. 1 Hcr 42 % *Co*	Expt. 2 Hcr 60 % *Ins*	Expt. 2 Hcr 60 % *Co*	Expt. 3 Hcr 72 % *Ins*	Expt. 3 Hcr 72 % *Co*
Extracellular ^{32}P (%)	3	76	82	60	68	44	56
	6	32	26	24	16	18	9
	10	30	28	22	20	16	18

TABLE 2. The effect of insulin on extracellular ^{32}P activity and on extracellular inorganic phosphate and glucose concentrations at different starting pH's (Expt. *D*). A single result is shown where the findings in parallel insulin and control systems did not differ significantly. The significance of the differences between glucose concentrations is doubtful (see text). There were no significant differences in extracellular Cl^-, Na^+ and K^+ concentrations between any of two corresponding systems. Haematocrits 60 %. Insulin concentrations, 3 μg/100 ml.

	Time (min)	Expt. 1 *Ins*	Expt. 1 *Co*	Expt. 2 *Ins*	Expt. 2 *Co*	Expt. 3 *Ins*	Expt. 3 *Co*
pH*	0	7·32		7·51		7·60	
	15	7·28		7·41		7·45	
Extracellular ^{32}P (%)	15	88	92	70	81	73	81
	60	70	84	50	61	45	54
IP (μg/ml.)	0	62		61		61	
	15	63		66	63	68	62
	60	63	62	63	62	62	61
Glucose (g/l.)	60	1·85	1·91	1·74	1·70	1·52	1·60

* Measured on whole cell suspensions.

Immediate P release

When insulin was added to unwashed red-cell systems incubated at 37° C there was an immediate release of cellular P (Figs. 1 and 2). The rise in extracellular phosphate was transient; and because of the inevitable delay of centrifugation the peaks were probably never recorded. The highest values (when the cell suspensions were spun within a minute of the addition of insulin) represented approximately 6–8 % of the estimated

TABLE 3. The effect of insulin on ^{32}P partition (Expt. *F*). All fractional activities are expressed as percentages of the total ^{32}P activity in the whole cell suspension (100 %) at a cell/extracellular-fluid-volume ratio of 1 (Hcr 50 %). Extracellular IP = percentage activity in the supernatant. Residual P = percentage activity in the TCA-insoluble fractions (precipitate). Insulin concentrations 3 μg/100 ml.

Hours...	0·75		2		3		6		8	
System...	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*
Extracellular P	69	84	42	52	24·5	28	21·5	15	20	22·5
Intracellular										
ATP	4·5	2	11	12·5	12	15·5	12	14	12	7·5
2,3-DPG	8·5	8	22	24	30	27	33	52	33	34
ADP	0·5	0	1	1	2	1·5	2·5	2	2·5	1·5
HEX-DP	1	0	3	1·5	3·5	1·5	4	2	3	1
IP	10	6	9	6·5	14	12·5	13	12	14	30
Total TCA-soluble P	24·5	16	46	45·5	61·5	68	64·5	82	64·5	74
Residual P	6·5	0	12	2·5	14	4	14	3	15·5	3·5

TABLE 4. Summary of two further experiments as described in Table 3 (Expt. *F*). Insulin concentrations 3 and 4 μg/100 ml.

	Hours...	0·5–0·75		2–2·5		3–4		5–6		7·5–8·5	
	System...	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*
Expt. 2	Extracellular P	64·5	71·5	36·5	48	26	32·5	30	22·5	28	25·5
	Intracellular TCA-soluble P	32	28·5	54	50	60	66·5	61	74	60	71
	Residual P	3·5	0	9·5	2	14	1	9	3·5	12	3·5
Expt. 3	Extracellular P	70·5	79	46	52	45	44·5	41·5	29·5	36	37
	Intracellular TCA-soluble P	25·5	20	48	46·5	46	54	50	68·5	54	60
	Residual P	4	1	6	1·5	9	1·5	8·5	2	10	3

TABLE 5. The effect of insulin on the relative specific activities of ^{32}P in intracellular P fractions (Expt. *G*). The results indicate counts/min/μg P. Haematocrits 45–55 %. Insulin concentrations 4–5 μg/100 ml.

	Hours...	0·5–1		2–3		5–6		8–9	
	System...	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*	*Ins*	*Co*
Expt. 1	2,3-DPG	136	114	271	242	464	742	456	511
	ATP	148	73	233	268	361	522	368	476
	ADP	60	22	93	144	202	200	204	192
	HEX-DP	58	14	236	165	364	252	360	208
	IP	312	208	325	247	338	351	342	556
Expt. 2	2,3-DPG	—	—	358	350	582	789	842	610
	ATP	—	—	364	380	698	739	710	623
	ADP	—	—	129	240	509	538	520	403
	HEX-DP	—	—	320	152	679	411	690	312
	IP	—	—	342	198	461	483	497	603
Expt. 3	2-3-DPG	212	164	546	448	840	1280	920	840
	ATP	240	110	432	583	751	1121	782	710
	ADP	99	37	165	323	411	509	406	490
	HEX-DP	89	20	710	356	731	481	750	505
	IP	342	310	454	312	585	467	561	980

total P content of the cells. Under otherwise comparable conditions the rise in extracellular P concentration was greater at an alkaline than at an acid pH (Table 2) and at a higher cell/extracellular volume ratio. When insulin was added to cell systems after pre-incubation with ^{32}P and fractional activities were measured in the extracellular fluid and in the various intracellular P pools an immediate discharge of unlabelled cell P was demonstrable; but there was no rise in extracellular ^{32}P activity and no fall in ^{32}P activity in any of the cellular fractions separated (Fig. 3).

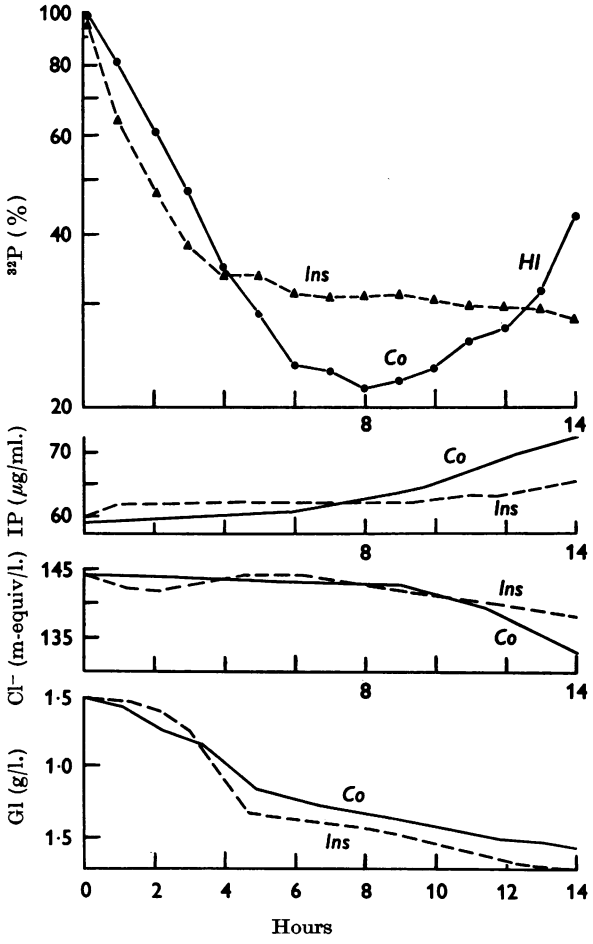

Fig. 1. The parallel effects of insulin on extracellular ^{32}P activity and extracellular inorganic phosphate (IP), Cl^-, and glucose (Gl) concentrations (Expt. *A*). The insulin concentrations were 2, 4 and 6 μg/100 ml. during the first, second, and third 4 hr periods respectively. Interrupted lines, insulin systems; full lines, control systems. Haematocrits 45%. *Hl* = haemolysis in two out of three triplicate samples.

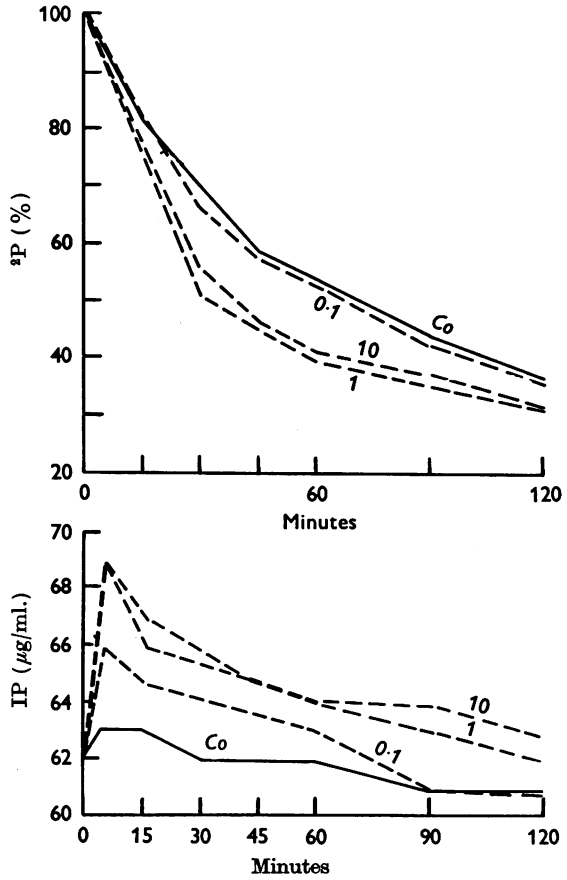

Fig. 2. The early effect of insulin on extracellular ^{32}P activity and extracellular inorganic phosphate concentration (Expt. *C*). Interrupted lines, insulin systems (insulin concentrations indicated in μg/100 ml.): full lines, control systems. Haematocrits 60 %.

P turnover pattern

The three factors which determine the shape of the dynamic equilibrium curves (Fig. 1) can be graphically expressed as the asymptote to the first, steeply inclining segment, the asymptote to the equilibrium or near-horizontal segment, and the 'breaking point'. The first asymptote approximates the uptake rate of ^{32}P by the cells. The second asymptote reflects the partition of ^{32}P between the cells and the extracellular fluid at near-equilibrium. The 'breaking point' is essentially a mathematical concept: it would exist as a sharp point only if P atoms traversed the cell as on a conveyor belt in a single file. Nevertheless, the nearest point on the equilibrium curve to the junction of the two asymptotes can be marked; and the distance (t) on the time scale between 0 and this point can be con-

ceived as the 'mean transit time' of P atoms from their moment of entry into the cell to their moment of discharge. Under all experimental conditions insulin altered all three variables.

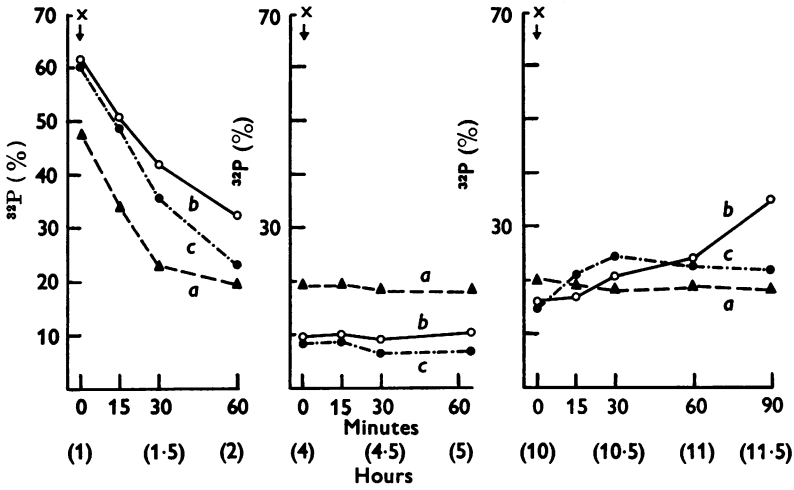

Fig. 3. The effect of insulin on extracellular ^{32}P activity when added to cell suspensions after varying periods of pre-incubation (Expt. *E*). The pre-incubation times are indicated in brackets. The insulin concentrations were 2–3 μg/100 ml. Full lines, control system: interrupted lines, insulin systems (insulin added at zero pre-incubation time): dotted, interrupted lines, delayed insulin systems (insulin added at zero incubation time). Haematocrits 70 %.

(*a*) The uptake rate of ^{32}P by the cells was markedly accelerated. Although this effect was recordable within 30 min of the addition of insulin, it was not immediate in the sense in which the initial P release discussed above was immediate. As reflected by extracellular ^{32}P activity, the difference between control and insulin systems was maximal 30–60 min after the simultaneous addition of insulin and ^{32}P. When insulin was added after prolonged pre-incubation with ^{32}P (i.e. after cell/extracellular ^{32}P equilibrium had been virtually established) there was little or no extra ^{32}P uptake. After 10 hr or longer pre-incubation the effect of insulin on extracellular ^{32}P activity was usually reversed—i.e. there was a small release of labelled P by the cells (Fig. 3).

(*b*) With a faster ^{32}P uptake the final cell/extracellular equilibrium could yet have remained unchanged if there had been a proportional shortening of transit time. This would have shown on the curve as a steeper initial decline, leading to an earlier breaking point, leading to the same equilibrium level as before. The breaking points on the insulin curves show that t was in fact shortened; but the shortening was disproportionately greater than the increase in the ^{32}P uptake rate.

(*c*) The disproportionate shortening of t led to a new equilibrium level, representing a relatively smaller intracellular and a relatively larger extracellular ^{32}P pool (Fig. 1, Table 1).

(*d*) Figure 1 shows a further divergence between the insulin and the control curves during the later stages of incubation. On incubation beyond 8–10 hr, even with an adequate supply of glucose in the medium, red cells began to lose both labelled and unlabelled P to the extracellular fluid: dynamic equilibrium gave way to a progressively negative P balance. The control curve is roughly U-shaped; but whereas the downstroke represents the exchange of equivalent unlabelled P for labelled ^{32}P the upstroke represents the loss from the cells of both. The onset of the disequilibrium phase was delayed by insulin.

Cellular P fractions

In the interpretation of the numerical results set out in Tables 3–5 a distinction must be made between the significance and reproducibility of the findings in successive and in parallel experiments. In successive experiments with different blood-cell suspensions (even when every effort was made to follow a uniform procedure) both fractional and relative specific activities showed wide variations. The causes were probably many and cumulative; but the chief reason may have been small discrepancies in the timing of the successive centrifugations. On the other hand, the scatter of the counts in duplicate or triplicate chromatographic fractions derived from synchronously incubated, spun and extracted cells was comparatively small. No attempt was made to calculate standard deviations for each fraction: differences of more than 20% between parallel insulin and control systems in each of three similar experiments are probably meaningful; and differences of over 40% are almost certainly significant.

Two parameters were calculated. Fractional activities expressed ^{32}P activities in the extracellular and in the various intracellular P fractions as percentages of the ^{32}P activity in the whole cell suspension (Tables 3 and 4). Relative specific activities expressed ^{32}P activities as percentages of the P content of individual P pools (Table 5). Two main differences arose from this distinction. Relative specific activities did not reveal 'lost' activity—i.e. activity in spots not analysed and activity in the TCA-insoluble cell precipitate. On the other hand, they provided some evidence of the sequence of events in time. Fractional activities showed that in all insulin systems there was an increase in ^{32}P activity in the TCA-insoluble cell fraction (i.e. in the fraction not subjected to chromatography).

Glucose, pH and E_h

Insulin had no *apparent* effect on glucose uptake, Cl^- distributions, or on extracellular pH; but it caused a sustained fall in extracellular E_h (as monitored with a platinum electrode) (Fig. 4).

DISCUSSION

Several differences may be noted between the effects of insulin on actively metabolizing red cells described in the foregoing sections and the 'immediate electrochemical' effects reported in the preceding paper (Dormandy & Zárday, 1965). The immediate Cl^-, HCO_3^- and pH changes which were the main findings in cell suspensions at metabolic rest were eclipsed when the cells were incubated in the presence of glucose at 37° C. On the other hand, the immediate discharge of cellular P observed under metabolically active conditions was revealed as part of a complex resetting of the rate and pattern of P turnover.

Hahn & Hevesi (1941), Prankerd & Altman (1954), Gourley (1952) and other workers (Prankerd, 1961) have shown that orthophosphate containing radioactive phosphorus (^{32}P) enters the metabolically active red cell from the extracellular fluid by way of an active (energy-consuming) exchange process without a net increase in total cellular P. While there has been some debate about the identity of the first organic compound formed, the turnover clearly involves at least two but more probably three parallel processes. (i) Orthophosphate is taken up by incorporation into one or several organic phosphate esters. (ii) There is an internal turnover of ester phosphate partly or mainly linked to glucose metabolism. (iii) P is released from organic ester bondage and passes into the extracellular medium as orthophosphate (IP). All three phases were affected by insulin.

The initial rise in extracellular phosphate was characteristically transient: either the P release by the cells was followed by a compensatory process of P uptake; or, more probably, a fast and a contrary but slower insulin effect overlapped. Attempts to identify the source of the discharged P were not successful; but three pointers may be noted. First, the fact that the addition of insulin to cell suspensions after varying periods of pre-incubation with ^{32}P led to a discharge of unlabelled but not of labelled P suggests that not only the break-down but also the synthesis of this P source is insulin-dependent. Secondly, the insulin effect on fractional ^{32} P activities indicates that in part at least it is firmly bound to TCA-insoluble cell material. It is possible that some of the P of the membrane or stromal phospholipids is in dynamic equilibrium with extra-

cellular inorganic or intracellular ester phosphates; but it is more likely that certain phosphorylation centres of TCA-soluble metabolic intermediates are complexed to or embedded in the lipid or lipoprotein structural framework of the cells and are precipitated by TCA. It is conceivable that such 'structural' enzymic centres may be specially concerned with the type of vectorial P transfer envisaged by Mitchell (1962, 1963). Thirdly, the relative specific activities shown in Table 5 suggest that the increased P uptake in the insulin systems is probably linked to an ADP/

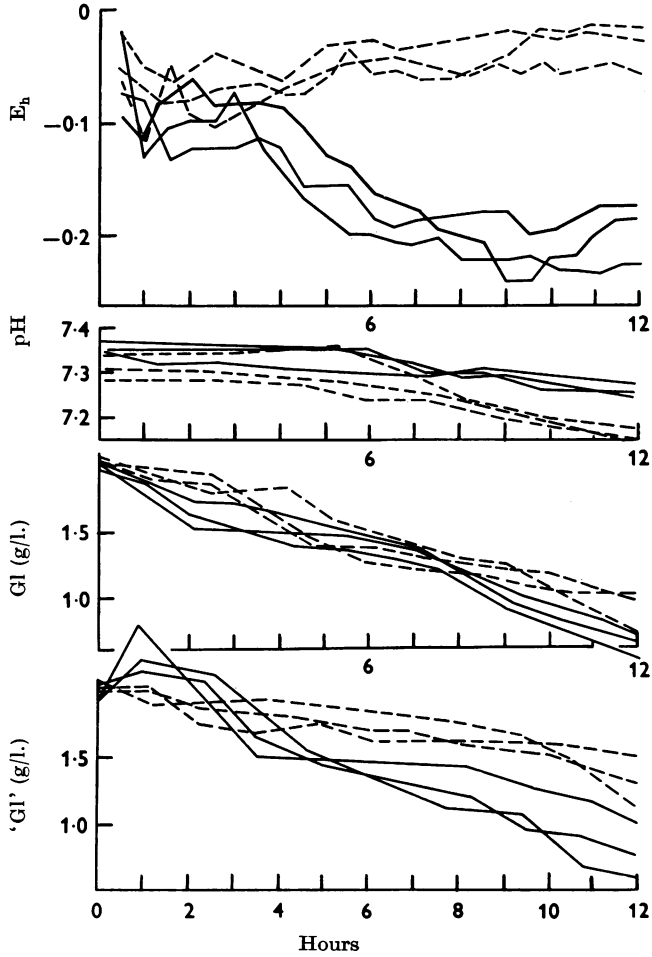

Fig. 4. The effect of insulin on extracellular E_h, pH and on extracellular glucose as estimated by two methods (Expt. *H*). The insulin concentrations were 2 and 4 μg/100 ml. during the periods of 1–8 and 8–12 hr respectively. Gl = glucose by glucose oxidase method. 'Gl' = glucose by copper-reducing method. Interrupted lines, control systems: Full lines, insulin system. Haematocrits 70 %.

ATP and/or a hexose diphosphate mechanism. A review of the literature underlines the need for interpreting these findings with caution.

From a cursory examination of the first two columns in Table 5 and from the consistently high relative specific activities of the IP fractions it might be argued that extracellular ^{32}P was probably taken up as inorganic phosphate. This would take no account of the fact that both ATP and 2,3-DGP contain more than one P atom. If extracellular ^{32}P were incorporated into the cell by way of the reaction ADP + IP → ATP, only one of the three P atoms would be taking part in the turnover cycle; and the relative specific activity of ATP would have to be three times lower than the relative specific activity of the terminal P alone.

A number of findings support the view that the insulin effects on P uptake and P release were part of an extensive realignment of intracellular intermediate pathways. Analysing the dynamic equilibrium curves (Fig. 1) it was convenient to express the differences in terms of P transit times; but in reality speed as such is almost certainly not the main variable. It is possible, of course, that certain enzymic reactions are actually accelerated by insulin; but more probably the reaction sequences themselves were different. (If there are several alternative and parallel routes of P turnover—a not unreasonable supposition—more P may have been shunted from a slow to a fast route.) The delayed onset of the P disequilibrium phase in the insulin systems points to a similar conclusion. (This phase coincided with the rapid swelling of the cells, with an accelerated K^+ loss and Na^+ uptake, with a falling pH, and with a striking increase in the tendency of the cell suspensions to haemolyse.)

In view of the close relations between P and glucose turnover the similar glucose-uptake curves in insulin and control systems are at first sight somewhat surprising. (Moreover, if the immediate electrochemical action of insulin does entail a resetting of the over-all free-energy level of intracellular metabolic processes an increased glucose uptake might reasonably be expected.) In fact, at least one explanation and one subsidiary reason may be advanced.

Rapoport & Luebering (1951) have pointed out that the exergonic glucolytic apparatus in the mature red cell may remain geared to the energy needs of an earlier nucleated state; and that the potential energy yield from glucolysis may outstrip its energy demand. An increased P turnover might then be associated with a changed internal balance of glucolytic intermediates rather than with an increase in total glucose consumption. It is perhaps significant that, while in insulin systems the total ^{32}P activity in the TCA-soluble cell extracts was lower than in the control systems, the relative specific activity of the hexose-diphosphate fractions was higher. Glucose-1,6-phosphate may represent the first step in an abortive glycogen-synthesizing pathway; and both glucose and fructose diphosphates are relatively high-energy intermediates.

The subsidiary reason is technical but may have wider implications. As shown in Fig. 5, one of the effects of insulin on extracellular fluid was a sustained fall in E_h (as monitored by the platinum electrode); and although the chemical basis of this effect remains obscure, it means that the results of glucose estimations in the insulin and control systems cannot be fairly compared. It is true that the first reaction in the glucose-oxidase procedure—the oxidation of glucose to gluconic acid and H_2O_2—is highly specific; but this step must be coupled to a peroxidase/dye reaction which is not. It seems that the general neglect of the E_h factor can be ascribed to one of two misconceptions. The first is to regard the E_h of the extracellular milieu as a linear function of the pH whose constancy is assured. (It can be readily verified that the addition of a reversible oxidant—e.g. a small amount of potassium ferricyanide—has no effect on the pH of the reaction mixture but markedly alters the apparent glucose concentration both by the reducing and by the enzyme method.) The second is to assume that the suspending media have virtually no reversible redox properties. This is probably justified for the type of solution used in the present series of experiments *before* they have been added to cells; but it is an unsound assumption once they have become the extracellular milieu of active metabolic systems.

SUMMARY

1. The effects of insulin were investigated in suspensions of unwashed red cells under active metabolic conditions. Doubts about the insulin effect on glucose uptake could not be resolved; but there were complex changes in the pattern of phosphorus turnover. The main findings were an immediate release of cell phosphorus, an increased ^{32}P uptake rate, a shortened intracellular ^{32}P 'transit time', and a change in the size and metabolic activity of the various cellular phosphate pools. The last effect included a marked increase in the turnover rate of ^{32}P in the TCA-insoluble cell fraction.

2. The immediate insulin effects on cell/extracellular Cl^- partition and on extracellular pH, previously observed under metabolically resting conditions, were no longer in evidence; but insulin caused a sustained fall in extracellular E_h as monitored by the platinum electrode.

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