Early Effects of Phytohaemagglutinin on Glucose Metabolism of Normal Human Lymphocytes

By C. J. HEDESKOV
Department of Medicine, Marselisborg Hospital, University of Aarhus Medical School, Aarhus, Denmark

1. Phytohaemagglutinin induced early changes in the catabolism of glucose by normal human lymphocytes suspended in a bicarbonate buffer. During 4 hr. incubation glucose utilization was almost doubled. 2. The rates of several reactions in the metabolism of glucose were estimated. Total pyruvate formation, lactate production and fatty acid synthesis were stimulated to the same degree as was glucose utilization. The pentose cycle and the glycogen synthesis were also stimulated but less than was glucose utilization. The pentose cycle was found to account for 1·4% and 0·9% of the total glucose utilization without and with phytohaemagglutinin respectively. In these cells rates of triose phosphate isomerization were at least six to seven times the rate of glucose phosphorylation. On an average 55–60% of the total carbon dioxide evolved was derived from decarboxylation of pyruvate, 25–30% from the tricarboxylic acid cycle and about 15% from the pentose cycle. Observed ratios of ^{14}C specific yields in glycogen from [1-^{14}C]- and [6-^{14}C]-glucose could possibly be explained by assuming the existence of two separate glucose 6-phosphate pools. 3. During 4 hr. incubation in bicarbonate buffer ^{14}C from [U-^{14}C]serine was incorporated into perchloric acid-insoluble material. This incorporation was stimulated by phytohaemagglutinin but was almost completely inhibited by puromycin. Puromycin also abolished phytohaemagglutinin-induced stimulation of glycolysis.

PHA,[*] a protein extracted from the kidney bean, *Phaseolus vulgaris*, stimulates cultured human blood lymphocytes to undergo mitosis between 48 and 72 hr. after its addition (Nowell, 1960). The mechanism for the action of PHA has not yet been established, but it has been suggested that it works as an antigen in stimulating lymphocytes to divide (Pearmain, Lycette & Fitzgerald, 1963; Elves, Roath & Israëls, 1963). Morphological changes preceding mitosis are barely detectable until the end of the first day of culture. In the next 24–48 hr. large pyronine-staining 'blast cells' are formed (Elves & Wilkinson, 1963; Schrek & Rabinowitz, 1963). The earliest metabolic changes are an exponential increase in RNA synthesis (Cooper & Rubin, 1965; Mueller & Le Mahieu, 1966) and an increase in the acetylation of histones (Pogo, Allfrey & Mirsky, 1966), which can be detected during the first 2 hr. after addition of PHA to the lymphocyte cultures. Somewhat later there is an increase in protein synthesis (Huber *et al.* 1967), which is claimed to include synthesis of γ-globulin (Parenti, Franceschini, Forti & Cepellini, 1966),

[*] Abbreviation: PHA, phytohaemagglutinin.

and after approx. 36 hr. in culture the lymphocytes begin to synthesize DNA (McIntyre & Ebaugh, 1962).

Some aspects of lymphocyte carbohydrate metabolism during the influence of PHA have also been investigated. After 24 hr. or more in culture there is a marked accumulation of glycogen in the cells (Quaglino & Hayhoe, 1965) and lactate in the medium (Parenti *et al.* 1966). The activity of some enzymes involved in glucose catabolism was found to be increased (Quaglino, Hayhoe & Flemans, 1962; Barker & Farnes, 1967; Hirschhorn, Hirschhorn & Weissmann, 1967), and a late effect of PHA on production of carbon dioxide (Hrachovec, 1966) and pentose-cycle activity (MacHaffie & Wang, 1967) has also been established.

The present experiments were undertaken to investigate whether PHA initiates changes in carbohydrate metabolism within the first few hours after isolation of the lymphocytes from the blood. The relative distribution of glucose carbon atoms on different metabolic pathways, in the absence and presence of PHA, and the effect on early carbohydrate metabolism of some substances that

are known to inhibit the blastogenic and mitogenic activity of PHA were investigated.

METHODS

Lymphocyte preparations. Suspensions of normal human blood lymphocytes were prepared as described by Hedeskov & Esmann (1967). The lymphocyte suspensions were contaminated with 2–3 red blood cells and less than 1 platelet/lymphocyte. These contaminations have been shown to be negligible with respect to the extent of glucose utilization and the evaluation of the contribution of various pathways to glucose metabolism (Hedeskov & Esmann, 1966; Hedeskov, Esmann & Rosell Pérez, 1966). The differential leucocyte count gave 95% lymphocytes and 5% polymorphonuclear leucocytes. Before incubation the lymphocytes were washed with a Krebs–Ringer bicarbonate buffer (De Luca & Cohen, 1964) and finally suspended in the appropriate amounts of the same buffer containing in addition 6·7 mM-glucose and 1% (w/v) gelatin.

Incubations. Incubations were carried out for 4 hr. with shaking in a Dubnoff-type apparatus with 3–5 ml. portions of the lymphocyte suspensions containing $16 \times 10^6 – 70 \times 10^6$ cells in different experiments. Conical flasks equipped with an empty vial in the centre well and closed by a rubber cap were used. The incubations were started after 5 min. equilibration of the medium with a mixture of $O_2 + CO_2$ (95:5) at 37° (pH 7·4) and terminated by injection of $HClO_4$ to a final concentration of 0·3 N. At the same time 2·0 ml. of ethanolamine-2-methoxy-ethanol (1:2, v/v) was injected into the CO_2-absorption vial in the centre well and the flasks were left overnight at room temperature (16–18 hr.).

Before incubation 2·5–15 μc of [1-^{14}C]-, [6-^{14}C]- and [U-^{14}C]-D-glucose or L-[U-^{14}C]serine was added to separate flasks and for each labelled compound parallel incubations with PHA-P were performed. The same batch of PHA was used in all experiments. PHA solutions in 0·9% NaCl were prepared immediately before use and added to the lymphocyte suspensions to a final concentration of 40–60 μg. of PHA/ml. After addition of PHA the lymphocytes quickly clump and adhere to the glass.

Isolation and fractionation. In experiments on glucose catabolism the following procedure was adopted. The deproteinized cells and medium were transferred quantitatively to 15 ml. centrifuge tubes. The cells were recovered by centrifugation and washed twice with 3% (w/v) HClO$_4$. The supernatant and washings were combined and neutralized with KOH. The KClO$_4$ was removed in the cold and carried negligible ^{14}C radioactivity. Portions of the combined supernatant and washings were subjected to continuous ether extraction for 72 hr. after addition of excess of H$_2$SO$_4$ and 0·3 m-mole of lactic acid carrier. The ether-extractable acids were chromatographed on a Dowex 1 (formate form) column (Busch, Hurlbert & Potter, 1952) and the radioactivity corresponding to the lactate peak was determined. This procedure does not completely separate lactate and succinate, but it has been ascertained by further purification of fractions from the Dowex columns on acid Celite (Swim & Krampitz, 1954) that incorporation of ^{14}C into succinic acid and other tricarboxylic acid-cycle intermediates is very low and, compared with incorporation of ^{14}C into lactate, negligible.

Portions of the residue after ether extraction were chromatographed on a Dowex 50 (H$^+$ form) column and residual radioactivity from glucose was washed out of the column with eight 20 ml. lots of water. The amino acids were then eluted with five 10 ml. lots of aq. 1·5 N-NH$_3$ and counted. In some experiments the amino acid fraction was chromatographed on buffered Whatman no. 1 paper with buffered phenol–water (McFarren, 1951) and counted in a Radiochromatogram scanner (Packard Instruments Inc.).

Lipids were extracted twice from the tissue residue by chloroform–methanol (2:1, v/v) for 12 hr. at 40°. After two further washings of the tissue with chloroform–methanol the combined lipid extracts were evaporated to near-dryness on a rotary evaporator (Büchi, Switzerland) or under N$_2$ and the residue was dissolved in 10 ml. of heptane. The heptane was washed three times with 3 ml. of water and dried with anhydrous Na$_2$SO$_4$. A portion of the heptane was evaporated and the lipids were saponified in ethanolic KOH at 80° for 20 hr. under reflux. The ethanol was evaporated under N$_2$, a few millilitres of water were added and the soap solution was acidified with HCl. The fatty acids were finally dissolved in 5·0 ml. of heptane and the water phase containing glycerol was made to 5·0 ml. Portions (1 ml.) of the fatty acid and glycerol fractions were taken for measurement of radioactivity.

Glycogen was extracted from the defatted tissue residue with 2 ml. of 30% (w/v) KOH on a boiling-water bath after addition of 30 mg. of glycogen carrier and precipitated with ethanol (final concn. 67%, v/v). The glycogen was dissolved in and precipitated from 10% (w/v) trichloroacetic acid twice and then hydrolysed with 2 ml. of N-H$_2$SO$_4$ on a boiling-water bath for 2½ hr. The hydrolysate was run through a Dowex 1 (OH$^-$ form) column and the radioactivity and glucose concentration of the eluate were determined.

In some experiments the lymphocytes were incubated with L-[U-^{14}C]serine and the incubation was terminated by addition to the cell suspensions of 0·1 vol. of 33% (w/v) HClO$_4$. The deproteinized cells and medium were left overnight at 4°. The precipitate was washed five times with 3% (w/v) HClO$_4$ containing 0·2% unlabelled L-serine, and extracted for 20 hr. at room temperature with ethanol–ether (3:1, v/v) and for 4 hr. with peroxide-free ether. The residue, representing protein and nucleic acids, was dissolved in N-KOH and Hyamine and counted.

Assay of glucose and lactate. Glucose was assayed spectrophotometrically in incubation medium and in glycogen hydrolysates with glucose 6-phosphate dehydrogenase and NADP$^+$ after reaction with ATP and hexokinase. Lactate was assayed spectrophotometrically with lactate dehydrogenase, hydrazine being a trapping agent in the assay.

Utilization of glucose was obtained by adding the ^{14}C recovery in ether-extractable organic acids, respiratory CO$_2$, glycogen, total lipids and amino acids. In each experiment glucose utilization with and without PHA was calculated as the average of glucose utilization in three separate flasks.

Recoveries. Recovery of glycogen added to the deproteinized sediment was between 30 and 50% and was corrected for in the calculations. Ether extraction of lactic acid from medium was substantially complete and recovery of lactate after chromatography on Dowex 1 (formate form) was 100%. Direct recovery of triglyceride glycerol was not ascertained, but it was found that after addition of glycerol tri[1-^{14}C]palmitate to a deproteinized cell residue, 102% of

the added radioactivity could be recovered in the fatty acid fraction. The incorporation of glucose carbon atoms into glyceride glycerol and fatty acids was so small that it possibly could be caused either by contamination of the glycerol and fatty acid fractions with radioactivity from glucose or by contamination of commercial [14C]glucose with [14C]glycerol in minute quantities. It was found that after drainage of the deproteinized incubation medium and two washings of the precipitate with $HClO_4$ 0·3–0·4% of the administered glucose radioactivity was left in the tissue residue. Further, experiments in which [1-14C]-, [6-14C]- and [U-14C]-glucose were added to tissue residue, and these preparations taken through the procedure for separation of radioactive glycerol, showed that the glycerol fraction contained radioactivity corresponding to 0·25%, 0·05% and 0·08% of the added glucose radioactivity respectively. These experiments permitted a calculation of the possible error in the measured 14C incorporation into glyceride glycerol in each experimental flask, and the error was estimated to be a maximum of 16, 6 and 10% respectively when [1-14C]-, [6-14C]- and [U-14C]-glucose were the substrates. This fairly small error was not corrected for in the calculations. The corresponding error for the fatty acid fractions was less than 2%. The absorption of respiratory 14CO2 in ethanolamine–2-methoxyethanol was found to be complete after 16–18hr. The recovery of [14C]serine after passage of a Dowex 50 (H+ form) column was better than 90%.

Counting procedures. Radioactivity was assayed in a Tri-Carb liquid-scintillation spectrometer (Packard model 2002). The scintillator was as described by Hedeskov *et al.* (1966). The background count rate was ascertained for each container with scintillator before addition of samples. Quench corrections were calculated by aid of the channels-ratio method. The largest correction was 50%. Usually more than 10000 counts were recorded. Hyamine solutions of $HClO_4$-insoluble material displayed no photoluminescence effects.

CALCULATIONS

The rates of several reactions in the catabolism of glucose and the distribution of utilized glucose carbon atoms on the different pathways were calculated according to a model of glucose metabolism presented by Katz, Landau & Bartsch (1966).

The incorporation of 14C from labelled glucose into a product is expressed as specific yield, i.e. the percentage of the utilized 14C recovered in the product. The number of μg.atoms of glucose carbon incorporated into lactate, CO_2, glycogen, fatty acids, glyceride glycerol and amino acids were calculated as the total number of counts/min. recovered in the respective fractions when [U-14C]glucose was the labelled precursor, divided by the number of counts/min./μg.atom of carbon in this labelled substrate, or as the number of utilized μg.atoms of glucose carbon multiplied by the specific yield in the product from [U-14C]glucose.

The contribution of the pentose cycle can be calculated according to Katz & Wood (1963) and Wood, Katz & Landau (1963) from:

$$\frac{G1_{CO_2} - G6_{CO_2}}{100 - G1_{CO_2}} = \frac{3PC}{100 + 2PC}$$

where PC is net flow of glucose carbon atoms through the

cycle as a percentage of utilized glucose carbon atoms and $G1_{CO_2}$ and $G6_{CO_2}$ are specific yields of 14CO2 from [1-14C]- and [6-14C]-glucose respectively.

The total pyruvate formation was calculated as (total number of utilized μg.atoms of glucose carbon) minus (μg.atoms of glucose carbon converted into CO_2 in PC) minus (μg.atoms of glucose carbon converted into glyceride glycerol). The amount of glucose carbon atoms oxidized to CO_2 via the pentose cycle was obtained by multiplying PC (measured in μg.atoms of carbon) by $\frac{1}{2}$, since for each 6μg.-atoms of glucose carbon flowing through the cycle 3μg.-atoms will be converted into 3μmoles of CO_2.

Formation of CO_2 via the tricarboxylic acid cycle was calculated as (total pyruvate formed) $\times G6_{CO_2} \times \frac{2}{3}$, since specific 14CO2 yield from [6-14C]glucose equals that fraction of C-3 of pyruvate which is oxidized in the tricarboxylic acid cycle, and since for each C-3 of pyruvate oxidized at least one C-2 of pyruvate has also been oxidized.

The amount of CO_2 formed by decarboxylation of pyruvate was calculated as total yield of CO_2, which is equal to the specific yield of 14CO2 from [U-14C]glucose, minus the amounts of CO_2 formed via the pentose cycle and the tricarboxylic acid cycle. Twice this amount equals the number of μg.atoms of glucose carbon converted into acetyl-CoA. From the total amount of pyruvate formed the amount of pyruvate converted into lactate, CO_2 and acetyl-CoA was subtracted. The remainder was taken as net pyruvate formation.

The rates of triose phosphate isomerization relative to the rate of glucose utilization, E_t (dihydroxyacetone phosphate → glyceraldehyde 3-phosphate) and E_{-t} (glyceraldehyde 3-phosphate → dihydroxyacetone phosphate), were calculated from y, x and PC:

$$E_t = \frac{y + yx(1 + 2PC)}{2(x - y)}$$

$$E_{-t} = \frac{(1 - PC)\left(y + \dfrac{1}{1 + 2PC}\right)}{2(x - y)}$$

where:

$$x = \frac{\text{specific yield of }^{14}\text{C in glycerol from [1-}^{14}\text{C]glucose}}{\text{specific yield of }^{14}\text{C in glycerol from [6-}^{14}\text{C]glucose}}$$

and

$$y = \frac{\text{specific yield of }^{14}\text{C in lactate from [1-}^{14}\text{C]glucose}}{\text{specific yield of }^{14}\text{C in lactate from [6-}^{14}\text{C]glucose}}$$

represent ratios of specific radioactivities from [1-14C]-glucose and [6-14C]glucose of dihydroxyacetone phosphate and glyceraldehyde 3-phosphate respectively. PC, x and y from three experiments were averaged before calculation of E_t and E_{-t}. The rates are expressed as μg.atoms of carbon/10^{10} lymphocytes/hr. by multiplying E_t and E_{-t} by the rate of glucose utilization.

MATERIALS

D-[1-14C]Glucose, D-[6-14C]glucose, D-[U-14C]glucose and L-[U-14C]serine were purchased from The Radiochemical Centre, Amersham, Bucks. PHA-P was obtained from Difco Laboratories, Detroit, Mich., U.S.A. Glycogen, puromycin hydrochloride, N-acetyl-D-galactosamine and L-serine were from Sigma Chemical Co., St Louis, Mo.,

U.S.A. Cortisol (as the sodium succinate) was a gift from Upjohn Co., Kalamazoo, Mich., U.S.A. Hyamine was obtained from Packard Instrument Co., Downers Grove, Ill., U.S.A. Dextran was kindly supplied by Pharmacia Ltd., Uppsala, Sweden. The reagents for enzymic glucose and lactate determinations were from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Extinction was determined on a C. Zeiss (Germany) spectrophotometer, model PMQII.

RESULTS

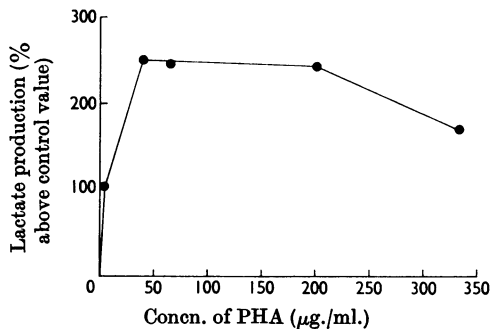Glucose utilization was almost doubled in lymphocytes incubated with PHA (Table 1). The

Fig. 1. Lymphocytes (26.7×10^6) were incubated for $4\frac{1}{2}$ hr. in 3 ml. of the buffer described in Table 1. One control flask was without PHA. To five flasks was added PHA to increasing final concentrations as stated. Lactate concentration was determined before and after incubation.

optimum stimulatory effect of PHA on the rate of lactate production was found at concentrations of PHA in the medium between 30 and $200\,\mu g./ml.$ (Fig. 1). A PHA concentration of $40–60\,\mu g./ml.$ was used throughout all recorded experiments.

Table 1 records the ^{14}C recovery in different products as specific yields and the ratios of ^{14}C yields from [1-^{14}C]- and [6-^{14}C]-glucose. By far the greater part of glucose carbon atoms was converted into lactate, with C-1/C-6 ratio 0.97. The dilution of the specific radioactivity of the hexose phosphate pool when [1-^{14}C]glucose was metabolized was thus shown to be small but significant. The increases in mean specific yields in lactate from all three labelled forms of glucose, when PHA was added, were not statistically significant.

Of the glucose carbon atoms 3–5% was converted into carbon dioxide. About 15% of the total carbon dioxide was derived via the pentose cycle, 25–30% via the tricarboxylic acid cycle and 55–60% via decarboxylation of pyruvate. Specific $^{14}CO_2$ yield from [1-^{14}C]glucose was significantly diminished in the presence of PHA, whereas specific $^{14}CO_2$ yield from [6-^{14}C]glucose was unchanged. PC was calculated to be 1.4 and 0.9% in the absence and presence of PHA respectively.

The fraction of utilized glucose carbon atoms incorporated into glycogen was diminished from 2.3% to 1.7% in the presence of PHA. Unexpectedly, the C-1/C-6 ratio of specific yields in glycogen was found to be greater than 1, with and without PHA. The incorporation of ^{14}C into total lipids was

Table 1. *Specific yields from* [1-^{14}C]-, [6-^{14}C]- *and* [U-^{14}C]-*glucose in products*

Lymphocytes were incubated for $4\frac{1}{2}$ hr. in 5 ml. of Krebs–Ringer bicarbonate buffer containing 6.7 mM-glucose and 1% gelatin. The concentration of PHA (when added) was $40\,\mu g./ml.$; $5–15\,\mu c$ of the differently labelled forms of glucose were added to separate flasks. The temperature was 37° and the pH was 7.4. The recovery of ^{14}C in products is expressed as a percentage of glucose ^{14}C utilized. Glucose utilized is expressed as percentage of initial amount of glucose. The results are means $\pm$ s.e.m. for three experiments. The average lymphocyte concentration in the three experiments was 13.4($\pm$0.6) $\times 10^6$/ml.

| PHA | Position of ^{14}C in glucose | Specific yields (%) | | | | | | Glucose utilization (%) |
		Lactate	CO_2	Glycogen	Fatty acids	Glyceride glycerol	Amino acids	
−	1	77.9 ± 3.1	5.4 ± 0.2	2.7 ± 0.3*	0.032 ± 0.008	0.046 ± 0.009	2.6 ± 1.1	18.5 ± 1.3
−	6	80.5 ± 2.3	1.4 ± 0.1	1.4 ± 0.2	0.035 ± 0.007	0.044 ± 0.011	3.7 ± 0.9	17.5 ± 0.1
−	U	77.8 ± 0.5	4.4 ± 0.7	2.3 ± 0.4	0.027 ± 0.007	0.038 ± 0.010	2.6 ± 0.2	17.6 ± 0.6
	C-1/C-6 ratio	0.97 ± 0.01	3.83 ± 0.42	2.12 ± 0.12	0.90 ± 0.05	1.11 ± 0.09	0.65 ± 0.21	
+	1	81.6 ± 3.2	4.2 ± 0.3†	1.8 ± 0.3	0.035 ± 0.009	0.025 ± 0.005	1.6 ± 0.3	35.5 ± 1.7†
+	6	84.9 ± 3.9	1.5 ± 0.2	0.9 ± 0.07	0.045 ± 0.009†	0.024 ± 0.006	1.9 ± 0.3	33.5 ± 0.5†
+	U	82.1 ± 2.9	3.2 ± 0.4	1.7 ± 0.3†	0.030 ± 0.005	0.018 ± 0.006†	1.5 ± 0.03†	35.0 ± 0.2†
	C-1/C-6 ratio	0.96 ± 0.01	2.92 ± 0.42	1.92 ± 0.19	0.80 ± 0.15	1.12 ± 0.35	0.86 ± 0.07	

* Two experiments.
† The mean differences between these values and the corresponding control values without PHA are statistically significant for paired data ($P < 0.05$).

Table 2. *Effect of PHA on rates of some reactions in normal lymphocytes catabolizing glucose*

Experimental conditions were as stated in Table 1. Rates are expressed as μg.atoms of glucose carbon/10^{10} lymphocytes/hr. The results are means $\pm$ s.e.m. for three experiments. The mean differences between control cells and PHA-treated cells are statistically significant for paired data with the indicated P values (N.S., not significant).

	Reaction rate		Stimulation (%)
	Without PHA	With PHA	
Phosphorylation of glucose	1364 ± 106	2620 ± 103	92 ($P < 0.001$)
Glucose 6-phosphate $\rightarrow$ ribulose 5-phosphate	57 ± 8	74 ± 10	30 ($P < 0.05$)
Pentose phosphate $\rightarrow$ hexose phosphate	38 ± 5	49 ± 7	30 ($P < 0.05$)
Glycogen synthesis	32 ± 8	45 ± 9	41 ($P < 0.025$)
Triose phosphate isomerase reaction	9680	16240	68
Synthesis of glyceride glycerol	0.5 ± 0.2	0.5 ± 0.2	0 (N.S.)
Total pyruvate formation	1354 ± 105	2607 ± 101	93 ($P < 0.001$)
Lactate formation	1060 ± 76	2147 ± 52	103 ($P < 0.005$)
CO_2 from decarboxylation of pyruvate	37 ± 10	46 ± 7	24 (N.S.)
Pyruvate $\rightarrow$ acetyl-CoA	74 ± 19	92 ± 13	24 (N.S.)
Net pyruvate formation	184 ± 42	323 ± 86	75 (N.S.)
Fatty acid synthesis	0.3 ± 0.1	0.7 ± 0.1	133 ($P < 0.01$)
CO_2 from tricarboxylic acid cycle	13 ± 1	25 ± 3	92 (N.S.)
CO_2 from pentose cycle	10 ± 1	13 ± 2	30 ($P < 0.05$)
Total CO_2 production	60 ± 9	84 ± 9	40 ($P < 0.01$)
Glucose carbon atoms incorporated into amino acids	35 ± 1	39 ± 3	11 (N.S.)

extremely low (less than 0·1%). PHA stimulated the incorporation of ^{14}C into fatty acids as compared with glyceride glycerol, in which specific yield from [U-^{14}C]glucose actually decreased when PHA was added.

A small fraction of utilized glucose carbon atoms was converted into amino acids with C-1/C-6 ratio approx. 0·8. Specific yield in amino acids from [U-^{14}C]glucose was decreased in the presence of PHA. By paper chromatography it was found that demonstrable radioactivity in amino acids was present only in glutamic acid and serine.

Table 2 summarizes the rates of glucose phosphorylation, glucose 6-phosphate dehydrogenation, triose phosphate isomerization, glycogen synthesis, glyceride glycerol synthesis, fatty acid synthesis, amino acid synthesis, carbon dioxide production and formation of pyruvate and lactate. Marked stimulation of glucose utilization by PHA is evident.

Total pyruvate formation, lactate production and fatty acid synthesis were stimulated to extents corresponding to the stimulation of glucose utilization.

The pentose cycle and glycogen synthesis were also stimulated, but less than glucose utilization, so that the percentage contribution of these reactions to glucose catabolism was decreased by PHA.

Pyruvate decarboxylation, tricarboxylic acid-cycle activity and incorporation of glucose carbon atoms into glyceride glycerol and into amino acids were not stimulated by PHA.

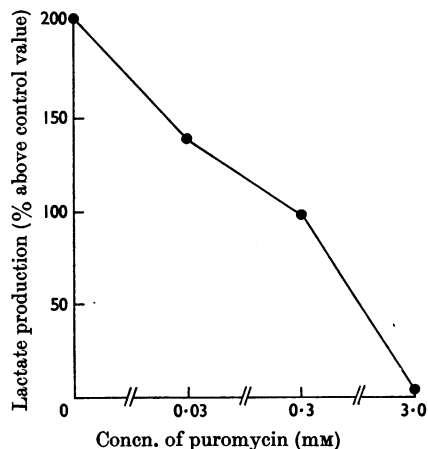E_t and E_{-t} were calculated as described in the

Fig. 2. Lymphocytes (16.2×10^6) were incubated for 4 hr. in 3 ml. of the buffer described in Table 1. One control flask was without PHA and puromycin. To the four experimental flasks was added PHA to a final concentration of 60μg./ml. and increasing amounts of puromycin as stated. Lactate concentration was determined before and after incubation. Puromycin by itself (3 mm) inhibited lactate production by 40%.

Calculations section from x, y and PC. The rates of triose phosphate isomerization given in Table 2 were obtained by averaging E_t and E_{-t}, which deviated from each other by no more than 10%.
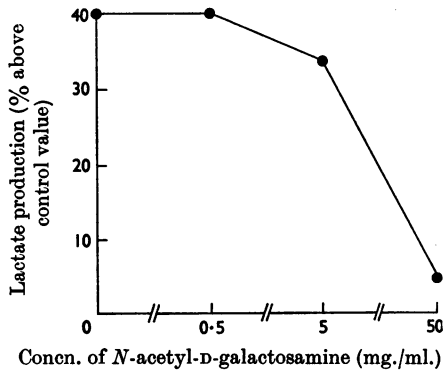
Fig. 3. Leucocytes ($58\cdot8\times10^6$; 85% lymphocytes and 15% polymorphonuclear leucocytes) were incubated for 4hr. in 3ml. of the buffer described in Table 1. Experimental conditions were as in Fig. 2 except that puromycin was replaced by N-acetyl-D-galactosamine in the concentrations stated. N-Acetyl-D-galactosamine by itself (5mg./ml.) inhibited lactate production by 6%. The relatively small PHA stimulation of lactate production in this experiment can be ascribed to the exceptionally large contamination with polymorphonuclear leucocytes, which themselves do not respond to PHA but increase the magnitude of the lactate production in the reference lymphocyte suspension.

Table 3. *Incorporation of ^{14}C from [U-^{14}C]serine into perchloric acid-insoluble material of normal lymphocytes*

Lymphocytes (34×10^6–74×10^6) were incubated for 4hr. in 3ml. of Krebs–Ringer bicarbonate buffer containing, in addition, 6·7mM-glucose and 1% gelatin. The temperature was 37° and the pH was 7·4. The concentration of PHA (when added) was 60μg./ml. The concentration of puromycin (when added) was 3mM. [U-^{14}C]Serine (2·5μc) was added to each flask. Results are means ± S.E.M. for four experiments.

| Additions | | Percentage incorporation/ |
PHA	Puromycin	10^8 lymphocytes/hr.
–	–	$7\cdot05\pm1\cdot19$
–	+	$0\cdot24\pm0\cdot03$
+	–	$11\cdot26\pm2\cdot04*$
+	+	$0\cdot22\pm0\cdot02$

* The mean difference between this value and the control value without PHA is statistically significant for paired data with $P<0\cdot025$.

The rates are, with and without PHA, six to seven times the rates of glucose utilization, which implies essentially complete isotopic equilibration of triose phosphate. The difference between the mean C-1/C-6 ratio in glycerol and the mean C-1/C-6 ratio in lactate was 0·16 and 0·14 with and without PHA, but these differences were not statistically signifi-

cant. If the differences are not real, $(x-y)$ will tend to zero and E_t and E_{-t} will increase beyond bounds (cf. the Calculations section). The values given here are therefore probably minimum values. It is unlikely that the triose phosphate isomerase reaction plays a role in metabolic regulation, but the knowledge of the extent of isotopic equilibration has some bearing on the interpretation of ^{14}C incorporation into products from [1-^{14}C]- and [6-^{14}C]-glucose. For example, in the calculation of PC from specific $^{14}CO_2$ yields from [1-^{14}C]- and [6-^{14}C]-glucose complete isotopic equilibration of triose phosphate is assumed.

Puromycin is known to inhibit protein synthesis at the level of transfer of amino acids from soluble RNA to protein (Yarmolinsky & de la Haba, 1959). Fig. 2 shows that puromycin (3mM) completely abolished the PHA-induced initial stimulation of lactate production. N-Acetyl-D-galactosamine at a concentration of 10–50mg./ml. has been shown to inhibit the leuco-agglutinating and mitogenic properties of PHA (Silber, Borberg, Miescher, Hirschhorn & Gesner, 1966), possibly in a manner akin to a hapten in preventing the attachment of PHA to the cell. Fig. 3 shows that the same concentration of N-acetyl-D-galactosamine also inhibited the early PHA stimulation of glycolysis in this system. Prednisolone inhibits PHA-induced lymphocyte mitosis (Nowell, 1961). In the present experiments it was, however, found that cortisol sodium succinate (1mM) gave only a slight inhibition (10–15%) of the PHA stimulation of lactate production.

In Table 3 are recorded the incorporations of ^{14}C from L-[U-^{14}C]serine into perchloric acid-insoluble material. The incorporation of ^{14}C into this fraction was significantly stimulated by PHA and was sensitive to puromycin, which gave a 96–98% inhibition.

DISCUSSION

One cannot assume *a priori* that PHA acts in these incubation experiments in a manner analogous to that found when PHA is added to lymphocytes in the more conventional short-term culture procedure, but it would seem to be very likely and some evidence for this assumption has been provided. In short-term cultures PHA causes an early increase in protein synthesis (Huber *et al.* 1967). In the incubation experiments reported in the present paper PHA stimulated the incorporation of ^{14}C from [U-^{14}C]serine into a perchloric acid-insoluble fraction and this incorporation was highly sensitive to puromycin. Since it has been shown that protein synthesis in rabbit spleen lymphocytes is inhibited completely by puromycin *in vitro* (van Furth, Schuit & Hijmans, 1966) and that in bacteria

puromycin inhibits protein synthesis but not RNA synthesis (Takeda, Hayashi, Nakagawa & Suzuki, 1960), it is considered highly probable that the ^{14}C incorporation into the perchloric acid-insoluble fraction represents new synthesis of protein. Further, some of the early PHA effects demonstrated in the present work are identical with late effects demonstrated in short-term cultures, namely increased lactate production and glycogen synthesis.

It should also be mentioned that the changes demonstrated in the metabolism of glucose by lymphocytes are not necessarily a direct effect of PHA but might be a consequence of the PHA-induced clumping of the cells.

The calculations of the rates of several metabolic reactions presented in this paper have been done according to a model of glucose metabolism (Katz et al. 1966) that contains a number of simplifying assumptions.

The model does not provide for metabolism by non-triose pathways. It was, however, found that glycogen synthesis was only about 2%, and the error introduced by neglecting it will be small. It is also assumed that glucose 6-phosphate and fructose 6-phosphate are in complete isotopic equilibrium, which condition appears fulfilled in lymphocytes, where a high specific yield of $^{14}CO_2$ from [2-^{14}C]-glucose has been found in previous experiments (Hedeskov & Esmann, 1967). Further, fructose 1,6-diphosphatase is considered to be absent. The earlier finding (Hedeskov & Esmann, 1967) that ^{14}C does not randomize from C-6 to C-1 when [6-^{14}C]glucose is metabolized by lymphocytes is consistent with this requirement. Finally, the transaldolase and transketolase reactions are assumed to be irreversible, although they are known to be reversible. The error introduced by this assumption is difficult to evaluate, but it has been shown (Katz & Rognstad, 1967) that, if the rates of the reversed transaldolase and transketolase reactions are as high as half the rate of glucose utilization, a true PC of 10% will be underestimated only by 15% when the method of PC calculation used in this paper is applied. If the reversed transaldolase and transketolase reactions are slower, the error will be still less.

Transaldolase exchange activity, which is extensive in lymphocytes (Hedeskov & Esmann, 1967), does not invalidate the calculations when metabolism by non-triose pathways is insignificant.

The rates of different reactions calculated from incorporation of ^{14}C from [^{14}C]glucose represent actual tissue rates only if metabolism of endogenous substrates does not take place. Particularly, glycogen breakdown is pertinent. Glycogen content before and after incubation was not investigated, but values for lactate formation determined enzymically were approx. 10% greater, with and

without PHA, than those predicted from ^{14}C incorporation, indicating that some endogenous dilution had taken place either from glycogen or other sources. This means that most of the reactions were probably underestimated, but the error will be fairly small.

The C-1/C-6 ratios of specific yields in glycogen were about 2. The expected value, with a small dilution of C-1 in hexose phosphates caused by PC activity, would be a little less than 1, as observed in lactate. Such a discrepancy suggests the existence of two separate glucose 6-phosphate pools, one for glucose breakdown and one for glycogen synthesis. The increased C-1/C-6 ratio in glycogen is probably produced by dilution of ^{14}C in C-4, C-5 and C-6 of glucose 6-phosphate in the pool for glycogen synthesis by the transaldolase exchange reaction, and consistent with this mechanism is the observation that the specific yield in glycogen from [U-^{14}C]-glucose was lower than the specific yield from [1-^{14}C]glucose, but higher than the specific yield from [6-^{14}C]glucose.

Also, discrepancies between the extent of randomization of ^{14}C from differently labelled forms of glucose in corresponding carbon atoms in glycogen and lactate, as observed by Hedeskov & Esmann (1967), are consistent with a two-pool hypothesis.

From the experiments reported the point of action of PHA cannot be deduced with certainty. Apparently PHA must first be bound to the cells for effect. This binding can be inhibited by N-acetyl-D-galactosamine (Silber et al. 1966) and possibly by serum (see Forsdyke, 1967), and the present experiments show that N-acetyl-D-galactosamine also inhibits the PHA effect on glycolysis. Kleinsmith, Allfrey & Mirsky (1960) have suggested that PHA causes extensive gene activation, which results in increased RNA and protein synthesis.

The observed early increase in glucose utilization may be brought about through a stimulatory effect of PHA on one or more steps in the route from free extracellular glucose to pyruvate. The fact that glucose utilization, total pyruvate formation, the pentose cycle and glycogen synthesis are all stimulated could suggest that the concentration of glucose 6-phosphate has been increased in the PHA-treated lymphocytes, and preliminary experiments have in fact revealed a two- to three-fold increase in the glucose 6-phosphate concentration after 2hr. incubation of the lymphocytes with PHA. This could be brought about if PHA facilitated either the hexokinase reaction or the transport of glucose over the cell membrane. Glucose transport has, however, been reported not to be rate-limiting for glucose metabolism in lymph-node cells (Helmreich & Eisen, 1959). An increased glycolytic rate in the presence of an increased concentration of glucose 6-phosphate could imply either that

lymphocyte hexokinase is not inhibited by glucose 6-phosphate or that PHA has effected an increase in the concentration of hexokinase protein sufficient to overcome the glucose 6-phosphate inhibition of the enzyme. Increased synthesis of hexokinase protein as an explanation of the reported results is compatible with the fact that puromycin abolished PHA-induced stimulation of glycolysis, but this need not be the only explanation. Changes in the concentrations of certain nucleotides and inorganic phosphate caused by increased protein synthesis could also change the rate of glucose phosphorylation. That the increased glycolysis has been mediated primarily through an increase in the rate of the phosphofructokinase reaction seems less likely, as this would imply increased glycogen synthesis and pentose-cycle activity concomitant with a decreased concentration of glucose 6-phosphate.

One or more of the extensive metabolic changes in lymphocytes that occur after a few hours' incubation in buffer, for example stimulation of lactate production, might eventually be used as a convenient and time-saving measure of lymphocyte responsiveness to PHA or specific antigens *in vitro* instead of the parameters now in common use.

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