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The Action of Certain Drugs on the Uptake of ^{32}P by Human Erythrocytes

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Apart from the uptake of a small fraction which is rapidly labelled, the uptake of phosphate by erythrocytes is a temperature-dependent, probably enzymically regulated, process (Gourley & Gemmill, 1950). There is some doubt whether the phosphate is first incorporated into the labile radicals of adenosine diphosphate and adenosine triphosphate (Gerlach, Fleckenstein & Gross, 1958) or is transferred to these molecules from diphosphoglycerate (Prankerd & Altman, 1954). The energy for this uptake is generally attributed to glycolysis. A search has been made for suitable agents which might block the uptake of ^{32}P by interfering with the formation of either adenosine triphosphate or 2:3-diphosphoglycerate. The results of these investigations are reported in this paper.

METHODS

Action of drugs on phosphate exchange. Blood was obtained by venepuncture from healthy adult males. Clotting was prevented with solid heparin (Pularin brand, Evans Medical Supplies Ltd., 0.5 mg./ml. of blood). For each observation, 1 ml. of blood was added to 10 ml. of the following medium: NaCl 142 mM, KCl 5.9 mM, MgSO_4 1.5 mM, CaCl_2 1.7 mM, NaH_2PO_4 0.5 mM, Na_2HPO_4 2.8 mM,

dextrose 5.6 mM. Drugs were incorporated into the medium as required and when necessary the pH was restored to 7.4 with NaOH or HCl. pH was measured with a Universal pH meter (W. G. Pye Ltd.) calibrated with standard buffer solutions (National British Standard formula) pH 6.99 and 9.15. All treatments were carried out in duplicate and compared with a pair of untreated controls in each experiment. Immediately after adding the blood to the medium the flasks were incubated in a water bath at 37°. After 60 min., 0.2 ml. samples of carrier-free ^{32}P -phosphate were added to each flask and after a further 60 min. the contents of the flasks were poured into centrifuge bottles at 0° and were centrifuged for 10 min. at 1200 g in a refrigerated centrifuge. The supernatants were removed, the cells were haemolysed and digested with 2.2 ml. of 60% (v/v) perchloric acid for 2 hr. The total phosphate and ^{32}P in the digest were then estimated. To find the specific activity of the whole suspension, 0.2 ml. samples of ^{32}P -phosphate were added to two further controls. These were left at -20° in order to haemolyse the cells. Samples were then taken and treated as above.

Estimation of total phosphorus. Phosphorus was estimated by Allen's (1940) method. The intensity of the blue colour, produced by the reduction of phosphomolybdic acid by 2:4-diaminophenol hydrochloride, was measured with a type H 760 Spekker absorptiometer (Hilger and Watts Ltd.) with Ilford 608 filters (maximum transmission 83% at 7000 Å). Cells 0.25 or 4.0 cm. long were used, depending on the concentration of phosphorus being estimated.

Estimations of radioactivity. ^{32}P was estimated with a liquid Geiger-Müller counter (type M6, 20th Century Electronics Ltd.) and conventional counting equipment (Dynatron Radio Ltd.). The counts were corrected for background and for lost counts due to the resolving time of

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the apparatus. No corrections were made for decay as all the estimates in each experiment were done within a period of 12 hr. and replicate counts were so arranged that the mean time of counting was practically constant. As ^{32}P has a half-time of 14.3 days, the decline in count in 12 hr. is approx. 2.5%; differences between replicates due to decay are included in the estimate of error of the experiment. Each sample was counted for a minimum of 10 000 counts, or for samples with very low activity for 5 min.

Experiments in which the ^{32}P uptake of the P fractions was estimated. The difference between these experiments and those described above were:

(a) The volumes of blood and medium used were 2.5 and 25 ml. respectively.

(b) In some experiments, one control and two treated flasks were removed 15 min. after the addition of ^{32}P as well as those taken after exposure to tracer for 60 min.

(c) The cells were washed as described above, and were then lysed with water (2 ml.) at 0° . Subsequent steps of the extraction process were carried out at 0° and with ice-cold reagents. Trichloroacetic acid [2 ml. of 32% (w/v) soln.] were added to the haemolysate. The mixture was ground thoroughly in the centrifuge bottle with a glass rod and centrifuged. The supernatants were poured into bottles suitable for freeze-drying. The protein precipitate was re-extracted twice with 10% trichloroacetic acid and, after centrifuging, each supernatant was added to the contents of the appropriate bottle. The extracts were then freeze-dried. The resulting solid was extracted with ether until no more acid could be removed. A volume (50 μl .) of ethylenediaminetetra-acetic acid (0.1 M) was added and the solution made up to 1 ml. with water.

In order to obtain sufficient phosphate for analysis in each fraction, the extracts were run on to the base-line in the form of a strip 5 cm. long by 1 cm. wide. After each addition of extract, the strip was dried in a stream of warm air from a hair-drier. In this way 180 μg . of total phosphorus could be placed on the paper. The phosphate fractions were then separated by descending paper chromatography at room temperature for 22 hr. with propanol-aq. NH_3 soln.-water (6:3:1, by vol.) (Hanes & Isherwood, 1949). Phosphate esters were detected by the method of Neil, M., Walker, D. G. & Warren, F. L. (1957, personal communication). The chromatogram paper was dried in air, dipped in a solution of FeCl_3 (0.025%, w/v, in acetone), re-dried in air and dipped in $\alpha\alpha'$ -dipyridyl (0.1%, w/v, in acetone). After evaporation of the acetone the paper was exposed to moist sulphur dioxide to reduce the free iron to the ferrous form, which reacts with $\alpha\alpha'$ -dipyridyl to give a pink colour, phosphates appearing as white areas.

The extracts regularly separated into six fractions. Since the two fastest moving fractions contained insufficient phosphate to estimate by the method used and contained negligible radioactivity, no attempt was made to identify them. The remaining fractions were identified by comparing their R_{OP} values (distance travelled by fraction or marker relative to the distance travelled by orthophosphate) with those of known markers run under identical conditions. In this way, the slowest moving fraction was identified as containing 2:3-diphosphoglycerate and fructose 1:6-diphosphate; the next was identified as adenosine triphosphate; the third contained inorganic phosphate and the fourth contained ethylenediaminetetra-acetic acid and hexose monophosphate. The last substance was identified by the method of Partridge (1949).

Estimation of total phosphorus and ^{32}P in fractions. A measured area of paper was cut out from the part which contained each fraction. This was then moistened with water and digested with 0.2 ml. of conc. H_2SO_4 and 2.0 ml. of 60% perchloric acid until the solutions were clear. The total phosphorus and ^{32}P were then estimated.

In addition to the fractions, two paper blanks of 6.0 cm^2 were cut out from apparently phosphate-free areas of each paper. These were treated in an identical manner and the total phosphorus/ cm^2 was estimated. The estimates of total phosphorus in the fractions were corrected according to the mean values of the blanks.

Adjustment applied to the relative specific activity of the phosphate fractions. For various reasons the phosphate of each fraction is not labelled uniformly. It is possible that molecules near the surface of the cell are labelled earlier than those nearer the centre: this is unproven, but its possible importance in connexion with other elements has been indicated by Harris & Pranker (1957). Within molecules the phosphate attached to C-3 of 2:3-diphosphoglycerate and the α -phosphate of adenosine triphosphate is unlabelled after incubation of erythrocytes with ^{32}P for more than 60 min. (Pranker & Altman, 1954; Gerlach *et al.* 1958). There is no reason to expect different labelling in the present experiments, and the estimated relative specific activities have therefore been doubled for 2:3-diphosphoglycerate and multiplied by 1.5 for adenosine triphosphate, to allow for the unlabelled atoms. In addition to these causes of heterogeneity, 2:3-diphosphoglycerate was not separated from fructose 1:6-diphosphate: the fructose 1:6-diphosphate is unlikely to have been appreciably labelled by incubation with ^{32}P phosphate for less than 2 hr. (Pranker & Altman, 1954) and probably contributed between one-third and one-tenth of the total phosphate of the spot (Pranker & Altman, 1954; Rohdewald & Weber, 1956).

As a rough correction for this unlabelled compound, the relative specific activity of 2:3-diphosphoglycerate was further multiplied by a factor of $\frac{7}{5}$, making a total adjustment $\times \frac{7}{5}$. A similar correction of the estimated relative specific activity of the inorganic phosphate because of the concurrence of adenosine diphosphate in the chromatograms (Caldwell, 1953) has not been made because of uncertainty about the amount of adenosine diphosphate present. As one of the phosphate radicals of this compound was presumably unlabelled, the relative specific activity attributed to the inorganic phosphate probably errs somewhat on the low side.

Experiments to investigate the action of drugs on glycolysis. Glycolysis was measured manometrically by the direct method of Warburg as described by Dixon (1951). Freshly taken human blood (1 ml., containing heparin, 0.5 mg./ml.) and saline (3 ml., containing NaCl 120 mm, KCl 5.9 mm, MgSO_4 1.5 mm, CaCl_2 1.7 mm, NaHCO_3 25 mm, NaH_2PO_4 1.2 mm, dextrose 5.6 mm) were placed in the main chamber of each of four Warburg flasks. The drug to be examined (in 1 ml. of the saline solution) was placed in the side arm of each of two flasks, and saline solution (1 ml.) in the side arm of each of the remaining pair of flasks as a control. Two thermobarometers were set up. The contents of the flasks were equilibrated with $\text{N}_2 + \text{CO}_2$ (95:5) for 15 min. at 37° . The flasks were shaken 120 times/min. and measurements of the change in gas volume at constant pressure were made at 10–20 min. intervals. After 60 min. the flasks were tipped and readings were continued for 2 hr. The readings were corrected for changes in the thermobarometer.

RESULTS

Reliability of methods

The recovery from plasma of added phosphate was examined. Phosphate, in amounts varying from 15.5 to 62.0 $\mu\text{g.}$ of total phosphorus, was added to plasma and the total phosphorus present was estimated as described above. After subtraction of

the total phosphorus present in untreated plasma, a recovery of $97 \pm 4.6\%$ (S.D.) of the added phosphorus was obtained. The total phosphorus in the chromatograph paper was found by analysing a known area of treated paper. The mean phosphate content of forty-four blanks was 0.07 $\mu\text{g.}$ of phosphorus/cm.² of paper. The mean recovery (after correction for blanks) of total added phosphorus

Table 1. *Percentage exchange of phosphate of human erythrocytes at 37°*

Values presented in column 2 indicate phosphate compounds found in blood taken from healthy male adults and incubated at 37° for 75–120 min. Such small differences in phosphate content as occurred between the two times are included in the estimate of error. The two fastest fractions have not been mentioned in this table for the reasons stated above. They accounted for only 0.2% of total phosphorus. R.S.A., Relative specific activity (counts/min./ $\mu\text{g.}$ of phosphorus in each fraction expressed as a percentage of counts/min./ $\mu\text{g.}$ of total phosphorus in the whole suspension). Overall R.S.A., R.S.A. obtained experimentally. Adjusted R.S.A., R.S.A. adjusted for unlabelled phosphorus (see text).

Phosphate fractions	Percentage of total P in fraction (mean of 14 values)	15 min. R.S.A. (means of four values)		60 min. R.S.A. (means of 12 values)	
		Overall	Adjusted	Overall	Adjusted
		(S.E.)	(S.E.)	(S.E.)	(S.E.)
2:3-Diphosphoglycerate	64.9 ± 1.1	0.8 ± 0.2	1.8 ± 0.5	4.7 ± 0.48	11.0 ± 1.12
Adenosine triphosphate	20.4 ± 0.5	5.4 ± 0.7	8.1 ± 1.1	16.9 ± 1.07	25.4 ± 1.61
Inorganic phosphate	11.2 ± 0.8	5.9 ± 0.8	5.9 ± 0.8	15.4 ± 0.87	15.4 ± 0.87
Hexose monophosphate	3.3 ± 0.4	1.0 ± 0.2	1.0 ± 0.2	2.5 ± 0.17	2.5 ± 0.17

* These values represent the total phosphate and R.S.A. of the combined 2:3-diphosphoglycerate and fructose 1:6-diphosphate fractions.

Table 2. *Action of drugs on the gross phosphate exchange of human erythrocytes*

Bold figures: difference between control and treated samples significant at 1% level. Italic figures: difference between control and treated samples significant at 5% level. R.S.A., see Table 1. Figures in parentheses give the number of observations.

Expt. no.	Donor	Treatment	Concn. (mm)	Total P mean $\pm$ S.E. ($\mu\text{g./ml.}$ of cells)	R.S.A. mean $\pm$ S.E.
5	D.J.	Nil	—	544.1 ± 24.3 (10)	6.7 ± 0.15
		Sodium thiopentone	0.25		5.1 ± 0.05
		Sodium thiopentone	1.0		2.2 ± 0.05
		Sodium pentobarbitone	0.25		6.7 ± 0.25
		Sodium pentobarbitone	1.0		5.6 ± 0.25
		Sodium barbitone	1.0		6.8 ± 0.20
6	D.K.	Nil	—	517.1 ± 20.5 (12)	7.4 ± 0.40
		Sodium hexobarbitone	1.0		7.1 ± 0.16
		Sodium hexobarbitone	2.0		5.8 ± 0.14
		Sodium barbitone	8.0		5.5 ± 0.10
		Urethane	1.0		7.2 ± 0.20
		Urethane	8.0		7.2 ± 0.00
8	D.P.	Nil	—	570.7 ± 6.0 (10)	6.0 ± 0.05
		Sodium thioquinalbarbitone	0.25		4.5 ± 0.05
		Sodium thioquinalbarbitone	1.0		3.2 ± 0.20
		Sodium quinalbarbitone	0.25		5.6 ± 0.00
		Sodium quinalbarbitone	1.0		5.1 ± 0.10
7	L.L.	Nil	—	479.1 ± 15.5 (8)	7.8 ± 0.05
		Sodium salicylate	2.0		5.8 ± 0.30
		Sodium salicylate	8.0		3.6 ± 0.15
		Phenylbutazone	2.0		2.2 ± 0.20
9	L.L.	Nil	—	569.1 ± 13.4 (8)	8.5 ± 0.40
		2:4-Dinitrophenol	0.5		5.5 ± 0.00
		Iodoacetic acid	0.5		4.6 ± 0.54
		Sodium fluoride	5.0		8.1 ± 0.20
10	D.C.	Nil	—	489.1 ± 8.5 (4)	6.7 ± 0.10
		2:4-Dinitrophenol	0.5		5.3 ± 0.20
3	R.S.	Nil	—	512.7 ± 14.3 (8)	5.3 ± 0.15
		Ethanol	0.25		5.0 ± 0.10
		Diethyl ether	0.02		5.3 ± 0.10
		Chloroform	0.02		3.9 ± 0.25

Table 3. *Effect of drugs on exchange of major phosphate fractions*

All estimates are based on duplicate samples. Bold figures: difference between control and treated sample significant at 1% level. *Italic figures*: difference between control and treated sample significant at 5% level. R.S.A., see Table 1.

Expt. no.	Treatment	R.S.A., mean $\pm$ S.E.			
		2:3-Diphosphoglycerate	ATP	Inorganic PO_4	Hexose monophosphate
8	Nil	2.2 $\pm$ 0.05	13.0 $\pm$ 0.0	12.8 $\pm$ 0.4	2.6 $\pm$ 0.15
	Sodium thiopentone (0.5 mM)	1.1 $\pm$ 0.0	8.8 $\pm$ 0.15	11.0 $\pm$ 0.3	1.7 $\pm$ 0.16
	Sodium salicylate (5.0 mM)	1.1 $\pm$ 0.05	6.3 $\pm$ 0.7	8.5 $\pm$ 0.7	1.6 $\pm$ 0.4
9	Nil	5.9 $\pm$ 0.3	17.9 $\pm$ 0.8	14.2 $\pm$ 1.1	3.3 $\pm$ 1.1
	2:4-Dinitrophenol (0.5 mM)	3.3 $\pm$ 0.2	7.9 $\pm$ 0.9	7.2 $\pm$ 1.6	—
	Phenylbutazone (2.0 mM)	1.1 $\pm$ 0.1	3.3 $\pm$ 0.1	3.0 $\pm$ 0.2	—

Table 4. *Action of drugs on glycolysis in human erythrocytes incubated at 37° in Krebs solution*

Gas phase: $\text{N}_2 + \text{CO}_2$ (95:5).

Drug	Concn. (mM)	$100 \times \left(\frac{\text{Production of } \text{CO}_2 \text{ by treated cells}}{\text{Production of } \text{CO}_2 \text{ by controls}} \right)$ (Mean $\pm$ S.E.*)
Sodium thiopentone	0.5	106.7 $\pm$ 6.7
Sodium salicylate	5.0	107.0 $\pm$ 1.5
Sodium fluoride	20.0	0.0

* Duplicate estimates.

and ^{32}P from each of twenty-three chromatograms was $98 \pm 11\%$ (S.D.) and $103 \pm 11.8\%$ respectively. It therefore appears that the methods described above for estimating the total phosphorus and ^{32}P in each fraction on the chromatograms are reasonably reliable.

Distribution of phosphorus and ^{32}P in fractions

About 65% of the total acid-soluble phosphorus was found in the fraction which contained 2:3-diphosphoglycerate and fructose 1:6-diphosphate: 20% as adenosine triphosphate; 11% as inorganic phosphate and adenosine diphosphate and 3% as hexose monophosphate. The amount of phosphorus in these fractions did not change in incubation between 75 and 120 min., and has been assumed constant during the period of exposure to [^{32}P]phosphate. Sixty minutes after adding [^{32}P]phosphate to the medium, the mean percentage of total intracellular phosphate exchanged was 6.9 ± 0.9 (S.D., n 17). Adenosine triphosphate was more heavily labelled than any other fraction (Table 1).

Effect of drugs. Agents found to depress the uptake of [^{32}P]phosphate are shown in Table 2, and include barbiturates, chloroform, sodium salicylate, phenylbutazone, dinitrophenol and iodoacetate. Substances found to be inactive were urethane (8 mM), ethanol (0.25 M), diethyl ether (0.02 M), sodium fluoride (5 mM), promethazine hydrochloride (0.05 mM), lysergic acid diethylamide (0.01 mM), cocaine hydrochloride (0.1 mM), chlorpromazine hydrochloride (0.1 mM) and 6-phosphogluconate (0.1 mM).

Action of drugs on the exchange of the major phosphate fractions. Sodium salicylate and sodium thiopentone depressed the ^{32}P uptake into 2:3-diphosphoglycerate and adenosine triphosphate more than that into the cellular inorganic phosphate fraction and hexose monophosphate. Neither drug affected the size of these fractions. Similar effects were seen with 2:4-dinitrophenol and phenylbutazone (Table 3).

Effect of drugs on glycolysis. Neither sodium thiopentone nor sodium salicylate had any effect on glycolysis (Table 4), whereas sodium fluoride completely inhibited this process in human erythrocytes.

DISCUSSION

Present estimates of the sizes of the fractions of the erythrocyte acid-soluble phosphate are compared with other published values in Table 5, and are in reasonable agreement with those already reported. Variation in uptake on different occasions (Table 2) may be due to differences between donors or to some other variation in experimental conditions; insufficient experiments were performed to settle this point. Of the various fractions, adenosine triphosphate is most heavily labelled after incubation for 15 and 60 min. This is in agreement with the results of Gourley (1952), Hoffman-Credner (1955) and Gerlach *et al.* (1958). It is therefore likely that adenosine triphosphate is the precursor of the other cell-phosphate fractions, presumably being formed by the combination of the inorganic phosphate of the medium with adenosine diphosphate or adenosine monophosphate. If adenosine

triphosphate were formed by the reaction of adenosine diphosphate and 1:3-diphosphoglycerate, as Prankerd & Altman (1954) suggest, it would be expected that inhibitors of triose phosphate dehydrogenase would reduce the uptake of [32 P]-phosphate by 2:3-diphosphoglycerate, with which the 1:3-diphosphoglycerate is assumed to be in equilibrium. It is unlikely that salicylate acted in this way, although it has been reported to inhibit triose phosphate dehydrogenase of muscle (Lutwak-Mann, 1942), because in the highest concentrations used here (5 mM) it did not depress glycolysis. Similarly, the uptake of [32 P]phosphate by barbiturates took place without inhibition of glycolysis, and it follows that phosphate uptake partly at least depends on some other process. The barbiturates and salicylates have been described as uncoupling agents of oxidative phosphorylation (Brody, 1955), and the effect of the classical uncoupling agent, 2:4-dinitrophenol, was therefore examined. This drug, and also phenylbutazone (reported by Adams & Cobb, 1958 to act as an uncoupling agent), acted like sodium salicylate and sodium thiopentone. It is reasonable to suppose that all these drugs inhibit the uptake of 32 P by uncoupling oxidative phosphorylation. It is possible, on the other hand, that they prevent the diffusion of phosphate into the cells, perhaps by blocking membrane pores. This possibility has not been excluded experimentally.

The difference in potency between the thio-barbiturates and their corresponding oxy-analogues reported above may be due to differences in the amount of drug fixed by erythrocytes. Gold-

baum & Smith (1954) found that homogenates of rabbit erythrocytes took up twice as much thiopentone as they did pentobarbitone. Such differences may be related to their relative fat solubility. Body fat takes up five to six times as much thiopentone as it does pentobarbitone. Thiopentone has an oil-water coefficient which is 11 times that of pentobarbitone (Mark *et al.* 1958). Since erythrocyte membranes are composed mainly of lipoprotein, it is probable that the difference in activity between the thio-barbiturates and their oxy-analogues is due to differences in their fat-solubility.

SUMMARY

1. The uptake of inorganic radioactive phosphate by fresh human erythrocytes has been observed over a period of 60 min.

2. Cell-phosphate fractions have been separated by paper chromatography and the total phosphate and 32 P contents of four fractions were estimated: (a) 2:3-diphosphoglycerate and fructose 1:6-diphosphate; (b) adenosine triphosphate; (c) inorganic phosphate; (d) hexose monophosphate.

3. Adenosine triphosphate was more heavily labelled than the other fractions at 15 and 60 min. after the addition of tracer.

4. Sodium thioquinalbarbitone (0.25 mM), sodium thiopentone (0.25 mM), sodium quinalbarbitone (0.25 mM), sodium pentobarbitone (1.0 mM), sodium hexobarbitone (2.0 mM), phenylbutazone (2.0 mM), 2:4-dinitrophenol (0.5 mM), iodoacetate (0.5 mM) and chloroform (0.02 M) reduced the phosphate exchange.

Table 5. *Phosphate content of the fractions of human erythrocytes according to several authors*

Where there are two lines of figures, the first line is the mean and the second is the range or the standard error. Where no range is given the values are calculated mean percentages. Dashes indicate that the substance was not sought. Separation methods: 1, differential solubility of barium and magnesium salts and hydrolysis; 2, paper chromatography of acid extract; 3, paper chromatography of neutral extract. ATP, adenosine triphosphate; ADP, adenosine diphosphate; F1:6-DP, fructose 1:6-diphosphate; HMP, hexose monophosphate; 2:3-DPG, 2:3-diphosphoglycerate.

Reference	Separation method	Percentage of total acid-soluble phosphate of erythrocytes					
		ATP	ADP	Inorganic PO_4	F1:6-DP	HMP	2:3-DPG
Gourley (1952)	1	32.2	Not detected	7.9	Not detected	13.0	34.3
Prankerd & Altman (1954)	2	13.5 11.2-14.5	6.5 5.5-7.7	19.3 14.9-24.5	4.4 3.5-5.6	Not detected	55.2 48.9-58.0
Rohdewald & Weber (1956)*	3	26.3 21.7-32.9	Not detected	14.0 11.2-18.0	17.0 4.0-27.0	Not detected	39.0 26.5-49.0
Gerlach, Fleckenstein & Gross (1958)	2	16.7 $\pm$ 0.54	4.69 $\pm$ 0.22	2.02 $\pm$ 0.22	6.98 $\pm$ 0.33	Not detected	58.13 $\pm$ 0.70
Whittam (1958)	2	16.7	6.0	—	—	—	57.2
Present author	3	20.4 $\pm$ 0.5 (S.E.)	Not detected	11.2 $\pm$ 0.8 (S.E.)	—	3.3 $\pm$ 0.4 (S.E.)	64.9 $\pm$ 1.1† (S.E.)

* These values are for whole blood.

† This fraction contains 2:3-diphosphoglycerate and fructose 1:6-diphosphate.

5. Sodium salicylate and sodium thiopentone depressed the ^{32}P uptake of 2:3-diphosphoglycerate and adenosine triphosphate to a greater extent than they depressed the ^{32}P exchange of inorganic phosphate and hexose monophosphate. These agents do not depress ^{32}P uptake by inhibiting triose phosphate dehydrogenase, for neither drug had any effect on glycolysis. The effects of 2:4-dinitrophenol and phenylbutazone resembled those of sodium salicylate and sodium thiopentone on the ^{32}P uptake of the phosphate fractions.

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Experiments on the Origin of Oak-Bark Tannin

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In earlier work (Hathway, 1958), it was concluded that the pyrogallol phenols are formed in oak leaves, translocated to the cambium and undergo oxidation there, and the resulting phlobatannin (which is a tail-to-tail polymer) is stored in the outer bark. In the present work an attempt has been made to study more closely the origin of 'tannins' in *Quercus pedunculata* Ehrh. Field experiments, which have been carried out throughout two seasons, were planned to throw light on (1) variation in the tannin content of stembark with age, (2) disturbances in the normal distribution of tannin in stembark, brought about by the ringing of the bark, (3) the translocation of phenolic precursors of the tannin in the sieve tubes of the phloem.

MATERIALS AND METHODS

Determination of the tannin in stembark. Young plants were obtained as required from Alice Holt nurseries, and the ages are calculated approximately from the time of the first transplantation. The entire stembark of each plant

was detached, dried to a 10% moisture content, and ground in a Wiley mill to 20-mesh. A portion of the bark was used for the analysis of moisture, and of tannin and non-tannin extractives by Grassmann, Endisch & Kuntara's (1951) semi-micro hide-powder method. Three different plants were utilized at 3-monthly intervals.

With older trees in the Forest of Dean, slivers of bark were detached from all sides of the main stem within a ring stretching from 3 to 4 ft. from ground level. The slivers were mixed and a representative sample was selected for analysis. Three different trees on each site were peeled in this way at 3-monthly intervals.

Method of ringing trees. During the first week in June 1957, two trees were selected on each site in Alice Holt forest, Hants, for bark-ringing experiments, and two further trees on each site for use as controls. A 6 in. ring of bark was detached at 1 ft. from ground level; care was taken not to impair the sapwood. All eight trees were felled during the last week in May 1958, when a sample of bark was immediately detached from the butt and crown of the main stem of each tree. Samples of stembark from the butts of the trees were detached from a cylinder, 1 ft. in height, immediately above the girdles on the ringed trees and from the corresponding location on the main stems of the controls. Samples of stembark from the crowns of the