16. Gregoire, C., Duchateau, G., *Arch. Biol.*, 1956, v67, 269.

17. Kaplan, H. S., Carnes, W. H., Brown, M. B., Hirsch, B. B., *Cancer Res.*, 1956, v16, 429.

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Effect of Oxalate on Turnover of Radioactive Phosphate by the Human Erythrocyte.*†‡ (26360)

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Although oxalate has been reported to be involved in glycolysis of human blood(1,2,3, 4,5,6,7), its specific role has not been completely determined. The present investigation was made to study further the effects of oxalate on uptake, distribution and loss of phosphate by the human erythrocyte using radioactive phosphate and radioactive oxalate. Distribution of oxalate between plasma and erythrocyte, turnover of phosphate by erythrocytes incubated with oxalate, role of potassium, magnesium, and calcium ions on the effect of oxalate upon phosphate metabolism, and the effect of oxalate on distribution of phosphate in some of the acid-soluble phosphate ester fractions of the erythrocyte were determined.

Method and materials. Blood drawn from young adults was stored in sterile bottles containing sodium heparinate. The pH of the fresh blood was measured with a Beckman Model G pH meter with a glass electrode. During incubation the blood was kept mixed and at a nearly constant pH of 7.4 by bubbling a gas mixture containing 95% oxygen and 5% carbon dioxide slowly in through a finebore glass tube.

Since Weinhouse and Friedmann(12) demonstrated that oxalate is not metabolized in whole blood, radioactive oxalate (0.00075 Molar) was added and its distribution in whole blood determined by measuring the radioactivity of plasma, hemolyzed whole blood, and hemolyzed washed red cells. Radioactive analysis of a paper electrophoretic chromatogram from the hemolyzed whole blood was done to establish whether or not the oxalate was bound to protein.

Radioactive phosphate was used to measure turnover of phosphate by erythrocytes as affected by oxalate alone or in conjunction with calcium, magnesium, and potassium ions. Fresh erythrocytes were washed with physiological saline phosphate-buffer solution of pH 7.4, incubated for 30 minutes at 37.5°C in their own serum or the above mentioned physiological solution containing radioactive P^{32} inorganic phosphate, washed, then 1 volume further incubated one hour at 37.5°C in 50 volumes of saline-glycine buffer (0.15 molar NaCl, 0.0024 molar glycine, pH = 7.4). After the second incubation the supernatant solution was withdrawn and erythrocytes were washed 3 times with saline, then hemolyzed with 0.01 Molar sodium carbonate in 27% ethanol. P^{32} activity per volume of supernatant and cell hemolysate was determined. These data were used in the equation below for determining the fraction loss. In view of the constancy of turnover rate at one hour, this single determination was used for the assay. Since loss-rates varied with concentration of the phosphate in the uptake media, a standard physiological saline phosphate-buffer medium [0.30% glucose, 0.09% NaCl, 0.15 molar NaH_2PO_4-Na_2HPO_4, pH 7.4], which was found to give reproducible turnover rates, was used throughout the experiment. All tested uptake media were compared to it. Radioactivity measurements were made on "infinitely thick liquid specimens" using a lead shielded

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thin-walled glass Geiger-Müller counter. Counting time sufficient to give a standard deviation of less than 3% was used.

Whole blood was incubated with or without added oxalate (0.00075 molar) over a period of 4 hours. Uptake of radioactive phosphate by the acid-soluble phosphate esters of the erythrocytes was determined after separation by chromatography(9). Total phosphate of the esters was determined on the wet washed samples(10) by the method of Berenblum and Chain(11).

TABLE I. Distribution of C^{14} Oxalate after 4 Hours Incubation at 37.5°C with Whole Blood.

Blood fraction	Counts/min./ml
Plasma	205 ± 6
Washed cells	415 ± 9
Initial counts for whole blood	288 ± 6
$\left(\frac{\text{cells}}{\text{plasma}} = 42\%\right)$	

Values indicated (±) are stand. dev. of means of 4 samples.

Results of the present studies are shown in Tables I, II, III, and IV and Fig. 1. Oxalate was distributed significantly in favor of the erythrocytes (Table I).

In Table II, per cent of radioactivity lost from erythrocytes previously incubated with radioactive phosphate is expressed by the equation:

$$\text{Fraction lost} = \frac{\text{Activity of supernatant sol.}}{\text{Activity of supernatant sol.} + \text{activity of erythrocyte hemolysate}}.$$

Rate of P^{32} lost from red cell is dependent only on the P^{32} present in the red cell.

$$\left(-\frac{dA}{dt} = kA;\ A = \text{remaining activity}\right)$$

Varying the concentration of oxalate in the incubation medium markedly affects loss of radioactive phosphate from the washed erythrocytes (Fig. 1). The results show that above a concentration of 0.00075 molar oxalate the loss rate becomes constant. There is no significant change in rate of radioactive phosphate uptake among the uptake incubation media containing different concentrations of oxalate.

TABLE II. Rate of Loss of P^{32} from Erythrocytes after Incubation with P^{32} Phosphate.

Time (hr)	% P^{32} remaining in cells after incubation in: Serum	Saline-phosphate buffer	Specific reaction rate constant [k = (conc./time) / (avg conc.) = hr^{-1}]: Serum	Saline-phosphate buffer
0	100	100		
.50	82	76	.397	.546
1.00	67	58	.400	.544
1.50	55	45	.399	.533

Values indicated are avg of 4 trials.

Potassium, magnesium, and calcium ions affect oxalate inhibition of phosphate loss (Table III).

The effect of oxalate on distribution of phosphate into the adenosine-triphosphate (ATP), adenosinediphosphate (ADP), combined hexose-triose phosphates (H – 6 – P + G – 3 – P), 2, 3-diphosphoglyceric acid(2,3-DPGA), and inorganic phosphate (Pi) fractions of the acid-soluble phosphate ester fraction of the erythrocyte was studied. Table IV shows distribution of phosphate and radioactive phosphate in these fractions. These results have interpretive value in view of the demonstration by Gourley(13) of a lack of non-metabolic phosphate exchange between inorganic phosphate and glycolytic intermediates. Other oxalate effects that have been described in erythrocytes, such as decreased glucose utilization(1) and decreased enolase activity(4), have been verified in this laboratory. In this investigation ADP and ATP concentrations, their P^{32} ac-

TABLE III. Relative Loss of P^{32} from Washed Human Erythrocytes after (1) Incubation in Physiological Phosphate-Saline Buffer (Control) and (2) Incubation in the Buffer Plus Added Cations.

Molarity of added ions in incubation medium: K	Mg	Ca	Relative loss: Control	Oxalate
			100	62
.006			100	56
	.0015		100	94
		.005	136	136
.006	.0015		100	62
		.005	136	117
	.0015	.005	100	100
.006	.0015	.005	103	100

Values are avg of 4 trials.

TABLE IV. Effect of Oxalate on Abundance of Erythrocyte Phosphates after 4 Hours Incubation of Human Blood at 37.5°C with $P^{32}O_4$.

Fraction	Control			With oxalate		
	P^{32}	P^{31}	P^{32}/P^{31}	P^{32}	P^{31}	P^{32}/P^{31}
ADP	125 ±11.0	46 ± 2.8	2.70 ± .32	86 ± 4.7	54 ± 3.7	1.60 ± .05
ATP	114 ± 1.8	48 ± .4	2.40 ± .04	76 ± 7.0	46 ± 2.8	1.70 ± .05
H-6-P – G-3-P	54 ± 4.2	24 ± 1.6	2.30 ± .25	92 ± 5.3	37 ± 3.7	2.50 ± .32
2,3-DPGA	472 ± 6.1	202 ±21.0	2.30 ± .20	612 ± 9.0	245 ± 3.7	2.40 ± .06
$P_{inorg.}$	64 ± 5.8	39 ± .4	1.60 ± .12	39 ± 2.2	22 ± 1.6	1.80 ± .19

P^{32} values in counts/sec./ml blood cells (vol of blood cells detn. from original hematocrit).
P^{31} values in μg/ml blood cells.
Values indicated (±) are stand. dev. of means of 4 samples.

tivities, and their specific activities were lowered: inorganic phosphate concentration and its P^{32} activity were lowered while its specific activity was slightly raised; the 2,3-DPGA concentration, its P^{32} activity, and its specific activity were all raised; and concentration of the combined (H – 6 – P and G – 3 – P) fraction, its P^{32} activity, and its specific activity were all raised.

Discussion. It was demonstrated that oxalate concentrates in the erythrocyte, and by paper electrophoresis that none of the oxalate of the plasma, hemolyzed washed cells, or hemolyzed whole blood migrated with the albumins, globulins, or hemoglobin. It is postulated that oxalate occurs in the erythrocyte in an unbound form capable of reacting with glycolytic cofactors to bring about a lowering of phosphate loss from the erythrocytes (Table II).

In general, magnesium or calcium, alone or in combination, removed the oxalate effect and potassium enhanced it slightly. Since the glycolytic metabolism of inorganic phosphate is considered to be magnesium-dependent, it might be postulated that oxalate produces its effect by tying up magnesium. Addition of excess magnesium or calcium overcomes all of the oxalate effect.

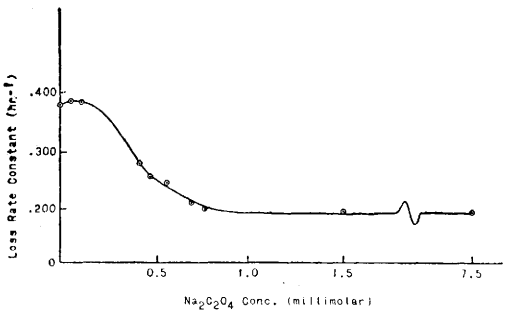

FIG. 1. P^{32} loss rates after incubation with P^{32} phosphate and varying amounts of sodium oxalate. Values on graph are avg of 4 samples with a maximum stand. dev. from means of ± 5%.

The effect of oxalate on distribution of phosphate in the acid-soluble phosphate ester fractions of the erythrocyte may in each case be attributed directly or indirectly to those reactions in the "Embden-Meyerhof" glycolysis scheme that depend on available magnesium. This is further evidence to support the theory that oxalate binds magnesium thus rendering it unreactive as a co-factor.

Summary. When incubated with human blood, C-14 oxalate is distributed between cells and plasma with a significant preference for the cells. After incubation with inorganic P^{32} phosphate and oxalate, rate of loss of P^{32} from the erythrocytes suspended in buffered-normal saline was lower in the case of erythrocytes pre-incubated with oxalate, reaching a minimum value with 0.00075 molar oxalate. Inhibition of P^{32} loss from erythrocytes by oxalate was slightly enhanced by potassium but was reversed by calcium and magnesium ions in physiological concentrations. Concentration of the cellular organic phosphates of the acid-soluble ester fraction changes significantly after incubation of erythrocytes with inorganic P^{32} and oxalate. The changes caused by oxalate may in each case be attributed directly or indirectly to a reduction in available magnesium.

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1. MacLeod, J. J. R., *J. Biol. Chem.*, 1913, v15, 497.

2. Pappius, H. M., Denstedt, O. F., *Can. J. Biochem. Physiol.,* 1954, v32, 338.

3. Lohmann, K., Meyerhof, O., *Biochem. z.,* 1934, v273, 60.

4. Bueding, E., Goodhart, R., *J. Biol. Chem.,* 1941, v141, 931.

5. Hahn, A., Ottawa, H., *Biol.,* 1937, v98, 81.

6. Long, C., *Biochem. J.,* 1944, v38, 447.

7. Reid, Allen, F., Jeanes, J. K., Gilmore, R. C., Jr., Robbins, M. C., *Am. Chem. Soc.* 128th Meeting, *Abst. of Papers,* 1955 Sept., 14C.

8. Reid, A. F., Gilmore, R. C., Jr., Jeanes, J. K., Robbins, M. C., *Cancer,* 1957, v10, 161.

9. Caldwell, P. C., *Biochem. J.,* 1953, v55, 458.

10. King, E. J., *ibid.,* 1932, v26, 294.

11. Berenblum, I., Chain, E., *ibid.,* 1938, v32, 295.

12. Weinhouse, S., Friedmann, B., *J. Biol. Chem.,* 1951, v191, 707.

13. Gourley, D. R. H., *Nature,* 1952, v169, 192.

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Constant Relation Between Blood Level of Thyrotropin and Total Thyrotropin in Thyroidectomized Mice. (26361)

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Several reviews concerning regulation of thyrotropin (TSH) release have recently appeared(1-4). A dual control seems to exist. One is a fundamental, non-neural regulatory mechanism, while the other is a neural mechanism for emergency release of TSH.

This paper presents quantitative data concerning the non-neural release of TSH. Levels of TSH in blood and pituitary or tumor were determined in two kinds of radiothyroidectomized mice, (1) those with enlarged pituitaries only, and (2) those with transplantable, TSH-producing tumors of pituitary origin. In both cases the TSH level in the blood was proportional to total amount of TSH in the pituitary or the tumor.

Methods. All mice were of the C57Bl strain and were radiothyroidectomized at about 3 months of age using approximately 200 μc of I^{131}. The 2 transplantable pituitary tumor lines 77D and 101D were obtained originally from Dr. Jacob Furth(5) and were carried for 11 and 7 transfer generations, respectively. For each transfer, suspensions of tumor tissue were injected subcutaneously into the hind leg of mice, radiothyroidectomized about one month previously. Tumors weighing 2 to 8 g developed after 6 or more months.

TSH was measured in baby chicks by thyroidal I^{131} depletion(6). Potencies are in terms of USP units. All pituitary and tumor tissues were frozen as soon as possible after removal and then lyophilized and milled to a 60 mesh powder in a Wiley mill. A suspension of this powder in 0.1 M $NaHCO_3$ was injected into chicks for estimation of potency. Heparin was added to all blood samples. Samples of blood ($>$ 1 ml) obtained from the vena cava of mice with tumors were pooled, centrifuged and the plasma used for bioassay. Blood samples, ($<$ 1 ml) obtained from individual mice with enlarged pituitaries, were assayed directly.

Results. Blood level of TSH in thyroidectomized mice with large pituitaries. The pituitaries of mice become greatly enlarged 12-18 months after radiothyroidectomy(7). Nineteen such mice with large pituitaries were killed. The blood was collected and assayed. The pituitaries were weighed and either assayed or transplanted to start new tumor lines. The positive relationship between pituitary weight and concentration of TSH in the blood is shown in Fig. 1. Most of the pituitaries that were assayed had a potency between 0.1 and 0.2 U of TSH per mg of dry pituitary weight. Hence, a unitage scale has been added to facilitate comparison with Fig. 2. The small square at the origin represents values estimated for TSH levels in normal mice (about 1 mU/ml of blood and 40 mU/2 mg of pituitary).

The milliunits of TSH per ml of blood