EVIDENCE OF ACTIVE TRANSFER OF CERTAIN NON-ELECTROLYTES ACROSS THE HUMAN RED CELL MEMBRANE

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In discussions of cellular permeability frequent reference is made to the peculiar sensitivity of some mammalian erythrocytes to small traces of copper, observed in studies of the penetration of glycerol into these cells. The retarding effect of copper on hemolysis of human red cells in isosmotic glycerol solutions was first reported by Jacobs and Corson (1934); Jacobs and his group (1938) have subsequently demonstrated this action in the rat, rabbit, and guinea pig, though not in many other mammals tested. With suitable precautions against interference by extraneous protein, Jacobs and Stewart (1946) detected the inhibition of entry of glycerol into the human red cell by copper at concentrations around 10^{-7} M. With 90 per cent inhibition, the amounts of copper required could not have covered more than about 1 per cent of the total cell surfaces involved; furthermore, no diminution in the effect was found when the cells were quickly removed and replaced from a fresh suspension, so that only a small fraction of even this small amount of copper was actually removed in the inhibitory processes. These considerations clearly suggested a special mechanism for glycerol entry into these cells, apparently localized in limited regions on the cell surfaces. Davson and Reiner (1942), in discussing the active transfer of sodium across the cat erythrocyte membrane, called attention to the evidence then at hand of an analogous active movement of glycerol in the human red cell.

Such small quantities of copper as those found effective in this connection typically show inhibitory effects on certain types of enzymatic reactions (Hellerman, 1937; Rapkine, 1938). Enzymes whose activity depends on readily available sulfhydryl (thiol) groups are often interfered with by small traces of copper, as well as by various other oxidizing agents, and by alkylating or mercaptide-forming agents (Barron and Singer, 1945). Thus it seemed expedient to test the effect on glycerol hemolysis of some of these agents which resemble copper in their inhibitory effects on other systems. The results of these experiments suggested that the entrance of glycerol into the human erythrocyte might involve the activity of a sulfhydryl-containing enzyme or at least of some sulfhydryl groups located at the cell surfaces.

Later experiments in which volume changes were followed by the photo-

metric method developed by Ørskov and Parpart lent further support to this suggestion. By this means it was also possible to study the rate of penetration of glucose, which enters the human red cell only very slowly, and in isosmotic solutions of which osmotic hemolysis is never attained. The experiments to be described seem to indicate an active transport, on the part of the cell membrane, of both glycerol and some hexoses, though not by identical mechanisms in the two instances.

Materials and Methods

Human erythrocytes were used throughout; blood was drawn from the antecubital vein and citrated, or by lancet and massage from the finger-tips into a large volume of saline solution. The cells were washed, in either case, by several successive centrifugations in relatively large volumes of the suspension medium; the final suspensions were refrigerated at about 5°C. until used. Cells were used for as long as 8 days following their withdrawal from the body, but were always washed several times and resuspended in fresh saline solution on the day of use. The concentrations of suspension used varied as the plan of the experiments and the details of the apparatus were changed, but were always such that the cells represented a very small fraction of the total volume.

The experiments on hemolysis were performed in pyrex test tubes of 7 ml. capacity; 1 to 3 drops of a cell suspension in saline solution were added to 5 ml. of the solution to be tested, and the time to hemolysis recorded with a stop-watch. The criterion for hemolysis was the visibility through the tube of a linear source of light; this crude method is more than sufficiently reliable to reveal differences of the order of magnitude of those to be reported.

The photometric cytometer used varied in exact form as the experiments progressed. The light source was a single straight-coil filament headlight bulb operated from a 6.3 volt Sola constant-voltage transformer. The light passed through a filter of $CaCl_2$ solution, or through the water in a constant-temperature jacket, and thence through a flat sided vessel of 7 to 40 ml. capacity, containing the cell suspension. The central beam passing through the suspension fell on a Weston photronic cell, type 3, model 594 GB, attached to a galvanometer, either Leeds and Northrup type R2500, or Rubicon type L, from which the deflection was observed on a frosted plastic scale. All experiments, except as specifically noted otherwise, were performed at the ambient room temperature.

The saline solution used in the earlier work was simply M/6 NaCl, buffered at pH 7.1 with M/50 sodium phosphates. Later, a balanced solution containing small amounts of $CaCl_2$, $MgCl_2$, and KCl, approximately at plasma concentrations, was used; and during one stage of the work, the medium was buffered at pH 6.5 rather than at pH 7.1. None of these alterations affected the processes studied in any obvious manner, except that the effects of the cupric ion were interfered with in any medium containing appreciable amounts of calcium ion and alkaline phosphate (see Lampitt, Clayson, and Barnes, 1945).

I. GLYCEROL UPTAKE

Results

(*a*) *Experiments with the Hemolysis Method.*—Significant delays in the hemolysis of human erythrocytes in isosmotic (0.3 M) glycerol solutions (buffered at pH 7.1 with M/50 sodium phosphates) were seen in the presence of Cu^{++}, Hg^{++}, I_2, and *p*-chloromercuribenzoate. These delays could always be prevented, and in many instances could be reversed, by the addition of suitable quantities of cysteine, glutathione, or other substances containing sulfhydryl or other groups which might compete for the inhibitors with the hypothetical active sulfhydryl groups at the cell surfaces. Typical data are presented in Table I; the concentrations of inhibitors and reactivators shown here are of the order of magnitude of the minimal effective concentrations usually found; though there was considerable variation in this respect, dependent primarily on the time elapsed between the last washing in saline solution and the use of the cells in the test. As noted by Jacobs, sensitivity of the cells to copper is much higher after thorough washing than after the suspension has stood for even a relatively short time, presumably because of the protective effect of small amounts of protein leaking from the cells. This same phenomenon was very evident with respect to the other inhibitors used. The effective concentrations of the reactivating agents were also subject to such variations, and were of course largely dependent on the concentration of inhibitor used. Usually, however, the molecular concentration of the reactivating substance had to equal, or exceed by 1 to 2 times, the concentration of the inhibitor used. Glutathione, cysteine, and thioglycolic acid, each of which contains a readily available thiol group, prevented inhibition whenever present in such concentrations. Alanine was ineffective against I_2 inhibition, and prevented Cu inhibition only at concentrations of about 30 times that of the inhibitor. Ascorbic acid reacted with iodine to prevent its effect on the cells, but had no influence on Cu inhibition. If addition of the sulfhydryl-containing substance was delayed until sometime after the inhibitor had made contact with the cells, rather than simultaneously with the inhibitor, the prevention of the inhibition was more difficult to accomplish (requiring a higher concentration of reactivator, and bringing about hemolysis less promptly). Instances of this procedure are also included in Table I; in one case (last section of the table) is shown the progressively diminishing effect of cysteine as continued exposure to *p*-chloromercuribenzoate is allowed. This relation is characteristic also of reactivation of various enzyme systems similarly inhibited (Barron and Singer, 1945).

The most pronounced effects in delaying glycerol hemolysis were obtained with the mercaptide-forming *p*-chloromercuribenzoate; this agent was there-

fore tested in isosmotic solutions of ethylene glycol, diethylene glycol, and monoacetin, (buffered like the glycerol solutions previously used) to determine

TABLE I

Reversible Inhibition of Osmotic Hemolysis by Glycerol

Inhibitor	Reactivator			Hemolysis time in isosmotic glycerol			
	Nature	Concentration	Time added	Without inhibitor	With inhibitor	With inhibitor and reactivator	
						Total time	Interval
		M.10^{-5}	*min.*	*min.*	*min.*	*min.*	*min.*
$CuCl_2$, 10^{-5} M	Thioglycolic acid	2.5	0	1.1	27	1.0	1.0
		4	2			4.4	2.4
	Glutathione	3	0			2.0	2.0
		4	2			4.6	2.6
	Alanine	30	0	1.5	19	1.2	1.2
		30	1			3.3	2.3
	Cysteine	3	0			1.2	1.2
		3	1			2.9	1.9
Iodine, $8 \cdot 10^{-5}$ M	Thioglycolic acid	3.5	0	1.1	90	0.7	0.7
		3.5	2			3.9	1.9
	Glutathione	3	0			1.1	1.1
	Ascorbic acid	2	0			1.1	1.1
	Cysteine	6	0	1.2	85	1.0	1.0
		30	2			2.3	0.3
$HgCl_2$, $8 \cdot 10^{-6}$ M	Glutathione	8	0	1.4	7	0.8	0.8
p-ClHg-benzoate, 10^{-3} M	Thioglycolic acid	400	10	1.9	29	12.7	2.7
	Glutathione	80	10			14.5	4.5
	Cysteine	130	0	2.0	250+	1.7	1.7
			0.3			2.1	1.8
			5			7.0	2.0
			20			24	4.0
			45			53.5	8.5
			180			200	20

whether the inhibitory effect on glycerol hemolysis applied generally to osmotic hemolysis by similar substances. Specimen results are presented in Table II.

Of the three substances, only monoacetin showed any similarity to glycerol in this respect, and the inhibition of monoacetin hemolysis is not so pronounced as is that of glycerol hemolysis. The process interfered with seems to be moderately specific for glycerol. This parallels the observations of Jacobs and Corson with respect to the Cu inhibition.

Other inhibitors tested failed to affect the rate of glycerol hemolysis; iodoacetate, though a strong inhibitor of many sulfhydryl-containing enzymes by reason of its alkylating reaction with such groups (Dickens, 1933), had no influence on glycerol hemolysis in concentrations up to 0.02 M. Other ineffective inhibitors, tested because of various suggestions, were hydroxylamine, up to $3 \cdot 10^{-3}$ M; NaF, up to 10^{-2} M; NaCN, up to 10^{-2} M; and H_2O_2, up to 1.2 per cent. The arsenical "mapharsen" (3-amino-4-hydroxyphenylarsineoxide hydrochloride), which reacts with some sulfhydryl enzymes to form an inactive

TABLE II

Effect of p-Chloromercuribenzoate on Osmotic Hemolysis by Several Related Penetrating Non-Electrolytes

Test penetrant	Hemolysis time in isosmotic penetrant	
	Without inhibitor	With p-ClHgB, 10^{-3} M
	min.	*min.*
Glycerol	0.7	60
Monoacetin	1.1	3.2
Ethylene glycol	0.3	0.3
Diethylene glycol	0.6	0.6

complex, appeared to inhibit glycerol hemolysis irreversibly at concentrations of 0.2 per cent or more (LeFevre, 1946), but this effect was later attributed to the osmotic protection afforded by the sucrose and Na_2CO_3 with which this drug is mixed in the medicinal ampoules from which it was obtained.

(*b*) *Experiments by the Photometric Method.*—The use of data based on the timing of hemolysis, in the interpretation of changes in cellular permeability, is always complicated by the possibility that observed differences may reflect changes in fragility or equilibrium conditions rather than actual changes in rate of penetration. Thus it was desirable to check the results of the above experiments by means of some other method which more definitely followed the changes in volume. This was provided in the photometric method developed by Ørskov and Parpart, which had the further advantage that the volume changes of the cells might be followed in media much more nearly normal for the cells than were the isotonic non-electrolyte solutions used with the hemolysis technique.

The inhibitory effects of cupric and mercuric ions, iodine, and *p*-chloromercuribenzoate upon the rate of entry of glycerol, and the reversibility of this

inhibition by sulfhydryl groups, were thus verified. Sample records of typical instances of inhibition are shown in Figs. 1 and 2. The concentrations of the mercaptide-forming inhibitors required to produce inhibition under these circumstances were somewhat higher than those necessary to inhibit hemolysis; these differences were minimized, if not absent, when the non-penetrating non-electrolyte sucrose in isotonic concentration was substituted for the electrolyte medium (Fig. 1). Parallel tests with thiourea, chosen because its normal rate of entry into the cells is somewhat less than that of glycerol, showed no such inhibition (Fig 2). Thus the inhibitory effects of these sulfhydryl reagents on glycerol hemolysis is attributable to a real decrease in the rate of entry of glycerol in their presence.

The effect of one particular inhibitor, the glucoside phlorhizin, may be of special interest, since this substance is so specifically active on the transfer mechanisms in the kidney tubule (Walker and Hudson, 1937) and the intestinal mucosa (Nakazawa, 1922; Wertheimer, 1933; Donhoffer, 1935), in which phosphorylation seems definitely to be involved (Lundsgaard, 1933; Laszt, 1935). The behavior of phlorhizin in delaying the entry of glycerol into human erythrocytes is shown in Fig. 2(*b*).

Other more or less likely inhibitors without demonstrable effect include mapharsen, alloxan, sodium arsenite, iodoacetate, fluoride, cyanide, maleate, and azide; Parpart, Barron, and Dey (1947) found no effect with *p*-carboxyphenylarsineoxide, iodoacetamide, iodosobenzoate, or cadmium, but inhibited glycerol hemolysis with chloropicrin.

Discussion

Parpart, Barron, and Dey (1947) suggested that the effect of inhibitors other than copper might be attributable not to interference with a transfer process, but to production of such a change in the hemoglobin structure that the cell is no longer free to alter its volume osmotically in the normal manner. More investigation of this possibility is required; it is evident that exposure of the cells to such agents as *p*-chloromercuribenzoate, at 10^{-3} M, is not without direct effect on the cell volume, inducing within a few minutes a slight shrinkage (or at least an increase in opacity) which is not apparently reversed upon addition of the reactivators. This shrinkage is noticeable in several of the figures. Iodine, at inhibitory concentrations, caused discoloration of the hemoglobin. However, the pronounced effects of the inhibitors on rate of volume change in glycerol were not observed with thiourea; and the delay in hemolysis, produced by *p*-chloromercuribenzoate in isosmotic solutions of glycerol or monoacetin, was not detectable in ethylene glycol or diethylene glycol. Further, the reversal of inhibition upon addition of substances furnishing sulfhydryl groups indicates that the action of the inhibitors in these experiments is through their effects on such chemical groupings; so that the interpretation of Parpart *et al.*

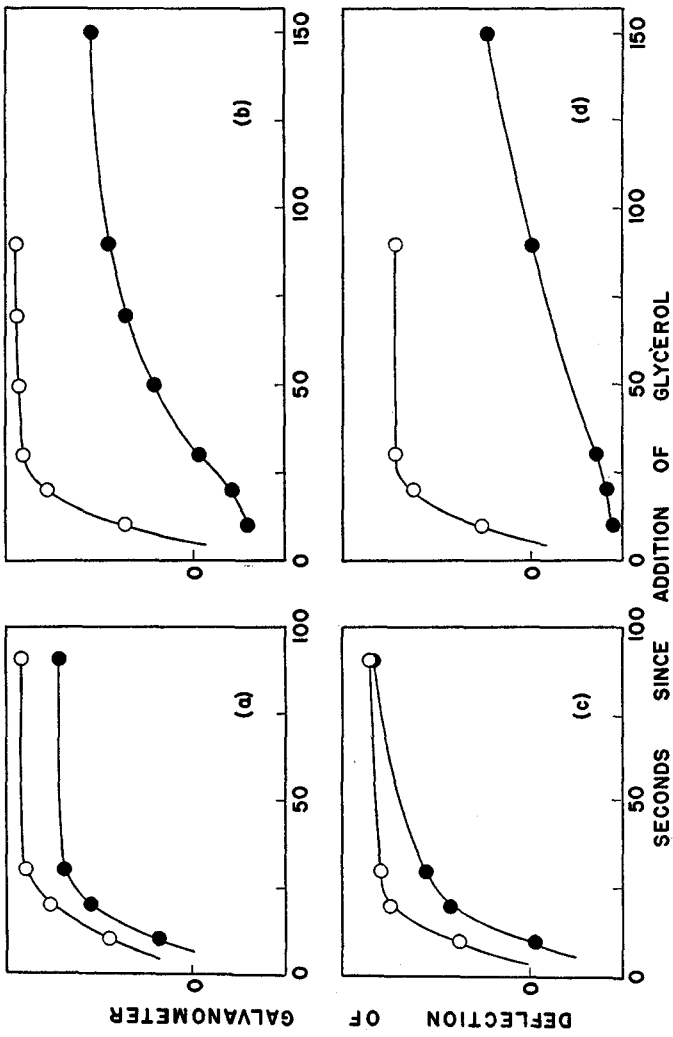

FIG. 1. Inhibition by *p*-chloromercuribenzoate of swelling in glycerol-saline solutions. At zero time, 5 ml. 2.4 M glycerol added to 35 ml. cell suspension.

Solid circles—with *p*-ClHgB,

(*a*) 0.001 M, in isotonic NaCl medium.

(*b*) 0.002 M, in isotonic NaCl medium.

(*c*) 0.001 M, in isotonic KCl medium.

(*d*) 0.001 M, in isotonic sucrose medium.

Open circles—without inhibitor, in same media.

Deflections plotted as movement from position prior to addition of glycerol; upward deflection indicates swelling of cells. Difference between initial and terminal levels results also from dilution of suspension.

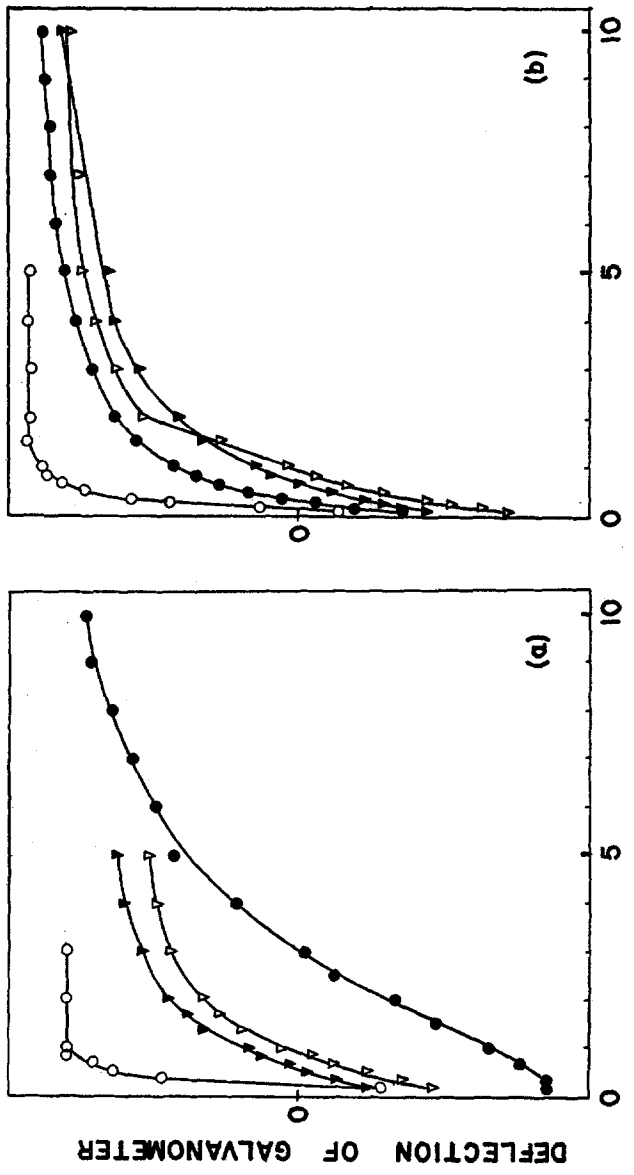

FIG. 2. Inhibition of swelling in glycerol-saline solutions, and absence of inhibition in thiourea-saline solutions.
Open symbols—without inhibitor. Circles—glycerol uptake.
Solid symbols—with inhibitor. Triangles—thiourea uptake.
(*a*) At zero time, 5 ml. 2.4 M glycerol or 1.2 M thiourea added to 35 ml. cell suspension in isotonic KCl, with or without *p*-chloromercuribenzoate, 0.002 M.
(*b*) At zero time, 1 ml. 1.8 M glycerol or 1.8 M thiourea, in the suspension medium, added to 5 ml. cell suspension in balanced saline medium, with dextrose at 0.05 M, with or without phlorhizin, 0.005 M.
Deflections plotted as in Fig. 1.

would necessitate postulation that the suggested paracrystalline state is readily reversible for some time by this means. Also, as will be reported in the next section, effective inhibitory concentrations of mercuric ion and of *p*-chloromercuribenzoate in respect to hexose uptake are on the order of 10^{-6} to 10^{-5} M, or 100 to 1000 times more dilute than those used in the experiments

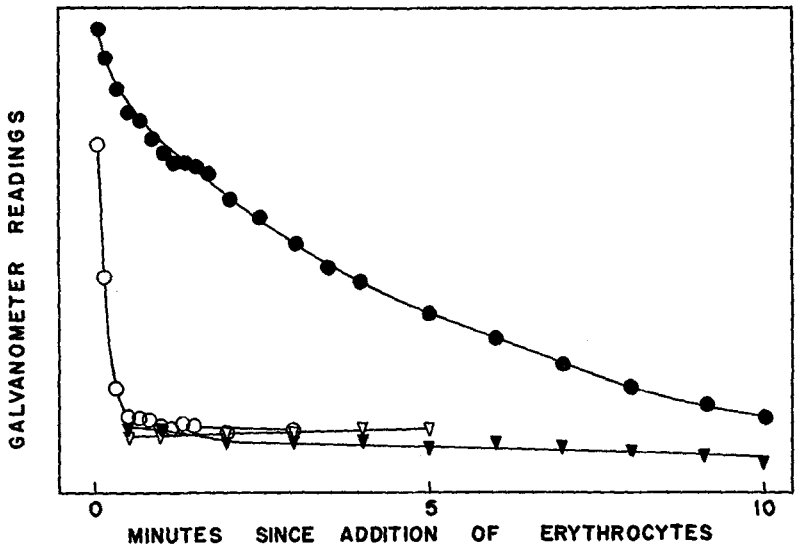

FIG. 3. Inhibition by *p*-chloromercuribenzoate of exit of glycerol from cell interior.

Circles—cells previously equilibrated with glycerol, 0.4 M, in buffered sucrose, 0.3 M.

Triangles—cells previously in sucrose medium alone.

At zero time, 1 ml. cell suspension added to 5 ml. sucrose medium.

Open symbols—without inhibitor.

Solid symbols—with *p*-ClHgB, 10^{-3} M.

Identical patterns were obtained with saline medium, but higher concentrations of inhibitor were required, with less pronounced effect, as also with respect to entry of glycerol.

with glycerol, in which the cells at least retain their identity for several hours. And the same inhibition by $CuCl_2$ or *p*-chloromercuribenzoate of volume changes in glycerol is seen with respect to the exit of this substance from the cell as with respect to its entrance (Fig. 3).

The inhibitory effects of the group of agents used suggested that the transport of glycerol into the human red cell was effected by an active metabolic system in which at least one essential link involved a sulfhydryl group. The pattern of effectiveness and ineffectiveness of the various types of sulfhydryl inhibitors tested indicated further, following Barron and Singer (1945), that

the sulfhydryl groups concerned were of the relatively unavailable type, requiring, except for the peculiar sensitivity to copper, rather drastic or specific chemical attack to be inhibited. The most obvious preliminary hypothesis, by analogy with known instances of active transport of polyhydric molecules in the kidney tubule and the intestinal mucosa, was that the critical process involved was a phosphorylation. Adenosine triphosphate can supply phosphate to glycerol (Gunsalus and Umbreit, 1945); and the apparent sulfhydryl-con-

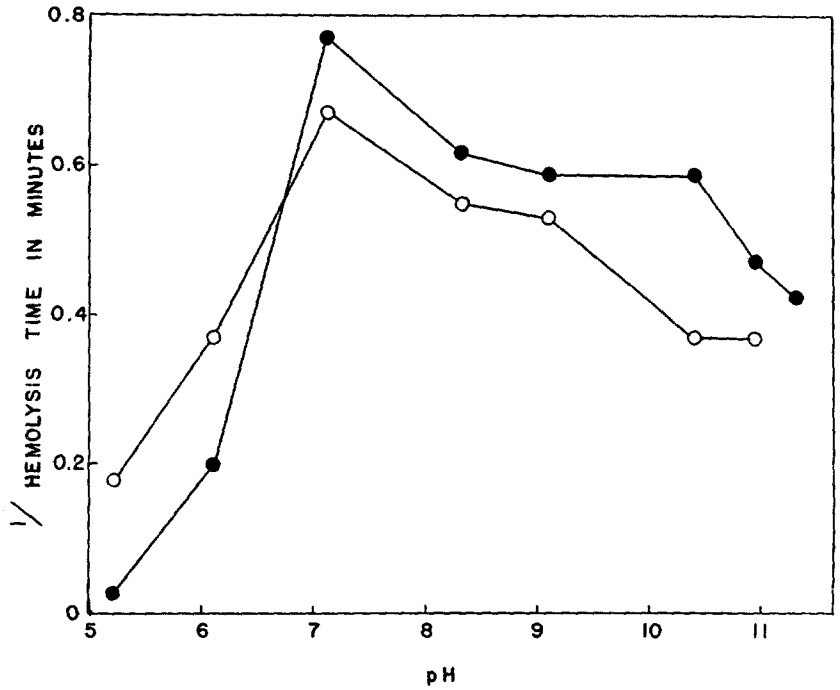

FIG. 4. Influence of pH on rate of attainment of hemolysis in buffered isosmotic glycerol (solid circles) and monoacetin (open circles).

taining enzyme concerned in these experiments may resemble adenosine triphosphatase. The latter is not affected by iodoacetate or iodoacetamide (Needham, 1942), but is reversibly inhibited by copper (Bailey, 1942; Binkley, Ward, and Hoagland, 1944) and by *p*-chloromercuribenzoate, though only questionably by the arsenicals (Singer and Barron, 1944); and is strongly inhibited by iodine (Ziff, 1944); these inhibitions being reversible by addition of cysteine, glutathione, or, to some extent, by addition of ascorbic acid. The failure of hydrogen peroxide, which prevents activity of ATPase (Mehl, 1944; Ziff, 1944), to inhibit the glycerol transport mechanism may be attributable to rapid destruction of the peroxide by red cell catalase.

A further particular in which the activity of the apparent glycerol transport mechanism may be compared with that of ATPase is with respect to the influence of pH. Fig. 4 shows the effect of pH on the rate of attainment of hemolysis in isosmotic glycerol and monoacetin solutions (with sodium phosphate buffer, M/50). These pH-activity curves resemble those of myosin ATPase in showing a pronounced inhibition on the acid side, but differ considerably in the position of the optimal pH; which for myosin ATPase lies in the neighborhood of pH 9 (Bailey, 1942; Singher and Meister, 1945). However, these curves parallel very closely those given by Kalckar (1944) for ATPase from potatoes; and Mehl (1944) describes a second optimum for myosin in the vicinity of pH 7.

Jacobs, Glassman, and Parpart (1935; 1938) have grouped the erythrocytes of a large number of mammalian species into two classes: those which show Cu sensitivity with respect to hemolysis in glycerol, and those which do not. Glycerol hemolysis in the sensitive group generally shares other properties absent in the other class: sensitivity to pH, CO_2, alcohols (Jacobs and Parpart, 1937); a low Q_{10}; and permeability to glycerol disproportionately high in comparison with general permeability. Investigation of the extension of this grouping to include sensitivity to the inhibitors used in the experiments reported here is indicated. Attention might also be directed toward other instances of inordinately high specific permeabilities, as of the mouse red cell for erythritol (Jacobs, Glassman, and Parpart, 1935), and to the *Chaetopterus* egg for glycerol (Lucké, Hartline, and Ricca, 1939).

II. GLUCOSE UPTAKE

Results

(*a*) *Experiments with Inhibitors.*—The use of the photometric method of following volume changes made possible a study of similar osmotic changes in glucose solutions. The procedure generally used was the addition to a cell suspension in isotonic saline solution of an additional 20 per cent of its volume of the same medium containing dextrose at 6 times the final concentration desired, usually isosmotic (0.3 M). The entrance of glucose proved to be as sensitive as that of glycerol to the presence of some of the inhibitors tested. The pattern of effectiveness of inhibitors was somewhat different for glucose and for glycerol. I_2, Hg^{++}, Hg_2^{++}, and *p*-chloromercuribenzoate affect both, but the glucose system is vastly more sensitive to the mercurials. Both are less strikingly inhibited by phlorhizin, at $5 \cdot 10^{-3}$ M; but the volume changes in glucose solutions are entirely unaffected by cupric ion up to $2 \cdot 10^{-5}$ M, much more than is required to effect a great delay in the swelling in glycerol solutions. Other inhibitors found ineffective on glucose uptake included alloxan, mapharsen, lead, iodoacetate, and arsenite. Fig. 5 shows typical instances of inhibition of swelling in glucose solutions by *p*-chloromercuribenzoate in con-

centrations of $2 \cdot 10^{-6}$—10^{-5} M, and a case of almost complete reversal of inhibition, by addition of cysteine shortly after application of the inhibitor, is given in Fig. 6.

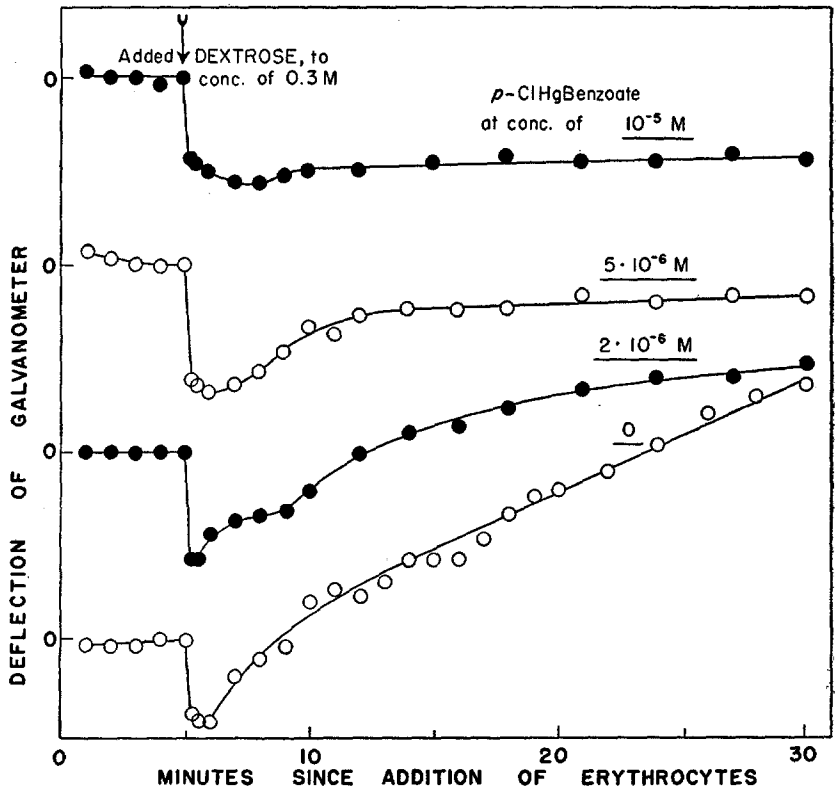

FIG. 5. Inhibition by *p*-chloromercuribenzoate of volume changes in glucose-saline solutions. Five minutes after exposure of cells to inhibitor in saline medium, at indicated concentrations, added 1 ml. of medium with dextrose, 1.8 M, to 5 ml. of cell suspension. Deflections plotted as in Fig. 1, as movement from position just prior to addition of glucose.

(*b*) *Experiments on Kinetic Relations.*—Aside from the action of inhibitors, there was reason to suspect that glucose was being carried into the cells actively, on the basis of the dynamics of the volume changes observed. Fig. 7(*a*) shows the shape of the curves relating cell volume and time following addition of various increments of dextrose to suspensions of cells in isotonic balanced saline solution. Note that equilibration is more rapid the less dextrose added, and that, with the larger additions, the initial rate of volume change increases as the concentration of sugar is decreased. Note also that the general form of the volume changes is a straight line when larger concentrations are in-

volved, but approaches the more usual exponential form with smaller concentrations. Qualitatively, this is exactly the set of relations that would be expected if there were an absolute limit to the amount of glucose that could enter

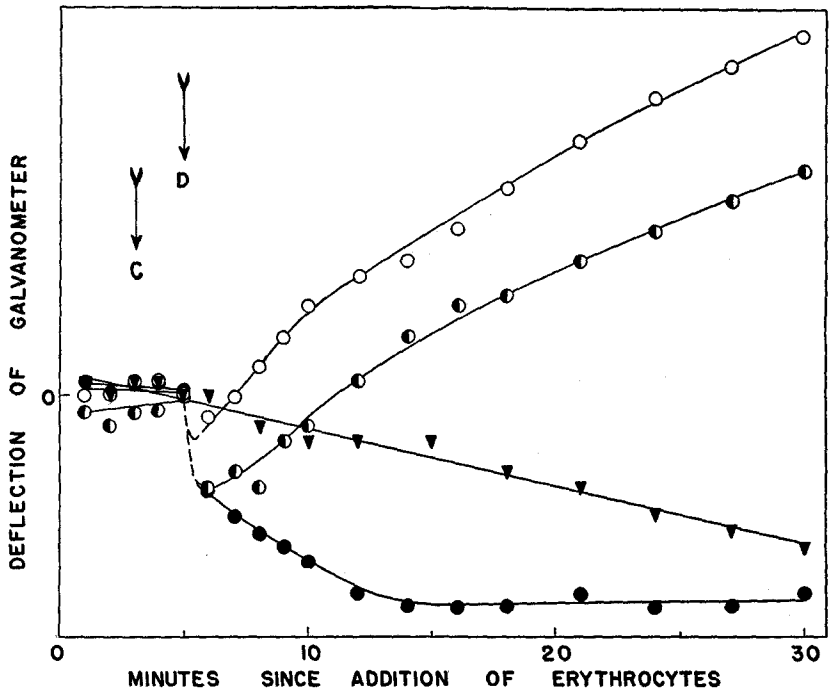

FIG. 6. Inhibition by $HgCl_2$ of uptake of glucose; reversal of inhibition by cysteine Five minutes after exposure of cells to inhibitor in saline medium (at *D*), added 1 ml of medium with dextrose, 1.8 M, to 5 ml. of cell suspension.

Triangles—control with $HgCl_2$, 10^{-6} M, without addition of dextrose.

Open circles—control without $HgCl_2$.

Solid circles—with $HgCl_2$, 10^{-6} M.

Half-solid circles—with $HgCl_2$, 10^{-6} M; at 3 minutes, (*C*), added one drop of medium with cysteine dihydrochloride to final concentration of $2.5 \cdot 10^{-4}$ M.

Deflections plotted as in Fig. 5.

the cell in a given time, regardless of the concentration gradient. The pattern found does not of course necessarily imply an active transfer mechanism, but would be a likely result of the functioning of such a mechanism.

At any rate, the pattern of these relations is clearly not compatible with the assumption of a simple unhindered passive diffusion process, following Fick's law:

$$\frac{dS}{dt} = kA\left(C_s - \frac{S}{V}\right),$$

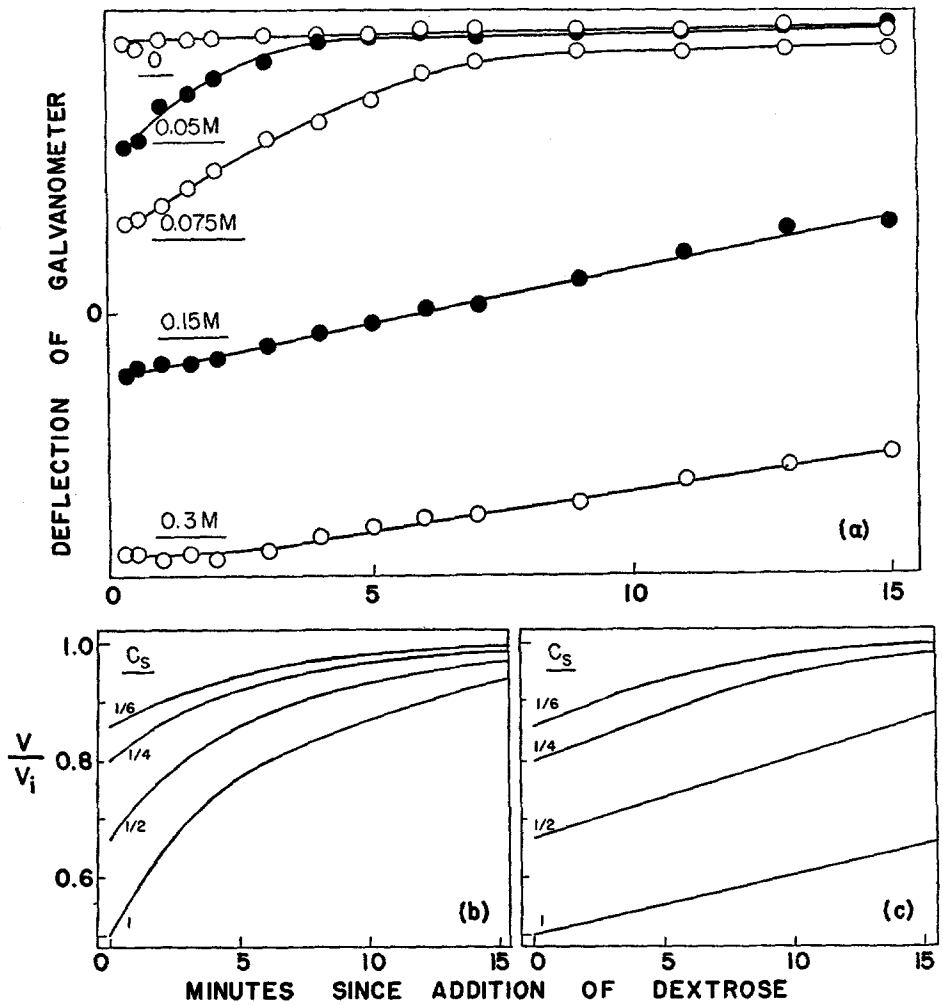

FIG. 7. Kinetics of swelling in glucose-saline solutions.

(*a*) At zero time, 1 ml. saline medium with dextrose at 6 times final concentration indicated added to 5 ml. cell suspension in medium. Deflections plotted as in Fig. 1.

(*b*) Predicted relations in similar experiment, assuming passive diffusion, with $dS/dt = k\ (C_e - S/V)$, with $k = 0.2$ iso-volumes per minute.

(*c*) Predicted relations in similar experiment, assuming diffusion rate is limited to maximal value, m, (= 0.02 iso-content per minute,) by process involving cellular component.

where S is the amount of the penetrating substance within the cell, k is a "permeability constant," A is the area across which the permeation occurs, C_e is the

external concentration of the penetrating substance, and V is the volume of the intracellular fluid in which S is dissolved. Since the cells make up only a small fraction of the total volume of fluid in these experiments, C_s may be regarded as constant. And since the manner in which A varies with V under these conditions in a cell having the form of an erythrocyte is at best uncertain, and since in other instances the area of these cells seems to remain constant in spite of volume changes, the factor A is commonly included in the permeability constant k. In the present instance, the penetrating substance can enter the cell only much more slowly than water, so that the osmotic pressure on the two sides of the membrane may be considered to be essentially identical at all times. Thus,

$$\frac{dS}{dV} = C,$$

where C is the total extracellular concentration, in osmotic units. Hence,

$$\frac{dt}{dV} = \frac{CV}{kC_i(V_i - V)},$$

where C_i is the external concentration of non-penetrating substances (in these experiments, isotonicity), and V_i is the volume of the intracellular fluid in an isotonic medium. Then

$$t = \frac{V_i}{k}\left[1 - \frac{CV}{C_i V_i} + \frac{C}{C_i}\ln\frac{C_s V_i}{C(V_i - V)}\right],$$

with initial conditions as in these experiments; with conditions at isotonicity as the units, this may be written

$$t = \frac{1}{k}\left[1 + (1 + C_s)\left(\ln\frac{C_s}{(1 - V)(1 + C_s)} - V\right)\right].$$

This relation gives a pattern of volume-time curves, with various values of C_s, such as those presented in Fig. 7(*b*). The behavior of the cells in the glucose solutions clearly cannot be harmonized with this pattern of passive diffusion.

If, however, it be assumed that the transfer involves temporary formation of a complex with some constituent of the cell membrane, or some other type of reaction limited by the quantity available of some such constituent, the simple diffusion will be complicated by this consideration. There may then be some limiting value, m, which dS/dt cannot exceed, regardless of the concentration gradient. This relation gives a pattern of volume-time curves such as that seen in Fig. 7(*c*). The form of these curves is qualitatively very similar to the observed behavior of the cells in glucose solutions. Quantita-

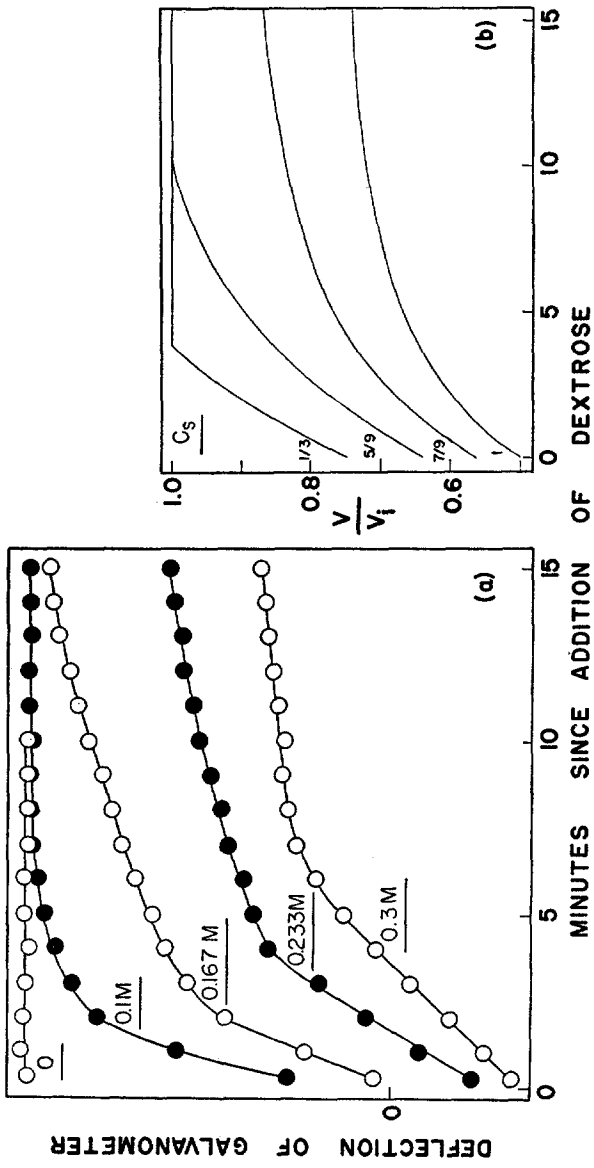

FIG. 8. Kinetics of swelling in glucose-saline solutions.

(*a*) As in Fig. 7(*a*), except that temperature here is 37°C.

(*b*) Predicted relations in similar experiment, assuming transfer rate proportional to degree to which "limiting concentration" has not been attained, with $dS/dt = k(L - S/V)$, with $k = 0.2$ iso-volume per minute, and $L = \frac{2}{3}$ isotones; terminating swelling at moment of attainment of even distribution of glucose.

tively, however, it is impossible, by this assumption, to account for the magnitude of the differences observed in initial rate, dV/dt, with different concentrations of dextrose. For this reason, and because of a further complication in the experimental curves, a more complex relation governing the course of the volume changes was suggested.

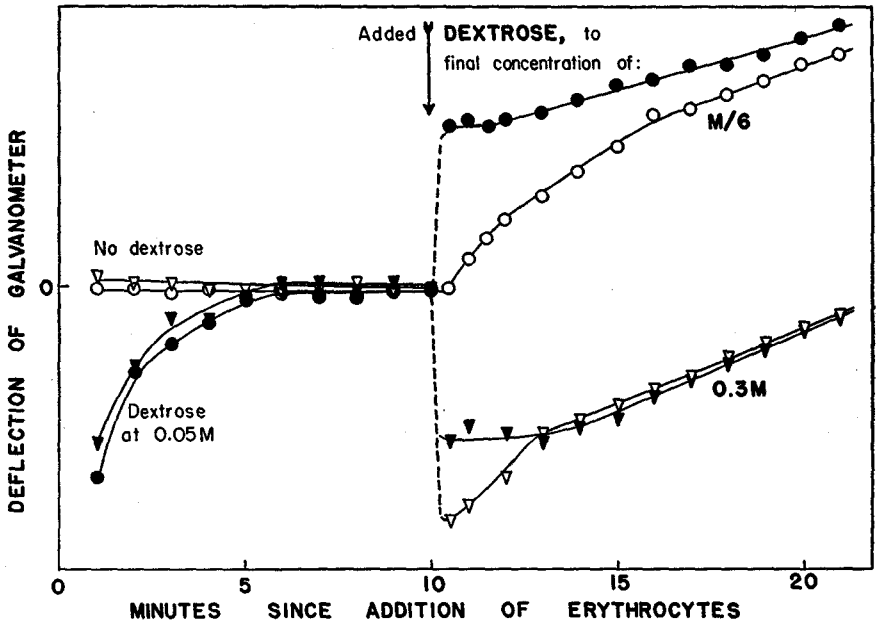

FIG. 9. Effect of previous equilibration with dilute glucose solutions on swelling in glucose-saline solutions. At zero time, 5 ml. cell suspension added to 5 ml. of medium.

Open symbols—without dextrose.

Solid symbols—with dextrose, final concentration 0.05 M.

At 5 minutes, added 2 ml. medium with dextrose, to made final concentration of 0.3 M (triangles), 0.167 M (circles). Deflections plotted as movement from reading just prior to final addition of glucose.

The additional complication is demonstrated in Fig. 8(*a*); with longer experiments than those shown in Fig. 7(*a*), or with increased temperatures, it is seen that the initial swelling in the more concentrated solutions slows down rather abruptly after a time, and that this effect is more pronounced the stronger the solution. In some instances, the swelling appears nearly to cease altogether while the cell volume is still considerably less than in an isotonic solution. Furthermore, as shown in Fig. 9, preliminary equilibration with dextrose at concentrations in the neighborhood of what appears to be the intracellular limit, 0.05 to 0.1 M at room temperature, markedly depresses the subsequent

rate of swelling during the initial period, but does not affect the later rate after the process has slowed down. Thus, it is suggested that the postulated carrier mechanism functions only when the intracellular concentration of glucose is below this critical level. This assumption might simply be added to either of the two previous suggestions, so that the theoretical curves would show the terminal levelling found experimentally; but this would not improve the fit of the initial stages of the curves. These compound assumptions may be replaced by a single new hypothesis, the predictions of which approach the experimental curves; this suggestion is: that the rate of transfer of glucose into the cell is proportional to the disparity between the intracellular concentration of glucose and some "limiting" concentration. Thus the rate of uptake would be independent of the gradient across the membrane (except that thus far no evidence of transport *against* a gradient has been observed). This relation may be written,

$$\frac{dS}{dt} = k\left(L - \frac{S}{V}\right),$$

where L is the "limiting" intracellular concentration for glucose. Then, again with isotonic conditions as the units,

$$t = \frac{1 - V(C_s + 1)}{k(C_s + 1 - L)} + \frac{C_s + 1}{k(C_s + 1 - L)^2} \ln \frac{L}{(C_s + 1)(1 - V[C_s + 1 - L])}.$$

This relation, as amended by the limitation that transport against the gradient does not occur, is presented in Fig. 8(*b*). This parallels the experimental pattern in the attainment of terminal volumes short of the isotonic volume, and in that the initial rate of swelling decreases with increasing dextrose concentration. However, aside from quantitative discrepancies, the early stages of the experimental curves appear to be much more nearly straight lines than would be predicted from this relation. Also, the terminal subnormal cell volumes under most circumstances continue to increase slowly; this may be simply the result of the steady glycolytic activity, the extent of which in these conditions has not been determined. A more detailed analysis of the applicability of various quantitative hypotheses concerning these relations will be taken up in a later report. Extension of the system proposed by Shannon (1939) for the transfer of glucose across the kidney tubule appears to be recommended: extracellular glucose in ready equilibrium with its combination product (with some membrane constituent present in constant limited amount), the slower decomposition of this combination at the interior depending on some activity which is suppressed in proportion to the increasing glucose concentration.

(*c*) *Accessory Experiments.*—Some attempts have been made to check by chemical analytic methods the interpretation of the volume changes followed

in the photometric apparatus. The rapid uptake of glucose from an isosmotic solution in the saline medium, to an intracellular concentration of less than 50 per cent of the external concentration, followed by almost no subsequent change, was verified by analysis of the glucose content of the medium. The inhibitory effects of the mercuric ion were also indicated in this manner; but the necessity of using very dense suspensions of cells, so that the cell-glucose uptake would be reflected in a lowering of the extracellular concentration, entailed use of much higher concentrations of mercuric ion than in the other methods. This involved increased initial cell damage by the poison, and perhaps little accord should be given the results thus obtained.

The possibility that phosphorylation might be involved in the transport into the red cell of either glycerol or glucose or of both led to a brief investigation of the possibility that inorganic phosphate might be consumed in the process (incorporated into the organic phosphate carried into the cell interior). Large quantities of human erythrocytes were thoroughly washed in the usual suspension medium, and the suspension finally brought to about 40 per cent. 1 ml. of glycerol or 1 gm. of glucose was added to 80 ml. of such a suspension, and the cells removed by centrifugation after a few minutes. Under these circumstances, the concentrations of phosphate remaining in the supernatant medium were identical with that of the original medium (within the experimental error of about 1 per cent), whether glycerol or glucose or neither had been added to the cells. If even distribution had taken place, as would be expected from the other experiments, about 0.5 gm. of glycerol, or 0.4 gm. of glucose must have been intracellular in these suspensions, neglecting the cell dead space. This would amount to about 5.5 mM of glycerol or 2.2 mM of glucose; but the entire 80 ml. of suspension contained less than 2 mM of inorganic phosphate, assuming equal distribution intra- and extracellularly; and this amount was not changed measurably by the transport. Thus it is evident that phosphate is not fixed by the processes involved in carrying glucose or glycerol into the cell. However, this evidence does not preclude the possibility of a temporary phosphorylation during the transit of the membrane, such as is postulated in the intestinal and renal transfer of some sugars; in fact, Wilbrandt and Laszt (1933) report similar evidence of no change in the intestinal epithelial content of hexose phosphate during active absorption as compared to starvation. This reversible sort of transfer system would also be indicated by the fact that the inhibitors delay the process in both directions.

Discussion

The anomalous behavior of glucose in entering the human erythrocyte has occasioned earlier comment; Klinghoffer (1935) reported rapid equilibration between cells and plasma when small amounts of the sugar were added to blood, yet the cells could be kept in isosmotic glucose almost indefinitely with-

out hemolysis. Klinghoffer set the concentration of about 2 per cent as the critical range, below which even distribution between cells and plasma is rapidly attained, and above which an extracellular excess is maintained almost indefinitely. This figure was also indicated by the observation that human red cells in isosmotic glucose solutions swell rapidly to about 140 per cent of their original volume, then much less readily.

Bang and Ørskov (1937) found that the permeability constant for glucose in these cells was reduced by about 60 per cent by doubling the external concentration of the sugar in the neighborhood of 0.05 M. These authors refer to theses by Ege and Bjering as the first reports on this anomaly; Bjering apparently also observed that the entry of glucose into the cell is prevented by $Hg(CN)_2$, and that the normal uptake is at first rather rapid, later much slower. Meldahl and Ørskov (1940) checked this latter point in finding a progressively diminishing permeability "constant" as the entry of glucose proceeded, as calculated from the volume-time relations. The constants for thiourea, glycerol, and malonamide, on the other hand, appeared in similar experiments to increase progressively; this was interpreted as indicating increased pore size with swelling of the cells. In the calculation of these constants, Meldahl and Ørskov applied a form of Fick's law which assumes a constant cell volume, although changing figures for the volume were substituted in the equation; this introduces considerable error. However, the oversight leads to an apparent decrease of the "constant" with time, so that the observed discrepancies iin the case of glycerol, thiourea, and malonamide are even greater than reported. With glucose uptake, the marked progressive decrease in the "constant" is not so great as originally calculated, but is still very pronounced; so that the conclusions drawn by Meldahl and Ørskov are qualitatively valid. The behavior in glucose solutions was attributed by these observers to adsorption of glucose on the cell membranes, blocking passage through the pores. Some such process may well be involved, but direct evidence is lacking. Wilbrandt, Guensberg, and Lauener (1947) recently showed that the "permeability constant" for glucose in the human red cell may be more than 1000 times larger in a dilute solution of glucose than in a concentrated solution.

The investigations of Cori, Lundsgaard, Wilbrandt, Verzár, Laszt, and others on the absorption of sugars from the intestinal lumen indicated that there was some degree of specificity in selection of sugars to be moved across the intestinal lining by means of a phosphorylating mechanism. Wertheimer (1933) reported inhibition by phlorhizin of the uptake from the rodent intestine of galactose, glucose, and fructose; to a lesser extent, of mannose and dioxyacetone; and practically not at all, of xylose and arabinose. Wilbrandt and Laszt (1933) found a similar arrangement with respect to iodoacetate inhibition, and Laszt (1935) showed that the phosphorylating activity of extracts of the intestinal epithelium exhibited parallel specificity. Verzár (1935) came to

similar conclusions on the basis of the comparative kinetics of absorption of various simple sugars. No extensive series of sugars has been tested with regard to the apparent transport mechanism in the red cell; however, inhibition by mercuric ion, at $1\text{–}5 \cdot 10^{-6}$ M, as described for glucose uptake, is equally effective with the slightly slower levulose, and the slightly more rapid galactose uptake. More complete investigation of the molecular specificity of the relations described in this report is planned, together with tests, suggested by

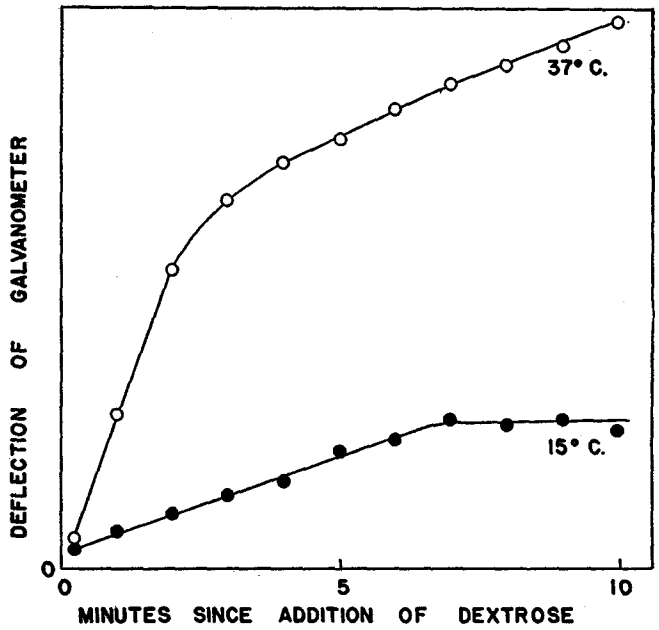

FIG. 10. Effect of temperature on rate of volume changes in glucose-saline solutions. At zero time, 1 ml. 1 M dextrose, in saline medium, added to 5 ml. cell suspension. Deflections plotted as in Fig. 1.

analogy with the intestinal and renal transport, of the possibility of competition between alternative penetrants (Cori, 1926; Shannon, 1938), the influence of hormones (Althausen and Stockholm, 1938), and of various glucosides (Abderhalden and Effkemann, 1934).

The temperature coefficient of the rate at which the cells take up glucose is indicated by a comparison of the initial slopes of the curves in Fig. 10. The Q_{10} computed from these and similar data is about 2.5, which is on the order of that reported by Bjering, although Bang and Ørskov (1937) found a figure of 5.5 with smaller glucose concentrations. Ørskov (1935) also reports a Q_{10} of about 2 for the uptake of glycerol by the human red cell, although Jacobs and coworkers (1935) found a very low Q_{10} for the same process. The figure on the

order of 2–3 would be compatible with the hypothesis of phosphorylation at the membrane as a prerequisite of penetration. However, there is some doubt as to the justifiability of comparing the initial rates of volume change under the same conditions at different temperatures, since the argument developed in the section on kinetics indicates probable involvement of other factors that may vary with temperature. Preliminary experiments indicate that the critical limiting concentration does in fact increase with the temperature, so that, according to the favored hypothesis, the effective gradient for glucose ($L—S/V$), in the same solution, increases with the temperature (compare Fig. 10). This may account for the extremely high Q_{10} reported by Bang and Ørskov, since comparison of the over-all rates at two temperatures would give a temperature coefficient larger than that of the permeability process itself, as indicated by changes with temperature in the value of "k", the magnitude of the discrepancy depending on the concentration of glucose used in the tests.

SUMMARY

1. Permeability of the human erythrocyte to glycerol, as indicated by the course of hemolysis and volume changes, is depressed by Cu^{++}, Hg^{++}, I_2, *p*-chloromercuribenzoate, and phlorhizin, without effecting general permeability changes. In so far as tested (Cu^{++}, *p*-ClHgB), these inhibitors delay exit of glycerol from the cell as well as its entry.

2. Permeability to glucose is similarly depressed by I_2 and phlorhizin, and is extremely sensitive to Hg^{++} and *p*-chloromercuribenzoate, but is not affected by Cu^{++}. An extensive series of other enzyme poisons is without effect in either system.

3. The effects of the sulfhydryl inhibitors are prevented or reversed in the presence of glutathione, cysteine, etc.

4. The kinetics of the volume changes in glucose-saline solutions indicates a mechanism for transport of glucose into the cell, regulated by the existing intracellular concentration, rather than by simple diffusion gradients.

5. The intermediation of a sulfhydryl group at the cell surface, probably an enzymatic phosphorylation, is suggested as an essential step in the passage of glycerol, glucose, and other like substances, across the human red cell membrane.

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