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Relationship of Erythrocyte Cholinesterase Activity to Cell Metabolism

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SERIAL MEASUREMENTS of cholinesterase activity of stored human red cells and of the ability of such cells to release gas from Krebs-Ringer bicarbonate solution in an atmosphere of 5 per cent CO_2–95 per cent N_2 has shown that both of these measurements exhibit fluctuations over the course of time of observation.[1] In a given study, comparison by inspection failed to reveal any generalization describing a constant relationship between the two measurements. The present report establishes such a relationship through a study of the release of gas from Krebs-Ringer bicarbonate solution by stored red cells with and without the addition of eserine. This study reveals the regulation of metabolic capacity in the presence of dextrose by cholinesterase. The data are not inconsistent with the suggestion of Lindvig, Greig, and Peterson[2] that cholinesterase is part of a cellular system governing the permeability of the erythrocyte membrane.

Methods

Blood collection and storage, Warburg manometry of the release of gas from Krebs-Ringer bicarbonate solution, and measurement of erythrocyte cholinesterase activity all were performed under the rigidly standardized conditions previously described.[1]

Comparison of Stored Erythrocytes with and without the Addition of Eserine

Erythrocytes taken from storage at −1.5 C. on the day of examination and were washed with 4 volumes of cold 0.85 per cent sodium chloride solution. The washed cells were suspended in 2 volumes of Krebs-Ringer bicarbonate solution and a cell count was made. To 10 ml. of suspended cells was added 1 ml. of a solution containing 0.2 per cent eserine (Physostigmine sulfate Merck) in 0.85 per cent sodium chloride. This provides a final concentration of eserine in the reaction mixture of 2.8×10^{-4} M. To a second 10 ml. sample was added 1 ml. of 0.85 per cent sodium chloride solution. The two mixtures stood approximately one-half hour at room temperature. Cholinesterase determinations were made and recorded as units per billion cells. Two ml. aliquots of each sample were placed in Warburg vessels containing 0.35 ml. of 0.25 per cent dextrose in Krebs-Ringer bicarbonate solution in the side arms. Similar samples were prepared in Warburg vessels containing 0.35 ml. of Krebs-Ringer bicarbonate solution in the side arms. All determinations were made in duplicate. A difference of manometry between duplicates greater than 15 per cent caused rejection of the measurement. The usual agreement was within 5 per cent. The Warburg flasks were placed in the water bath at 40 C. and were agitated at 90 to 110 strokes per minute while being gassed for 20 minutes with 5 per cent CO_2-95 per cent N_2. Substrates were then tipped into the main chambers and the manometers were adjusted at 120 mm. Shaking was resumed and manometric observations were made at intervals of 30 minutes, 1, 2, 3, 4, and 5 hours. Manometric changes were corrected for thermobarometer readings and were recorded as cu. mm. of CO_2 per 10^9 erythrocytes.

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TABLE 1.—*Release of Gas from Krebs-Ringer Bicarbonate Solution and Cholinesterase Activity of Normal Stored Erythrocytes*

Time of storage	Interval of manometry	cu. mm. of CO_2 per billion cells			Cholinesterase units per 10^9 cells
		Added dextrose (1)	No added dextrose (2)	Manometry due to dextrose (1) — (2)	
3 hr.	1 hr.	13.71	14.76	−1.05	1.23
	2-5 hr.	6.98	4.97	2.01	
	1-5 hr.	20.69	19.73	0.96	
1 day	1 hr.	10.37	10.24	0.13	
	2-5 hr.	2.38	2.53	−0.15	0.92
	1-5 hr.	12.75	12.77	−0.02	
2 days	1 hr.	4.40	3.72	0.68	
	2-5 hr.	4.64	3.41	1.23	0.83
	1-5 hr.	9.04	7.13	1.91	
3 days	1 hr.	9.21	9.80	−0.59	
	2-5 hr.	3.66	2.65	1.01	0.36
	1-5 hr.	12.87	12.45	0.42	
4 days	1 hr.	8.88	6.20	2.68	
	2-5 hr.	3.96	4.78	−0.82	0.48
	1-5 hr.	12.84	10.98	1.86	
7 days	1 hr.	8.77	8.06	0.71	
	2-5 hr.	5.65	4.12	1.53	0.37
	1-5 hr.	14.42	12.18	2.24	
11 days	1 hr.	6.09	5.50	0.59	
	2-5 hr.	4.59	4.03	0.56	0.0
	1-5 hr.	10.68	9.53	1.15	
16 days	1 hr.	14.46	15.18	−0.72	
	2-5 hr.	2.00	0.00	2.00	0.36
	1-5 hr.	16.46	15.18	1.28	
18 days	1 hr.	6.68	8.06	−1.38	
	2-5 hr.	2.92	1.84	1.08	0.37
	1-5 hr.	9.60	9.90	−0.30	
25 days	1 hr.	3.64	0.77	2.87	
	2-5 hr.	−2.11	−3.98	1.87	0.39
	1-5 hr.	1.53	−3.21	4.74	

RESULTS

A complete set of measurements for a whole blood storage study is given in tables 1 and 2.

For the purpose of this study attention should be focused on two features of the data obtained with normal cells without added eserine. Gas is usually released during the first hour by normal cells at a rate several times that of the

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TABLE 2.—*Release of Gas from Krebs-Ringer Bicarbonate Solution and Cholinesterase Activity of Eserinized Stored Erythrocytes*

Time of storage	Interval of manometry	cu. mm. of CO_2 per billion cells			Cholinesterase units per 10^9 cells
		Added dextrose (3)	No added dextrose (4)	Manometry due to dextrose (3) — (4)	
3hr.	1 hr.	9.4	5.98	3.42	
	2-5 hr.	9.83	4.14	5.79	0.35
	1-5 hr.	19.23	10.12	9.21	
1 day	1 hr.	11.83	6.48	5.33	
	2-5 hr.	3.34	3.23	0.11	0.0
	1-5 hr.	15.15	9.71	5.44	
2 days	1 hr.	2.73	2.27	0.46	
	2-5 hr.	5.02	4.48	0.54	0.0
	1-5 hr.	7.75	6.75	1.00	
3 days	1 hr.	9.68	3.62	6.06	
	2-5 hr.	5.00	3.82	1.18	0.0
	1-5 hr.	14.68	7.44	7.24	
4 days	1 hr.	10.13	−1.88	12.01	
	2-5 hr.	5.16	3.90	1.26	0.0
	1-5 hr.	15.29	2.02	13.27	
7 days	1 hr.	5.72	3.65	2.07	
	2-5 hr.	8.06	5.59	2.47	0.0
	1-5 hr.	13.78	9.24	4.54	
11 days	1 hr.	4.32	2.59	1.73	
	2-5 hr.	6.55	4.41	2.14	0.0
	1-5 hr.	10.87	7.00	3.87	
16 days	1 hr.	15.79	12.23	3.56	
	2-5 hr.	3.04	−0.21	3.25	0.0
	1-5 hr.	18.83	12.02	6.81	
18 days	1 hr.	4.96	1.52	3.44	
	2-5 hr.	4.62	0.60	4.02	0.0
	1-5 hr.	9.58	2.12	7.46	
25 days	1 hr.	2.24	−1.20	3.44	
	2-5 hr.	−0.04	−3.67	3.63	0.19
	1-5 hr.	2.20	−4.87	7.07	

hourly rate during the two to five hour interval. In fact, the gas released during the first hour is usually greater than the total released during the two to five hour period. Essentially, all of the gas is released by normal cells in both the 1 hour and the 2 to 5 hour intervals in the absence of added dextrose (column 2), the total gas attributable to added dextrose being very minor (column 1 − 2).

The data for eserinized cells shows that when cholinesterase activity is blocked,

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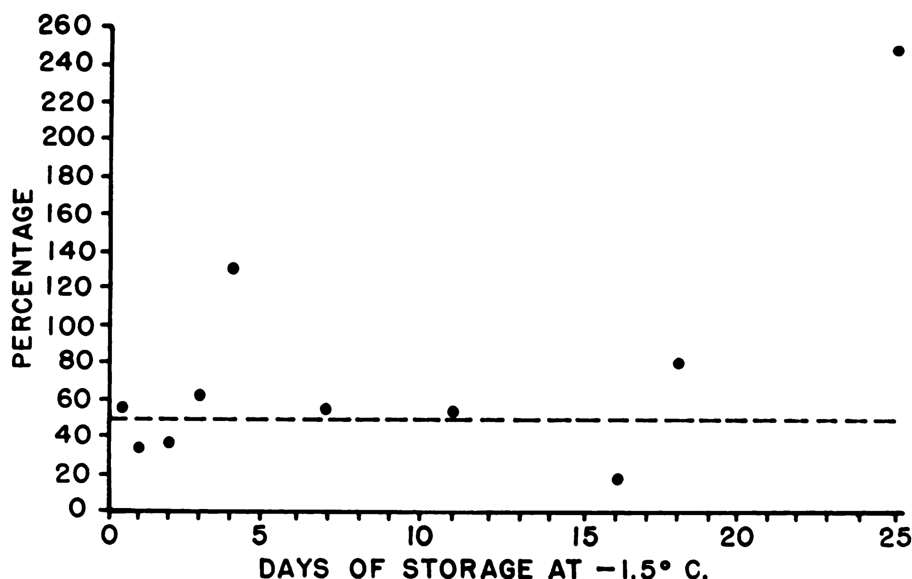

FIG. 1.—Percentage of first hour gas release (no added dextrose) referable to cholinesterase.

the CO_2 liberated, due to dextrose substrate, is considerably increased (column 3 − 4). This increased CO_2 release is often 10 or more times that for normal cells.

By comparison of column 4 with column 2 it is evident that, in most instances, the major portion of the first hour gas release by normal cells is attributable to some function of cholinesterase (fig. 1). The nature of this function is suggested when one recalls that the manometric determination of cholinesterase activity of Ammon[3] follows closely the technic that has been employed in this study for determination of metabolic activity, with the exception that the period of observation is 20 minutes and acetylcholine is added as substrate. In the present study, of course, no substrate for cholinesterase is added. The present data can be explained most readily by postulating the presence in the red cell of acetylcholine, or of some other substrate for cholinesterase, the hydrolysis of which releases an acid, or, much less likely, releases a gas directly. The ability of red cells to form acetylcholine has already been reported.[4] Attempts to demonstrate acetylcholine production by intact erythrocytes in our own laboratory by both the Hestrin reaction and frog rectus muscle contraction have met with only sporadic success.

Table 3, prepared by subtracting column 4 (endogenous CO_2 released from eserinized cells) from column 2 (endogenous CO_2 liberated from normal cells), gives the values for gas due to active cholinesterase. These values might be considered to represent cholinesterase substrate present in the red cells. Figure 2 shows that these CO_2 values vary directly with the CO_2 release, attributable to dextrose in the eserinized cell study. This would indicate that the substrate normally hydrolyzed by cholinesterase can govern the amount of dextrose utilized by the erythrocytes.

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TABLE 3.—*Gas Release Due to Cholinesterase**

Time of storage	Interval of manometry		
	1 hr.	2-5 hr.	1-5 hr.
3 hr.	8.78	0.83	9.61
1 day	3.76	−0.70	3.06
2 days	1.45	−1.07	0.38
3 days	6.18	−1.17	5.01
4 days	8.08	0.88	8.88
7 days	4.41	−1.47	2.94
11 days	2.91	−0.38	2.53
16 days	2.95	0.21	3.16
18 days	6.54	1.24	7.78
25 days	1.97	−0.31	1.66

* Column 2 of table 1 minus column 4 of table 2.

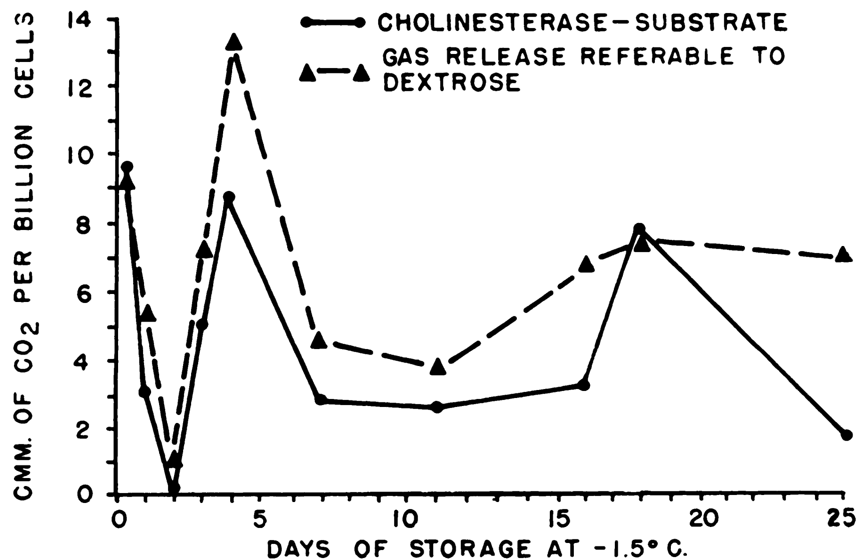

FIG. 2.—Comparison in eserinized erythrocytes of cholinesterase substrate content and gas release referable to dextrose.

In the normal cell, at body temperature, the coexistence of enzyme and substrate must be so balanced as to keep the dextrose utilization at a very low level. With fresh erythrocytes, which have never been subjected to refrigeration, no divergence is noted between the normal and eserinized cell release during the first hour.

The accumulation of substrate during the storage at low temperature, even for three hours, must indicate a widely divergent temperature coefficient for synthesis and hydrolysis of the substrate. Either the hydrolysis is inhibited by lowered temperature, or accessibility of substrate to enzyme is changed. This produces the imbalance which is rapidly corrected upon the cell being returned to a state simulating its natural one. The data presented show that essentially

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TABLE 4.—*Micromoles of Acetylcholine Represented by Alles-Hawes Titrimetric Cholinesterase Determination and by Gasometric Measure of Cholinesterase Substrate*

Time of storage	μM. of acetylcholine	
	Calculated from Alles-Hawes det'n of table 1	Calculated from 1st hr. values of table 3
3 hr.	24.6	0.39
1 day	18.4	0.167
2 days	16.6	0.064
3 days	7.2	0.275
4 days	9.6	0.360
7 days	7.4	0.196
11 days	0.0	0.129
16 days	7.2	0.131
18 days	7.4	0.292
25 days	7.8	0.087

all of the cholinesterase substrate is normally hydrolyzed in the first hour interval. Actually, if this interval is broken into 15 minute periods, the cholinesterase substrate hydrolysis is seen to occur within the first 15 minutes of observation. The imbalance of this enzyme system, produced by lowering the temperature, is a factor that might be employed to advantage in future studies of cholinesterase substrate production by erythrocytes.

The data do not furnish direct evidence as to whether the increased dextrose utilization in eserinized cells is due to a better transport of dextrose metabolism within the cell. If the effect were on metabolism within the cell, one might suppose that endogenous metabolism (metabolism in the absence of added dextrose) would also be stimulated. This would result in increased gas release in the 2 to 5 hour period by eserinized cells with no added dextrose. This does not seem to be the case, since the 2 to 5 hour values in table 3 indicate no consistent increase of gas release. Hence, the dextrose effect appears to increase permeability of the cell to dextrose, or to intensified transport of dextrose across the cell membrane.

That cholinesterase hydrolytic capacity in the normal cell is always in considerable excess of substrate is illustrated in table 4. For this calculation, the cu.mm. of CO_2, assumed to be due to cholinesterase substrate (table 3), and the cholinesterase Alles-Hawes unit have been converted to μM. of acetylcholine.

The accumulation of cholinesterase substrate during storage, the residual endogenous metabolism after correction for cholinesterase substrate, and the gas due to dextrose in normal cells all exhibit the same type of undulant fluctuation during storage that has previously been noted for other metabolic measurements.[1] This undulant fluctuation was the source of initial confusion in this study. The first use of eserinized cells was made with twenty-four hour erythrocytes. A stimulation of dextrose utilization was noted as shown in the current data. The second application of the technic was with cells on their thirty-fifth day of storage. In this determination there was actually a depression of dextrose utilization. The true meaning of these divergent observations is to be deduced from

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the present study. The quantitative effect of cancellation of cholinesterase activity varies with the state of the cell at the time of cancellation. On the second and eleventh days of the current study stimulation of dextrose utilization in the eserinized cells is at a minimum. On the second day, one is approaching complete reversal of the usual stimulation of dextrose utilization. The earlier study with thirty-five day old cells happened to be made on a day when there was no accumulation of cholinesterase substrate and, consequently, no stimulation of destrose utilization by the eserinized cells. The fluctuating values for cholinesterase-substrate suggests one explanation for erratic data obtained in the attempts to demonstrate acetylcholine synthesis.

Discussion

The first hour burst of gas release noted in this study is consistent with the observations of Bird[5] made under similar circumstances with whole blood. The absolute amount of gas obtained in this study with washed erythrocytes from citrated blood in Krebs-Ringer bicarbonate solution is much smaller than that seen by Bird with whole heparinized blood. This may be due, in part, to the collection in citrate. Other data from our laboratory show that the metabolic capacity, as measured, is appreciably lowered in cells collected in citrate as compared to that of cells collected through the resin Dowex 50. In our studies, there appears also to be proportionately less oxygen released during manometry than was reported by Bird. On at least two occasions the first hour burst is completely absent (column 4), and if oxygen were released in these instances, it must be assumed to have been reutilized.

Lindvig, Greig, and Peterson suggested that erythrocyte cholinesterase must be actively engaged in acetylcholine hydrolysis for the maintenance of normal cell fragility.[2] They showed, with human cells in buffer containing no dextrose, that when erythrocyte cholinesterase is inactivated, the cell becomes more fragile, or at least more readily permeable to sodium ions, potassium ions, and hemoglobin. The present data permit further correlation of cholinesterase with erythrocyte permeability in that when cholinesterase is blocked by eserine, the red cell is more readily permeable to dextrose. Moreover, the data demonstrate the presence in stored erythrocytes of a cholinesterase substrate which has a direct influence on cell permeability. If this substance is also acetylcholine, as is perfectly possible in view of the demonstration of the choline acetylase system in erythrocytes,[4] one necessity for keeping the cholinesterase hydrolytically active is revealed as that of prevention of accumulation of acetylcholine.

Paradoxically, the data also suggest that active cholinesterase is essential for the synthesis of acetylcholine. Thus, the increased permeability to dextrose of eserinized cells varies directly with the quantity of acetylcholine present before addition of the eserine. There is no evidence of further substrate formation during the study in the presence of eserine.

One seeming contradiction appears if one compares the control cells in this study with those reported by Lindvig, et al. In their study, control cells with no added acetylcholine slowly increased in permeability. In this study, the control cells with no added acetylcholine did not exhibit increased permeability to dextrose during the time of study. It could be suggested that hydrolysis of the

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accumulated acetylcholine substituted the hydrolysis of added acetylcholine, but the data indicate quite conclusively that the accumulated substrate was hydrolyzed in the first few minutes of the study. The difference must lie, then, in the presence of added dextrose. The dextrose must permit the synthesis of new substrate as rapidly as the accumulated substrate is exhausted, thus providing continued activity for the cholinesterase.

It appears from the data presented that the cholinesterase substrate relationship is a delicately balanced system of considerable importance to cell metabolism. This balanced system might be due either to a synthetic-hydrolytic equilibrium mediated by a single enzyme, or to equilibrated but separate synthetic and hydrolytic systems. In this equilibrium, synthesis is dependent on the presence of active cholinesterase and dextrose. Hydrolysis is dependent on active cholinesterase alone.

The action of acetylcholine in contracting the fibrous protein of certain types of muscle comes to mind. The structural proteins of the erythrocyte are known to be fibrous in nature. It is attractive to imagine that the production of acetylcholine by the red cell provides a means of regulation of cell permeability by alternate contraction and relaxation of these fibrous proteins.

It should be noted that added acetylcholine differs in its action from acetylcholine produced by the cell. In the study of Lindvig, et al. added acetylcholine, when protected by eserine, cannot increase the permeability of the red cell membrane above normal. The present data show that a lesser buildup of acetylcholine within the cell produces a profound increase in permeability when protected by eserine.

LeFevre and Davies[6] have suggested an active transport mechanism for aldoses and ketoses. In this suggestion it is shown that aldoses selectively inhibit one another's transport, the most active in this respect being dextrose. It has been seen in this study that dextrose can control permeability through its linkage with the cholinesterase-cholineacetylase system. The other aldoses of LeFevre's study, galactose, mannose, xylose, and arabinose, are all metabolized by the red cell, but much more slowly than dextrose. Ketoses, on the other hand, have no inhibitive effect on the subsequent transport of aldoses into the cell, presumably because they are unable to serve as the essential link with the permeability controlling system. It is known, for instance, that fructose is phosphorylated by fructokinase, a separate enzyme from that initiating glycolysis of the aldoses. LeFevre's observations might thus be explainable by the mechanism suggested in this study.

Summary

Production by normal human erythrocytes of a substrate for cholinesterase has been demonstrated.

At body temperature, the formation and hydrolysis of the cholinesterase substrate is delicately controlled.

At lowered temperatures, 0 to -1.5 C., production of cholinesterase substrate greatly exceeds its hydrolysis.

The quantity of cholinesterase substrate capable of exerting its influence governs the quantity of dextrose converted to lactic acid by the erythrocyte under anaerobic conditions.

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The observations corroborate and extend the suggestion of Lindvig, Greig, and Peterson that cholinesterase is part of a cellular system governing erythrocyte permeability.

The balance between production and hydrolysis of cholinesterase substrate at low temperature exhibits the same undulating fluctuations during red cell storage that have been noted for other erythrocyte metabolic functions.

Summario in Interlingua

Un previe reporto de mesurationes in vitro ha establite le character fluctuante, con alternationes de valores alte e basse, tanto del activitate cholinesterasic de erythrocytas immagazinate como etiam de lor capacitate, in le presentia de dextrosa, de relaxar gas ab le solution de bicarbonato typo Krebs-Ringer intra un atmosphera de 5 pro cento CO_2 e 95 pro cento N_2. Le presente studio establi un relation constante inter le duo phenomenos.

In normal erythrocytas hyman le production de un substrato pro cholinesterase ha essite demonstrate.

Al temperatura del corpore, le formation e le hydrolyse del substrato de cholinesterase es delicatemente regulate. Si le temperatura es reducite a inter 0 e −1,5 C., le production del substrato de cholinesterase grandemente excede su hydrolyse.

Le quantitate del substrato de cholinesterase que es capace de exercer su influentia regula le quantitate de dextrosa convertibile a acido lactic per le erythrocyta sub conditiones anaerobic.

Iste observationes corrobora e amplifica le suggestion de Lindvig, Greig, e Peterson que cholinesterase es parte de un systema cellular que governa la permeabilitate de erythrocytas.

Le balancia inter le production e le hydrolyse del substrato de cholinesterase a basse temperaturas exhibi le mesme fluctuationes durante le immagazinage del erythrocytas que ha essite constatate pro altere functiones metabolic del erythrocytas.

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