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Contract No. DA-49-007-MD-632

Studies on Metabolism and Mechanism of Destruction of the Formed Elements of the Blood

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Supported by:
Research and Development Division
Office of the Surgeon General
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Washington 25, D.C.

University of Rochester School of Medicine and Dentistry

Rochester 20, New York

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During the period covered by this report, the following work has been carried out:

A. Further studies on membrane changes occurring in erythrocytes stored in ACD solution at 4 degree C.

Previous progress reports have outlined our finding that during blood storage a progressive loss of lipid from the red blood cell occurs, and that the loss of each erythrocyte lipid is proportional to its initial concentration. During the past year we have used the hemoglobin free erythrocyte "ghost" preparation previously described (see publications -1) further to characterize membrane changes occurring during blood storage. Ghosts were made from erythrocytes stored for periods of up to 42 days, and the following parameters studied: membrane lipid, membrane protein, membrane reactive sulfhydryl groups, membrane bound ATP-ase and glucose 6-phosphatase dehydrogenase. It was found that no measurable quantitative changes in membrane protein, sulfhydryl groups and enzymic activities occurred during storage, but, as was expected, the loss of lipid found in the intact stored red blood cell was reflected exactly in the membrane preparation. The results at 42 days of storage are summarized in the following tables.

TABLE I

Lipid Composition of Intact Cells and "Ghosts" Before and After 42 Days of Storage.

	Pre-Storage		Post-Storage	
	Intact Cell	Ghost	Intact Cell	Ghost
Total Lipid/cell ($g \times 10^{-13}$)	4.91	4.95	3.75	3.79
Lipid Phosphorous/cell ($ug \times 10^{-9}$)	11.5	11.3	8.99	8.62
Cholesterol/cell	1.13	1.14	0.82	0.79

TABLE II

Protein and Enzymic Activities in Erythrocyte "Ghosts" Before and After 42 Days of Storage.

	Pre-Storage	Post-Storage
Protein/ghost ($g \times 10^{-13}$)	5.80	5.92
Reactive sulfhydryl groups/ghost ($mM \times 10^{-14}$)	1.5	1.6
ATP-ase activity/ghost ($mM\ P_i \times 10^{-10}$ /hr.)	1.78	1.88

These studies indicate that membrane changes produced during storage effect primarily or exclusively the lipid components of the membrane. This is additional evidence for our previous suggestion that alteration in the membrane lipids might constitute a central feature of the "storage lesion".

We have previously reported that some evidence for repair of membrane lipid loss, during post-storage incubations at 37° C, could be found in blood stored for less than 21 days. This consisted of observing that the rate of movement of lipid phosphorous from plasma to red blood cells during post-storage incubations was increased substantially over that found in the fresh cell. This suggested that a process of repair of lipid loss, analogous to the repair of cation gradients might be operative.

During the past year, we have attempted to delineate this process further, and to study the relation of lipid repair to the energy metabolism of the erythrocyte. Blood was stored for 42 days, and then incubated at 37° in the presence of P^{32}-labelled plasma lipids. All other plasma-labelled phosphorous compounds were removed by prior dialysis of the plasma, as previously described.

The addition of adenosine to such post-42 day storage incubations caused a prompt regeneration of cellular ATP, as has been described by other workers (Shafer, W.A. and Bartlett, G.R., J. Clin. Invest., 41, 690, 1962). The regeneration of cellular ATP produced a striking increase in the movement of inositol phosphatide from the plasma to the red blood cell membrane, as shown in the following table.

TABLE III

Movement of Plasma Inositol Phosphatide P^{32} into Red Blood Cells During Post-42-Day Storage Incubation at 37° C.

(Relative Specific Activity - as Per Cent)

Incubation Time	Additive	
	Adenosine & glucose	Glucose
6 hours	17	5
12 hours	21	7

No consistent increased movement of other plasma phopholipids could be detected between the two types of experiments over the time periods studied. It should, however, be noted that repair of cationic gradients during post-storage in vivo circulation may take a matter of days. Thus, a net increase in the movement of other lipids, in the presence of adenosine might be found, if the process could be observed for longer periods.

The elaboration of membrane lipid loss during storage and subsequent repair will continue to be one of the central interests in our studies.

B. Further studies of phospholipid synthesis from inorganic P^{32} by various cellular elements.

The question of incorporation of inorganic P^{32} into phospholipids continues to appear important for the understanding of various membrane functions in mitochondria, and because of the continued suggestions that such incorporation plays a role in active transport in the erythrocyte. Interpretations of such experiments vary because (1) the role of the small amounts of non-lipid contaminants which are present in lipid extracts, and are potentially highly labelled by the radioactive precursor used, has not been fully evaluated, and (2) the degree of incorporation, in relation to the amount of radioactivity introduced, has not been clearly quantitated. Such quantitation would provide some indication of the magnitude of phospholipid synthesis.

These two problems of contamination and quantitation were studied by following the in vitro incorporation of inorganic P^{32} into the phospholipids of duck erythrocytes, bone marrow cellular elements and human red blood cells. Various extraction and purification procedures were evaluated, as well as the behavior of non-lipid phosphorous containing compounds of the red blood cell, (inorganic phosphorous and the various phosphorylated glycolytic intermediaries), in the chromatographic systems used for lipid separation. This made possible the evaluation of the role of potential non-lipid phosphorylated contaminants in interpreting results suggesting phospholipid synthesis by non-nucleated red blood cells.

Results obtained from lipid extracts known to be free of measurable non-lipid phosphorylated contaminants are shown in the next table. Incorporation of radioactivity is expressed as the relative specific activity of the given phospholipid in relation to the specific activity of the added inorganic phosphorous.

TABLE IV

Relative Specific Activities (as %) of Duck and Human Erythrocyte Phospholipids During Incubation With P-32.

	Duck			Human		
Time (hours) / Lipid	6	12	24	6	12	24
Inositol Phosphatide	0.6	1.1	2.0	0.008	0.022	0.046
Sphingomyelin	0.05	0.1	0.3	0.006	0.01	0.006
Lecithin	0.08	0.26	0.55	0.014	0.02	0.014
Phosphatidyl Serine	0.05	0.14	0.23	0.001	0.002	0.001
Phosphatidyl Ethanolamine	0.10	0.27	0.49	0.01	0.01	0.003
Phosphatidic Acid	1.91	2.37	3.16	0.5	1.5	2.8

The pattern of P^{32} incorporation into marrow cellular phospholipids is similar to that in the duck erythrocyte, though of greater extent. The observed relative specific activities of marrow phospholipids after 24 hours of incubation with inorganic P^{32} are shown in the next table.

TABLE V

Relative Specific Activities of Marrow Phospholipids After 24 Hours of Incubation with Inorganic P^{32}

Lipid	RSA (%)
Inositol Phosphatide	2.64
Sphingomyelin	0.67
Lecithin	1.90
Phosphatidyl Serine	0.89
Phosphatidyl Ethanolamine	1.00
Phosphatidic Acid	1.43

In the duck erythrocyte the marrow cellular elements true incorporation of P^{32} into phospholipids was characterized by a progressive increase in the specific activity over a period of 24 hours, and by a relative specific activities exceeding 0.1% in every case. This incorporation could be confirmed by radioautograms.

In the human red blood cell evidence for incorporation of P^{32} could be found only in the case of phosphatidic acid. This is quantitatively minor constituent of the red blood cell membrane, accounting for 3% of the total lipid phosphorous, i.e. about 0.13 mM per L of red blood cells. Unless the turnover of this compound were very rapid, its renewal could not account for active solium transport from the erythrocyte which amounts to approximately 3 mM per L of red blood cells.

During the past year, we have studied in some detail the degree of P^{32} incorporation into human erythrocyte phosphatidic acid in relation to other phosphorylated cellular compounds. Typical results of such experiments are shown in the next table. Incorporation is again expressed as the specific activity relative to the specific activity of the added inorganic P^{32} at time 0.

TABLE VI

Relative Specific Activities (%) of Various Phosphorylated Intracellular Compounds During 6 Hours of In Vitro Incubation in the Presence of Inorganic P^{32}

Intracellular Compound	Time				
	30 min.	1 hour	2 hours	4 hours	6 hours
Phosphatidic Acid.	0.03	0.065	0.15	0.37	0.5
Inorganic P.	2.0	2.5	6.5	9.4	6.2
ATP	2.0	2.5	6.4	8.5	6.0
2,3- DPG	0.9	1.8	5.3	8.9	7.2

It can be seen from these data that during 6 hours of incubation the phosphatidic equilibrates with neither the intracellular inorganic phosphorous nor with intracellular ATP, whereas the relatively large pool of 2,3-DPG does achieve essentially the same specific activity as these latter two compounds. The almost linear, increase, over 6 hours, in the specific activity of the phosphatidic acid is very much against the idea that this compound exists in several compartments, and that one of these, only, turns over extremely rapidly in connection with active sodium transport. Thus, these quantitative data on the turnover of phosphatidic acid are against the view that this compound is related to the carrier mechanism in active sodium transport.

Additional studies on this point are in progress. In a preliminary way, the following factors seem not to influence the rate of turnover of phosphatidic acid: the introduction of caridac glycosides, such as ouabain, and consequent inhibition of active cation transport, prior depletion of cellular ATP and blood storage at 4° C. On the other hand, we have found that phosphatidic acid does not become labelled during incubations of emulsified cellular lipids with P^{32}, or during incubations of intact or ultrasonically solubilized ghosts with this labelled compound. These results indicate that although ATP does not seem necessary for the turnover of phosphatidic acid, other intracellular constituents are necessary, and that the labelling of this compound is not simply a n exchange phenomenon. The exact significance of phosphatidic acid labelling in the erythrocyte will be studied further.

Recently, the suggestion has been made (Kirschner, L.B. and Barker, J., Fed. Proc. 22, 333, 1963) that membrane inositol phosphatide became highly labelled- to a much greater extent than phosphatidic acid-when previously cold stored porcine erythrocytes were incubated at 37° C with inorganic P^{32}. The conclusion was drawn that such turnover was intimately related to active sodium extrusion. It can be seen from Table III that in the human red blood cell incorporation of inorganic P^{32} into inositol phosphatide probably does not occur. The exchange of this compound with plasma inositol phosphatide, however, is increased in stored human erythrocytes, when adenosine is added and ATP regeneration facilitated, as described above. More detailed studies of the turnover of inositol phosphatide, using rigorous isolation procedures are in progress.

C. Further studies on the chemical ultrastructure of the erythrocyte membrane.

The development of a reproducible ghost or membrane preparation has permitted us, during the past year, to continue our studies on the basic problem of the organization of the lipid molecules in the erythrocyte membrane. Variation in extraction procedures appeared unfruitful in developing information in this area using intact erythrocytes, as described in previous progress reports. Using the ghost preparation, however, we have established, during the past year, that about 50% of the total lipid is extractable from the erythrocyte membrane with non-polar solvants, such as hexane, di-ethyl ether and chloroform. The distribution of the lipids extractable with non-polar solvants, determ ined to date, is shown in the next table.

TABLE VII

Membrane Lipids Extracted with Non-Polar Solvants

Lipid	Per cent of total present extractable
Cholesterol	90-100
Lecithin	45-55
Sphingomyelin	15-25
Phosphatidyl Serine	10-15
Phosphatidyl Ethanolamine	50-60

The amounts of the quantitatively minor, but potentially important, compounds, such as inositol phosphatide and phosphatidic acid, which can be extracted with non-polar solvants have not as yet been determined with sufficient accuracy.

The extractability of a membrane lipid depends in part on its participation in a lipoprotein complex. Those lipids extractable with non-polar solvants can be throught not to exist in intimate relation with the protein moiety of the membrane. Along with our previous demonstration of the existance of exchangeable and non-exchangeable pools of various membrane lipids, these extraction studies lend further support for the concept of the non-homogeneous distribution of lipids within the erythrocyte membrane. Further studies of this sort are in progress.

D. The use of C-14 as an isotope in studying the dynamic state of membrane lipids.

Our basic aim in this area continues to be the study of individual portions of the lipid molecule in the dynamic behaviour of membrane lipids. To achieve a degree of precision comparable to the results obtained with P^{32}, certain technical problems require sdution. This will entail the use of liquid scintillation counting of radioactivity, and partial isolation of individual phospholipids by refrigerated silicic acid column chromatography. The requirement for this are outlined in the application for support for the coming year.

E. Study of membrane lipids in abnormal cells.

Analyses of erythrocyte and plasma lipids in abnormal states continues as opportunities present themselves. During the past year we have confirmed the previously reported abnormalities of lipid distribution in erythrocyte and plasma lipids of patients with acanthocytosis. We have further confirmed the absence of such abnormalities in various relatives of affected persons.

Essentially normal quantitative and qualtative lipid distributions have been found in the plasma and erythrocytes of four patients with Tay-Sach's disease.

Publications

1. Weed, R.I., Reed, C.F. and Berg, G. Is hemoglobin an essential structural component of human erythrocyte membranes? J. Clin. Invest., 42, 581, 1963.

2. Ways, P., Reed, C.F. and Hanahan, D.J. Red Cell and Plasma Lipids in Acanthocytosis. J. Clin. Invest. (in press-August 1963).