Sulphydryl Reagents and Rh Activity of Erythrocyte Stroma

IT has recently been shown that the Rh activity of lyophilized human Rh-positive stroma could be abolished with certain sulphydryl reagents, that this could be prevented by the concurrent addition of excess thiol, and that the loss was reversible with one such reagent[1]. It was not established whether the antigenic activity of non-lyophilized erythrocytes shows such an effect. The resolution of this problem was hampered in the case of intact cells by the presence of soluble sulphydryl systems, including haemoglobin and glutathione[2], and in the case of non-lyophilized stroma both by the occlusion of these soluble components, and by the difficulty of quantitation of wet stroma. The results of the present experiments suggest that the loss of Rh activity of lyophilized stroma brought about by sulphydryl reagents is equally true of stroma which has not been lyophilized.

Two methods of obviating occlusion were used. The first may be termed a *p*H shuttle, and consisted of washing the intact cells in 0·9 per cent sodium chloride, rupturing in distilled water, adjusting the *p*H under continuous monitoring to 8·3 with trisodium phosphate, then adding citric acid to *p*H 5·5, followed by centrifugation. The residue was then taken up in 15–20 times its volume of distilled water and the process repeated. At the higher *p*H the suspended phase assumed approximately colloidal dimensions and occluded material was released. Five or six such shuttle steps were required and it was noted that some loss of Rh activity resulted. A second method was then used which did not appear to result in loss of activity. Rapid freezing at the temperature of solid carbon dioxide was substituted for the *p*H shuttle, and the material was washed with distilled water. The freeze-thaw procedure was repeated twice.

Using stroma washed with the freeze-thaw method a fine uniform suspension was made up volumetrically in distilled water, a measured portion removed, lyophilized and weighed, and the relation between dry weight and volume calculated. Twenty mg of the lyophilized suspension was matched with the corresponding volume of non-lyophilized stroma. The samples were washed with 0·1 M sodium phosphate buffer (*p*H 7·0), centrifuged and treated with 10^{-4} M *p*-chloromercuribenzoate (Nutritional Biochemicals) in the same buffer for 60 min at 22° C with agitation. A similar set was treated with phosphate buffer only. The experiment was repeated using 3×10^{-4} M reagent. The specimens were then centrifuged, washed twice with phosphate buffer, and 1 or 2 ml. human isoimmune anti-D suitably diluted in normal human serum were added. The dilution was standardized so that 20 mg of untreated O Rh positive lyophilized stroma could just remove all anti-D activity after 60 min incubation at 37° C, and subsequent centrifugation. Similarly for the present experiments the supernatants after centrifugation were studied for residual anti-D activity against trypsinized O positive erythrocytes, as previously described[1]. The results are summarized in Table 1. The partial loss of activity with the lower concentration of *p*-chloromercuribenzoate and the complete loss with the higher concentration were indistinguishable for lyophilized and non-lyophilized stroma. In each case no loss in activity was noted when 0·1 M 2-mercaptoethanol (Eastman Organic) was present with the sulphydryl reagent. The results of similar experiments with *N*-ethylmaleimide (Nutritional Biochemicals) are shown in Table 2.

Lyophilization of washed erythrocyte stroma results in the solubilization in 0·9 per cent sodium chloride of one-third its weight, consisting mostly of hexose-containing material of large molecular size together with some peptides[3]. Since this probably indicates significant disruption of the cell wall the question arose whether the sensitivity of the Rh (D) antigen to sulphydryl reagents in the case of lyophilized cells might not be dependent on the lyophilization process itself. These results show that such sensitivity does not depend on lyophilization. Freezing of intact erythrocytes without drying is not associated with significant disruption under certain circumstances[4] and it would not be anticipated that freezing and thawing of stroma would result in changes comparable to lyophilization. The present investigation does not show whether the Rh antigen of the intact erythrocyte is capable of reacting with sulphydryl reagents.

Table 1. RH ACTIVITY AND *p*-CHLOROMERCURIBENZOATE

O positive stroma	Treated with 5 ml.	Resulting loss of Rh activity
Non-lyophilized	Phosphate buffer, *p*H 7	None
Non-lyophilized	10^{-4} M PCMB in phosphate	Partial
Non-lyophilized	3×10^{-4} M PCMB in phosphate	Complete
Non-lyophilized	3×10^{-4} M PCMB and 0·1 M 2-ME* in phosphate	None
Lyophilized	Phosphate buffer	None
Lyophilized	10^{-4} M PCMB in phosphate	Partial
Lyophilized	3×10^{-4} M PCMB in phosphate	Complete
Lyophilized	3×10^{-4} M PCMB and 0·1 M 2-ME in phosphate	None

Twenty-mg specimens were all from the same donor, and were incubated at 22° C for 60 min with agitation. After washing twice with phosphate, anti-D serum was added and incubated for 60 min at 37° C. After centrifugation, the supernatant was studied for residual anti-D activity.
* 2-mercaptoethanol.

Table 2. RH ACTIVITY AND *N*-ETHYLMALEIMIDE

O positive stroma	Treated with 5 ml.	Resulting loss of Rh activity
Non-lyophilized	Phosphate buffer	None
Non-lyophilized	10^{-3} M NEM in phosphate (at 22°)	Partial
Non-lyophilized	10^{-3} M NEM in phosphate (at 37°)	Complete
Non-lyophilized	10^{-3} M NEM and 0·1 M 2-ME in phosphate (at 37°)	None
Lyophilized	Phosphate buffer	None
Lyophilized	10^{-3} M NEM in phosphate (at 22°)	Partial
Lyophilized	10^{-3} M NEM in phosphate (at 37°)	Complete
Lyophilized	10^{-3} M NEM and 0·1 M 2-ME in phosphate (at 37°)	None

Procedure as in Table 1 with the indicated incubation temperatures.

This investigation was supported by grants from the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health and the National Foundation.

I thank Gary L. Rodney for technical assistance.

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Copper Transport in Mammalian Tissues

RECENTLY, Neumann and Silverberg[1] presented results of experiments which were very similar to those which we had briefly reported earlier[2,3]. They concluded that amino-acids facilitate the transport of copper at the cellular level and presented conclusive evidence to support this. They further concluded that the facilitation of copper transport by amino-acids fulfils the criteria of an "active transport system", as it is dependent on temperature, concentration and oxygen, and operates against a chemical concentration gradient.

We believe that the latter conclusion cannot be based on the evidence presented by Neumann and Silverberg. Their first two criteria, dependence on temperature and on concentration, apply equally to the processes of simple or facilitated diffusion. Dependence on oxygen may indicate a dependence on chemical energy—but this is not necessarily so. The last criterion—the operation of the copper transport against a chemical concentration gradient—cannot be used as an argument in favour of this being an "active transport" as the concentrations of free copper ions on either side of the membrane are not known. In fact, it is well known that most of the copper in both the extracellular and the intracellular compartments is bound to various ligands. Therefore, concentration of the label in the slices, the development of a ratio