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ENZYME ACTIVITIES AND ULTRASTRUCTURE OF A MEMBRANE FRACTION FROM HUMAN ERYTHROCYTES

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SUMMARY

The homogeneity of an isolated membrane fraction of human erythrocytes was studied by density gradient centrifugation in silica solution. A main component (about 90 % on a nitrogen basis) was obtained as a single band at a density of 1.08.

When analyzed by electron microscopy, this component was found to consist of a membranous structure which, in contrast to isolated ghosts, appeared to be essentially devoid of cytoplasmic structures.

The main component, obtained at a density of 1.08 of the gradient, was shown to contain glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase. Thus, the present results strongly support the view that part of the glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase of the human erythrocyte is intimately associated with the membranous structure.

INTRODUCTION

The plasma membrane of a mammalian cell generally consists of lipids and proteins which are intimately associated in such a way that they form a physical lipoprotein boundary separating the cell from its environment. This is the site of much of the physical and chemical activity involved in the transport of substances in and out of the cell. It has been established by several authors, for example, that the ATPase enzyme system, which is concerned with the active transport of sodium and potassium across the plasma membrane, is located in the membrane (for review, see ALBERS[1]). Furthermore, there is some evidence that, like the mitochondrial enzyme complex involved in oxidative phosphorylation, enzyme activity may depend on an organized lipoprotein structure[2].

It has been shown that isolated membranous structures of human erythrocytes are capable of forming adenosine triphosphate when incubated with substrates and cofactors of the first energy-yielding step of glycolysis[3–6]. In studying an isolated membrane fraction, RONQUIST[5] found that the activity of glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12) was firmly attached to the membranous structure and that this activity was 10-fold larger than that of phosphoglycerate kinase (EC2.7.2.3).

In the present investigation, the isolated membrane fraction was further purified by density gradient centrifugation in a colloidal silica solution[7]. The membrane fraction obtained after this purification procedure was analyzed for glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase (EC 2.7.4.3) activity. In addition, electron microscopy of the ghost material and membrane fraction of human erythrocytes was performed to examine the purity and ultrastructure of the isolated membranous material, as well as any changes of the membranous structure which may have been caused by the silica solution.

MATERIAL AND METHODS

Analytical methods. Nitrogen was determined by a micro-Kjeldahl method.

Special chemicals. Dithiothreitol (Cleland's reagent) was obtained from Calbiochem, Los Angeles. Glutathione (reduced form) was purchased from Sigma-Chemical, St. Louis. NADH, AMP, ATP, 3-phosphoglycerate (sodium salt), phosphoenolpyruvate (tricyclohexylammonium salt) and all enzymes used in the enzyme assay systems were obtained from Boehringer and Soehne, Mannheim.

Preparation of ghost material and membrane fraction. Ghosts and membrane fraction of human erythrocytes were prepared as previously described[5].

Enzyme assay. The glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase activities of the membrane fraction were determined at 25° according to a previous work[5], except that the assay media also contained 10^{-4} M dithiothreitol or $5 \cdot 10^{-4}$ M reduced glutathione.

Adenylate kinase activity was estimated by coupling ADP formation with pyruvate kinase, phosphoenolpyruvate, lactate dehydrogenase and NADH[8], and using a similar technique to that applied for glyceraldehyde-3-phosphate dehydrogenase and phosphoglycerate kinase[5]. The activity in the absence of AMP in the assay medium was determined concomitantly and subtracted from the adenylate kinase activity. An enzyme unit is defined as that amount which oxidizes 1 μmole of NADH per min at 25°.

Colloidal silica density gradient centrifugation. Continuous density gradient centrifugation was performed according to PERTOFT AND LAURENT[7], after mixing membranous material corresponding to 1.7–2.0 mg of nitrogen with the colloidal silica solution (16% (w/v) of Ludox HS, pH 7.4, without added electrolytes). If not otherwise stated, the silica solution contained 10^{-3} M dithiothreitol or $5 \cdot 10^{-3}$ M glutathione (reduced form). Gradient centrifugation was carried out in a Spinco Model L ultracentrifuge for 20 min at 102000$\times g$. The well-defined band obtained (see Fig. 1), consisting of the membranous material, was washed 4 times with a 0.01 M Tris–acetic acid buffer (pH 7.4) before use.

Electron microscopy. The ghost material and the membrane fraction before and after silica gradient centrifugation (20 preparations in total), were suspended in 0.01 M Tris–acetic acid buffer (pH 7.4) and fixed by addition either of an equal vol. of 2% osmium tetroxide or 2 vol. of 2.5% glutaraldehyde in 0.01 M Tris–acetic acid buffer (pH 7.4). Fixation time ranged from 30 to 60 min. The glutaraldehyde-fixed material was rinsed and then post-fixed and stained in the solution of osmium tetroxide for 30–60 min with a final osmium tetroxide concentration of 1%. After osmium fixation, the material was sedimented by centrifugation for 5 min at 450$\times g$. Sub-

sequently, the sedimented material was washed 3 times with the Tris–acetic acid buffer. Dehydration was performed with increasing concentrations of ethanol, and the samples were embedded in Epon[9]. During the course of fixation and dehydration, the membranous material was transformed into a dispersed flocculation, and centrifugation at low speed was necessary at each step of the procedure, because of the very slow sedimentation by gravity alone.

Sections were cut, stained by uranyl acetate and lead citrate[10], and examined in an RCA EMU 3-B electron microscope.

RESULTS

Density gradient centrifugation

On density gradient centrifugation of the ghost material and the membrane fraction, a main component was recovered at a density of 1.08 in the gradient (Fig. 1). The banding of the main component at this unusually low density may be explained by an exclusion effect[7]. A faintly visible layer at a density of 1.05 was also formed, comprising less than 10% of the main fraction (calculated on a nitrogen basis). When ghosts were centrifuged, a clear, hemoglobin-containing layer, well separated from the main component, was recovered at the bottom of the tube (at density 1.12–1.14). Thus, the main component was distinctly separated from the other layers in the silica gradient and was easily obtained without any contamination by the other layers.

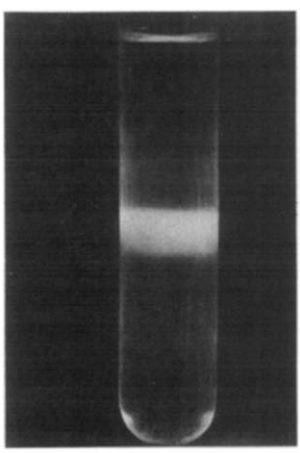

Fig. 1. Photograph of 12-ml centrifuge tube, showing the main fraction at density 1.08 resulting from density gradient centrifugation at 102000 $\times g$ for 20 min of 1.0 ml of membrane fraction (corresponding to 1.7 mg nitrogen).

Enzyme acitivity of membrane fraction

Table I shows that 75% of the glyceraldehyde-3-phosphate dehydrogenase activity was retained in the membrane fraction after gradient centrifugation, provided that sulfhydryls had been added to the medium. It seems likely that the partial loss of activity of glyceraldehyde-3-phosphate dehydrogenase was due to contact of the membrane fraction with the silica solution only, since the same recovery of the enzyme activity was obtained after mixing the membrane fraction with the silica solution

(containing sulfhydryls), irrespective of subsequent gradient centrifugation. If SH groups were not present in the medium, nearly all the glyceraldehyde-3-phosphate dehydrogenase was inactivated by the silica solution.

It is also evident from the table, under the conditions used, the activity of phosphoglycerate kinase and adenylate kinase was not influenced by the silica medium, nor did any activity separate from the main fraction during gradient centrifugation.

TABLE I

ENZYME ACTIVITIES OF MEMBRANE FRACTION AFTER COLLOIDAL SILICA DENSITY GRADIENT CENTRIFUGATION

Enzyme activities are given in units per mg nitrogen.

	Membrane fraction (not ultracentrifuged)	*Ultracentrifuged membrane fraction (no SH reagent in medium)*	*Ultracentrifuged membrane fraction (Cleland's reagent in medium)*	*Ultracentrifuged membrane fraction (reduced glutathione in medium)*
Glyceraldehyde-3-phosphate dehydrogenase	1.11	0.01	0.85	0.78
Phosphoglycerate kinase	0.12	0.10	0.13	0.11
Adenylate kinase	0.10	0.09	0.09	0.09

Electron microscopy

The ghost material had a similar appearance, irrespective of the method of fixation. This also applied to the membrane fraction. The ultrastructure differed, however, between the samples which had not been subjected to density gradient centrifugation and those which had.

(*1*) *Ghost material*

Ghosts, not subjected to density gradient centrifugation, contained material in irregularly distributed formations. The size of each formation varied, the larger ones being 5–10 μ (see Fig. 3). Each formation was composed of both membranes and granules (Fig. 2). The membranes appeared in groups more or less free from contamination by granules. The membranes mostly formed rounded, irregular structures, but sometimes they also ran parallel to each other. The granules, which had a maximum size of about 300 Å, were assembled irregularly, and usually lay inside closed compartments of the membranes (Fig. 2).

The ghost material subjected to density gradient centrifugation had a general appearance similar to that of the ghosts which had not been centrifuged (Fig. 3), although an ultrastructural difference was revealed. The membranes in the resolution used, seemed to carry attached particles, often on only one side (*cf.* Figs. 4 and 6). The rounded particles had a maximum diameter of about 150 Å. The particles had a more regular shape and size than the afore-mentioned granules and were presumably composed of a material differing from that of the granules.

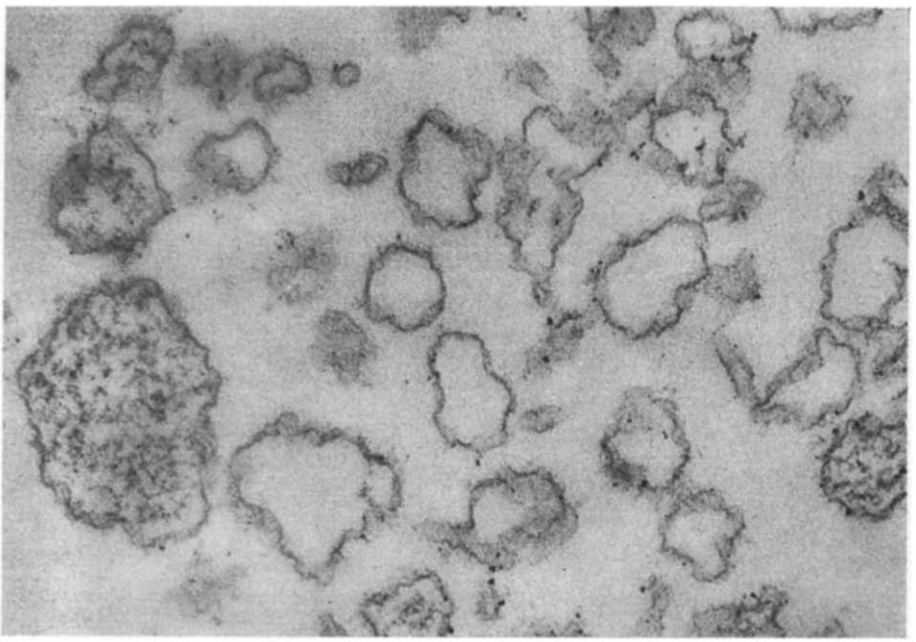

Fig. 2. Ghost material, not subjected to density gradient centrifugation. The membranous component is observed as small, rounded structures. The granular component appears enclosed in some of the membrane structures. Magnification 46000 ×.

(2) *Membrane fraction*

The membrane fraction also contained irregular formations of material. They were, however, smaller than those of the ghost material and were mainly devoid of granules (Fig. 5). The membranes ran irregularly, but were mostly circular and enclosed each other.

The membrane fraction subjected to gradient centrifugation had a general appearance similar to that not gradient centrifuged, but the membranes exhibited a particulate ultrastructure (Figs. 4 and 6) similar to that of the ghost material after centrifugation in silica.

DISCUSSION

The present results give additional evidence for the view that part of the glyceraldehyde-3-phosphate dehydrogenase, phosphoglycerate kinase and adenylate kinase of human erythrocyte is intimately associated with the membranous structure.

The ghost material and the membrane fraction (*i.e.*, disintegrated and further purified ghost material) intended for electron microscopy were chemically fixed and treated with various dehydrating agents and plastic solutions. Consequently, the ultrastructure of the membranous material studied by microscopy is probably not identical with that of the material used for enzymatic analyses. Electron microscopy can, however, indicate the purity of the fractions used for the enzymatic analyses and structural changes, if any, caused by different approaches during preparation of the samples. The methods used for fixing and embedding the material were the customary

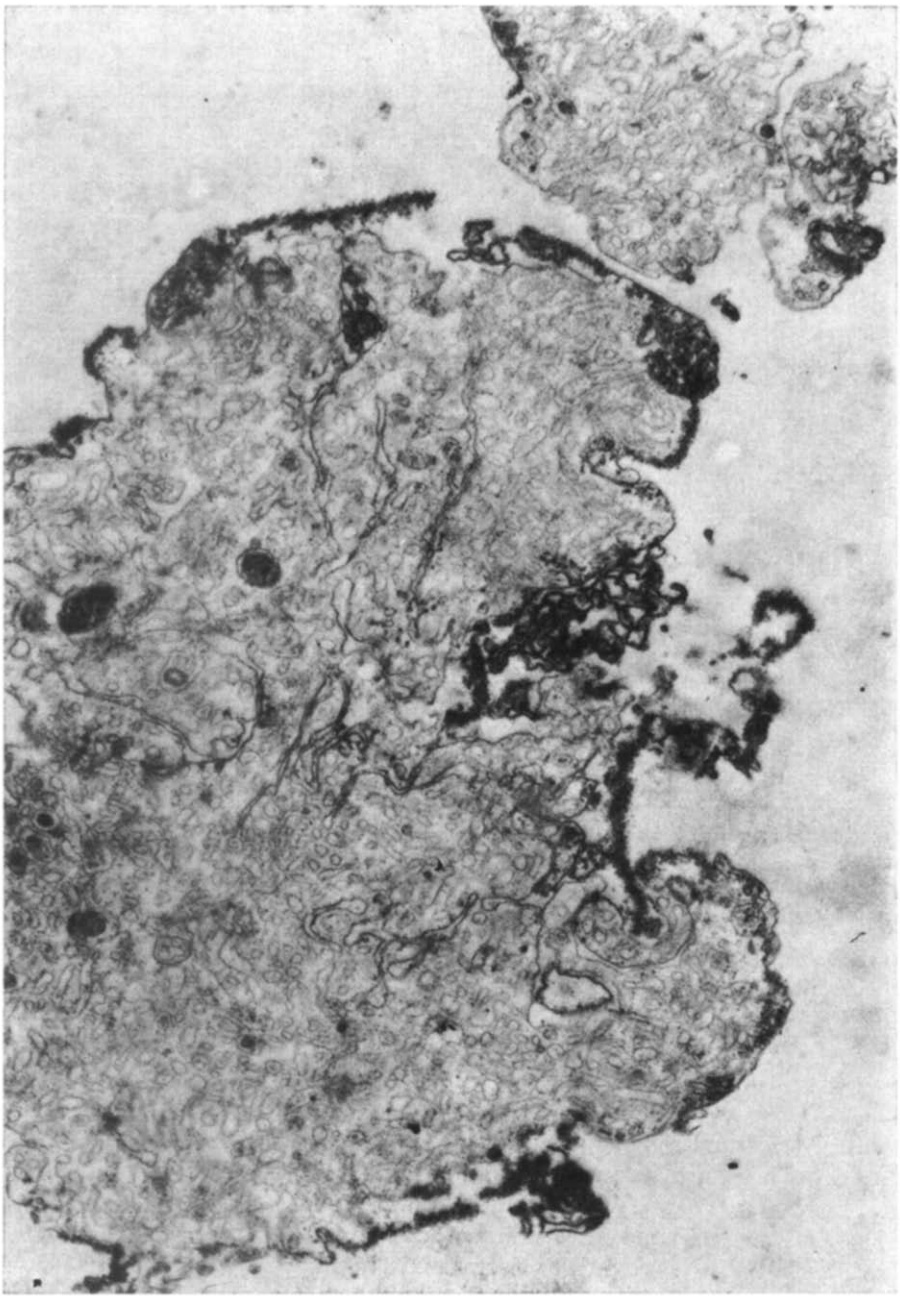

Fig. 3. Formations of ghost material subjected to density gradient centrifugation. Magnification 12000 ×.

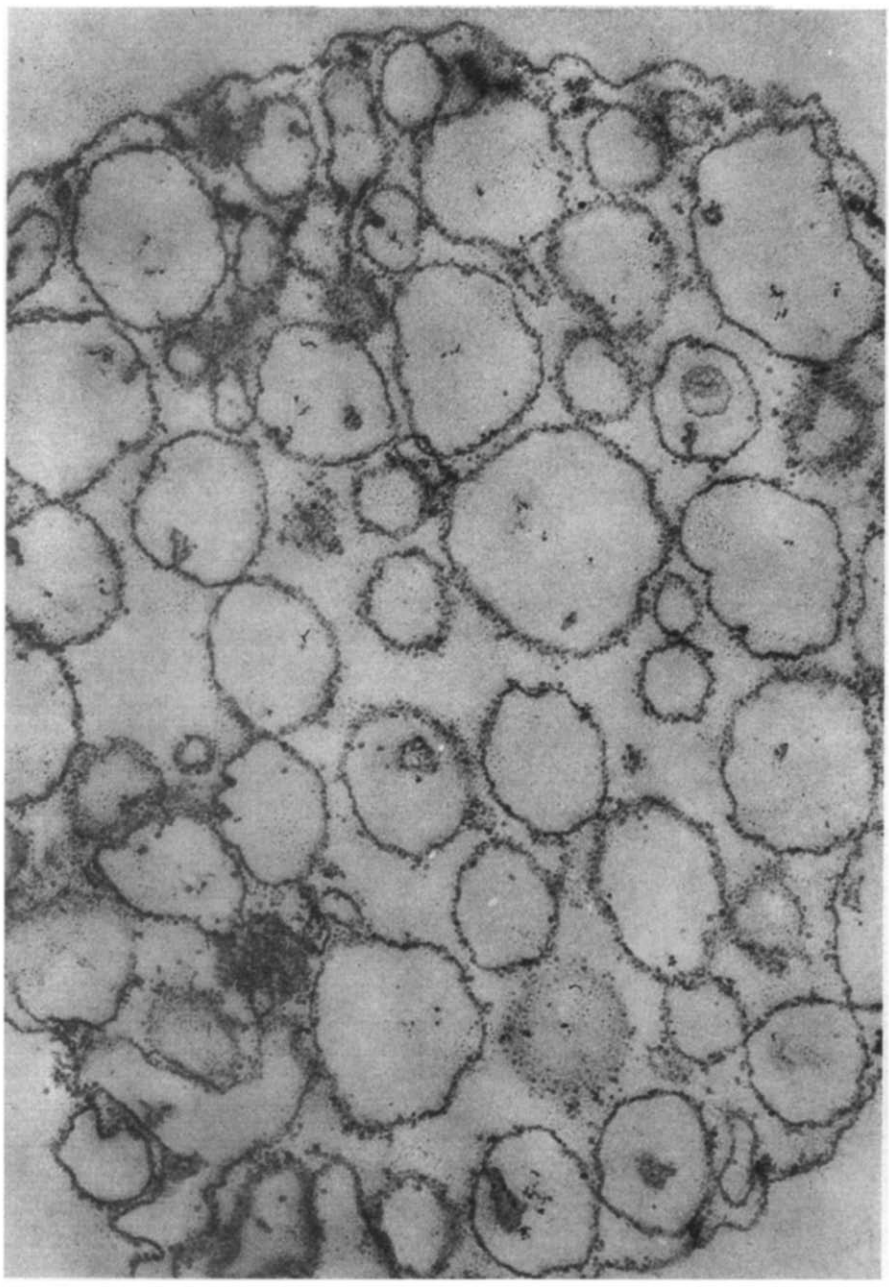

Fig. 4. Formation of membrane fraction, after density gradient centrifugation. The membranous component has a particulate appearance. Magnification 46000 ×.

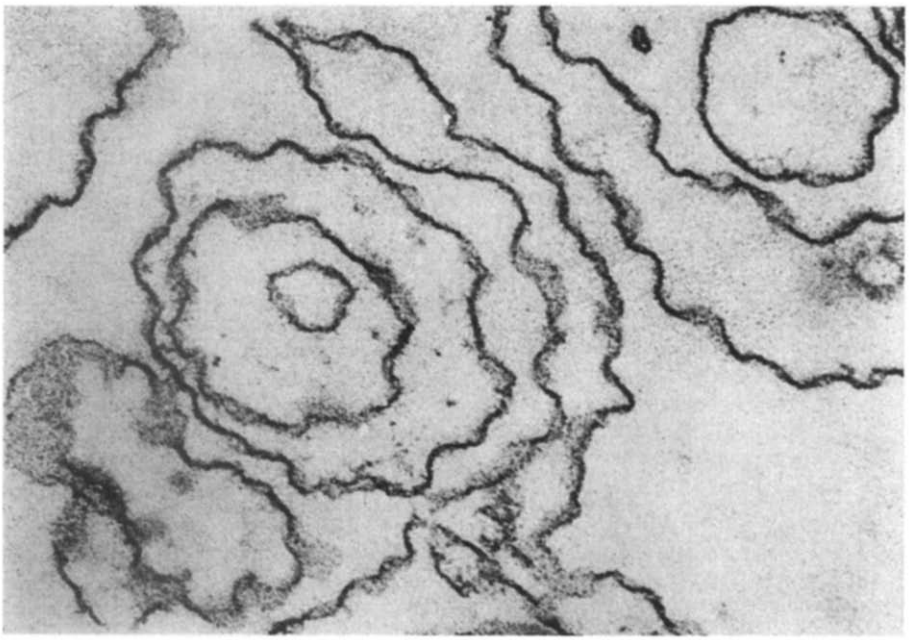

Fig. 5. Membrane fraction, not subjected to density gradient centrifugation. Magnification 72000 ×.

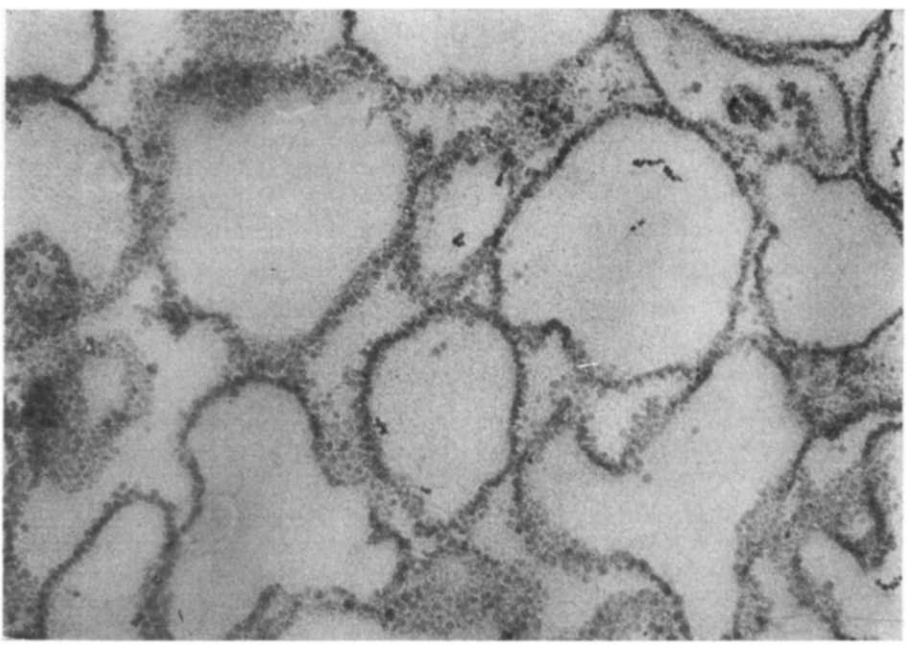

Fig. 6. Membrane fraction, subjected to density gradient centrifugation. Magnification 72000 ×.

ones, and no attempts have so far been made to examine the components of the various fractions with high resolution techniques.

The granules, which were more commonly seen in the ghost material, occurred only rarely in the membrane fraction. Since the membrane fraction had been more purified[5], it is therefore probable that they represent a contamination of cytoplasmic origin or of lipoprotein complexes formed during the early steps of the preparation.

The membranous component of both the ghost material and the membrane fraction was often present as circular structures after the two methods of fixation used. Although no definite conclusion concerning the circular formations is possible from the present data, it is probable that the isolated membranous structures have rearranged into smaller rounded structures during the preparation procedures.

Particles observed in the ghost material and the membrane fraction which had been subjected to gradient centrifugation had a size of about 150 Å. The silica particles in the colloid silica solution used have been estimated to range from 80 to 250 Å in size[11]. Consequently, the particles noticed may have been silica particles attached in some way to the membranous structure. The adsorption of silica may facilitate the inactivation observed of glyceraldehyde-3-phosphate dehydrogenase in the absence of sulfhydryls.

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