MAMMALIAN RED CELLS AS A SOURCE OF "SMALL PARTICLES"

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In recent years several authors have reported the isolation of small particles or heavy proteins from animal tissues (1–4). By differential centrifugation particles of different size ranges have been obtained (2), and their chemical composition and immunological specificity studied (2, 4).

The immediate importance of this discovery is that material is made available for the study of certain cell constituents in isolated form. The method thus opens a new way of approach to the investigation of the structure of cells. In addition the small particles are of indirect significance in connection with attempts to isolate filtrable viruses from infected tissues by physical methods. The size of particles obtained from normal tissue seems to vary over the same range as the size of viruses. Cell constituents must, therefore, be expected to be present in virus preparations obtained by selection according to size alone (centrifugation, filtration).

It is of interest to learn more about the origin and nature of these particles in view of their importance in both theoretical and practical respects. The fundamental question about their origin is whether they are preformed in the living cell or are formed through a breakdown of the cell structure. The general opinion seems to favor the first view, and it has been suggested on the basis of their contents of lipids that they represent the chondriosome (2). No conclusive evidence concerning their origin one way or another is available.

In work so far reported, whole organs have been used as a source. These, of course, contain besides various types of cells, blood and connective tissue. This report deals with attempts to isolate similar particles from red blood cells. Mammalian erythrocytes were chosen because they represent cytoplasm in "pure" form without a nucleus or microscopically visible granules. Furthermore, they can be obtained in large quantities without admixture of non-cellular elements.

From the beginning two objectives were kept in mind. The first was to see whether particles of a size comparable to those isolated from whole organs could be found. If so, the second objective was to test whether the yield could be increased by methods which might be expected to break down cell stroma. It was found that by resuspending hemolyzed blood cells in isotonic

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NaCl solution small particles could be obtained by differential centrifugation. The yield varied in different experiments, but by heating the resuspended "ghosts" at 50°C. for 30 minutes the yield could be increased several times. About one-third of the dry weight of the particles is lipids. They contain antigens that will give rise to hemolysins on inoculation into a foreign host. The yield, while varying in different experiments, was always increased several times when the resuspended ghosts were heated at 50°C. for 30 minutes.

Materials and Methods

Horse blood that had been stored at 12–14°C. for a period varying from a few days to 2 weeks was used. The cells were sedimented in an ordinary centrifuge, resuspended in saline, and washed in the centrifuge 3 times. Saline amounting to about 3 times the volume of the cells was used each time.

In the earlier experiments the further procedure was as follows: A 5 per cent suspension of the packed cells was made up in 0.05 M NaCl solution (which is approximately one-third isosmotic towards animal cells). After 30 minutes at room temperature the hemolyzed suspension was centrifuged for 1½ hours at about 8000 R.P.M. in the cold. The centrifuge used was that described by Pickels (5) with a 10 inch rotor. The clear, dark red supernatant was then carefully sucked off and the sediment resuspended in the same volume of isotonic NaCl solution. "Reversal of hemolysis" (Ponder) then took place and the suspension was again opaque.

10 ml. samples of resuspended ghosts were then heated in a water bath at different temperatures for 30 minutes. Control samples were kept on ice. Immediately after the heating the tubes were cooled and kept in the refrigerator. They were sometimes stored at this temperature for 1 or 2 days before further treatment took place, as this seemed to increase the final yield.

The next step was to centrifuge the samples for 10 minutes in an ordinary laboratory centrifuge running about 1500 R.P.M. The sediment was discarded and the supernatant run in an angle centrifuge at 4700 R.P.M. for 15 minutes. Considerable sediment was formed. The supernatant was then run in the air-driven centrifuge at 30,000 R.P.M. for 30 minutes (6).

FINDINGS

A typical experiment is summarized in Table I. The defibrinated blood had been kept in the refrigerator for 15 days. The cells were washed and lysed as described. Samples of the resuspended ghosts were kept at 40°, 50°, 60°, and 70°C. for 30 minutes. An extra sample was included: "50°C. old." The resuspended ghosts in that sample had been kept for 2 days in the refrigerator before being heated.

The first three columns in Table I show roughly the relative amounts of sediment formed during each centrifugation. The fourth and fifth columns show the nitrogen content of the sediments and the supernatants from the ultracentrifuge, respectively. It was found that non-protein N was negligible and the figures therefore represent total N.

The figures in Table I indicate that the heating at 40–50°C. decreased the amount of material sedimenting at low speeds but increased the amount of sediment obtained in the ultracentrifuge and the proteins remaining in the final supernatant, *i.e.*, the heat had a dispersing effect on the suspended structures. This effect was more pronounced at 50°C. than at 40° or 60°C. The sediment in these samples was grayish, translucent with a slight red tinge.

TABLE I

Amount of Material in Sediments and Final Supernatants after Centrifuging Samples of Resuspended "Ghosts" Kept at Different Temperatures

Resuspended "ghosts" kept 30 min. at:	Amount of sediment formed after centrifuging at:		After centrifuging at 30,000 R.P.M.	
	1500 R.P.M.	4700 R.P.M.	Total N in final sediment	Total N in final supernatant
°C.			*mg.*	*mg.*
0 (control)	+++	+++	0.06	1.8
40	++	+	0.16	2.2
50	+	+	0.36	2.4
50 ("old")	+	+	0.61	2.3
60	+++	+++	0.16	1.7
70	++++	++	0.02	0.1

TABLE II

Distribution of Nitrogen in Samples of Heated and Unheated "Ghost" Suspensions

Total nitrogen in	Heated*	Cold
Sediment in Swedish centrifuge	4.3	4.8
Sediment in ultracentrifuge	0.8	0.3
Supernatant in ultracentrifuge	2.0	1.7

* For 30 minutes at 50°C.

In the experiment described an examination of the final sediment from the 50°C. samples in the dark field microscope revealed myriads of small particles, which impressed one as being at the limit of visibility. In the unheated (control) sample the same particles were seen and an occasional ghost was found, but no ghosts were found in the heated samples. In the sample that had been heated at 70°C. almost all the protein was denatured and was sedimented at low speed.

Later experiments confirmed this finding. One is summarized in Table II. In that case distilled water was used for hemolysis instead of hypotonic salt solution.

35 çc. of washed and packed red cells were suspended in 140 cc. distilled water in the cold. 30 minutes later NaCl was added to bring the solution to isotonicity. The

suspension was then run for 2 hours in the cold at 8000 R.P.M. The sediment was then resuspended in 140 cc. distilled water and this process repeated twice. The final sediment, which was almost white, was then suspended in 75 cc. of 0.9 per cent saline. Samples of this suspension were then heated at 50°C. for 30 minutes and others kept on ice.

Both heated and unheated samples were then run first in the Swedish centrifuge at 4800 R.P.M. for 20 minutes and the supernatant from that run again in the ultracentrifuge as described. The material used in this experiment did not contain visible amounts of hemoglobin.

Red cell stroma was prepared according to a method reported by Parpart (7) using the precipitating effect of CO_2 in water. A whitish, tough product was obtained which could not be resuspended or dissolved in water or 0.9 per cent NaCl solution and on heating did not yield any of the small particles.

Content of Lipids

Two experiments were performed to learn about the lipid contents of the particles.

The red cells were hemolyzed and washed 3 times with 0.05 M NaCl solution, after which they were almost colorless. They were then heated for 30 minutes at 50°C. After centrifuging off the remaining ghosts in the angle centrifuge the suspension was run in the ultracentrifuge 30,000 R.P.M. for 30 minutes and the sediment frozen with dry ice and dried from the frozen state. The dry powder was then extracted first with acetone and then with alcohol-ether, each for 3 days at room temperature.

In the first experiment the total amount of dried particles weighed 80.6 mg. of which 27.6 mg. or about 34 per cent were extracted. In a second experiment, particles whose dry weight was 396 mg. yielded 123 mg., or about 32 per cent.

Erickson *et al.* (8) found an average of 24 per cent lipids in the dried stroma of horse erythrocytes. The figures given by Claude for the lipoid content of particles isolated from tumors and whole organs varied between 25 and 51 per cent (9, 10). The figures presented here lie between these values. The percentage of lipids found depends on the amount of potentially soluble protein (hemoglobin) that may be left in the ghosts or particles, and on the effectiveness of the method of extraction. Both are liable to vary with small differences in technique, and some of the discrepancies in the literature are undoubtedly due to such irregularities.

Antigenicity

Two rabbits were immunized with a suspension of these small particles, to see whether they carried the antigens responsible for the production of hemolysins.

The suspension given contained per milliliter approximately 5 mg. particles by dry weight. Three injections of 1 ml. each were given intravenously. The rabbits' blood was tested for hemolysins to horse erythrocytes before the immunization was begun and again 4 weeks later, *i.e.*, 10 days after the last injection. An excess of guinea pig complement was used, but in a preliminary test it was ascertained that threefold the dose of guinea pig serum used in the experiment did not by itself produce hemolysis of the horse cells.

The two rabbit sera hemolyzed horse cells in a dilution of 1:20 before immunization was begun and in dilutions of 1:1600 and 1:3200, respectively, after immunization. In other words, on injection into a foreign host, the particles isolated as described produced hemolysins effective against the whole cells in a reasonably high titer.

DISCUSSION

Small particles were isolated from horse erythrocytes, which had the same general characteristics as regards size and lipid content reported by various workers for particles from other tissues. That they were of the same nature has not been definitely proved, but a statement to the contrary would not seem to be justified. The evidence at present is that all broken down animal cells yield particles varying in size over a considerable range. The amount of these particles seems to be influenced by the treatment to which the cells are subjected.

The findings reported in this paper indicate that the heating in the presence of salt solution had a dispersing effect on previously insoluble structures; that is, the stroma must be looked upon as the source of the particles. The high lipid content of the particles is further evidence that they were derived from the stroma and, furthermore, the ghosts had been washed free of soluble proteins as well as possible.

Furchgott (11) has observed a disintegration of red blood cells under the influence of lyotropic salts like $LiClO_4$ and KCNS. The cells or ghosts usually began by forming long threads which later broke down into very small particles. He further found that under certain conditions red cells would disintegrate in the presence of NaCl. His results suggest that this disintegration is a complex reaction occurring in more than one stage. These important findings of Furchgott should be of value in further attempts to elucidate the nature of the process.

The natural conclusion from all these observations is that some at least of the small particles are not preformed in cells but appear through disintegration of cell structure. It would therefore be natural to expect to find them differing in quantity and in quality, according to the influences to which the cells have been subjected before and after death. This should be kept in mind when isolation of viruses from animal tissues is attempted. Organs from healthy

animals can hardly be relied upon as controls to diseased tissue, since it seems probable that pathological processes bring about changes in the quantity and quality of the particles obtained. There is no obvious reason to believe without direct proof that particles found in virus-infested tissue represent the virus itself rather than abnormal cell constituents. Their absence in the corresponding normal tissue should not alter that fact.

Hurst (12) used the Bielschowsky method to show that during an attack of poliomyelitis the neurofibrils are broken down and appear as tiny granules filling the pericaryon. This is an instance in which, under the influence of a virus, organized cell structures disintegrate into small fragments.

It should be pointed out that Claude (2) employs a 0.005 M phosphate buffer solution of pH 7.1 or 0.0002 N solution of NaOH instead of a physiological NaCl solution as a solvent. In his experience the particles did not remain in good suspension when NaCl solution was used. Other authors used NaCl solutions (3, 4). It should further be mentioned that Claude and others prefer working in the cold "in order to lessen the effect of autolysis and 'spontaneous' deterioration of the material" (2). No quantitative data on the effect of room temperatures are available. It has been found (13) however that heating at 54°C. and 95°C. may bring about a decrease in the opalescence of the suspension. This indicates that under certain conditions heating will have a dispersing effect on small particles from whole organs as it does on those isolated from red blood cells.

Until more exact data are available on the effect of temperature and solvents on the yield, these findings do not seem to justify a conclusion as to the similarity or dissimilarity of the particles from red cells and whole organs.

The question of the origin of the particles from red cells would appear to be bound up with the question of the nature and distribution of the "stroma." The existence of a submicroscopic (molecular) structural pattern inside the cell is still controversial and since the results reported throw no light on that matter it will not be discussed. (For discussion, see 14–17.)

Evidence is increasing that cytoplasmic constituents which can be sedimented in the centrifuge and hence belong to the "insoluble stroma" contain substances of functional importance. This has been found to be true of cytochrome oxidase (18–21), catalase (19), some antigenic components of cytoplasm necessary for cell growth *in vitro* (22, 23); and conceivably some of the concentrated virus preparations should be included. The antidiuretic, chloruretic, and pressor substances can be sedimented from crude extracts of pituitary tissue by a 7 hour run in the ultracentrifuge (24). After chemical purification the active pituitary substances can no longer be sedimented. The finding of respiratory enzymes in sedimentable matter from animal cells and the necessity of postulating an internal structure for the normal functioning of the respiratory processes has been recently discussed by Commoner (25).

If there is an internal reticulum in cells,—and the evidence for it is increasing,—the small particles might well be fragments of these structures, varying in quantity and size according to the extent and nature of the breakdown.

SUMMARY

1. Small particles essentially similar to those previously isolated from other tissues have been isolated from mammalian red blood cells (horse blood).

2. About one-third of the dry weight of the particles is lipids.

3. The particles produce hemolysins against the homologous erythrocytes when inoculated into a foreign species.

4. The fact that the particles can be isolated from mammalian red cells which do not contain visible granules is taken to indicate that some at least of the particles isolated from whole organs represent disintegrated "stroma."

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