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STUDIES ON ISOLATED CELL COMPONENTS

X. STRUCTURE FORMATION BY CLEAR LIVER PROTEIN SOLUTIONS

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BASED on the original concepts of Bungenberg de Jong [8], it has been proposed that some cell structures may exist in equilibrium with their constituent macromolecules in solution [3, 4, 6]. Such a mechanism would allow changes in structure to occur in response to alterations in the composition of the dissolved or soluble phase of the cell during growth, starvation, differentiation, in response to external agents and stimuli, and during cell division. The formation of specific structures as opposed to simple coacervates requires that surface specificities exist on the building blocks from which the structures are formed and that different microenvironments occur throughout the cell, resulting in the formation of different structures in different places, e.g., the cell membrane and cortex at the edge of the cell and the nuclear envelope at the surface of the nucleus.

The present study reports a series of structures observed to form in uncontaminated solutions of liver proteins under a variety of conditions. These structures are at least superficially similar to those found in cells. The observations are of interest because they suggest that some of the structures observed in tissue breis may be formed during or after cell breakage and may not have existed as such in the cell. They further raise the question whether *in vitro* formation of these structures may not involve some of the same intermolecular forces, specificities, and conditions that give rise to similar structures *in vivo*. The condensation of "protomorphs" from clear bacterial extracts by the addition of manganese [7] appears to be an example of a somewhat similar effect.

EXPERIMENTAL

Clear solutions of rat liver soluble proteins (ratlivsol) were prepared from rapidly perfused liver by ultracentrifugation as previously described [5]. The final solution obtained by centrifugation at 105,000 × *g* for 1 hour was pale yellow and perfectly transparent. No structure or particles of any kind were seen with the phase contrast

[1] Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission.

microscope. After 105 minutes to 4 days at 2°C, either undialyzed or during dialysis against a variety of solutions indicated in the figure legends, a gradually increasing turbidity that produced a small white pellet after centrifugation at 18,000 × *g* for 5 minutes was observed. For examination under dark-medium phase contrast, the pellet was resuspended in either a small amount of the supernatant protein solution or in the dialysis buffer. Reference phase contrast photomicrographs of a fresh liver brei in 0.25 *M* sucrose are shown in Figs. 1 and 2.

The first effect observed microscopically was the appearance of a very few small granules ranging from the limits of resolution of the microscope used to approximately 1 μ in diameter. Most of these appeared solid, but a few were tiny hollow spheres. Occasionally a crescent form that resembled swollen isolated mitochondria was seen [1, 10]. Most of the observable material in all preparations was in the form of an amorphous precipitate. In preparations studied at various intervals, six discrete types of structure were observed, as follows:

Small granules.—Most of these were solid and ranged from the limits of resolution to 1–2 μ in diameter. A few were hollow or had a crescent form. There appears to be an upper size limit for the dense solid particles (Fig. 3).

Refractile granules.—Highly refractile granules appeared that were generally about 1–2 μ in diameter and resembled lipid droplets (Figs. 3, 4, and 18). Since they readily sediment in aqueous solution they cannot be composed entirely of lipid.

Round vesicles.—These hollow structures were generally smaller than liver nuclei and ranged from 2.5 to 8 μ in diameter. Although most were completely empty, but with a few granules adhering to their outer edge (Fig. 15), many exhibited an internal structure (Figs. 5–10, 12–16). In a few instances small granules in rapid Brownian motion were observed inside the vesicles. A few vesicles were observed having sufficient internal phase contrast density to make them resemble superficially isolated liver nuclei, but the vast majority were empty (Fig. 11). Not infrequently two or more of the vesicles coalesced to form multiple compartmented structures.

Thin translucent sheets.—These resemble somewhat the bits of cell membrane sometimes seen in rat liver breis. The continuity of the sheets, which allows them to be distinguished from fibers, may be demonstrated by focusing up and down. The cross section in focus as the objective is moved is seen as a fine continuous line (Figs. 19 and 20).

Fibrous material.—This material was observed under a variety of conditions. In whole breis, very fine fibrils often formed on the under surface of the coverslip during observation. Fibrils observed here in suspension are often quite irregular with many embedded granules (Fig. 22) or occur as a "brush heap". Fibers formed in solution frequently contain occluded granules, giving them a pseudo-banded appearance. Fine fibers and fibrils extending down to the limits of resolution of the light microscope may be readily produced by lowering the pH of the liver protein solution to between 5 and 6 (Fig. 23). This is accomplished by brief dialysis against pH 5.0 sodium acetate buffer, 0.1 ionic strength.

Matrix material.—A diffuse matrix substance may enmesh granules and fibrils to form islands of granular material. In some preparations these islands have diffuse edges, in others the borders are as well defined as those of large cells (Figs. 21 and 25).

For comparison, precipitates produced by the interaction of rat serum with rabbit antirat serum and bovine serum albumin with its rabbit antiserum are shown in

Figs. 26 and 27. These precipitates do not resemble ratlivsol precipitates, and indicate that the origins are not comparable. All illustrations except Fig. 22 are enlarged to the same magnification. The results presented were obtained with samples of ratlivsol prepared at intervals over a period of one year. The results are not caused by bacterial contamination since bacteria would be removed by ultracentrifugation and structure formation occurs in only a few hours in the cold.

DISCUSSION

A variety of structures have been shown to form from clear solutions of rat liver soluble proteins (ratlivsol). Many of these structures resemble superficially those seen in fresh breis and in living cells. This at once raises the question of the true identity of isolated cell fractions.

In previous studies (Anderson, unpublished), it was noted that breis left for several hours at room temperature showed two distinct types of "nuclei". One type had the usual phase contrast density; the other appeared optically empty and resembled isolated nuclei treated with deoxyribonuclease [2]. The latter objects, in the light of the present study, may have been formed *in vitro*. In electron micrographs of cell fractions, structures have occasionally been observed that have no evident counterpart in micrographs of intact cells. Especially suspect are particles obtained only after prolonged ultracentrifugation (which actually is often sufficient to sediment a large part of the soluble protein). Since a variety of structures appear when a ratlivsol preparation is

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Fig. 1.—Rapidly perfused rat liver homogenized with 2 ml 0.25 *M* sucrose per gram of tissue. For microscopy, preparation was diluted with 8 volumes of 0.25 *M* sucrose. High dry objective.

Fig. 2.—Same as Fig. 1, but with oil immersion.

Fig. 3.—Ratlivsol granules after dialysis for 25 hours at 4°C against pH 7.5 sodium phosphate buffer, 0.02 ionic strength.

Fig. 4.—Highly refractile granules seen in ratlivsol preparation dialyzed 22 hours at 4°C against 0.25 *M* sucrose.

Fig. 5.—Vesicle observed after 24 hours at 4°C.

Fig. 6.—Vesicle observed after 18 hours at 4°C.

Fig. 7.—Vesicle observed after two days at 4°C.

Fig. 8.—Vesicle observed in ratlivsol containing 0.001 *M* $CaCl_2$ after 18 hours at 4°C.

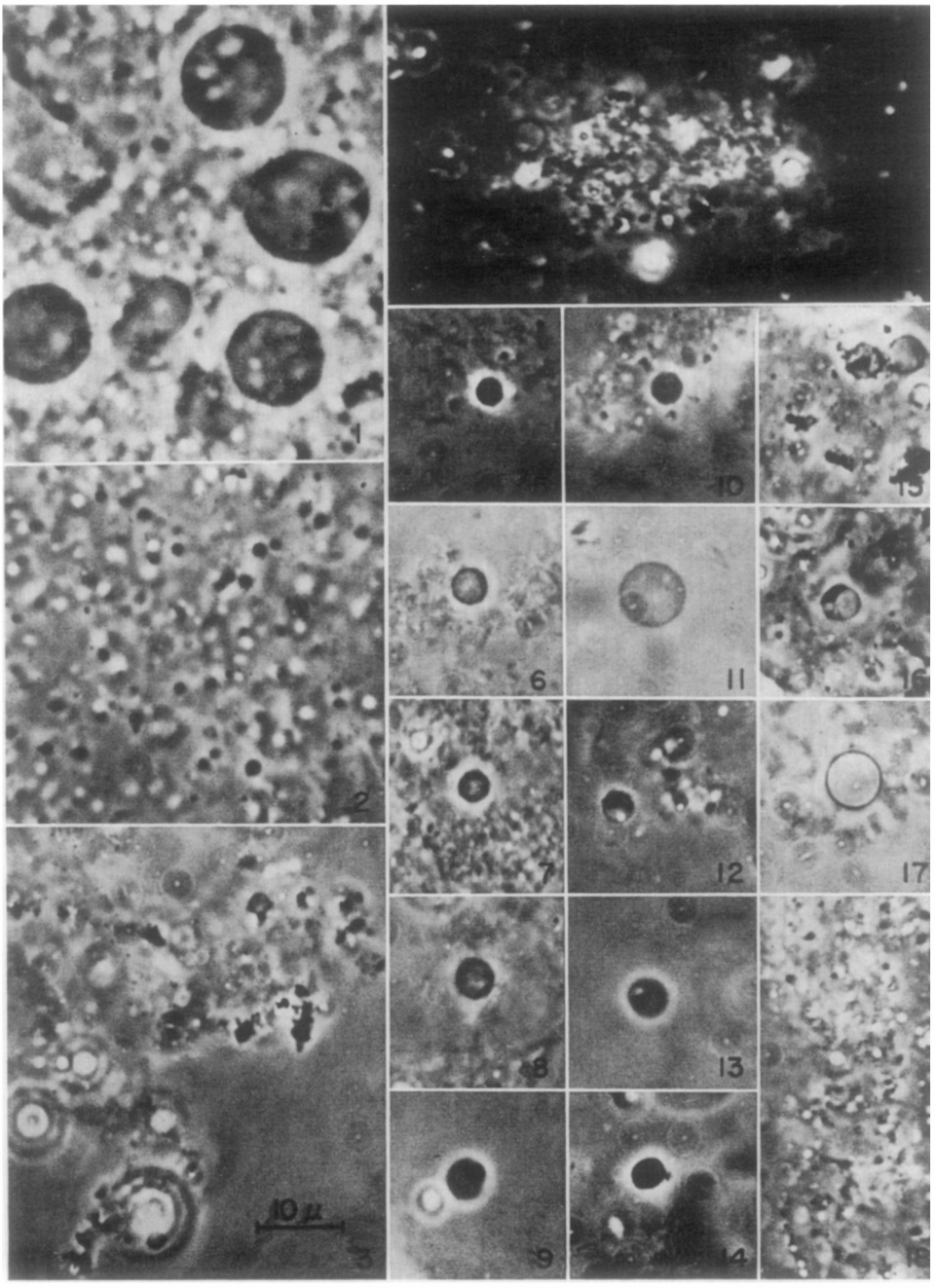
Figs. 9–12.—Vesicles observed after dialysis for 105 minutes against 0.25 *M* sucrose.

Figs. 13–14.—Structures seen after dialysis for 105 minutes against pH 7.5 sodium phosphate buffer, 0.02 ionic strength.

Figs. 15–16.—Same as Fig. 14, but dialyzed 26 hours.

Fig. 17.—Same, dialyzed 4 days.

Fig. 18.—Granules obtained by dialysis against sodium phosphate buffer pH 7.5, ionic strength = 0.02. Separated by centrifugation for fifteen minutes at 2500 rpm.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left overnight in the cold, there is no reason to assume that structures would not be similarly formed during extended centrifugation. The structures observed in centrifuged and noncentrifuged preparations may not be identical since small particles would be sedimented as rapidly as they appear and may therefore not be able to continue to increase in size.

Although the results presented cannot be directly related to the problem of alterations in cell structure during fixation, the demonstration that complex structures can be formed from soluble cellular protein solutions suggests that the effects of fixatives on such solutions deserves careful study. The solutions used here contain a large portion of the total liver nitrogen. In electron micrographs of liver cells, it is not obvious that this much material is diffusely distributed throughout the cell.

The vast majority of the structures seen in breis undoubtedly preexisted in the cell, but it will be difficult to prove that any individual particle is not formed *in vitro*. A clear distinction should be made between structures formed as a result of the action of fixatives such as were demonstrated in the early work of O. Butschli, Fischer, and Hardy [9, 11] and structure formation *in vitro* without fixation. The former is of interest in the interpretation of objects seen in sections, although the term "vital artefact" has been used to stress the concept that there are degrees of relation between the section and the cell. The structures described here, though they may certainly be termed artefacts, are such in a somewhat different sense since they are formed by very different mechanisms.

It would be fundamentally important to know whether the same mechanisms and some of the same molecular species that form the structures described also interact *in vivo* to produce structures in living cells. Obviously,

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Fig. 19.—Membranous structure formed after dialysis against pH 7.5 sodium phosphate buffer, ionic strength = 0.02.

Fig. 20.—Membrane formed after dialysis for 105 minutes against 0.24 *M* sucrose.

Fig. 21.—Granules embedded in matrix material. Dialyzed 25 hours against pH 7.5 sodium phosphate buffer, ionic strength = 0.02.

Fig. 22.—Fibrous material formed after dialysis for 25 hours against pH 7.5 sodium phosphate buffer, ionic strength = 0.1.

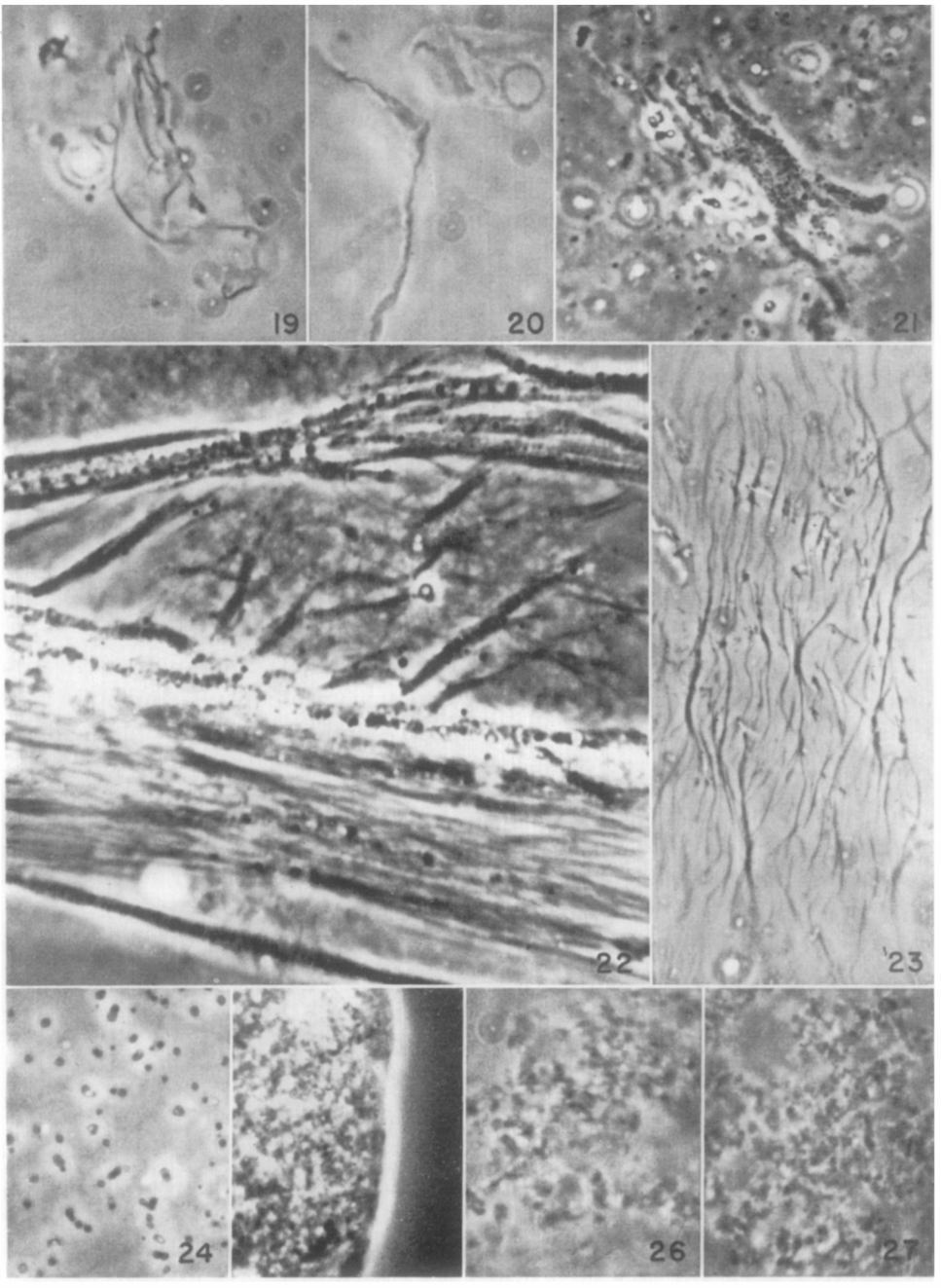
Fig. 23.—Fibrils formed against coverslip in preparation dialyzed against pH 5.0 Miller Goldner acetate-NaCl buffer, ionic strength = 0.1, for 6 hours.

Fig. 24.—Original granule preparation observed September 1, 1956 after dialysis against sodium phosphate buffer, ionic strength = 0.02.

Fig. 25.—Same preparation as 24 showing structure formed by aggregation of granular material.

Fig. 26.—Amorphous precipitate formed in mixture of rat serum and rabbit antirat serum.

Fig. 27.—Precipitate formed by bovine serum albumin mixed with its rabbit antiserum.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the question cannot be answered until the macromolecular constituents of the various cells structures have been isolated and characterized. Extensive serological cross reactions have however been observed among soluble tissue proteins, microsomes, mitochondria, and nuclei (suggesting that similar molecular species may be widely distributed through the cell). Furthermore, exclusive localization of enzymes in a given structure, as opposed to the dissolved or soluble phase, is rarely seen. This may be attributable to disruption of particulates during isolation, but we might also suggest that cell structures exist in equilibrium with their constituents in the cell. The implications of this concept in growth and differentiation have been previously discussed [3, 4, 6].

SUMMARY

A variety of structures, many of which resemble components of liver cells, have been shown to form in clear ultracentrifuged extracts of rat liver. The structures appear after dialysis against isotonic sucrose, pH 7.5 phosphate buffers, or after standing in the cold. When the pH is lowered to 5 or 6, fibrils are readily formed. It is suggested that these structures may form in rat liver breis.

The possibility is discussed that cell components exist in equilibrium with their constituent molecules in solution and that mechanisms similar in part to those involved in structure formation in solution may also obtain in the living cell.

Note added in proof: Since this manuscript was submitted two papers have appeared describing the formation of many similar structures from cytoplasmic protein preparations and from mixtures of hemoglobin and deoxyribonucleic acid. (NELSON, E. L., *J. Exptl. Med.* **107**, 755 and 769 (1958)).

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