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STUDIES ON ISOLATED CELL COMPONENTS

VII. A REEXAMINATION OF THE PREPARATION AND PROPERTIES OF RAT LIVER HOMOGENATES[1]

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THE techniques of differential centrifugation of tissue homogenates, introduced by Bensley and Hoerr (4), and developed by Claude (6), Hogeboom *et al.* (12) and many others, has found wide application in the study of enzymes and enzyme systems, in the investigation of certain physiological properties of various cell components, and in the initial stages of the purification of nucleic acids and various proteins. The methods appear for the most part to have been empirically devised and have been found to result in a degree of separation which does not approach that which would be expected from purely theoretical considerations. It has been considered desirable therefore to reexamine a number of aspects of these techniques. This paper will be concerned with the preparation and properties of rat liver homogenates; a subsequent paper will deal with the techniques of high resolution differential centrifugation.

HOMOGENATE PREPARATIONS

Tissue homogenates, such as those used in the present series of studies for the preparation of isolated nuclei, may be considered as true solutions containing a number of micro-ions, as colloidal suspensions containing particles covering the entire range of sizes customarily ascribed to colloids, and as slurries with particles large enough to settle out in the course of a few hours. In such a complex system a number of factors may be expected to have marked effects. These include the pH, density, viscosity, and composition of the suspending medium; the size distribution, density, and colloidal properties of the particulates, and the method and solutions used for tissue perfusion. Furthermore, it is not possible to eliminate completely

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the rapid changes which occur during and after cell death. It is important to determine as far as possible whether homogenization involves the rapid rupture of healthy cells, or the maceration of injured, cytolyzing ones—in short, whether or not the technique is really an exercise in necrobiology.

EXPERIMENTAL

Perfusion.—In the nuclear isolation method previously described (20) livers were perfused via the dorsal aorta using cold 0.15 *M* NaCl. Recently the use of cold sucrose-$CaCl_2$ solution introduced through the hepatic portal vein has been advocated (13). Other workers have generally used either saline or a balanced salt mixture such as Locke's solution. The dilemma at this point is whether to be concerned with the state of intactness of the cells or with changes occurring in cell components after cell breakage in unsuitable solutions. The amount of perfusion fluid remaining in the liver is probably around 20 g per 100 g of liver since the mass of extracellular material in the rat liver is about 24 g per 100 g of tissue (10). In mice, the values may be expected to be somewhat higher since the volume occupied by blood is 27 per cent of the liver volume for males and 31 per cent for females (17). When a 10 per cent homogenate is prepared, the concentration of the perfusion medium will be 2–3 per cent. It has been considered of interest therefore to study the effects of different solutions on cell breakage and on the amount of residual stroma.

Perfusion technique.—The following method has been used for some time and has been found to provide essentially blood-free livers in a very short space of time.

The rat is stunned or killed by cervical luxation, rapidly decapitated, and allowed to bleed for a few seconds while suspended by the tail. The skin covering the entire left side of the body is quickly removed using heavy curved stainless steel autopsy scissors. An incision is made on the left side below the ribs, continued around to the spine, and along the spinal column to the top of the pleural cavity severing all ribs in between. The diaphragm is cut close to the chest wall around to the midline. The aorta is then cut halfway through about $1\,^1/_2$ cm above the diaphragm, leaving a flap pointing anteriorly. A thin-walled glass cannula is then inserted with the perfusion fluid streaming out, thus washing away surrounding blood during insertion. The perfusion fluid is maintained under a pressure of 120 cm of water to approximate the normal rat blood pressure (9). The left lobe of the lung is then removed to allow free outflow of blood. The inferior vena cava is also nicked. The vagus nerve is generally severed during this procedure, but this has been shown to have no obvious effects on liver circulation (18). Perfusion is generally completed within 1–2 minutes, and is continued while the liver is being removed.

For complete perfusion it has been found advisable not to chill the liver until after perfusion has been started. Cold Locke's solution is poured over the liver as soon as it begins to clear. The success of perfusion through the aorta, in view of the double circulation of the liver, is evidently due to the fact that the perfusion solution flows in through the hepatic artery and out both the hepatic vein and the hepatic portal vein, since the posterior half of the animal contains considerable blood when the liver is blood free. In some instances it has been found advantageous to cut the hepatic portal vein near the end of perfusion.

Perfusion is thus begun 30–50 seconds after death, the liver is cooled a minute or so later, and removed for weighing by the end of about 4 minutes. Every effort is made to minimize the time between death and the completion of perfusion. That rapid changes may occur is shown by the finding that a 15-minute interruption of liver circulation in the dog generally proves fatal (19), while in the rat kidney enspherulation and fragmentation of mitochondria occur in a few proximal tubules within 6 minutes after closure of the renal artery (8). The perfused livers are weighed in tared beakers of cold Locke's solution, blotted, and transferred to a cold graduate cylinder where sufficient homogenization medium is added to give the desired final homogenate volume.

If perfusion is omitted, a number of aggregates of cytoplasmic particulates are seen which sediment with the nuclei. These are probably bound together by fibrin. Perfusion therefore appears essential.

Effects of perfusion with various solutions.—The central role of divalent cations in the surface precipitation reaction (11) suggests that homogenization in the presence of substances such as citrate which bind calcium might result in better homogenization and fewer partially broken cells. Dounce (7) has suggested that this may be one of the reasons for the success of his citric acid method. To study this possibility livers were perfused with solutions containing 1 part of calcium-free Locke's solution and 1 part of isotonic sodium citrate. Homogenates were then prepared in Solution I (20) and were found to consist chiefly of microscopically intact whole cells. Similar results were obtained with sodium pyrophosphate, adenosinetriphosphate, and ethylenediamine tetraacetate. A method for preparing suspensions of whole cells based on these observations has been reported elsewhere (2). The concentration of calcium-binding substances may be reduced to about 0.01 *M* and still be effective. It is evident that under these conditions the removal of calcium softens the intercellular cement with results identical with those long known in similarly treated embryos (2).

In view of these results it was considered of interest to study the effects of perfusion with Locke's solution, both with and without calcium, and with sucrose containing calcium (0.25 *M* sucrose + 0.0018 *M* $CaCl_2$), and sucrose alone (0.25 *M*). Perfusion was accomplished as described and weighing was done in a cold beaker of the perfusion medium. Livers perfused with Locke's solution were homogenized in Solution I, while those perfused with sucrose solutions were homogenized in the perfusion solution. All homogenates were prepared in the same homogenizer (1.005 inches inside diameter, 10 inches long, with a plastic pestle 0.986 inches in diameter), and were given 70 even up-and-down strokes by hand with the homogenizer set in ice. Fifty to sixty milliliters of 10 per cent homogenate was prepared from each animal and strained through 12 ×× bolting silk.

As will be shown in a subsequent communication, a number of centrifugation artefacts exist which prevent cell components from sedimenting ideally. For this reason, the amount of material which might be expected to sediment with or ahead of liver nuclei (here referred to as contamination) was estimated by making direct counts with the phase contrast microscope rather than by centrifugal techniques. Homogenates were diluted with three volumes of the suspending medium. An average of 800 nuclei on five slides was counted from each preparation. The numbers of free nuclei (N), whole cells (WC), and broken cells (BC) or particles of debris which might sediment with the nuclei were tabulated separately. The number of free nuclei plus the number of whole cells gave the total number of nuclei present. Each cell fragment or particle of debris was considered to be the equivalent of one nucleus, while the amount of cytoplasm present in each whole cell was considered to be the equivalent of seven nuclei. The contamination present was considered to be equal to the sum, (WC × 7) + BC, and the percentage contamination was found from the equation:

$$\frac{(WC\times 7)+BC}{N+WC+(WC\times 7)+BC} = \text{percentage contamination.}$$

This method of calculation involves several simplifying assumptions, since the presence of about 31 per cent of binucleate cells (16) is disregarded, and all broken cells and debris particles are obviously not the same size as nuclei. However it is considered useful for comparative purposes.

The results obtained with sixteen male Sprague Dawley rats (213–270 g) are shown in Table I. The findings with Locke's solution both with and without calcium, and with sucrose plus calcium are similar, while a definite decrease in the amount of contamination is seen with 0.25 *M* sucrose.

TABLE I

Effect of perfusion with various solutions on the amount of material present which may sediment with nuclei and on the number of whole cells present.

Complete Locke's		Ca-Free Locke's		0.25 *M* Sucrose 0.0018 *M* $CaCl_2$		0.25 *M* Sucrose	
% Contam.[1]	WC/100 N[2]	% Contam.[1]	WC/100 N[2]	% Contam.[1]	WC/100 N[2]	% Contam.[1]	WC/100 N[2]
30.3	2.5	24.1	2.6	29.1	3.5	21.6	2.7
29.0	2.4	29.1	3.0	22.5	1.6	13.4	1.7
20.9	0.6	37.1	5.7	23.6	2.1	13.4	1.9
25.2	1.8	29.3	2.1	19.7	1.6	7.4	1.0
Average:							
26.3	1.8	29.9	3.3	23.7	2.2	13.9	1.8

The effect of pH on homogenization.—It has been suggested that best cell breakage is obtained at an alkaline pH (4). Dounce (7) found that nuclei disappeared when the pH was raised to 6.5 and suggested that this may be due to the action of cytoplasmic enzymes. Since no salts were used it is evident that, as pH 7.0 is approached with citric acid, the medium becomes essentially like distilled water which has been shown to cause marked swelling and disruption of fresh nuclei (3). The effect of pH on homogenization appeared therefore to merit further investigation.

Two complete sets of experiments were performed, the first with Osborne-Mendel, the second with Sprague-Dawley strain rats. Since the results are almost identical, only the findings with the latter strain will be reported. The livers of nine male rats (249–304 g) were perfused as described with complete Locke's solution. These were homogenized (70 strokes with the homogenizer described in the preceding section) in solutions of varying pH made by mixing different proportions of a solution containing 0.023 *M* KH_2PO_4 and 0.145 *M* sucrose with a solution containing 0.023 *M* K_2HPO_4 and 0.145 *M* sucrose. The lowest and highest pH's were achieved by using one or the other of these solutions alone. The solutions resemble Solution I in total salt concentration and in sucrose content. All homogenates were prepared in such a manner as to contain 1 g of liver per 10 ml of homogenate, and were strained through bolting silk.

The results are shown in Fig. 1 where contamination, calculated as described, is plotted against homogenate pH. It is evident that a sharp decrease

[1] Percentage calculated as described in text.
[2] WC/100 N = Number of whole cells counted per 100 nuclei.

in the amount of contamination occurs as the pH is raised from 5.8 to 7.8. This was found to be due to a decrease in the number of whole cells at the higher pH's and also to the fact that the cell wall material is free of attached cytoplasm at pH 7.8, and consists of very small shreds of material which probably would not sediment with nuclei. In the lower pH range the cell-wall material is generally fairly thick and often has attached coagulated

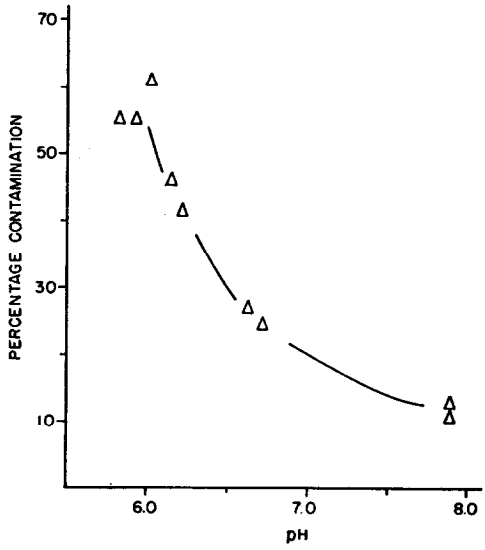

Fig. 1. Percentage contamination found in rat liver homogenates prepared in 0.023 *M* potassium phosphate buffers containing 0.145 *M* sucrose. The technique for preparing homogenates and the method for calculating contamination are described in the text.

cytoplasm. Very few half-broken (and therefore coagulated) cells were seen at pH 7.8. It is evident that under these conditions optimal cell breakage and dispersion of the cytoplasm occurs at a pH above 7.0.

Density of rat liver.—Relative density measurements were made on three livers from 2-month-old female rats of the Sprague-Dawley strain. These were rapidly perfused with Locke's solution and then weighed in air and in 0.28 *M* NaCl. The data of Opie (15) indicate that rat liver does not gain or lose water in this solution. The values obtained were 1.084, 1.085, and 1.088 g per cc with an average of 1.086. All measurements were made at 20° C. These values are in excellent agreement with those reported by Mori and Momoki (14) which were 1.088 for male and 1.084 for female rat livers. Perfused rat liver therefore has approximately the same density as 0.62 *M* sucrose. Homogenates prepared in more concentrated sucrose solutions may be layered over the homogenization medium since its density will be greater than that of the homogenate. Conversely, homogenates prepared in

TABLE II

Viscosities and densities of various solutions used for nuclear isolation. Densities are expressed in grams per cubic centimeter *in vacuo* at 0° C of solutions made up in double distilled water at 20°. Viscosities are calculated from the data of Bingham and Jackson (5). Addition of indicated salts to various sucrose solutions was found to have no detectable effect on viscosities.

Solution			Viscosity Centipoises at indicated temperatures			Density g/cc at 0° C
Description	Molarity of sucrose	Sucrose g/liter	0°	5°	10°	
3 × isotonic	0.88	301.2	5.75	4.61	3.80	1.1185 (1.1207[2])
"isotonic"	0.25	85.57	2.34	1.97	1.68	1.0338
Solution I[1]	0.145[2]	49.6	2.07	1.75	1.50	1.0224
Solution II[1]	0.218[2]	74.6	2.25	1.90	1.63	1.0323
Solution III[1]	0.272[2]	93.1	2.39	2.02	1.72	1.0395
Used in layering experiments	0.190[2]	65.0	2.18	1.85	1.58	1.0285
,,	0.219[2]	75.0	2.26	1.90	1.63	1.0326
,,	0.234[2]	80.0	2.29	1.93	1.65	1.0345[3]
,,	0.248[2]	85.0	2.33	1.96	1.68	1.0364[3]
,,	0.263[2]	90.0	2.38	2.00	1.71	1.0385
,,	0.584[2]	200	3.57	2.98	2.50	1.0819
,,	1.169[2]	400	10.0	7.58	6.25	1.1593
,,	1.753[2]	600	41.7	26.3	20.4	1.2350
Distilled water	—	—	1.7921	1.5188	1.3077	0.9998

solutions of lower concentration can be layered only over a solution containing a higher concentration of sucrose.

Physical properties of various media.—In order to calculate the theoretical sedimentation rates of various cell components it is necessary to know their size and density, and the density and viscosity of the solutions used. The viscosity of several sucrose-containing solutions used in studying isolated cell components has been calculated from the data of Bingham and Jackson (5) and the results are presented in Table II. It has been determined experimentally that the addition of small amount of salts (0.0125 *M* K_2HPO_4, 0.0094 *M* KH_2PO_4, and 0.0015 *M* $NaHCO_3$) to sucrose solutions over the range 0.145–0.88 *M* does not alter the viscosity detectably. The absolute densities of the same series of solutions have been experimentally determined

[1] Solutions used in nuclear isolation procedure previously described by Wilbur and Anderson (20).

[2] These solutions contain in addition to the sucrose concentration shown the following salts: 0.0125 *M* K_2HPO_4, 0.0094 *M* KH_2PO_4, 0.0015 *M* $NaHCO_3$.

[3] By interpolation.

at 0° C by use of a specially designed 100-ml pycnometer which could be filled accurately at 0° C and weighed after warming to room temperature. All weighings were corrected to *in vacuo* conditions and are expressed in terms of grams per cubic centimeter. Determinations were made in triplicate. These results are included in Table II. Solution viscosities are given at several temperatures since the values diminish rapidly with rising temperature

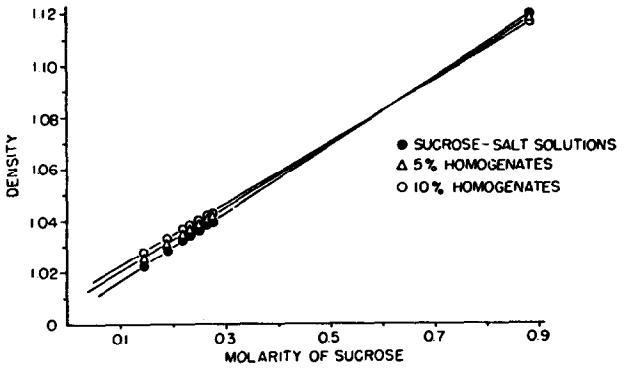

Fig. 2. Density of solutions containing 0.0125 *M* K_2HPO_4, 0.0094 *M* KH_2PO_4, 0.0015 *M* $NaHCO_3$, and concentrations of sucrose as indicated. Curves with open symbols show calculated densities of 10 and 5 per cent homogenates prepared in the same solutions. Since all solutions contain the same amount of salt, and liver tissue is present in preparations represented by two of the curves, none of the curves will intercept the zero point. All data for 0° C.

for any given solution. The densities, however, are given for 0° C only since little change occurs over the range 0–10° because the density of water passes through a maximum at 4° and is the same at 8.1° as at 0.0°.

Requirements for layering homogenates.—Assuming a density of 1.086 for rat liver, the densities of homogenates containing 1 g and 0.5 g of liver for each 10 ml of homogenate may be readily calculated, and are shown in Fig. 2. From these curves the density of a sucrose solution required to support a layer of homogenate made in any other salt-sucrose solution may be readily read. A salt-sucrose solution having the same density as a homogenate made in Solution I would have to have a density greater than Solution I by a value of at least 0.0045, while a similar solution having the same density as a homogenate prepared in Solution III would have to have a density greater by 0.0035. In the neighborhood of a sucrose molarity of 0.62 the salt-sucrose solution would have the same density as the solution used in making it. These values set the general limits of any layering procedure which employs sucrose, and allow a system to be set up at whatever sucrose concentration may appear desirable. As will be shown in a subsequent paper, simple layering procedures are subject to several artefacts which are more evident when the density differences are low.

Colloidal properties of homogenates.—The sign of the charge on proto-

plasmic colloids has been the subject of considerable disagreement (11). The following observations, however, support the conclusion that the colloidal constituents of a homogenate bear a *negative* charge. Addition of very small amounts of protamine, Janus green B, or other polycations has been found to flocculate homogenates and to cause rapid sedimentation of all homogenate constituents in very low centrifugal fields (1). The addition of strong polyanions such as heparin, ATP, or polysulfonated dyes (Erie fast rubine, solantine red, wool red 40F, brilliant scarlet 3R) produces no comparable effects. The gradual flocculation of homogenates observed on standing may well be due to depolymerization of nucleic acids and a destruction of ATP, resulting in a decrease in colloid associated negative charges. It is not unlikely that similar changes occur in cytolyzing cells.

DISCUSSION

The results presented allow a more rational approach to the problem of the preparation and fractionation of tissue homogenates. The inclusion of calcium-binding substances in the perfusion solution increases markedly the number of whole cells appearing in the finished brei. No appreciable difference was found, however, when cell breakage after perfusion with both complete and calcium-free Locke's solution were compared. When 0.25 *M* sucrose was used, addition of 0.0018 *M* $CaCl_2$ actually increased the amount of contamination observed.

Variations in the pH of the homogenization medium were found to have a marked effect on the composition of the homogenate. At a slightly acid pH many whole cells, partially disrupted cells, and cell walls with associated coagulated cytoplasm were seen. In contrast, above pH 7 few whole cells and almost no broken cells were found, whereas the intercellular material was represented by small shreds with no adherent cytoplasm. These findings suggest that homogenization is best accomplished above pH 7. The application of the data presented on the physical properties of homogenates and suspending media to the development of high resolution differential centrifugation is described in a subsequent communication.

SUMMARY

Several aspects of the preparation of homogenates and certain physical properties of suspending media have been examined in an attempt to develop a more rational approach to the problem of the differential centrifugation

of tissue homogenates. The addition of calcium-binding agents to the perfusion solution has been found to result in the presence of a large number of whole cells in liver breis. No difference in cell breakage was observed when calcium-free Locke's solution was compared with complete Locke's solution. The addition of 0.0018 *M* $CaCl_2$ to a perfusion medium consisting of 0.25 *M* sucrose resulted in an increase in the number of whole cells and cell wall material when compared with sucrose alone.

Comparison of the composition of homogenates identically prepared except for variations in the pH of the homogenization medium showed that at pH 6 a large number of whole cells, broken cells, and cell wall fragments were present. The number of these contaminants decreased markedly as the pH was raised to 7.88.

Data on the viscosity and density of a number of suspending media, on the density of perfused liver, and on the density of homogenates are presented. From these the requirements for the preparation of layered systems for differential centrifugation can be calculated.

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