STUDIES ON ISOLATED CELL COMPONENTS

IX. THE SOLUBLE PHASE[1]

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It is evident that the physical properties and reactions of the cytoplasm are a reflection of the interactions of the many molecular species and particulates of which it is composed. While emphasis has long been laid on the lability of the cytoplasm and its rapid reaction to injury, it is difficult to conclude that the substances or structures that give rise to these properties are necessarily so labile that it is impossible to isolate them. It appears justified, therefore, to explore brei fractions for both the molecules or structures and the reactions which give rise to such fundamental properties as the sol-gel reaction and cytolytic reaction to injury. In previous studies by Hultin and Gross [14, 15, 8, 9, 10] the possibility that whole brei may react in a manner parallel to that of whole cells has been examined. The addition of calcium was shown to stimulate markedly the formation of acid and to cause clumping of subcellular particulates in sea urchin egg breis. These effects have been explained on the basis of Heilbrunn's theory of the central mediating role of calcium in stimulation and cytoplasmic "gelation" [9, 10, 11].

In the present series of studies, an attempt is made to explore the soluble phase of the perfused rat liver to see: (*a*) whether it is capable of exhibiting reactions analogous to those of the cytoplasm of intact cells; (*b*) whether the components of a sol-gel system can be isolated from it; and (*c*) to ascertain whether or not any organization of the constituent macromolecules survives the isolation procedure. Although a number of studies have been carried out on the soluble phase,[2] previously reviewed by Barry [4] and Sorof *et al.* [26], the results obtained in these studies differ in many respects from those previously reported. The differences are thought to be caused, at least in part, by the rapid method of preparation.

In this paper, the preparation of the soluble phase is described, together with data on its stability and composition.

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[2] This term emphasizes the concept that many molecular species may exist in several phases in the cell, i.e., they may be dissolved or in a soluble state, absorbed on particulates, or bound as parts of cell structures.

MATERIALS AND METHODS

Sprague Dawley rat livers were perfused *in situ* with complete Locke's solution by the rapid perfusion method previously described [2]. Weighing and homogenization were done in cold 0.25*M* sucrose. Except as otherwise noted, the final volume of the brei in milliliters was equal to three times the liver weight in grams. The brei was immediately centrifuged in an International Equipment Co. high speed head No. 295 at 3500 rpm (13,330 × *g* at the center of the tube, $R_{min} = 2.5$ cm, $R_{max} = 6.7$ cm) for 20 minutes. The average time between stunning the rat and the beginning of centrifugation in 44 preparations was 10.1 minutes. The temperature of the brei was 7°C immediately after preparation but had dropped to 2.5° during the first centrifugation, which was carried out at − 5 to − 8°. When two livers were perfused, the first brei was centrifuged as soon as prepared. When the second brei was ready (approximately 10 minutes later), the centrifuge was stopped and the tubes containing the second brei added, after which it was run for the full 20 minutes. The centrifuge head was allowed to decelerate free at the end of the run. The lipid film at the top was carefully removed with a suction pipette and the supernatant fluid transferred to tubes for the Spinco No. 40 rotor (prechilled to − 5°) and spun at 40,000 rpm for one hour (105,000 × *g* at the center of the tubes, $R_{min} = 3.8$ cm, $R_{max} = 8.1$ cm). The lipid layer was again removed and the clear yellow supernatant fluid (final temperature 10°C), referred to as the soluble phase (SP), recovered.

Nitrogen analyses were done by the method of Ma and Zuazaga [18].

RESULTS

If the preparation described is contaminated with microsomes or other particulate material, then the composition of the SP should vary with centrifugation time. However, if centrifugation is carried out until a range of centrifugation times is reached where very little change in composition occurs, then the preparation may be assumed to be homogeneous and contain only soluble molecules or isopyknic particles.

The results of a study of supernatant fluid nitrogen content versus centrifugation time are shown in Fig. 1. The entire supernatant fluid was removed in each instance after pipetting off the fatty layer. Although the density of the solution near the bottom is greater than that near the top, owing to sedimentation of protein during the longer runs, the amount of nitrogen in the whole SP varies little over a wide range of centrifugation times. It is evident that the treatment chosen is not critical, and lies in a broad plateau where little change in nitrogen content occurs when the treatment is varied.

To ascertain whether particles might be present that were not sedimented because they had the same density as the SP, a preparation was diluted with equal volumes of *M*/15 sodium phosphate buffer, pH 7.8, and centrifuged

for one hour at 40,000 rpm in the Spinco No. 40 rotor. A very tiny pellet believed to be sedimented protein was observed in both the experimental and undiluted control. There do not appear to be particles of microsomal dimensions isopyknic with the SP as prepared here.

Analysis of the nitrogen content of 14 different breis gave an average nitrogen content of 9.51 mg of N/ml (maximum 10.75, minimum 7.70). Analysis

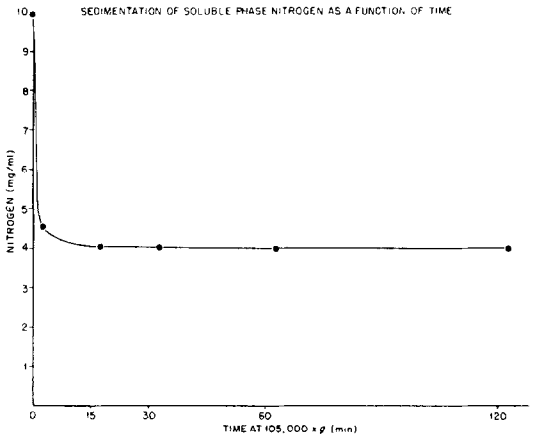
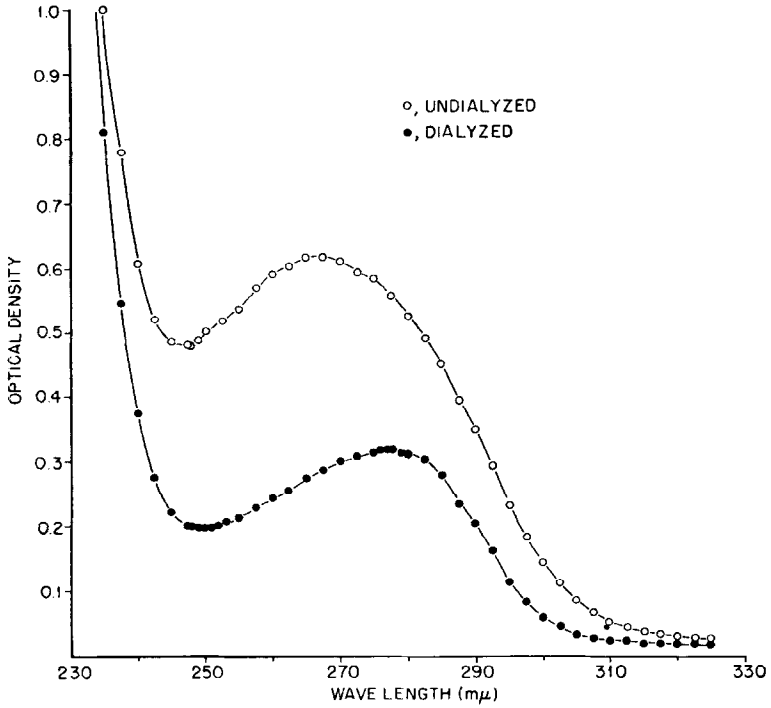


Fig. 1. Concentration of nitrogen in supernatant solutions removed after centrifugation equivalent to 105,000 × g for the times indicated. Centrifugation done during acceleration and deceleration was disregarded.

of 8 SP preparations gave an average of 4.12 mg of N/ml (maximum 4.49, minimum 3.75). An average of 43 per cent of the total brei nitrogen was therefore recovered in the soluble phase. Dialysis of 3 preparations against 0.02M sodium phosphate (pH 7.8) showed that 87.5, 87.6, and 87.9 per cent of the SP nitrogen was nondialyzable.

The absorption spectra of the SP before and after dialysis are shown in Fig. 2. The undialyzed sample, diluted 1:80 with phosphate buffer, shows a curve in the ultraviolet that is typical of a protein solution containing a small amount of either nucleic acid or nucleotides. The ratio of absorption at 280 mμ to that at 260 mμ was 0.89. After dialysis for 22 hours against M/15 sodium phosphate at 5°C, pH 7.8, the ratio was increased to 1.28, closer to the value of 1.75 characteristic of many proteins [27]. The maximum difference between the two absorption spectra was observed at 260 mμ. The volume of the dialyzed sample was adjusted in such a manner that it was comparable to the undialyzed sample. The absorption spectrum from

Fig. 2. Absorption spectrum of undialyzed (○) and dialyzed (●) soluble phase preparations. Solutions diluted 1:80 with M/15 phosphate buffer pH 7.8 for measurements in the deep ultraviolet (top). Near ultraviolet through infrared spectrum done on samples diluted 1:1 with same buffer (bottom). Dialysis was against buffer used for dilution.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•, DIALYZED
OPTICAL DENSITY
1.0
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250
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290
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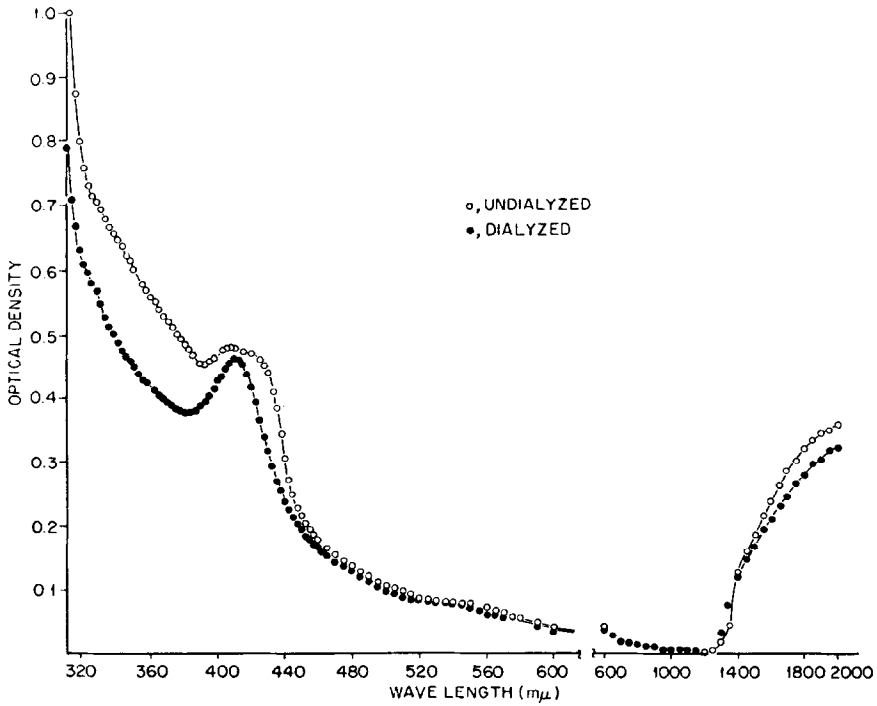
330
WAVE LENGTH (mμ)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OPTICAL DENSITY
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WAVE LENGTH (mμ)

310–2000 mμ was done on an undialyzed preparation diluted 1:1 with pH 7.8 phosphate buffer. The peak at 407 mμ is characteristic of metallo enzymes such as catalase. After dialysis, this peak was sharpened considerably, suggesting that several dialyzable compounds with peaks in this region exist. No evidence of hemoglobin was found. The rise in absorption from 1300–2000 occurs in both dialyzed and undialyzed samples.

The results of these experiments suggest that only a very small amount of 260 absorbing material exists in the SP which is nondialyzable. Since the very real possibility exists that the results may be caused by depolymerization of ribonucleic acid during prolonged dialysis, the experiment was repeated using electrodialysis in a modified electroconvection apparatus for 42 minutes. The untreated SP was diluted 1:50 with phosphate buffer and the electrodialyzed sample, which increased in volume during treatment, was diluted to give a dilution comparable with that of the original material. The 280:260 ratio for the SP was 0.81; after electrodialysis it rose to 1.22. The greater part of the 260 absorbing material is therefore in a readily dialyzable form.

The pH stability of the SP was studied by mixing equal volumes of SP and sodium phosphate buffers in the cold, allowing them to stand 30 minutes (in the cold) and then centrifuging them for 60 minutes at 2400 rpm (1460 × g at bottom of tube). The pH of the supernatant solution and the nitrogen content of the supernatant and pellet were then determined. The results, expressed as percentage of the original total nitrogen remaining in the supernatant solution with buffers prepared as indicated, are shown in Fig. 3. The preparation was completely soluble under these conditions from pH 1.3–3.5, and from 6.3–11 (the highest pH examined). No precipitation occurs at low pH with phosphate even if the SP is mixed with equal volumes of concentrated phosphoric acid. If, instead of mixing with buffer, dilute phosphoric acid is added slowly, a precipitate is formed which redissolves on the addition of more acid. When a series of very dilute hydrochloric acid solutions were used in place of phosphate buffers, a curve similar to that of Fig. 3 was obtained with the exception that in the more acid ranges (pH 1) a voluminous precipitate occurred. The addition of NaCl to clear phosphoric acid solutions of soluble proteins also produced precipitation. Sulfuric and acetic acids produced effects resembling those of phosphoric acid. In very acid solutions the chloride ion appears to have a specific precipitating effect.

Further experiments showed that the protein precipitated at pH 4.8 in phosphate could be redissolved at pH 6.9 or higher if handled quickly and in the cold. If kept at the lower pH for any length of time, denaturation occurred. Purification of this fraction will be described in a subsequent paper.

When the pH of the SP was changed by dialysis against $M/15$ phosphate buffers of various pH's; somewhat different results were obtained. No precipitation was seen at pH 1–2. However, precipitation occurred in varying amounts from pH 3–8 with a maximum in the 3–6 range, suggesting that some proteins may be solubilized by dialyzable substances.

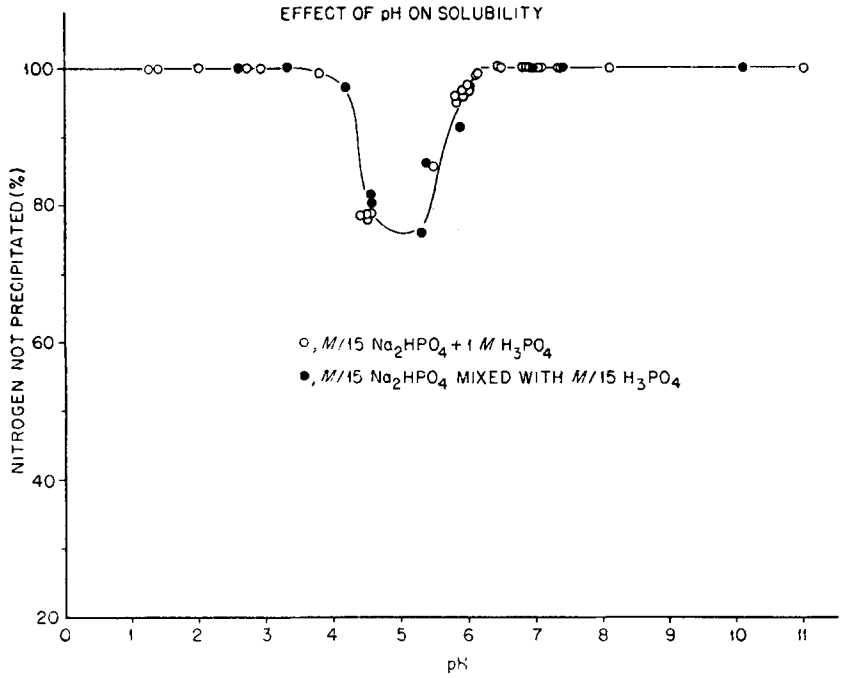

Fig. 3. Effect of pH on solubility of soluble phase proteins. Soluble phase mixed with equal volumes of phosphate buffers, allowed to stand for 30 minutes in the cold, and then centrifuged. Results expressed as percentage of nitrogen remaining in solution. Buffers prepared by mixing varying proportions of $M/15$ Na_2HPO_4 and $1M$ H_3PO_4 (○); buffers prepared from $M/15$ Na_2HPO_4 and $M/15$ H_3PO_4 (●). Extreme pH values were studied using either phosphoric acid or tribasic sodium phosphate.

The solubility of SP constituents as a function of NaCl concentration was studied by dialyzing fresh preparations against various concentrations of NaCl in the cold. As much as 42 per cent of the undialyzable nitrogen is precipitated in distilled water, whereas 6.2 per cent was precipitated in $0.1M$ NaCl and only 0.56 per cent in $1M$ NaCl.

The problem of the nature and amount of 260 mμ absorbing material in the SP was investigated further, since there is little agreement in previous work as to the amount of nucleic acid in this fraction. If the 260 mμ absorbing

material consists of a spectrum of compounds ranging from simple nucleotides to long RNA chains, the results of attempts to separate various fractions would be expected to show considerable variations. The acid soluble 258 mμ absorbing material was, therefore, studied first and was found to be highly reproducible in parallel extractions of any one brei or SP preparation. In

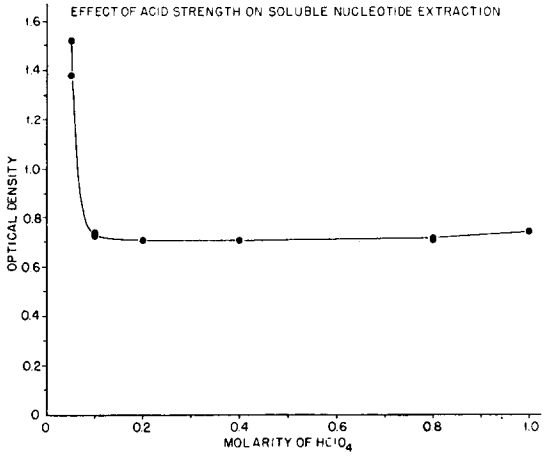

Fig. 4. Effect of acid strength on acid-soluble nucleotide extraction. Brei samples (3-ml) containing 50 mg of liver per milliliter extracted twice with 6-ml volumes of $HClO_4$ of molarities indicated. Extracts diluted to 25 ml and read against acid blank in 1 cm cells at 258 mμ.

one experiment, for example, 16 3-ml brei samples (each containing 50 mg of the same liver per milliliter) were extracted with 0.2M $HClO_4$ [20]. The extracts were diluted to 25 ml with 0.2N $HClO_4$ and the density at 258 mμ determined in 1 cm light path cells. The average for the 16 determinations was 0.753, S.D. $\pm$ 0.011. The effects of variation in strength of acid used for extraction was examined by extracting 3 ml brei samples with cold $HClO_4$ solutions whose final concentrations varied from 0.05–1.0N (Fig. 4). Identical results are obtained between 0.2–0.4M with a very slight rise at 0.8–1.0M; 0.05M was insufficient to precipitate the protein and RNA present. Although the actual amount of acid used will depend on the amount of protein and other buffering substances present, it does not appear that the strength of the acid used is critical for the extraction of acid soluble nucleotides.

In three successive experiments with three different livers the $E_{258}^{1\,cm}$ of preparations containing the extract from 1 ml of homogenate (0.33 g liver), prepared as described and diluted to 50 ml, were 0.840, 0.785, and 0.874, whereas the SP from the same livers, similarly extracted and diluted, yielded solutions whose densities were 0.540, 0.555, and 0.594. The per cent of the total acid soluble material of the brei, which was recovered in the SP was 64.3, 68.0, and 67.0 per cent (average 66.4). It appears that a considerable

fraction of the acid soluble 258 mμ absorbing material is bound to particulate material. Further experiments showed that the amount recovered in the SP varied considerably with the dilution of the brei, suggesting that part of it was adsorbed.

RNA of the SP was determined by prolonged hydrolysis, in 1*N* $HClO_4$ in the cold, of samples previously extracted with dilute acid to remove acid soluble nucleotides [20]. The amounts of RNA found in four successive preparations were 237, 247, 224, and 241 μg (average 237) of RNA/ml of soluble phase. This amounts to approximately 10 per cent of the RNA of the whole liver.

As an alternate method, the RNA was extracted by heating samples of the SP made 1*M* with respect to NaCl in a boiling water bath for 10 minutes [6]. The extraction was repeated once. After centrifugation, two volumes of alcohol was added to the clear supernatant fluid. A fine precipitate formed, which was centrifuged down, after standing 24 hours in the cold. The precipitate was extracted for 24 hours with 1*N* $HClO_4$. The absorption spectra of the alcohol solution and the acid extract showed that nearly all of the RNA was sufficiently highly polymerized to be insoluble in 70 per cent alcohol.

No deoxyribonucleic acid could be detected in the SP by the Dische reaction [22].

DISCUSSION

The data presented suggest that a reproducible fraction, the soluble phase (SP), can easily be obtained from rapidly perfused rat liver by high speed centrifugation. No evidence of insoluble particulate matter was found in it.

Study of the SP preparation described here is considered of interest for the following reasons. In studies aimed at elucidating the "intracellular distribution" of enzymes, substances, or activities, the SP is generally composed of the supernatant fluid left after sedimentation of the microsomes plus the washings from particulate fractions. These washings may be expected to include a maximum of loosely adsorbed materials. By preparing the SP in the highest practical concentration with the greatest possible speed, and by excluding washings from sedimented material, a better approximation of the intracellular SP is obtained. A comparison of the constituents of the SP prepared by the two different methods should yield a better indication of which substances are loosely bound to particulate matter. For work at high concentrations it is also unnecessary to add salts since the components are subjected to the same ions in a lower concentration as in the cell (except for the perfusion solution included). Furthermore, as shown by Sorof and

Cohen [24], the protein concentration is in the proper range for electrophoretic study.

Only very gentle "homogenization" is used and every effort is made to handle the material as gently as possible in the hope that a minimum number of mitochondria and nuclei will be macerated. Autolytic effects, which occur rapidly in the liver, are minimized by sedimenting all the nuclei and the greater part of the mitochondria within 13–14 minutes after death of the animal. These two fractions contain 77 per cent of the catheptic activity of the liver [19].

In the present study an average of 43 per cent of the total nitrogen of the brei was recovered in the soluble phase. This finding is in the general range reported by previous workers who found 31 per cent [13]; 41.3 per cent [23]; 42 per cent [1]; 38.6 per cent [19]; and 55 per cent [25] for rat liver. Barnum and Huseby [3] found 29.2 per cent for mouse liver.

The pH stability studies are of interest from several points of view. A number of studies on the viscosity of the cytoplasm have shown that a decrease in pH results in an increase in cytoplasmic viscosity. In these studies, a fraction of the SP has been found to be insoluble over the range of pH 3.5–6.3 with a maximum precipitation around pH 5. This fraction may well be the basis for the changes observed in intact cells. During cytolysis after injury, the cytoplasmic pH may drop to this range with accompanying coagulation. The precipitation observed *in vitro* resembles the intracellular change in that it is at first reversible in the cold, but becomes irreversible with time. The finding of Barth [5] that cells immersed in acid solutions below pH 5 coagulated more rapidly when the acids were dissolved in NaCl than in sea water may be related to the precipitating effects of the chloride ion in acid solutions found here.

From the point of view of the isolation of cell components, it is evident that the pH range 3.5–6.3 should be avoided since the particulate fractions will be grossly contaminated with precipitated protein. This was found to be the case by Dounce [7] who has frequently pointed out that nuclei may be isolated above and below this general range but not within it. In addition to the SP protein precipitated, microsomes are agglutinated at pH 5 and have been prepared in this manner by Hers *et al.* [12]. The method does not appear suitable for the removal of microsomes from the SP however, since soluble proteins are also precipitated.

The results of the dialysis experiments suggest that some of the cytoplasmic proteins are solubilized by dialyzable substances. This phenomenon will be discussed in detail in a subsequent paper on cytoplasmic complex formation.

Only a small fraction (approximately 10 per cent) of the liver RNA has been found in the soluble phase. Previous reports [3, 13, 17, 21] indicate that between 19–33 per cent of the liver RNA was in the soluble fraction. The discrepancy may possibly be due to loosely adsorbed RNA removed from particulate material during washing, where washings have been included in the SP, or to incomplete sedimentation of microsomes. The SP prepared as described here would be expected to contain a minimum of material desorbed from particles because (*a*) the dilution of the original cellular SP has been kept to a minimum, (*b*) the particulate fractions have been sedimented as rapidly as possible, and (*c*) the sedimented material has not been reextracted.

SUMMARY

The soluble phase (SP) of rat liver has been prepared by a method in which the liver is rapidly perfused and centrifuged so that the nuclei and nearly all of the mitochondria are sedimented within 13–14 minutes after the death of the animal. An average of 43 per cent of the total nitrogen of the brei is recovered in the SP together with approximately 10 per cent of the RNA. An average of only 66 per cent of the acid soluble nucleotides of the liver were recovered in the soluble phase.

In phosphate buffers the SP was stable between pH 1.26 and 3.5, and between 6.3 and 11. Between 3.5 and 6.3 marked precipitation occurred with a maximum of 24 per cent of the SP nitrogen precipitated near pH 5. An average of 12.3 per cent of the SP nitrogen was dialyzeable. The relation of the pH stability of the SP to viscosity changes in cells in slightly acid media is discussed.

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