FREE AMINO ACIDS AND NUCLEIC ACID CONTENT OF CELL NUCLEI ISOLATED BY A MODIFICATION OF BEHRENS' TECHNIQUE

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It has already been found that a considerable number of enzymes occur in nuclei of rat liver cells which have been isolated at pH 6.0 by a procedure involving the use of the Waring blendor with very dilute citric acid for breaking the cells, followed by differential centrifugation (1–3). As a complementary type of work, studies have been started to determine the relative concentrations of some important substrates in the cell nucleus as compared to the corresponding concentrations in the whole cell or in the cytoplasm. Since many of the substrates of the cell are water-soluble it was necessary to avoid aqueous solutions in the preparation of the nuclei. For this purpose a modification of the method of Behrens (4), which employs only non-polar organic solvents, was developed. The free amino acid pattern of these nuclei was determined by paper partition chromatography, and their nucleic acid content estimated. The method of Behrens has already been used in a study of the distribution of vitamins between nucleus and cytoplasm (5).

EXPERIMENTAL

A. Source of Material.—Wistar strain rats fed *ad libitum* on a "fox chow" diet were killed by decapitation. The livers were removed as quickly as possible, easily visible fibrous tissue was removed, and the livers were then immediately frozen by being placed in beakers cooled in an acetone-dry ice bath. The frozen livers were allowed to stand at −15° in a cold room for about an hour, and then were broken into as small pieces as possible. The resulting material was then immediately lyophilized continuously during an interval of 36 to 48 hours. When nearly all the water had been removed, the material was powdered with a mortar and pestle and lyophilization was continued for 5 to 6 hours. The final powder was immediately used in the cell fractionation procedure described below.

B. Preparation of Cell Nuclei, Whole Cells, and the Cytoplasmic Fractions.—Five preparations of nuclei were isolated from liver powder lyophilized as above, three from rat liver and two from rabbit liver. Fifty gm. of the lyophilized powder were ground in a ball mill of 1 liter capacity at −15° for 24 hours in the presence of 200 ml. of 50–60° petroleum ether with about 160 irregular pebbles of 15 to 20 mm. average diameter. The petroleum ether facilitated grinding so that relatively small proportions of unbroken cells remained even though the grinding time was not prolonged. The

ground suspension was filtered through four layers of fine cheese-cloth to remove fiber. The ball mill and pebbles also were washed with petroleum ether and the washings filtered. The fibrous residue was then washed several times with small portions of petroleum ether, and subsequently discarded.

The combined filtrates were centrifuged at about 2000 R.P.M. (in large centrifuge tubes) at 0° for 10 minutes (International No. 2 centrifuge). Practically all the suspended material was thereby sedimented, leaving an opalescent supernatant fluid which was decanted from the sediment and discarded.

The sediment was thoroughly suspended in about 250 ml. of a 50 per cent mixture of benzene-carbon tetrachloride, and was subjected to centrifugation at about 2000 R.P.M. at 0° for 15 minutes. The supernatant containing ground cytoplasm was decanted from the sediment which contained whole cells, nuclei, cell fragments, and some ground cytoplasm. The supernatant was discarded and the sediment was again thoroughly suspended in about 250 ml. of 50 per cent benzene-carbon tetrachloride and centrifuged as before. The supernatant, containing more ground cytoplasm, was discarded. The sedimentation in 50 per cent benzene-carbon tetrachloride was then once more repeated (using thoroughly dispersed sediment obtained from the preceding centrifugation).

The washed sediment was then suspended in about 250 ml. of a mixture of benzene-carbon tetrachloride of specific gravity 1.30 (made by mixing 200 ml. of benzene with 288 ml. of carbon tetrachloride). This suspension was centrifuged for 1 hour at 2000 R.P.M. at 0°. The material in the centrifuge tube was found to be stratified into three principal layers: (*a*) a dense crust of dark colored floating material, which was removed mechanically from the centrifuge tube and was found to consist mainly of whole cells and large broken cell fragments, with a liberal admixture of nuclei, many of which however bore some adherent pieces of cytoplasm; (*b*) an intermediate liquid suspension, which after filtration through fine cheese-cloth was found to consist mainly of ground cytoplasm with some nuclei, and (*c*) the bottom layer which was found to contain a high proportion of cell nuclei. It was from the bottom layer (*c*) that purified nuclei were later obtained.

The sediment obtained from the benzene-carbon tetrachloride mixture of specific gravity 1.30 was again carefully suspended in 250 ml. of this same solvent mixture and was again centrifuged at 2000 R.P.M. for 1 hour at 0°. The small amount of floating crust and the supernatant liquid were removed and discarded. The sediment was then thoroughly resuspended in 250 ml. of benzene-carbon tetrachloride mixture of specific gravity 1.345 (made by mixing 200 ml. of benzene with 372 ml. of carbon tetrachloride), and was centrifuged at 2000 R.P.M. for 50 minutes at 0°.

The nuclei were recovered as a mat of material floating at the top of the suspending solvent mixture after this centrifugation, and the sediment at the bottom of the centrifuge tube and the solvent mixture (which contained only a small amount of suspended material) were both discarded.

These nuclei were sedimented in about 100 ml. of a benzene-carbon tetrachloride mixture of specific gravity 1.30 (in two 60 ml. centrifuge tubes) at about 2400 R.P.M. for 25 minutes at 0°. The supernatant liquid was discarded and the nuclei were recovered as the sediment. At this stage, microscopic observations showed the presence of some fiber and fine material as contaminants.

The nuclei were next ground in a mortar for a few minutes with a few milliliters of a 50 per cent benzene-carbon tetrachloride mixture, and the resulting suspension was then diluted to about 125 ml. with the same solvent mixture and strained, first through 4 layers of fine cheese-cloth and then through one layer of cotton flannel. The latter procedure caused some loss of material but removed practically all the fiber.

Finally the nuclei were recovered from the solvent mixture by centrifugation in two 60 ml. centrifuge tubes for 8 minutes at about 2400 R.P.M. The supernatant was discarded and the nuclei were again suspended in about 100 ml. of 50 per cent benzene-carbon tetrachloride and were centrifuged for 5 minutes at about 2400 R.P.M. The

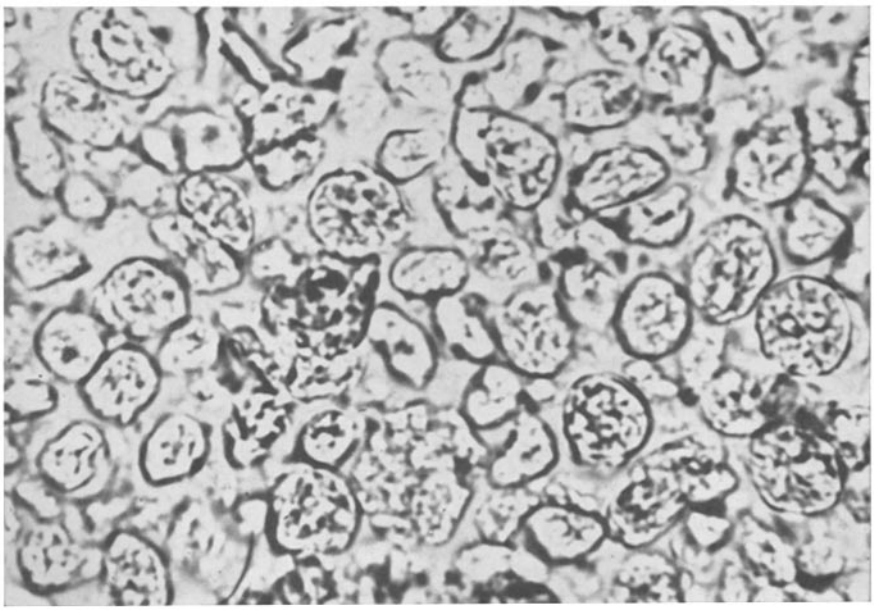


FIG. 1. Photomicrograph of cell nuclei isolated by the modified Behrens' technique. Very slight staining with toluidine blue.

supernatant fluid was discarded. The nuclei thus obtained, which formed a yellowish or light brown sediment, were allowed to dry thoroughly in the air. They were then placed in a tightly stoppered test tube and stored in the cold at −15°.

Since it was not found possible to carry out the preparation of nuclei starting from the lyophilized powder in 1 day, the preparation was interrupted at points where the nuclei were in the form of sediments. Apparently the partially purified nuclei can be dried in the air at almost any stage in the preparation and stored at −15° for 12 hours or longer without appreciable deterioration. However, care must be taken to disperse the dried material very thoroughly in the next batch of suspending medium before proceeding with the preparation.

A photograph of nuclei obtained by the procedure described above is given in Fig. 1. In making this photograph, it was necessary to add dilute citric acid to keep the

nuclei from agglutinating on the slide. Even in the dilute citric acid, it was very difficult to obtain a thin dispersion of the nuclei, and for this reason some of the smaller nuclei and fragments of nuclei appear out of focus.

In making the benzene-carbon tetrachloride mixtures, C.P. benzene free of thiophene and C.P. carbon tetrachloride were used. Centrifugation at 0° was accomplished by using a refrigerated centrifuge.

C. Properties of Nuclei Obtained as Described Above.—When suspended in distilled water, 0.85 per cent NaCl, or very dilute citric acid at pH 6.0, the nuclei formed a rubbery gelatinous mass. A fairly clear, highly viscous gel was formed when a small amount of ammonia or dilute NaOH was added to the nuclei suspended in distilled water. The addition of 5 or 10 per cent NaCl to the nuclei also caused the formation of a similar gel. The Behrens' nuclei are thus similar in regard to gel formation to nuclei prepared at pH 4.0 or lower by means of the wet citric acid method (6), and to chicken erythrocyte nuclei prepared in aqueous solution at pH 6.8 (7).[1] On the other hand, nuclei from the preparation described in footnote 1 were similar to liver cell nuclei prepared at pH 6.0 in very dilute citric acid (6). It seems probable that normally cell nuclei should form a highly viscous gel if suspended in strong saline or dilute alkali, and that if they do not do so, an autolytic action of some sort has taken place.

The nuclei were found to contain the enzyme arginase in as high concentration as is found in nuclei prepared at pH 6.0 with dilute citric acid. Catalase was present in about 50 to 60 per cent of the concentration found in whole fresh liver. Esterase also was present, but was not determined quantitatively. Aldolase was detectable but evidently had been largely destroyed by the procedures involved in isolating the nuclei. Thus the Behrens' type nuclei are suitable for studies of at least some enzymes.

D. Nucleic Acid Content of Behrens' Type Nuclei.—In view of recent reports (8, 9) that the DNA content of cell nuclei is considerably lower in nuclei as they exist within the cell than in isolated nuclei, it appeared desirable to in-

[1] The first batch of nuclei to be obtained by the modified Behrens' technique was prepared in a slightly different manner from the procedure described above, and yielded no gel whatsoever with any reagents, presumably because of slight autolysis. In brief, the departures from the above-described method were as follows: (*a*) the livers were frozen slowly by being placed in a refrigerator at −15°; (*b*) lyophilization was carried out intermittently over a period of 1 week, the material being kept at −15° when lyophilization was not proceeding; and (*c*) grinding in the ball mill was done using the dry powder without solvent, for a period of about 6 days. Grinding without solvent is to be advised only if one wishes to obtain large quantities of purified whole cells, which can then be recovered as a floating mat after centrifugation in a benzene-carbon tetrachloride mixture of specific gravity 1.30, after preliminary centrifugations in a 50-50 benzene-carbon tetrachloride mixture.

vestigate the desoxyribonucleic acid content of nuclei isolated by the Behrens' procedure, since these nuclei could not have lost sufficient material while being prepared to result in appreciably high DNA values.

The DNA was assayed by the procedure of Schneider (10). RNA was also estimated from phosphorus analyses of the hot trichloracetic acid extract used to extract total nucleic acid after removal of acid-soluble phosphorus.

TABLE I

Analysis of Nucleic Acids and Phosphorus in Behrens and Aqueous Type Nuclei (Per Cent, Dry Weight)

Constituent	Type of nucleus						
	Behrens No. 1	Behrens No. 2	Aqueous citric acid pH 4.0 Not defatted	Aqueous citric acid pH 4.0 Defatted	Aqueous citric acid pH 6.0 Not defatted		
Water	8.9	6.5	—	—	—	—	—
Acid-soluble phosphorus	0.45	0.47	0.21	0.49	0.29	0.47	0.85
Total phosphorus: By direct analysis	1.36	1.69	—	—	—	1.74	2.61
Total phosphorus: By adding total nucleic acid P to acid-soluble P	1.36	1.59	1.97	3.06	1.60	—	—
Nucleic acid phosphorus: By subtracting acid-soluble P from total P	0.86	1.22	—	—	—	—	—
Nucleic acid phosphorus: By analyzing total P in Schneider hot trichloracetic extract	0.86	1.02	1.76	2.57	1.31	1.27	1.76
DNA (Schneider colorimetric method)	4.1	6.0	12.9	15.4	11.2	13.9	14.01
Total nucleic acid, calculated from nucleic acid P	11.4	13.6	—	—	17.44	—	—
RNA calculated from total nucleic acid less DNA	7.3	7.6	—	—	6.2	—	—

The results are shown in Table I in which are included other phosphate analyses. Table I also gives corresponding analyses of nuclei prepared in aqueous solution with very dilute citric acid, for purposes of comparison.

The results show that the DNA content of the Behrens' type nuclei is about half that of nuclei prepared in aqueous media. Since it is unlikely that the Behrens nuclei contain an amount of impurity approaching 50 per cent, it appears that the recent work on the DNA content of the nucleus as it exists within the cell (8, 9) is confirmed by an independent procedure.

It is also of interest that the RNA content of the Behrens' type nuclei is higher than that of nuclei prepared at pH 6.0 in aqueous medium. This has been confirmed by Dr. C. Barnum who kindly analyzed some of our nuclei for RNA by the Schneider colorimetric procedure. If this RNA is the result of cytoplasmic contamination, the contaminant would necessarily have to be a microsome or submicrosome fraction, since large granules contain too little RNA to account for the results. Contamination of this sort would seem improbable, however, in consideration of the method used to obtain the nuclei.

Our results indicate that considerable material including protein and possibly RNA must be lost when nuclei are prepared even by very mild procedures in aqueous solution.[2] If this is true, it would seem that the Behrens' type

TABLE II

Desoxyribonucleic Acid Per Cell Nucleus of Normal Rat Liver together with Per Cent DNA (Schneider Analysis)

Type of nucleus	Per cent DNA Dry weight basis	DNA per cell nucleus
		micrograms
Aqueous, citric acid pH 6.0, not defatted	13.9 14.0	10.2×10^{-6} 11.1×10^{-6}
Aqueous, citric acid pH 4.0, not defatted	12.9	11.1×10^{-6}

nuclei should lose material when suspended in water under conditions used to prepare the "aqueous" type nuclei. This was actually found to be the case, since approximately 50 per cent of the dry weight of the Behrens' type nuclei was extracted when the nuclei were suspended in water adjusted to pH 6.0 with very dilute citric acid. Nine-tenths per cent NaCl solution also extracts much material from the nuclei, including a considerable fraction of the arginase of the nuclei.

In order to find out whether DNA itself is extracted from nuclei prepared at pH 6.0 or 4.0 in aqueous medium, analyses were made for the DNA content per average nucleus. This work involves estimating the number of nuclei per milliliter of suspension by means of a blood cell-counting chamber, and determining the DNA per milliliter of suspension by the Schneider technique (10).

The results are shown in Table II. The values are of the same order of magnitude as those published for nuclei of other species (17–20). Since the DNA

[2] We have not investigated nuclei obtained by means of hypertonic sucrose (11–15) but these nuclei are said to lose nucleic acid while being prepared, unless a small amount of citric acid is also added (8, 16).

per nucleus is the same for nuclei prepared at pH 6.0 as for nuclei prepared at pH 4.0 close to the isoelectric point of nucleohistone, it seems unlikely that any appreciable amount of DNA is lost at pH 6.0 or pH 4.0 and that DNA can be used as a reference material in studying the composition of nuclei obtained in aqueous medium.

E. Extraction of Free Amino Acids from the Various Tissue Fragments.—The free amino acids were obtained by extraction of the lyophilized cell fractions with 78 per cent ethyl alcohol, in a ratio of one part of cell fraction by weight to 5 parts of the alcohol by volume. A similar technique has been employed by Dent and collaborators (21).

F. Preparation of Chromatograms.—The analytical technique used was essentially that of Consden, Gordon, and Martin (22) as modified by Dent (23). The two solvents consisted of highly purified phenol, and a mixture of collidine (2,4,6-trimethylpyridine) and lutidine (2,4-dimethylpyridine). A sample containing approximately 50 micrograms of amino nitrogen (the amount of extract from 100 mg. of nuclei) was partitioned first with phenol, followed by partition with collidine-lutidine mixture. Positions of the amino acids were made visible by spraying the paper (after drying) with a 0.1 per cent solution of ninhydrin in *n*-butyl alcohol.

The individual amino acids were identified by their relative positions on the square, and the identity was established by comparison with a reference chromatogram (24). In making this comparison, a consideration of the relative positions of the spots was found to be somewhat more reliable than a consideration of the exact R_f values.

Rough estimates of the relative concentrations of the amino acids also were made, by noting the intensities of the spots together with their sizes.

Chromatograms of hydrolysates of nuclei also were prepared from material obtained by heating samples of nuclei with 6 N HCl in sealed tubes at 100° for 24 hours. These nuclei were subjected to one extraction with 80 per cent alcohol before being hydrolyzed.

G. Results of Paper Chromatography Studies.—It was found that the amino acid pattern of the isolated nuclei was similar to, or identical with, that of whole liver cells. A sample of recovered finely ground cytoplasm was also found to yield the same pattern.

The occurrence of glutathione in extracts of nuclei as well as whole cells is of interest. The glutathione spot was sometimes single as can be seen in Fig. 2 (preparation of nuclei mentioned in footnote 1) and sometimes double as seen in Fig. 3. Whole cells from the preparation mentioned in footnote 1 contained very little glutathione, presumably owing to hydrolysis, but a faint glutathione spot could nevertheless be observed. When a small amount of glutathione was added to the amino acid extract from the whole cells of this same preparation, it migrated to the position of the faint glutathione spot already observable.

Hydrolysis with 6 N HCl of a 78 per cent alcoholic extract from nuclei caused the disappearance of the glutathione spot. The glutathione spot diminishes greatly in intensity if a sample of nuclei is allowed to stand for several hours in the 78 per cent alcohol used as extractant. The glutathione of whole liver cells is even more unstable in this respect.

The double glutathione spot (Fig. 2) was caused to migrate as shown in Fig. 4 by treatment of the evaporated alcoholic extract of the nuclei (on the paper)

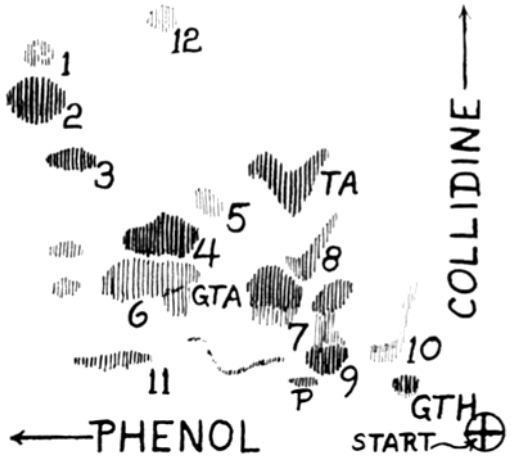


FIG. 2. Chromatogram of extractable free amino acids from Behrens' nuclei, preparation mentioned in footnote 1.

1 = phenylalanine	9 = glutamic acid
2 = leucines	10 = aspartic acid
3 = valine	11 = lysine
4 = alanine	12 = tyrosine
5 = threonine	TA = taurine
6 = β-alanine	GTA = glutamine
7 = glycine	GTH = glutathione
8 = serine	P = peptide

with 30 per cent hydrogen peroxide. The same phenomena occurred when a relatively large amount of glutathione was added to a hydrolysate of casein, as can be seen from Fig. 5 (no peroxide added) and Fig. 6 (peroxide added), except that insufficient peroxide was added in this case to oxidize all the glutathione so that a residual double spot could be seen in the original position (Fig. 6). We believe that these observations are sufficient to make the identification of the glutathione spot quite certain. The cystine spot migrates somewhat similarly upon the addition of hydrogen peroxide, but this spot is nevertheless readily distinguished from the glutathione spot. It is not yet known

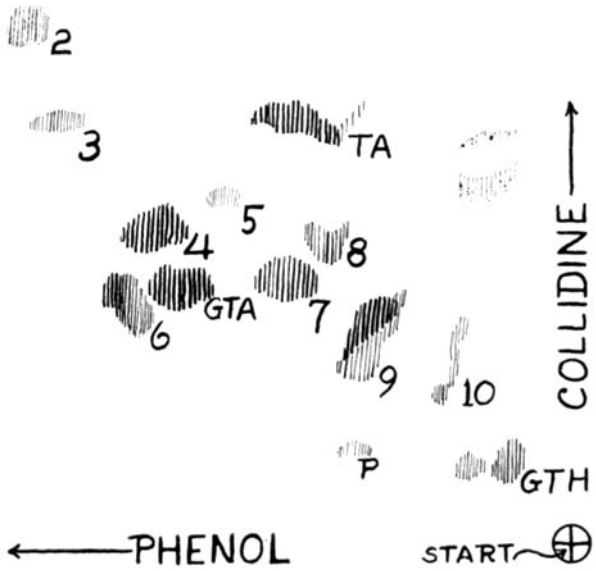


FIG. 3. Chromatogram of extractable free amino acids of Behrens' nuclei, best preparation obtained. Numbers same as for Fig. 2.

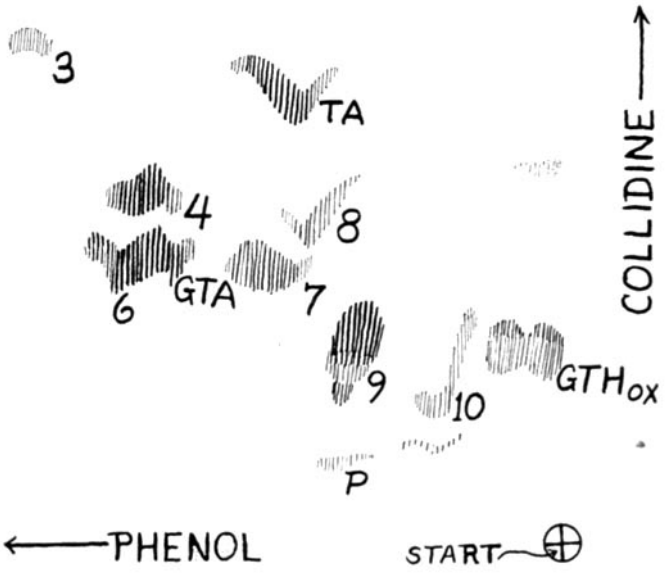


FIG. 4. Same preparation as shown in Fig. 3 but with H_2O_2 added. GTH = oxidized glutathione. Other numbers and letters same as in Fig. 2. The spot due to the leucines, not shown, was barely visible on the original chromatogram.

why large amounts of glutathione are apt to give a double spot while small amounts give a single spot. It can, however, be surmised that the double spot

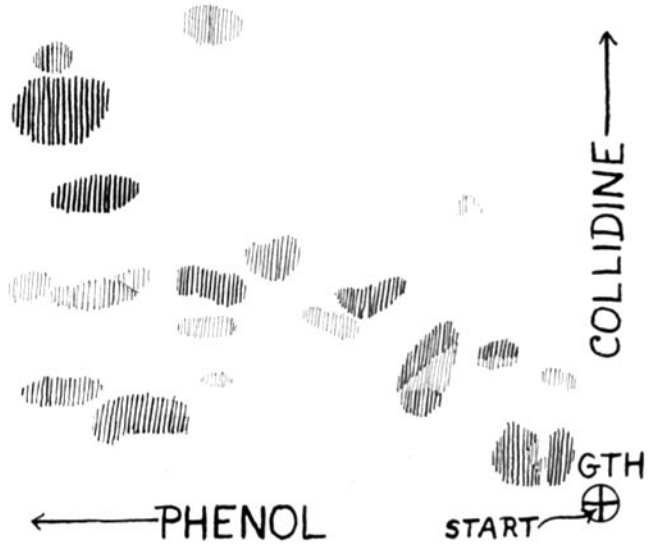


FIG. 5. Chromatogram made from amigen with added glutathione.

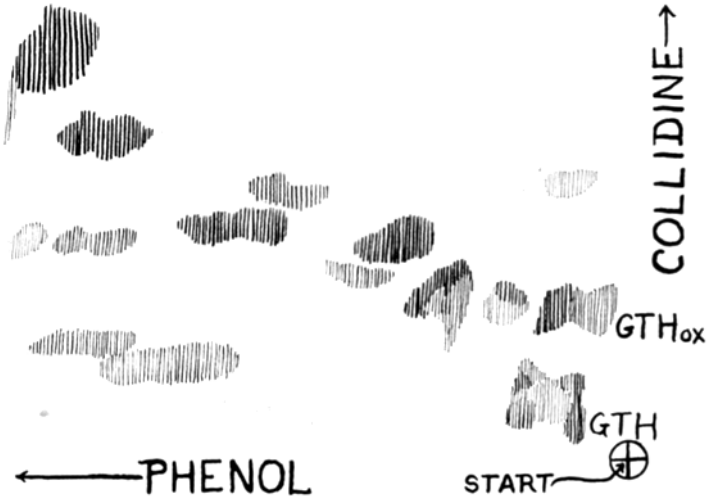
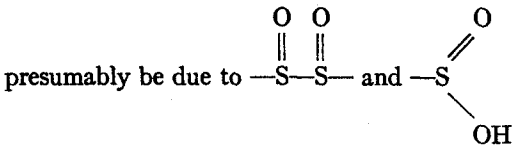
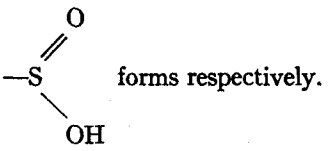


FIG. 6. Chromatogram made from amigen with glutathione and H_2O_2 added.

is caused by the presence of both the —SH and —S—S— forms of glutathione, while the single spot corresponds to the —S—S— spot only. If this explana-

tion is correct, the double spot observed after treatment with H_2O_2 would presumably be due to  and  forms respectively.

A second point of interest is that many of the extractable essential amino acids are very low in concentration in the recovered whole cells and the nuclei. For example, phenylalanine, tyrosine, histidine, threonine, arginine, lysine, and methionine are very low in concentration as compared with glycine, alanine, and glutamic acid. This can be seen from Figs. 2 and 3. In the chromatogram obtained from the microscopically best appearing nuclei obtained from rat liver, the essential amino acids were particularly low (Fig. 3). In the case the spot due to leucine plus isoleucine was detectably fainter than that from the whole cells.

Certain faint spots also appear in the chromatograms from nuclei and whole liver cells which indicate the presence of peptides of unknown constitution. One such spot, labelled *P* in the figures, is especially prominent. These spots disappear completely if the extracts are hydrolyzed and then subjected to further chromatography.

An analysis was made of the amino acids obtained by hydrolyzing the residue of nuclei of rat liver remaining after extraction with 78 per cent alcohol. This showed that practically all the amino acids are present and that the essential amino acids are present in amounts comparable to those found in ordinary proteins. An unknown spot adjacent to that caused by arginine also was present.

The chromatogram of the extractable amino acids of whole lyophilized rabbit liver was practically indistinguishable from that of rat liver, except that the spot thought to be due to taurine was weaker than the taurine spot in the chromatograms of the free amino acids of rat liver.

DISCUSSION

This work represents in part the commencement of attempts to obtain information concerning the distribution of substrates between nucleus and cytoplasm. Results obtained from using whole cells can be taken to represent roughly results that would be obtained from isolated cytoplasm, owing to the small percentage of cell volume occupied by the nucleus.

The pattern of amino acids of the isolated cell nuclei of the animals fed as described seems to be practically the same as that of whole cells or cytoplasm. It should be remembered that in this work the tissue is merely being brought into equilibrium with the 78 per cent alcohol solvent which is subsequently

subjected to chromatographic analysis. The amino acids, therefore, are not being quantitatively extracted. However, we have ascertained that the amino acids studied are all completely soluble in the solvent at the concentrations in question.

A number of the essential amino acids are low relative to the concentrations of glycine, alanine, and glutamic acid in both the nuclear fraction and the whole cells. The fact that the essential amino acids are much lower in concentration than some of the non-essential amino acids may reflect the limit on the concentrations of essential amino acids which is imposed by the diet, in contrast to the greater availability of amino acids such as glycine, alanine, and glutamic acid from the metabolism of the animal.

We are aware that diffusion of amino acids between the cytoplasm and nucleus might occur sufficiently to be a disturbing factor in this work, although it does not seem highly probable. We know that the liver was at all times at a temperature at least as low as 0°, while being lyophilized, and from the experience of others it was probably at a temperature of about −20° as long as appreciable water vapor was evaporating. The flasks always were surrounded by a thick continuous block of ice at the end of the lyophilization as the result of freezing of water in the Dewar flask. It might at first sight seem necessary to lyophilize the tissue at a temperature lower than the lowest eutectic point of the water-soluble materials in the nuclei, in order to block diffusion between nuclei and cytoplasm. Scott (25) has recommended that temperatures as low as −78.5° be employed for lyophilizing tissue sections preparatory to microincineration and localization of metals. Such a low temperature, however, would probably make the lyophilization of a large bulk of tissue extremely difficult and time-consuming, if not completely impractical. It may not be necessary to resort to such low temperatures in studying nuclei, which are of fairly large dimensions relative to the whole cell, since it is by no means certain that residual unfrozen liquid would form a continuous liquid phase, and moreover the viscosity of any unfrozen liquid might be so high even at −20° that diffusion from nucleus to cytoplasm and *vice versa* would be slow. The problem of ice crystal growth seems to be the major difficulty of histologists, but this difficulty does not seem to be involved in chemical work of the sort described in this paper. Nevertheless, it may be desirable in the future to investigate experimentally the effect of temperature of lyophilization by working at an intermediate temperature in the neighborhood of −40 or −50° if possible.

The possibility that proteolysis could have contributed significantly to the results reported seems rather remote in view of the marked differences between the amino acid patterns of the nuclear extracts and those of the hydrolyzed nuclei; although in the case of the preparation mentioned in footnote 1, some of the peptide spots might have been due to a slight proteolytic action.

SUMMARY

1. Nuclei were prepared from frozen rat liver by a modification of the technique of Behrens, and were studied with regard to the content of free amino acids and nucleic acid.

2. Under rigorously controlled conditions, preparations of nuclei are obtained by the Behrens' method which form a gel in the presence of 5 or 10 per cent NaCl or of water plus a small amount of dilute alkali; whereas when conditions are less rigorously controlled, nuclei are obtained which form no such gel. The property of forming gels with alkali is probably characteristic of all cell nuclei which have not undergone autolysis.

3. Nuclei prepared by the Behrens' technique contain the enzymes arginase, catalase, and esterase in very appreciable concentrations.

4. The free amino acids of the isolated cell nuclei, as well as of other liver cell fractions, have been investigated using the technique of paper chromatography.

5. The chromatographic patterns of the free amino acids of whole cells, ground cytoplasm, and isolated cell nuclei were very similar or identical. A feature of interest in these chromatograms was the faintness or absence of the spots due to a number of the essential amino acids, as compared to the intensities of the spots due to glycine, alanine, and glutamic acid. Glutathione was present in the isolated nuclei as well as in the whole cells.

6. Chromatograms made from hydrolysates of nuclei showed high concentrations of the essential amino acids and were similar to chromatograms of hydrolysates of typical proteins.

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