STUDIES OF TWO TYPES OF ALKALINE PHOSPHATASE IN NUCLEI ISOLATED FROM THE LIVERS OF FED AND FASTED RATS BY A MODIFICATION OF THE BEHRENS TECHNIQUE*

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It has been shown that two types of alkaline phosphatase occur in the nuclear and cytoplasmic fractions obtained from normal rat liver by differential centrifugation of aqueous homogenates. One type, not activated by magnesium, is quite insoluble and is relatively unextractable from cellular particulates, whereas the other type, which can be activated by magnesium, is soluble and to a large extent is extractable. The latter type occurs predominantly in the "soluble aqueous phase" of the cell and may pass between the nucleus and cytoplasm by diffusion *in vivo*.

In order to obtain further information on the significance of the distribution studies carried out with these two types of alkaline phosphatase (1), it was decided to isolate nuclei from the livers of fed and fasted rats by a modification of the Behrens technique, and to compare the alkaline phosphatase activity of the nuclei to the corresponding activity of the whole cell fraction. There is some disagreement at present as to whether the Behrens technique will yield nuclei of acceptable quality from rat liver (2, 3), but in our opinion nuclei of sufficient purity for the purposes of this work definitely can be obtained. The results of our investigation in addition to supplementing work with the "aqueous type" nuclei, fit in so well with the data from the latter type of nuclei that it seems apparent that the Behrens type nuclei isolated by us are sufficiently good for the purpose in question. The desoxyribonucleic acid (DNA) content of the Behrens type nuclei from fasted rats, moreover, was so high (ca. 18 per cent) that gross contamination seems very unlikely on this score alone.

We shall show that the Behrens type nuclei are similar to those isolated in aqueous media in respect to the ratio of the two forms of alkaline phosphatase

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and to the extractability of these two enzymes. The work with fasted animals moreover will indicate that the "insoluble" enzyme, not activated by magnesium, is far more stable to protein depletion than is the soluble form of the enzyme. Finally, it will be shown that depletion of the latter seems to occur in a parallel manner in the cytoplasm and nuclei.

EXPERIMENTAL

Procedure for Isolating Rat Liver Nuclei Using a Modification of Behrens' Technique.— Methods for preparing isolated nuclei from rat liver and other organs by modifications of Behrens' non-aqueous technique were published by Dounce *et al.* (4) and Allfrey *et al.* (2). The modification used in this work was similar to that used by Allfrey *et al.* (2) with the following exceptions.

The livers from 40 Wistar strain rats fasted for 36 hours (water allowed *ad libitum*) or from normal rats were removed immediately after decapitation and bleeding of the animals and were at once placed in liquid nitrogen. The rapid freezing brought about by this procedure should prevent or minimize intracellular translocation of diffusible material within the cells or tissue. The livers were removed from the liquid nitrogen, powdered as described by Dounce (5), and lyophilized until 65 to 70 per cent of the wet liver weight had been removed as water. The tissue was then essentially devoid of moisture. The dry liver powder thus obtained was ground in a mortar and sifted through a No. 20 and then a No. 40 mesh wire sieve in order to remove the bulk of the fiber and thereby facilitate the milling procedure that followed. 50 gm. of the sifted powder was then ground in a ball mill at −15°C. according to the directions of Dounce (5).

After the milling process, the liver suspension was again sifted, first through a No. 40 mesh wire sieve and then through four layers of curity cheese-cloth No. 120 (Kendall Mills, Walpole, Massachusetts).

Thereafter, the procedure of Allfrey *et al.* was followed quite closely, using mixtures of cyclohexane (sp. gr. = 0.779) and carbon tetrachloride (sp. gr. = 1.595) to obtain the solutions of desired specific gravity. The specific gravities of the mixtures were determined at 4°C. as apparently was done by Allfrey *et al.* (2). The solvent mixtures suggested by Allfrey *et al.* were not however always correct for optimal recovery of nuclei in every step of the procedure, and therefore it was necessary to examine the supernatant fluid and sediment after each centrifugation and to modify the procedure as required (6).

Allfrey *et al.* stated that they were unable to prepare nuclei from normal rat liver, although they obtained nuclei of fair quality if the animals were first fasted. Using the method as described above, the authors were able to prepare reasonably clean rat liver nuclei from normal animals and animals fasted 36 hours, but these preparations were not quite as good as the best material prepared previously by Dounce (4) from normal rats as shown by direct comparison of the two products. It was difficult to determine the exact state of purity of these preparations microscopically, whether the material was stained or not, because of the extremely irregular form and the fragility of the nuclei. When stained with methyl green–pyronine (7, 8) or aceto-orcein (9), very little cytoplasmic contamination could be seen, but a fair number of broken nuclei were observed. The nuclei obtained from the livers of fasted rats were remarkably shrunken in appearance as compared with those obtained from the livers of fed animals.

The "whole homogenate" was prepared according to the procedure of Allfrey *et al.* (2) after first employing the preliminary steps described previously under the procedure for isolating rat liver nuclei using a modification of Behrens' technique. The whole homogenate represents an aliquot of the whole liver tissue after removal of fiber and lipide; it was treated with the same solvents for identical times and temperatures as the nuclei isolated by the

Behrens technique from the same original liver preparation. This homogenate differs somewhat from the homogenate obtained by the aqueous techniques of cell fractionation, for in the latter case little or no lipide is removed. Fiber is removed also in the case of the aqueous homogenate.

Alkaline Phosphatase Assay Methods.—The alkaline phosphatase activity of the isolated nuclei was determined both in the presence and in the absence of magnesium ions by the calcium phenolphthalein phosphate method, sodium β-glycerophosphate method, *p*-nitrophenyl phosphate method, and the naphthylamine Bordeaux (azo dye) method described previously (1).

The specific activity of alkaline phosphatase is expressed as the micrograms (γ) of inorganic phosphorus or Klett units of naphthylamine Bordeaux (azo dye) produced per milligram of dry weight of nuclei (or homogenate) per hour of incubation at 37°C.

Extraction of Alkaline Phosphatase from Behrens Type Nuclei.—The extraction of the rat liver nuclei isolated by the Behrens procedure was slightly different from the extraction of rat liver nuclei isolated in aqueous media and described in the preceding paper (1). The Behrens nuclei were suspended in cold isotonic saline without previous suspension in distilled water and without adjustment of the pH to 7.0. The remainder of the procedure was identical with the procedure described for the nuclei isolated in aqueous media (1). Behrens nuclei could not be extracted in distilled water at a pH of 7 because of extensive agglutination and disruption of the nuclei under these conditions.

RESULTS

Typical data obtained from the numerous assays for the specific activity of alkaline phosphatase in rat liver cell nuclei isolated by a modification of the Behrens method are summarized in Table I.

TABLE I

Typical Results with Regard to Specific Alkaline Phosphatase Activity of Rat Liver Nuclei Isolated by Behrens' Technique from Normal and Fasted Animals

Specific alkaline phosphatase activity is expressed as the micrograms of phosphorus or Klett units of naphthylamine Bordeaux (azo dye) produced per milligram of dry weight of nuclei (or homogenate) per hour of incubation at 37°C.

Values in parentheses indicate the specific alkaline phosphatase activity of the homogenate corresponding to the adjacent value given for the specific activity of the nuclei from the same preparation. Values obtained from the same preparation of nuclei when assayed by the different methods are shown on the same line.

Source of nuclei	Assay of alkaline phosphatase activity				
	Phosphorus test method				Naphthylamine Bordeaux test method
	Calcium phenolphthalein phosphate substrate	Sodium β-glycero-phosphate substrate	Calcium β-naphthyl phosphate substrate	Sodium *p*-nitrophenyl phosphate substrate	
	γ of P	*γ of P*	*γ of P*	*γ of P*	*Klett units*
Normal rat liver	0.4	1.9 (1.9)	2.3	2.9 (1.8)	42
Rat liver after 36 hours' fasting	1.5 (0.8)	4.6 (2.7)	3.0 (2.4)	4.5 (2.6)	46

The results given in this table, when compared with the average activity of whole homogenates, which are also included in this table, show that the enzyme is definitely present in the isolated nuclei, and that the specific activity is generally higher than the specific activity of the corresponding whole homogenate. These data confirm the findings on nuclei isolated in an aqueous media which were reported in the preceding paper (1).

Studies of the Extraction of Alkaline Phosphatase from Rat Liver Nuclei Isolated by the Behrens Technique from Fed and Fasted Animals.—Nuclei obtained by the Behrens technique from normal rats and from rats that had been fasted for 36 hours were extracted with isotonic saline as described in the Experimental section. Typical results obtained from extraction of these nuclei are shown in Fig. 1.[1]

The data indicate that approximately 50 per cent of the protein can be extracted from the isolated nuclei by isotonic saline. This amount is appreciably greater than the 25 per cent that can be extracted from normal rat liver cell nuclei isolated in aqueous media (1). These findings help to explain why a much lower percentage of DNA is found in nuclei isolated by the Behrens technique than in the nuclei isolated in an aqueous medium. The data also indicate that nuclei prepared in an aqueous medium must lose a fair amount of protein during the isolation procedure. The nuclei isolated by the Behrens technique cannot lose appreciable protein during the isolation procedure, owing to the use of non-polar solvents instead of aqueous solutions throughout the isolation procedure.

Studies of the specific activities of nuclei isolated by the Behrens technique from fed rats before and after extraction with saline (Table II) indicate that the nuclei lose the magnesium-activated alkaline phosphatase faster than they lose other protein, as the result of the extraction. The reverse is true of the alkaline phosphatase not activated by magnesium. In the case of the fasted rats, however, saline extraction caused protein to be removed faster than either the magnesium-activated enzyme or the enzyme that is not activated by magnesium. Table II also shows that the specific activities of both forms of phosphatase in nuclei isolated by the Behrens technique from rat liver always increase upon fasting, but that such increase is greater for the enzyme that is not activated by magnesium than for the magnesium-activated enzyme. Finally it can be deduced from consideration of Table II and Fig. 1 that the magnesium-activated form of alkaline phosphatase is more labile both to fasting and to extraction by saline than is the form of the enzyme not activated by magnesium. Both fasting and saline extraction in fact tend to cause a similar

[1] It will be noted on examination of Fig. 1 that in two cases the recovery of protein is greater than 100 per cent, and that the same is true in one case for the form of alkaline phosphatase that is activated by magnesium. No adequate explanation for these discrepancies has been found other than the possibility that they represent some sort of experimental error.

depletion of the magnesium-activated form of the enzyme relative to the form of the enzyme not activated by magnesium, and therefore extraction by saline has less effect in changing specific activity ratios of the enzymes for nuclei from fasted animals than for nuclei from fed animals.

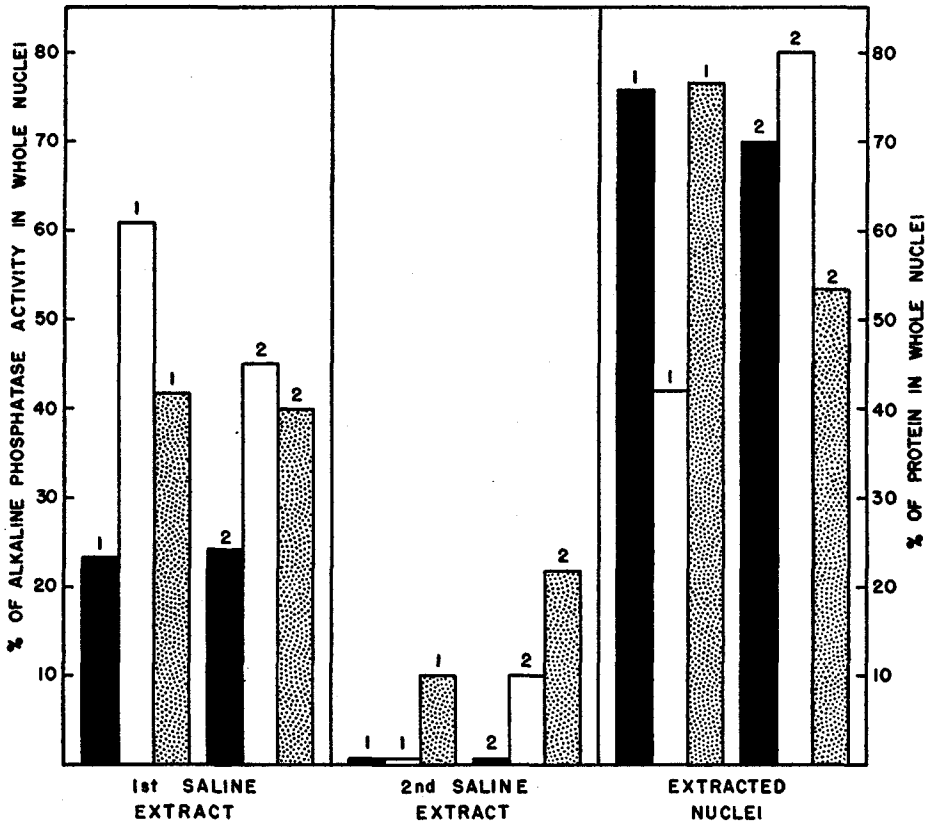

FIG. 1. Extraction of protein and alkaline phosphatase from rat liver nuclei isolated by the Behrens technique from normal and fasted animals. 1, Behrens nuclei isolated from normal rats; 2, Behrens nuclei isolated from rats fasted for 36 hours. The solid column indicates alkaline phosphatase activity in the absence of Mg^{++}; the white column, alkaline phosphatase activity in the presence of Mg^{++}; the stippled column, protein content.

These results therefore would seem to suggest that the form of the enzyme not activated by magnesium is more strongly bound within the nucleus than the magnesium-activated form of the enzyme, and can perhaps therefore be considered as an intrinsic part of the nuclear structure. This fact, along with the previous finding that the magnesium-activated enzyme was mainly in the soluble fraction of the rat liver cell (1), suggests that the soluble, magnesium-

activated enzyme may diffuse back and forth between nuclei and cytoplasm. It should be kept in mind that no doubt some of the magnesium-activated form of alkaline phosphatase is measured together with the form not activated by magnesium, even if no magnesium is added, since there must be some magnesium ion normally present in the cellular components. This means that our separation of enzyme into two forms may not be as clean cut as if no magnesium were originally present in the cellular components.

Fasting has been known previously to cause a considerable loss of protein from rat liver cells (3, 10, 11). Miller (10, 11) found that the alkaline phosphatase activity of the whole liver of fasted rats, with full activation by magnesium, was lost at approximately the same rate as the liver protein, and therefore

TABLE II

Specific Alkaline Phosphatase Activity of Normal and Saline-Extracted Rat Liver Nuclei Isolated by the Behrens Technique

Specific activity is defined as the micrograms of P liberated from β-glycerophosphate per milligram of dry weight of nuclei per hour of incubation at 37°C.

	Fed rats		Rats fasted for 36 hours		Ratio of: Specific activity of nuclei from fasted rats / Specific activity of nuclei from fed rats	
	Mg^{++}	No Mg^{++}	Mg^{++}	No Mg^{++}	Mg^{++}	No Mg^{++}
Whole nuclei	1.4	0.3	3.0	1.5	2.1	5.0
Saline-extracted nuclei	1.1	0.4	4.6	2.0	4.2	5.0
Ratio of: Specific activity of extracted nuclei / Specific activity of whole nuclei	0.8	1.3	1.5	1.3	—	—

the specific activity of the total alkaline phosphatase in the whole liver remained relatively constant during a 7 day fast. Miller made no attempt however to investigate the changes of enzyme activity that may have occurred in the various cell fractions. His results for whole cell homogenates do not parallel our results for isolated nuclei, but this does not necessarily indicate any discrepancy.

DISCUSSION

The experiments that were carried out with Behrens type nuclei showed that nuclei prepared in this manner had a specific activity which was approximately identical with that of homogenates prepared in aqueous media (1). It is quite certain that Behrens nuclei isolated from livers of normal rats contain a higher content of non-alkaline phosphatase protein than do nuclei isolated in an aqueous medium. One must also remember, however, that the Behrens

nuclei and the homogenate control have been treated with organic solvents which extract lipides. Cell nuclei isolated in aqueous media contain about 5 to 10 per cent lipide and since the concentration of the lipides in cytoplasm can undoubtedly be higher than this, it is reasonable to conclude that the specific activity of the whole cell fraction might be increased more than the specific activity of the nucleus upon extraction of the cell lipides.

Nuclei isolated from the livers of rats that had been fasted for 36 hours were found to have specific activities approximately double the activity of the homogenate. These nuclei are remarkably shrunken when isolated by the Behrens technique. The per cent of DNA is also very high, so that one may conclude that the nuclei tend to lose protein faster than they lose alkaline phosphatase. This finding is an additional indication that at least some part of the alkaline phosphatase present in the nucleus of the liver cell is firmly bound to the structural material of the nucleus.

The data in Fig. 1 show that a maximum of 25 per cent of the magnesium-activated alkaline phosphatase is extractable from Behrens nuclei isolated from the livers of rats fasted for 36 hours. Similar results were obtained from rat liver nuclei isolated in 0.44 M sucrose adjusted to pH 6.2 with dilute citric acid (1). A maximum of 50 per cent of the magnesium-activated alkaline phosphatase could be extracted from liver nuclei prepared from normal rats by the Behrens technique. Little of the enzyme that is not activated by magnesium can be extracted by saline from any type of isolated nuclei studied.

These data obtained on the alkaline phosphatase activity and the saline extraction of alkaline phosphatase and protein from Behrens type of nuclei isolated from normal rats and rats fasted for 36 hours lend support to the previous hypothesis that there are two types of alkaline phosphatase in the rat liver cell nuclei. The data further show the effect of fasting in causing preferential removal of the soluble, magnesium-activated form of the enzyme from the nucleus *in vivo*.

SUMMARY

1. Rat liver nuclei were isolated from normal rats and rats fasted for 36 hours by a slight modification of the Behrens technique.

2. The nucleus of the rat liver cell contains two types of alkaline phosphatase. This confirms the previous findings on rat liver nuclei isolated in aqueous media.

3. The one type of alkaline phosphatase is not activated by magnesium ions, and this enzyme is very strongly bound to structural material of the nucleus. The other type of alkaline phosphatase is activated by magnesium ions, and this enzyme is probably free to diffuse from cytoplasm to nucleus and *vice versa* through the nuclear membrane.

4. Fasting caused a pronounced decrease of protein in general and of the alka-

line phosphatase which is activated by magnesium ions from the nucleus of the rat liver cell, while the alkaline phosphatase that is not activated by magnesium was less affected.

BIBLIOGRAPHY

1. Emery, A. J., Jr., and Dounce, A. L., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 315.
2. Allfrey, V., Stern, H., Mirsky, A. E., and Saetren, H., *J. Gen. Physiol.*, 1952, **35,** 529.
3. Allfrey, V., Stern, H., Mirsky, A. E., and Saetren, H., *J. Gen. Physiol.*, 1952, **35,** 559.
4. Dounce, A. L., Tishkoff, G. H., Barnett, S. R., and Freer, R. M., *J. Gen. Physiol.*, 1950, **33,** 629.
5. Dounce, A. L., *in* The Nucleic Acids, (E. Chargaff and J. N. Davidson, editors), New York, Academic Press, Inc., 1955, **2,** chapter 18.
6. Dounce, A. L., *Internat. Rev. Cytol.*, 1954, **3,** 199.
7. Kay, E. R. M., Ph.D. Thesis, The University of Rochester, 1952.
8. Kay, E. R. M., *Stain Technol.*, 1953, **28,** 41.
9. Kurnick, N. R., and Ris, H., *Stain Technol.*, 1948, **23,** 17.
10. Miller, L. L., *J. Biol. Chem.*, 1948, **172,** 113.
11. Miller, L. L., *J. Biol. Chem.*, 1950, **186,** 253.