INTRACELLULAR DISTRIBUTION OF ALKALINE PHOSPHATASE IN RAT LIVER CELLS*

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During the course of a study to determine the validity of the various histochemical methods for intracellular localization of alkaline phosphatase, the results of which will be presented elsewhere, it became necessary to determine the distribution of this enzyme in the various cellular particulates and soluble fraction. In such a study, accurate results can be obtained only if the method of cell fractionation prevents disruption of cell particulates and translocation of the various components of each cell fraction. Novikoff and coworkers (1–4) studied the distribution of alkaline phosphatase in rat liver cell fractions using the best techniques available at that time. It was the opinion of the present authors that their method of procedure could be materially improved, and therefore a further study of the distribution of this enzyme in rat liver cell fractions was undertaken. A new type of manually operated all glass homogenizer described previously (5, 6) was used in preparing the cell fractions. The method used by us prevents disruption of mitochondria, but the translocation of unbound, soluble protein between nuclei and the soluble portion of the cytoplasm must be assumed (7). Since the conclusion of this study, Dounce *et al.* (8) have described a similar but improved isolation technique, which was used in some of the isolations of nuclei described in this paper.

The present paper reports the finding of two forms of alkaline phosphatase having different intracellular distributions in rat liver cells. This finding is largely in agreement with the work of Allard *et al.* (9, 10) which was reported during the course of our work, and also with studies published by Cloetens (11, 12) and Rosenthal *et al.* (13, 14). The findings of Watson (15), who recently demonstrated the existence of pores in the nuclear membrane of mouse pancreas cells, and those of Holtfreter (16), who demonstrated the permeability of the nuclear membrane of the frog egg to hemoglobin, make it possible

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to interpret certain of the results described in this paper by postulating that nuclear membranes in general are permeable to soluble protein by simple diffusion. This will be more fully covered in the discussion.

EXPERIMENTAL

Preparation of the Rat Liver Homogenate.—The rat livers were quickly removed after the animals had been decapitated and bled, and were at once placed in an ice bath. After being chilled, they were weighed and pulped with a cold mortar and pestle. The pulped livers from two rats were diluted to a volume of 95 ml. with cold 0.44 M (15 per cent) sucrose. The suspension was then homogenized with 12 strokes in the new type manually operated glass, ball type homogenizer of Dounce, having a clearance between the pestle and homogenizer wall of approximately one-thousandth of an inch,[1] with the homogenizer immersed in a bath of ice and water (17). An aliquot of this suspension was removed and stored in an ice bath for later analysis. This aliquot was called the homogenate.

Isolation of Nuclei.—Rat liver nuclei were isolated in 0.44 M sucrose without adjustment of the pH (18), using the ball type homogenizer cited above. This procedure was similar to that of the more recently described method of Dounce *et al.* (8) except that in most of the work the 0.44 M sucrose medium was not adjusted to pH 6.2 with dilute citric acid as described by these authors. In a later study, also to be included in this paper, rat liver nuclei (but none of the other cell fractions) were also isolated by the newer method (8).

This procedure employing 0.44 M sucrose without pH adjustment does not generally produce a nuclear preparation that is 100 per cent free of whole cells, but relatively few whole cells and no red cells or mitochondria remain. Nuclei prepared in this manner have a morphological appearance similar to their appearance in unbroken cells.

Procedure for Isolating Rat Liver Nuclei in 0.44 M Sucrose with Citric Acid to Adjust to Various pH Values.—Isolated nuclei were prepared in 0.44 M sucrose adjusted to various pH values with citric acid, essentially as described by Dounce *et al.* (8) for the 0.44 M sucrose–pH 6.2 method. The only modifications of the method were in the amounts of 0.1 M citric acid added to adjust the pH to the desired value; slightly higher centrifugation speeds or longer periods of centrifugation were required at times when the pH was greater than 6.2.

Isolation of Rat Liver Nuclei Using 0.25 M Sucrose with 0.004 M $CaCl_2$.—A modification of the Schneider and Petermann (19) method was used as described by Dounce (6) with the following exceptions. The concentration of the sucrose-$CaCl_2$ mixture was 0.25 M sucrose–0.004 M $CaCl_2$ instead of 0.005 M $CaCl_2$ as used by Dounce. The only other change was that the supernatant fluid from the first centrifugation was saved and used for subsequent isolation of the mitochondria, microsomes, and soluble fraction of the rat liver cells.

This procedure produced a preparation of isolated nuclei that was quite clean and relatively free of whole cells, red blood cells, mitochondria, and microsomes. The $CaCl_2$ appeared to cause some toughening of the cell membranes of whole cells, and prevented complete elimination of whole cells and cellular debris from the preparation. The nuclei retained approximately their native shape and size, but the intranuclear material appeared slightly reticulated.

Isolation of Mitochondria.—Mitochondria were isolated in 0.44 M sucrose by further fractionation of the final supernatant fluid obtained by the procedure described previously for the isolation of nuclei in 0.44 M sucrose. The method was very similar to that described by Witter *et al.* (20) with the following exceptions.

The supernatant fluid after the first centrifugation was saved for the isolation of microsomes and the soluble fraction. The sediment obtained under the conditions of the method contained most of the mitochondria from the homogenate. This sediment was resuspended in approxi-

[1] Hereafter this homogenizer will be referred to as the "loosely fitting homogenizer."

mately 100 ml. of cold 0.44 M sucrose by homogenizing with 6 strokes in the loosely fitting homogenizer. The centrifugation was repeated and the supernatant fluid was discarded. The sedimented mitochondria were resuspended in approximately 25 ml. of cold 0.44 M sucrose.

Mitochondria isolated by this procedure retain their original rod shape.

Isolations of Microsomes.—After sedimenting the nuclei and mitochondria from the 0.44 M sucrose rat liver homogenate as described above, the supernatant fluid was centrifuged at 30,000 R.P.M. (78,000 *g*) for 1 hour at 33–35°F. in a Spinco model L preparative ultracentrifuge. The sedimented microsomes were resuspended in 15 ml. of 0.44 M sucrose by homogenizing with 3 strokes in a loosely fitting ball type homogenizer.

Soluble Fraction.—The soluble fraction was the fraction remaining after the nuclei, mitochondria, microsomes, and all particulates sedimenting from 0.44 M sucrose solution at 78,000 *g* had been separated from the original homogenate.

Assay Methods for Alkaline Phosphatase

1. Calcium Phenolphthalein Phosphate Method.—The calcium phenolphthalein phosphate assay method for alkaline phosphatase was a modification of the method of Dounce and Lan (18). The calcium phenolphthalein phosphate was synthesized according to the method of King (21) with the addition of several washes by 0.1 N HCl to free the preparation of inorganic phosphate.

The incubation mixture consisted of the following concentrations of the various reagents: 0.13 M sodium veronal, 0.001 M $MgSO_4 \cdot 7H_2O$, 0.01 M $CaCl_2$, and 0.27 per cent calcium phenolphthalein phosphate. The final mixture had a pH of approximately 9.7. 6.5 ml. of this buffer-substrate mixture was incubated for 15 minutes at 37°C. for temperature equilibration, then further incubated for 15 minutes to 1 hour after the addition of 0.5 ml. of the enzyme preparation. The amount of phenolphthalein liberated by the enzyme during incubation was then determined in a manner similar to that described by Dounce and Lan (18).

This method was found to be somewhat less sensitive than the others mentioned below, and sometimes turbidities caused difficulty.

2. Sodium β-Glycerophosphate Method.—The method used for determining alkaline phosphatase activity with sodium β-glycerophosphate as substrate is a modification of the methods of Bodansky (22), Gomori (23, 24), and Kabat and Furth (25).

The incubation mixture consisted of the following: 0.4 ml. of 0.8 M sodium β-glycerophosphate, 0.1 ml. of 0.1 M $MgSO_4$, and 7.0 ml. of 0.1 M sodium veronal (pH 9.68) buffer. This incubation mixture was brought to the desired temperature (37°C.) and then incubated at this temperature after adding 0.5 ml. of the cell fraction being analyzed. The incubation flasks were continuously shaken at a rate of 200 times per minute through a distance of 2 inches during the entire incubation time (usually 1 hour).

After the incubation period, the reaction was stopped by the addition of 1 ml. of ice cold 20 per cent trichloroacetic acid. The mixture was then transferred quantitatively to a 15 ml. graduated centrifuge tube containing 1 ml. of cold 20 per cent trichloroacetic acid, and the volume was adjusted to 10 ml. with distilled water. The mixture was next centrifuged and a 5 ml. aliquot of the supernatant fluid was analyzed for phosphorus by the method of Fiske and SubbaRow (26). The trichloroacetic acid was added immediately after the enzyme in the blank determination.

Alkaline phosphatase activity was expressed as the micrograms (γ) of inorganic phosphorus liberated from sodium β-glycerophosphate per milligram of dry weight of the enzyme preparation per hour.

Beer's law of dilution was found to hold over the concentration range of inorganic phosphate used.

3. p-Nitrophenyl Phosphate Method.—The *p*-nitrophenyl phosphate method used for the

determination of alkaline phosphatase activity was a modification of the method used by Bessey *et al.* (27). The substrate, disodium *p*-nitrophenyl phosphate, was obtained from the Sigma Chemical Company, St. Louis. In the present study, the amount of inorganic phosphorus rather than the amount of *p*-nitrophenol liberated by the enzyme was determined by means of the method of Fiske and SubbaRow (26).

The substrate solution consisted of the following reagents: 7.0 ml. of 0.1 M sodium veronal buffer (pH 9.68), 0.1 ml. of 0.1 M $MgSO_4$, and 0.4 ml. of 4 per cent sodium *p*-nitrophenyl phosphate. 0.5 ml. of the homogenate or cell fraction in question was added to the substrate solution (previously brought to the proper temperature) and incubation was carried out at 37°C., usually for 1 hour, with continuous shaking. Subsequent steps in the determination of phosphorus were the same as previously described under the β-glycerophosphate method.

4. Naphthylamine Bordeaux (Azo Dye) Method.—The naphthylamine Bordeaux method for the quantitative determination of alkaline phosphatase activity was based on the histochemical method of Menten, Junge, and Green (28, 29). The substrate is calcium β-naphthyl phosphate.

Modifications necessary to adapt the method to cytochemical studies were made. For example, since the diazonium salt of α-naphthylamine is quite unstable and is believed to be somewhat inhibitory toward the enzyme (30), it was omitted from the substrate mixture and was added only after the incubation time was completed.

The substrate mixture consisted of the following reagents: 50 mg. of calcium β-naphthyl phosphate, 1 ml. of 1 per cent $MgSO_4$, 15 ml. of 0.1 M sodium veronal buffer (pH 9.68), and 83.5 ml. of distilled water. 0.5 ml. of homogenate or cell fraction was used as enzyme source. A blank was made at the same time and under the same conditions by substituting 0.5 ml. of water for the enzyme source. The final pH of the reaction mixture was approximately 9.6.

The solutions were incubated for 1 hour at 37°C. with continuous shaking, followed by the addition of 50 mg. of α-naphthyl diazonium-1,5-disulfonate. The synthesis of naphthylamine Bordeaux was then allowed to take place at room temperature over a period of 10 minutes although the reaction actually is completed in approximately 5 minutes.

After the formation of the dye was completed, the reaction mixture was filtered through a No. 613 Eaton-Dikeman (Mt. Holly Springs, Pennsylvania) filter paper, and the filtered material was allowed to *dry completely* at room temperature on the filter paper.

Chloroform was next passed through the dried material on the filter paper and collected quantitatively in a 200 ml. volumetric flask until all of the red azo dye was eluted, and the solution of dye was then diluted to exactly 200 ml. with more chloroform. After thorough mixing, the amount of dye present was determined colorimetrically using a Klett colorimeter with a No. M 515 filter. All results were corrected by subtracting the amount of dye formed in the blank as a result of non-enzymatic hydrolysis of the substrate.

The Klett units were not converted to milligrams of naphthylamine Bordeaux because no accurate extinction coefficient for this dye has yet been established. The alkaline phosphatase activity was expressed as Klett units corresponding to a given amount of enzyme after 1 hour's incubation at 37°C. under the conditions stated previously.

The calcium β-naphthyl phosphate and α-naphthyl diazonium-1,5-disulfonate used in this assay method were synthesized by the methods of Manheimer and Seligman (29).

Extraction of Alkaline Phosphatase from Nuclei Isolated in Aqueous Media

Small aliquots of a preparation of rat liver nuclei isolated in the aqueous medium were assayed for protein content (micro-Kjeldahl method) and for alkaline phosphatase with sodium β-glycerophosphate as substrate. The protein content and alkaline phosphatase activity of a 10 ml. portion of the nuclear suspension were then calculated. A 10 ml. aliquot was adjusted to a pH of 7 with 0.1 M sodium bicarbonate, and after thorough stirring was

allowed to remain at 4°C. for 30 minutes with occasional further stirring. At the end of the 30 minute period, the preparation was centrifuged at 2750 R.P.M. (1500 *g*) for 5 minutes in a refrigerated centrifuge at 33–34°F. The supernatant fluid was carefully decanted, labelled as the "aqueous extract," and stored at 4°C. for later analysis for protein and alkaline phosphatase.

The sedimented nuclei were then resuspended in 10 ml. of cold isotonic saline (0.9 per cent) and the suspension was again allowed to stand at 4°C. for 30 minutes with occasional stirring. At the end of the 30 minute period the suspension was centrifuged as described previously to sediment the nuclei. The supernatant fluid was carefully decanted, labelled as the "first saline extract," and stored at 4°C. for later analysis.

The sedimented nuclei were again resuspended in 10 ml. of cold isotonic saline and the extraction procedure was repeated. The supernatant fluid obtained from this extraction was labelled the "second saline extract" and the sedimented nuclei were resuspended in cold distilled water in a total volume of 100 ml.

The extracted fractions were then assayed for protein content and alkaline phosphatase activity. The amount of protein and alkaline phosphatase present in each extracted fraction was expressed as the per cent of the total amount of protein and alkaline phosphatase, respectively, present in the original aliquot of isolated nuclei.

RESULTS

In the first part of our investigation, all determinations of alkaline phosphatase were made in the presence of an optimal concentration of magnesium ion, in order to obtain results giving the maximal amount of enzyme. All data to be presented will be of this kind until definite statements are made introducing studies which show the effect of leaving out the magnesium ion.

Specific Activity of Alkaline Phosphatase in Nuclei Isolated by Different Methods.—Typical data obtained from the numerous assays for the specific activity of alkaline phosphatase in isolated rat liver cell nuclei are summarized in Table I. Since the analysis for desoxyribonucleic acid (DNA) was not made on most of the nuclear preparations, it is impossible to express the alkaline phosphatase activity on the basis of the DNA content. It will be noted that occasional results do not seem to be in line with the rest. No satisfactory explanation can be given for this, except that in some instances there may have been slight turbidities in the solutions used for colorimetry. In general, the test for inorganic phosphorus is more sensitive and reproducible than the other colorimetric tests used, and therefore the data obtained by this method are probably more reliable.

The results for isolated nuclei shown in Table I, when compared with the average activity of whole homogenates, also included in this table, show that the enzyme is definitely present in the isolated nuclei, and that the specific activity (activity per milligram of dry weight) is generally higher than the specific activity of the corresponding whole homogenate.

The Distribution of Alkaline Phosphatase Activity in the Various Cell Fractions.—Mitochondria, microsomes, and the soluble fraction were prepared from the same homogenate by the methods described in this paper. These

preparations were then assayed for the specific activity and the total activity of alkaline phosphatase.

TABLE I

Summary of the Specific Alkaline Phosphatase Activity of Isolated Nuclei

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.

Isolation method	Assay of alkaline phosphatase activity	
	Phosphorus test method sodium β-glycerophosphate substrate	Naphthylamine Bordeaux test method
	γ—P	*Klett units*
0.44 M sucrose	3.3	40
	3.5	—
0.25 M sucrose—0.004 M $CaCl_2$	1.5	56
	7.3	—
	2.3	15
	—	231
0.44 M sucrose—pH 5.8	5.4	37
	8.0 (2.0)	56 (4)
0.44 M sucrose—pH 5.9	8.0	55
	8.0	33
0.44 M sucrose—pH 6.0	3.7–4.6 Av. 4.2	—
	1.1	63
	4.6	44
	8.0	15
	8.0	79
	2.6 (0.9)	47 (2)
0.44 M sucrose—pH 6.2	5.9 (0.9)	50
	6.0	40
0.44 M sucrose—pH 6.3	4.4 (1.4)	65 (2)

The results of the assays of isolated mitochondria, microsomes, and soluble fraction for the specific activity of alkaline phosphatase are shown in Table II. The relative distribution of alkaline phosphatase in the nuclei, mitochondria, microsomes, and soluble fraction from normal rat livers is summarized graphically in Fig. 1.

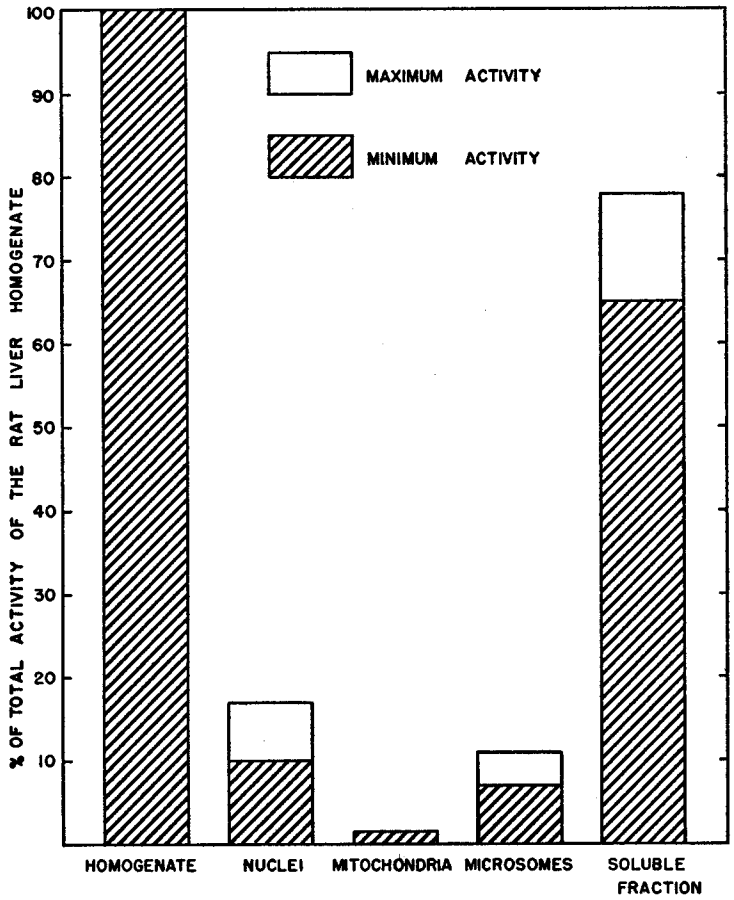

FIG. 1. Relative distribution of alkaline phosphatase activated by magnesium ions in normal rat liver cell fractions. Fractions prepared in 0.44 M sucrose. Assayed by the β-glycerophosphate method.

TABLE II

Summary of the Specific Activities of Alkaline Phosphatase of Isolated Rat Liver Fractions

Specific alkaline phosphatase activity is expressed as the micrograms of phosphorus per milligram of dry weight of rat liver fraction per hour of incubation at 37°C.

Liver cell fraction	Isolation method	Assay of alkaline phosphatase activity by the phosphorus test method (sodium β-glycerophosphate substrate)
Mitochondria	0.44 M sucrose	0–2.5 Av. = 1.3
	0.44 M sucrose—pH 6.2	1.8
Microsomes	0.44 M sucrose	2.0–7.0 Av. = 4.0
Soluble fraction	0.44 M sucrose	10.0–20.0 Av. = 15.0

The results obtained by the determination of the inorganic phosphate liberated by the enzyme from β-glycerophosphate, the most sensitive assay procedure, show that the specific activity of the alkaline phosphatase of rat liver mitochondria is the lowest of the activities of all the cell fractions. The specific activity of the microsomes is approximately of the same order of magnitude as that of the nuclei, while the soluble fraction of the liver cells shows a considerably higher specific activity than that of any of the particulate cell components.

It can be seen from Table I that the specific activity of the rat liver cell nucleus when measured by sodium β-glycerophosphate hydrolysis tends to be considerably higher than the specific activity of the homogenate. It can be calculated from the data that the nucleus should contain approximately 30 per cent of the alkaline phosphatase of the cell, assuming that the nucleus comprises about 10 per cent of the total weight of the cell (31–33). However, on the basis of the assumption that the supernatant fraction contains about one-third of the total weight of the cell, if we assume 25 per cent for the mitochondria, 25 per cent for the microsomes, and 10 per cent for the nucleus, with a few per cent for fat and glycogen, it can be calculated that the specific activity of the homogenate ought to be higher than it is, perhaps by a factor of 2 or 3.

The ratio of specific activity of the nuclear fraction to the specific activity of the homogenate is even greater when the hydrolysis of β-naphthyl phosphate is used to measure the activity, but in view of the fact that this procedure is rather new, we are not yet willing to speculate as to the possible reasons for this difference of the ratios.

From the statements just made, one might be inclined to suspect some inhibition of enzyme activity in the homogenate which does not act in the nuclear fraction or the supernatant fraction. On the other hand, the data in Fig. 1 do not substantiate such a conclusion, since the recoveries obtained by adding the total activities of each fraction do not seem to add up to anything significantly different from 100 per cent. The question could only be settled definitely by the accumulation of more data, and does not in any case seem to affect the main conclusions drawn from the results presented in this paper.

It can be concluded that mitochondria contain very little, if any, alkaline phosphatase. The microsomes contain approximately the same total amount of alkaline phosphatase as the nuclei, while most of the enzyme is present in the soluble fraction of the rat liver cell.

pH Optimum of Alkaline Phosphatase from Various Cell Fractions.—After consistently finding alkaline phosphatase in the rat liver nucleus, it was desired to obtain, if possible, some evidence as to whether the enzyme might be in diffusion equilibrium between the nucleus and cytoplasm. Since it seemed possible that the alkaline phosphatase of the nucleus might be different from

the alkaline phosphatase of the cytoplasm, the enzymes of the isolated nuclei and the soluble fraction were tested first in regard to pH optima. The results of these experiments are shown in Fig. 2. It is apparent that the alkaline phosphatase of the nuclei cannot be distinguished from that of the cytoplasm on the basis of a difference in pH optimum. The optimal pH of both the nuclear enzyme and the enzyme of the soluble fraction is approximately 9.6, when 0.44 M sucrose at pH 6.2 is used in the isolation of the nuclei and in the preparation of the soluble fraction.

It can be seen from Fig. 2 that the alkaline phosphatase of nuclei isolated in 0.44 M sucrose at pH 6 seems to have a slightly higher optimal pH (9.7)

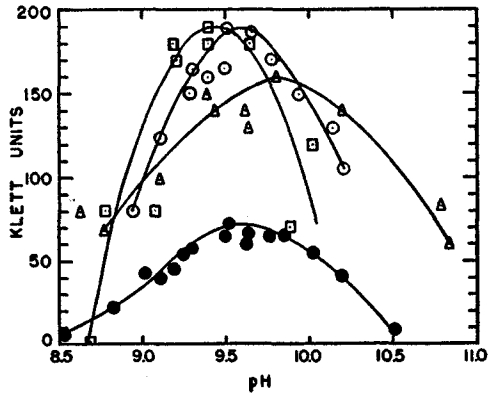

FIG. 2. pH optimum of alkaline phosphatase in isolated nuclei and soluble fractions from rat liver. Samples were assayed by the β-glycerophosphate test method in the presence of magnesium ions. Buffer solutions were 0.1 M sodium veronal-HCl or 0.1 M sodium veronal-NaOH. Klett filter No. 66 was used. ⊙, nuclei prepared in 0.44 M sucrose. ⊡, nuclei prepared in 0.25 M sucrose containing 0.004 M $CaCl_2$. △, nuclei prepared in 0.44 M sucrose adjusted to pH 6.0 with citric acid. ●, rat liver soluble fraction prepared in 0.44 M sucrose.

than that of nuclei isolated in 0.44 M sucrose without pH adjustment, or in 0.25 M sucrose containing 0.004 M $CaCl_2$. The pH optima of both the latter two types of nuclei were close to 9.5. However, this small difference may not be significant.

Substrate Specificity and Magnesium Activation of Alkaline Phosphatase in Rat Liver Cell Fractions.—Studies of possible different alkaline phosphatases in the cell fractions were next made by comparing the behavior towards activation by magnesium of the alkaline phosphatases of the various isolated cell fractions in the presence of each of four substrates, namely sodium β-glycerophosphate, sodium *p*-nitrophenyl phosphate, calcium β-naphthyl phosphate, and calcium phenolphthalein phosphate. All assays in this experiment were based on the amount of phosphorus liberated from these substrates per hour

of incubation at 37°C., in the presence and in the absence of magnesium ions, regardless of whether the substrate yielded a colored product or not. The alkaline phosphatase activity was then expressed as micrograms of phosphorus liberated per milligram of dry weight (specific activity). The results are plotted in Fig. 3.

It can be seen from this figure that the behavior of alkaline phosphatase among the various cell fractions toward activation by magnesium depended upon which of the four substrates was used. If only one enzyme were present in the different cell fractions, it would be expected that a similar pattern would be obtained with each substrate.

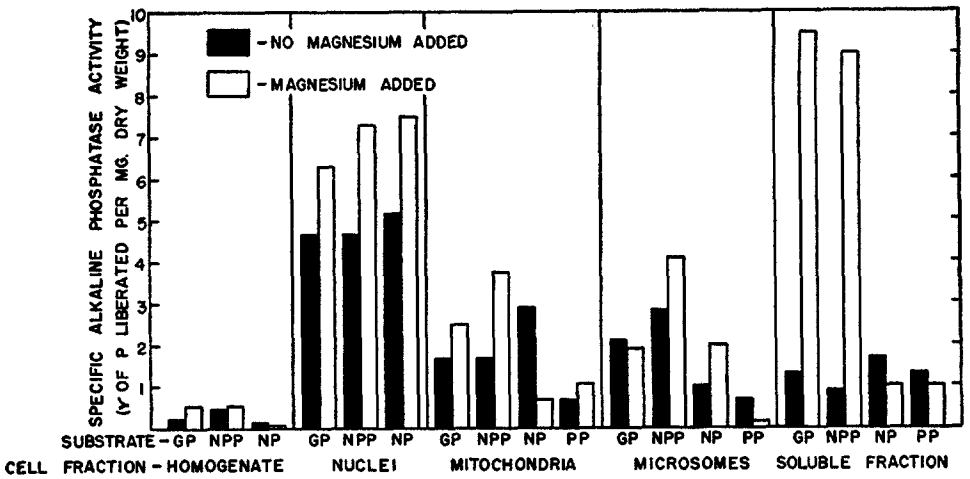

FIG. 3. Substrate specificity and magnesium activation of alkaline phosphatase in cell fractions prepared from rat liver. Isolation medium—0.44 M sucrose. GP, sodium β-glycerophosphate. NPP, sodium *p*-nitrophenyl phosphate. NP, calcium β-naphthyl phosphate. PP, calcium phenolphthalein phosphate.

The behavior of the alkaline phosphatase of mitochondria and microsomes towards activation by magnesium is similar, and it is very probable that the slight alkaline phosphatase activity found in some of the mitochondrial preparations may be a result of contamination by the microsomal fraction. Support for this statement may be found in the fact that most of the preparations of mitochondria that were tested exhibited no detectable alkaline phosphatase activity, either in the presence or absence of magnesium.

Very marked differences were observed when the activating effects of magnesium on the soluble fraction and the isolated nuclei were studied, as can be seen from Fig. 3. The activation by magnesium of the alkaline phosphatase of the nuclear fraction amounted only to 10 to 30 per cent, regardless of the substrate used. In contrast, the activity of the enzyme in the soluble fraction

was increased approximately ninefold in the presence of magnesium ions when the substrates sodium β-glycerophosphate and sodium *p*-nitrophenyl phosphate were employed. On the other hand, magnesium ions apparently caused a slight inhibition of the alkaline phosphatase activity of the soluble fraction when either calcium β-naphthyl phosphate or calcium phenolphthalein phosphate was used as the substrate.

These results lend support to the hypothesis that more than one type of alkaline phosphatase exists in the nuclear and soluble fractions of rat liver cells. It might be concluded from the results shown in Fig. 3 that there is one type of enzyme in the soluble fraction and another in the nucleus. However, it seems more likely from the results of the experiments concerned with the activating effect of the magnesium ion that there are at least two types of alkaline phosphatase in the cell nuclei, one of which is soluble and corresponds to the major portion of the enzyme of the soluble fraction, and the other of which is insoluble and apparently represents a different type of enzyme. The soluble phosphatase appears to require magnesium for maximal activity, while the insoluble enzyme is apparently not activated by magnesium ions. It is not possible from the results reported in this paper to deny that still other types of alkaline phosphatase might exist in the cell, for instance in the microsomal fraction, but no evidence in support of this idea has been found.

Similar results on the effect of magnesium ions in activating the alkaline phosphatase of rat liver nuclei and soluble fractions were recently reported by Allard *et al.* (9, 10) and Rosenthal *et al.* (13, 14). Both of these groups found one form of alkaline phosphatase requiring magnesium ions in the soluble fraction of a preparation fractionated in 0.25 M sucrose. Another form of the enzyme, which did not require magnesium ions, was present and firmly bound in the nuclear, mitochondrial, and microsomal fractions but was absent from the soluble fraction.

The results of the research by the present authors agree fairly well with the findings of Allard *et al.* and Rosenthal *et al.*, except that no cell fraction was found to contain one type of the enzyme to the complete exclusion of the other, and that mitochondria probably do not contain alkaline phosphatase.

Studies of the Extraction of Alkaline Phosphatase from Isolated Rat Liver Cell Nuclei.—The results of the previously described studies are believed to show rather conclusively that one component of the alkaline phosphatase of the rat liver cell nucleus is different from the major portion of the alkaline phosphatase of the soluble fraction of the cytoplasm.

Further information was desired on the extractability of the alkaline phosphatase or phosphatases of the nuclei. It seemed possible for example that it might prove feasible to remove soluble phosphatase completely, leaving behind the insoluble or firmly bound enzyme. Accordingly, experiments con-

cerned with extraction of the enzyme were carried out with nuclei isolated from normal rat liver in 0.44 M sucrose at pH 6.2.

Typical results obtained from extraction of these nuclei with distilled water and with isotonic saline are shown in Fig. 4.

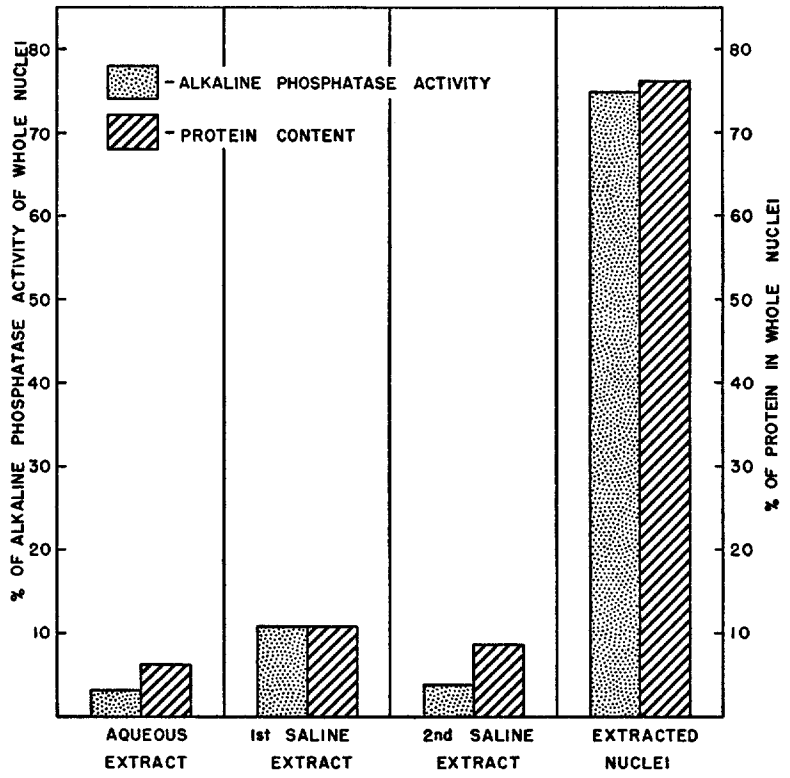

FIG. 4. Extraction of protein and alkaline phosphatase from rat liver cell nuclei by isotonic saline. Nuclei were prepared from normal rat liver in 0.44 M sucrose adjusted to a pH of 6.2 with dilute citric acid. Alkaline phosphatase activity was tested in the presence of magnesium ions. Samples were assayed by the β-glycerophosphate test method.

The data indicate that very little protein and alkaline phosphatase were removed from the rat liver nuclei isolated in 0.44 M sucrose at pH 6.2 when the latter were extracted with cold distilled water adjusted to pH 7. One extraction with cold isotonic saline, however, removes an appreciable amount of protein and alkaline phosphatase. A second extraction with isotonic saline removes only a very small additional amount of the enzyme but does remove more protein. This seems to indicate that a part of the alkaline phosphatase present in the cell nuclei is readily soluble in isotonic saline and is easily re-

moved in a single extraction. The relative amount of the "magnesium-activated alkaline phosphatase"[2] extracted from the rat liver nuclei isolated by the aqueous method was found to parallel approximately the relative amount of protein that could be extracted.

Recently Dr. Michael L. Watson of the Atomic Energy Project at The University of Rochester (15) has studied with the electron microscope very thin sections of mouse pancreas previously fixed with buffered osmic acid, and has clearly shown the presence of quite regularly spaced interruptions in the nuclear membrane, with diameters of 200 to 300 A. This finding is the climax of a considerable amount of work indicating the likelihood that the nuclear membrane is in general permeable to protein molecules (34). The work includes recent studies by Dr. J. Holtfreter of the Biology Department of The University of Rochester, who found that the nuclear membrane of the frog egg cell apparently is permeable to hemoglobin (16), as well as earlier work by Anderson (cited in reference 16). If there are also pores in the membrane of the rat liver nucleus, as seems highly probable, the results reported in this paper on the extraction of nuclei, as well as the results of studies of the distribution of alkaline phosphatase activity among the various cell fractions, become easily understandable. The presence of pores in the nuclear membrane of the size found by Watson (200 to 300 A in diameter) should permit the passage by passive diffusion through the nuclear membrane of the soluble forms of alkaline phosphatase and of other soluble protein, thus establishing a diffusion equilibrium for such material between nucleus and cytoplasm. Only protein or enzyme firmly bound to insoluble components of nuclear or cytoplasmic structure could be expected to escape this process of equilibrium by diffusion.

DISCUSSION

The results given in Fig. 3 show the following order of specific activities for alkaline phosphatase (after activation by magnesium): soluble fraction $\gg$nuclei$>$microsomes$>$mitochondria$>$homogenate. The amount of alkaline phosphatase in nuclei isolated by procedures involving aqueous solvents may be a minimal amount, owing to the certain loss of some protein and the probable loss of a portion of the soluble form of phosphatase from the nuclei during the isolation procedure.

The data show that a maximum of 25 per cent of the magnesium-activated alkaline phosphatase is extractable by saline from rat liver nuclei isolated in 0.44 M sucrose adjusted to pH 6.2 with dilute citric acid. It is known that all

[2] The term "magnesium-activated alkaline phosphatase" is used to represent that portion of the alkaline phosphatase which was activated upon the addition of magnesium ions over and above that portion of the alkaline phosphatase which was active in the absence of magnesium ions.

of the cellular DNA is present in the cell nucleus and it is also known that DNA is a polyelectrolyte and as such must be able to act like an ion exchange resin in binding proteins under suitable conditions. If a protein is to be bound, however, the pH of the reaction mixture must be below the isoelectric point of the protein but still above that of DNA, so that the protein will be cationic and the DNA anionic. Such conditions of pH are generally very easy to find, since the isoelectric point of DNA is very low. Under the proper conditions of pH, salt linkages will be formed between the protein (or enzyme) and the DNA. These salt linkages might be rather difficult to disrupt until the pH of the reaction became equal to or greater than the isoelectric point of the protein. However, strong saline should suffice to displace the protein entirely or partially from the DNA even below the isoelectric point of the protein.

The inability to extract the alkaline phosphatase that is not activated by magnesium from the nucleus of the rat liver cell might therefore be explained by assuming that the pH of the extraction mixture was not high enough. But if the pH of a suspension of isolated nuclei that has not been subjected to the action of enzymes from broken mitochondria is raised much above 7.0, a gel forms and the nuclear structure is altered or destroyed. Formation of this gel makes it difficult to work at elevated pH values with isolated nuclei.

It is thus impossible to say at the present time what type of linkage holds the alkaline phosphatase that is not activated by magnesium to the structural material of the nuclei. If the isoelectric point of the enzyme is not high, it would seem that the binding must be through covalent bond formation with some material in the nuclei. If the isoelectric point of the enzyme is high, however, the binding conceivably could be of a salt type.

The findings reported in this paper which should perhaps receive the greatest emphasis are the relative amounts of each type of enzyme in the different cell fractions and the ease of extractability of the magnesium-activated alkaline phosphatase from the rat liver nucleus, as compared with the greater difficulty in extracting the phosphatase that is not activated by magnesium. The ease with which the magnesium-activated alkaline phosphatase can be extracted may explain the lack of this enzyme in the rat liver nuclei isolated by Allard *et al.* (9, 10) in 0.25 M sucrose without adjustment of pH. Finally, it should be apparent from our work that the finding of an enzyme in isolated nuclei that is not easily extracted from them cannot be used at the present time as a valid reason for considering the nuclear membrane to be impermeable to protein.

SUMMARY

1. Cytochemical studies of the intracellular distribution of alkaline phosphatase in rat liver have been made, using a fractionation procedure recently developed in this laboratory (8) and a similar but modified method not described previously. Aqueous media were used in both cases.

2. The alkaline phosphatase was found to consist of two forms, one of which is strongly activated by magnesium and one of which is not sensitive to this metal.

3. The form of the enzyme that is not activated by magnesium occurs mainly in the nuclear fraction, where it seems to be rather firmly bound. Some of this form of the enzyme is also found in the microsomes, but very little if any occurs in the soluble supernatant fraction.

4. The form of alkaline phosphatase which is activated by magnesium occurs mainly in the soluble supernatant fraction, but what is believed are significant amounts also occur in nuclei. A significant portion of this form of the enzyme can be extracted from the isolated nuclei with cold, isotonic saline solution. Some activity of this form of the enzyme is also found in the microsomal fraction.

5. Mitochondria appear to contain relatively little alkaline phosphatase of either kind.

6. The concept of a porous nuclear membrane has been invoked to explain some of the results obtained in this work. It is postulated that part at least of the form of the enzyme that is activated by magnesium is free to diffuse back and forth through pores in the nuclear membrane, whereas this is considered not to be possible for the form of the enzyme that is insensitive to magnesium as a result of the firm binding of the latter to nuclear substance.

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