The Proteases of Isolated Cell Nuclei

ALEXANDER L. DOUNCE* AND ROBERTO UMAÑA†

From the University af Rochester School of Medicine and Dentistry, Rochester 20, New York

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The existence of considerable proteolytic activity in rat liver nuclei isolated in aqueous media under various conditions has been confirmed. Peaks of maximum activity have been detected at *p*H values of 3.0, 3.5, 4.5, 7.0, and 9.0. Under the experimental conditions used, no activity was evident at *p*H 5.8. Under certain conditions, the proteolytic activity of rat liver nuclei can be increased. These conditions generally correspond to increased damage to cell organelles during the isolation of the nuclei. Conditions which allow increased proteolytic activity of the nuclei manifest themselves by increased degradation of the nuclear proteins. There seems to be a direct relationship between the degree of proteolytic activity and the amount of soluble histone present in the nucleus. Under conditions in which proteolytic activity has been minimized, the amount of soluble histone can decrease to a value of 1.25% of the dry weight of rat liver nuclei and represents only 5% of the total histone content of the nuclei. The ratio of the total amount of histone to the DNA content of the nuclei on a dry weight basis has been found to be approximately 1.8.

Studies of the composition of isolated nuclei reported by Monty and Dounce (1959) have demonstrated that nuclei prepared by different methods yield different amounts of the chief protein fractions. During the course of the present study, it has been found not only that the relative amounts of all of the protein fractions of the nuclei can be caused to vary as the result of slight changes in the *p*H used during the isolation, but that this is also true of the ratio of total histone to DNA. The purpose of this paper is to investigate causes of the latter phenomenon with the ultimate aim of establishing the correct histone to DNA ratio and of isolating the total histone of the nuclei in undegraded form.

Crampton *et al.* (1957), Moore (1959), and Satake *et al.* (1960) have studied the amino acid composition of histones obtained from calf thymus and have pointed out the possibility that some of the fractions obtained by column chromatography may be products of enzymatic degradation. Very recently Rasmussen *et al.* (1962) have studied the complexity of histone obtained from thymus and isolated thymus nuclei. Phillips and Johns (1959) have shown the existence of proteolytic activity at neutral and alkaline *p*H values in histone preparations. Sarkar and Dounce (1961) have demonstrated the existence in calf thymus nuclei of at least four different proteolytic enzymes which under the conditions employed presented maximal activity at *p*H values of 3.8, 5.6, 7.0, and 9.0. All these studies have led us to believe that, under certain conditions, a good deal of proteolytic activity can exist in preparations of nuclei and can be one of the causes of discrepancies in the analytical values for the amounts of the various nuclear protein fractions. In order to test this possibility, the proteolytic activities of different types of nuclei have been studied and protein analyses have been performed. A comparison between the proteolytic activity at a given *p*H and the analysis of nuclei isolated at that *p*H has been made and the effects of some protease inhibitors on the proteolytic activity and protein composition of liver cell nuclei have been studied. Although considerable proteolytic activity has been found in nuclei isolated from rat liver under various conditions, it is not yet possible to state with certainty that proteases occur in the nuclei *in vivo*. However, the latter point is not the main purpose of this paper, which has been written to show the observed effects of proteolysis on proteins extracted from previously isolated nuclei.

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EXPERIMENTAL PROCEDURES

Preparation of Nuclei.—Nearly all of the studies reported in this paper were carried out with rat liver nuclei, although some work was done with calf thymus nuclei (as mentioned subsequently), and a few confirmatory experiments were done with calf liver.

Isolation of Rat Liver Nuclei.—Rat liver nuclei were isolated by the following methods:

1. Method of Dounce (1943) using dilute citric acid medium with the Waring Blendor for homogenization at *p*H values varying from *p*H 3.0 to 3.8. Appropriate amounts of citric acid were used to achieve the desired *p*H values.

2. A modification of the above method in which the medium for homogenization was changed to 0.44 M sucrose and the ball-type homogenizer was used instead of the Waring Blendor for homogenization. In this procedure, the

nuclei were isolated at *p*H 3.6 and 3.8. Nuclei were also prepared at *p*H 3.6 and 3.8 by this method in the presence of 0.1 M phenylhydrazine hydrochloride.

3. A very slight modification of the method of Dounce *et al.* (1955) using 0.44 M sucrose as medium for homogenization with the ball-type homogenizer, wherein sufficient citric acid was added to adjust the *p*H to 5.8 instead of 6.0 to 6.2.

4. A slight modification of the method of Schneider and Peterman (1950) in which the Waring Blendor was used for homogenization. The medium for homogenization in this case was 0.25 M sucrose–0.003 $CaCl_2$. The Waring Blendor was run at 110 volts AC during the first homogenization and at 50 volts after the homogenate was filtered through cheesecloth. The schedule for centrifugation, together with the medium used for washing, is given by Sarkar and Dounce (1961).

Analyses of nuclei isolated at *p*H 3.6 with citric acid alone used in the Waring Blendor were found not to differ from those of nuclei isolated in 0.44 M sucrose with the ball-type homogenizer at the same *p*H. In both cases, there is a loss of mitochondrial proteases into the supernatant solution owing to the low *p*H.

Calf thymus nuclei were prepared in dilute citric acid in the *p*H range 3.0 to 3.8 according to the method described by Sarkar and Dounce (1961). Protein and nucleic acid analyses were carried out as described for rat liver nuclei.

Protein Extraction and Fractionation.—The different protein fractions were extracted more or less according to the schedule described by Monty and Dounce (1959). The first fraction was extracted with 0.14 M NaCl at *p*H 5.8 or 7.0.[1] Histones were extracted with 0.2 N HCl and fractionated into precipitable histone (precipitation with 0.1 volume of concentrated ammonium hydroxide at *p*H 10.6[2]) and soluble histone, precipitated with three volumes of 95% ethanol from the supernatant solution from the previous fraction (Monty and Dounce, 1959). The two histone fractions thus obtained are operationally defined, and the distinction between them is very useful in attempting to determine the amount of autolysis of histones during the isolation procedure. It will be seen subsequently that the soluble histone is probably an artifact produced by proteolytic autolysis. Residual protein was obtained as follows: The precipitate left after histone extraction was washed once with 95% ethanol, and the lipids were then extracted with two portions of ether-alcohol (1:1). After this treatment, the precipitate was washed twice with cold 10% trichloracetic acid and then was resuspended in 5% trichloracetic acid and heated to 95° for 15 minutes. The insoluble material was then washed four times with 95% ethanol and twice with ether, dried at 105°, and weighed. DNA was determined in an aliquot of the whole nuclei by the method of Schneider and Dische as described by Monty (1955).

Assay for Proteolytic Activity.—The proteolytic activity of the nuclei was determined by a modification of the Anson technique (Anson, 1938) as described by Koszalka and Miller (1960) and Sarkar and Dounce (1961). One ml of a suspension of nuclei was incubated with 1 ml of a 2.5% solution of hemoglobin in 8 M urea and 1 ml of 0.1 M buffer for 2 hours at 37°. Acetate buffers were used in the *p*H range from 3.0 to 5.6, phosphate buffers from 6.0 to 7.6, and glycylglycine from 8.0 to 9.0. After the incubation, 2 ml of 5% trichloracetic acid was added and the solution was filtered, the OD at 280 mμ was taken, and the specific activity was expressed as increase in OD per mg of nuclei per minute of incubation time. The control experiment was run by incubating the nuclei in the buffer solution and adding at the end of the incubation period 2 ml of 5% trichloracetic acid and subsequently 1 ml of the substrate solution. The OD of this control experiment was then subtracted from the OD of the assay. Some of the curves were checked by using the reagent of Folin and Ciocalteu (1927) to measure acid-soluble peptides.

The histone fractions obtained from our preparations were tested in 1.88 M H_2SO_4–0.33 M $HgSO_4$ solution as described by Mirsky and Pollister (1946). In certain experiments, the total histone fraction was determined by the colorimetric method described by these authors.

Results

Analyses of Rat Liver Nuclei Isolated in Sucrose–$CaCl_2$ for Proteolytic Activity.—In Figure 1 is presented the qualitative assay for proteolytic activity performed on rat liver nuclei prepared in 0.25 M sucrose–0.003 M $CaCl_2$ (method 4 above) with the Waring Blendor used for homogenization. Five different peaks of activity are evident, which occur at *p*H values of 3.0, 3.8, 4.8, 7.0, and 8.8. There is a point of very reduced activity at *p*H 5.8. The positions of all of the peaks occurring in neutral and acid regions were very constant for most of the types of preparations investigated. In preparations isolated at *p*H 3.6 and 3.8, there appear to be slight differences in location of the peaks of activity, but some residual citric acid remained in the preparation and this may have lowered the *p*H of the acetate buffer used and thus caused an apparent displacement of the peaks. There is a large peak of activity at *p*H 3.0 in preparations in which citric acid has not been used, and the fact that this peak is also obtained when isolated mitochondria are used as

[1] This fraction has subsequently been termed the globulin fraction, but, in addition to globulins, the fraction may contain a small quantity of RNA and some albumin-like protein.

[2] Not a true *p*H value, but the reading obtained with the Beckman *p*H meter with the ordinary glass electrode.

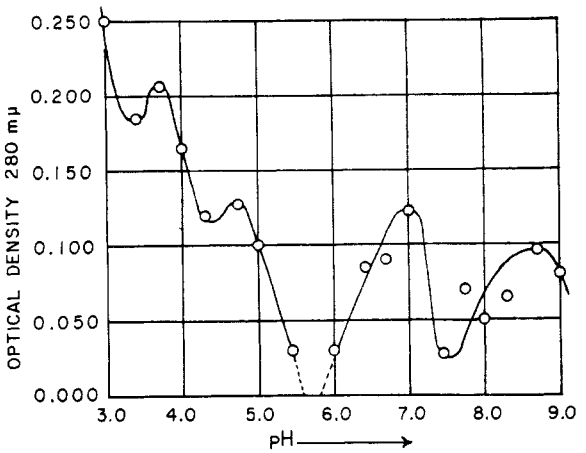

FIG. 1.—Proteolytic activity of rat liver nuclei isolated in 0.25 M sucrose − 0.003 M $CaCl_2$ without *p*H adjustment. (Concentration of nuclei not measured in this case—curve shows positions and relative heights of peaks only.) Small peaks at acid *p*H values are reproducible; variations of similar magnitude above *p*H 7.0 are not.

enzyme source makes us believe that the strong activity at *p*H 3.0 is real and probably represents a certain degree of contamination of the nuclei with mitochondrial or lysosomal enzymes. Whether a single enzyme produces the peaks at *p*H 3.0 and 3.8 should be a matter for further investigation.

The peaks of activity in the alkaline range have been found to be less constant than those in the acid range. More shifts have been found, and several times it has been difficult to decide whether there was a single peak or several.

Some curves also were obtained with bovine serum albumin used in a concentration of 2.5% in the acid range instead of urea-denatured hemoglobin. These curves showed the existence of peaks at *p*H 3.0, 4.4, and 6.4. At *p*H 5.8 there was again a minimum of activity, and the peak at *p*H 3.8 disappeared. No further investigations were performed to explain the lack of the latter peak.

From these studies, it is evident that rat liver nuclei isolated in sucrose–calcium chloride present proteolytic activity throughout the *p*H range studied, with several peaks of maximal activity that show a certain degree of overlapping. It is also evident that in the region about *p*H 5.8 there is a minimum of proteolytic activity. From this fact, we have concluded that nuclei should be isolated at this *p*H value when minimal proteolytic activity is essential. Moreover when nuclei are isolated at *p*H 5.8 in 0.44 M sucrose, there appears to be minimal damage to mitochondria and lysosomes, so that the latter can be discarded while still intact, taking with them any proteases which they may contain. From studies to be presented elsewhere, it appears that the bulk of the proteolytic enzymes of the liver cell normally are located in the mitochondrial fraction (including lysosomes), so that removal of the latter without damage is very important if one is to obtain nuclei that have been minimally subjected to protease action.

Analyses of Rat Liver Nuclei Isolated at Different pH Values and Correlation of the Results with Protease Activities at the pH Values in Question.— In order to study the effect of various degrees of proteolytic activity on the composition of rat liver nuclei, nuclei were isolated at different *p*H values and the results of analysis for the principal protein fractions were compared with the known proteolytic activities at the *p*H values used in the isolation. In the *p*H range from 3.0 to 3.8, the dilute citric acid method of Dounce was used (method 1 above). It has not been possible up to the present time to prepare nuclei in the *p*H range of 4.0 to 5.6 owing to increased resistance of the cells to breakage and nearly complete agglutination of the different particles contained in the homogenate, which makes it impossible to separate the nuclei. However, nuclei were also isolated at *p*H 5.8 (method 3 above), the ball-type homogenizer being used to avoid disruption of mitochondria and other organelles with subsequent liberation of proteolytic enzymes.

The analytical results of the above experiments are presented in Figure 2. The ordinate represents per cent of the constituent in question based on the total dry weight of the nuclei, while the abscissa represents the *p*H at which the nuclei were isolated. It is important to note in this graph the constancy of the DNA content of the nuclei throughout the *p*H range investigated. This means that any significant loss of total protein that occurred during the isolation must have been the same in all the cases. This fact does not preclude the possibility that enzymatic degradation may occur during the isolation of the nuclei, since the different peptides produced could remain within the nuclei (possibly complexed with

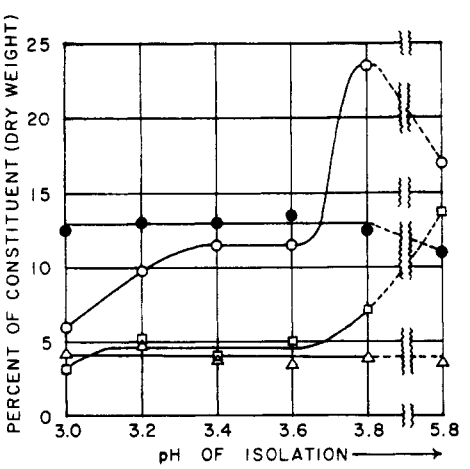

FIG. 2.—Analysis of rat liver nuclei isolated in 0.44 M sucrose at various *p*H values. O = P-histone; ● = DNA; □ = globulin; △ = S-histone.

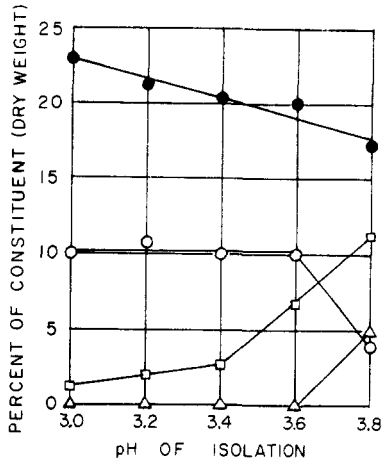

FIG. 3.—Analysis of calf thymus nuclei isolated at various *p*H values. O = P-histone; ● = DNA; □ = globulin; △ = S-histone.

DNA) and subsequently be removed during the extraction of the protein fractions.

When rat liver nuclei are isolated at *p*H 3.8, there is an increase in the content of precipitable histone to a value of 24%. On correlating this finding with the occurrence of a maximum of proteolytic activity at *p*H 3.8, it can be concluded that this increase in precipitable histone is probably due to enzymatic hydrolysis of other proteins with subsequent redistribution of material among the various protein fractions during the extraction and fractionation procedures, so that the precipitable histone fraction increases because of contamination with proteolytic products from the other fractions. This observation is strengthened by the observation that the DNA content remains constant at this *p*H (3.8).

When nuclei are prepared in 0.44 M sucrose at *p*H 5.8 (the point of minimal proteolytic activity) with the glass homogenizer being used to minimize the disruption of mitochondria and other organelles, the content of precipitable histone is 17.3%. The fact that nuclei of the latter type of preparation appear to be subject to a minimum of proteolytic activity makes it seem probable that this value of 17.3% may be close to the true histone content of nuclei isolated in aqueous medium.

Since there is no appreciable loss of DNA from nuclei isolated by any of the methods described in this paper, the amount of histone can probably be best expressed as the histone to DNA ratio. The ratio of total histone to DNA is close to 1.8 for the *p*H 5.8 nuclei.

The content of soluble histone remains constant in all preparations made in the *p*H range of 3.0 to 5.8. This constancy is not easily interpretable, considering the differences in enzymatic activity that occur over this *p*H range.

The effect of *p*H on the globulin content of the nuclei is variable and not entirely interpretable. It is likely that at *p*H values below 4.0, part of the globulin becomes denatured and insoluble and therefore appears in the residual protein fraction. This is supported by analysis of nuclei isolated at *p*H 3.8 previously published by Monty and Dounce (1959).

We have further investigated this point by extracting nuclei isolated at *p*H 3.8 with 0.2 N HCl without previous extraction with 0.14 M NaCl. In this case, the total globulin fraction (denatured and non-denatured) appears to dissolve together with the histone. Analysis of the extract for total protein by dry weight determination and for histone by use of the Mirsky colorimetric procedure gave a value of about 20% for histone and 15% for globulin (by difference). Direct determination of the residual protein yielded a value of 40%, which is close to that found for nuclei isolated in 0.44 M sucrose at *p*H 5.8.

Analyses of Calf Thymus Nuclei.—The analysis of calf thymus nuclei has also shown that there exists a certain relationship between the proteolytic activity of the nuclei and the percentages of the various protein components. The proteolytic activity of this kind of nuclei has been studied by Sarkar and Dounce (1961), who showed definite peaks of activity at *p*H 3.8, 5.6, 7.0, and 9.0. In Figure 3, the analytical results for calf thymus nuclei isolated in the *p*H range from 3.0 to 3.8 according to the general procedure of Sarkar and Dounce (1961) are presented. One of the outstanding features in Figure 3 is the absence of a soluble histone fraction over the *p*H range of 3.0 to 3.6 and the sudden appearance of this fraction when the *p*H of isolation was raised from *p*H 3.6 to 3.8. It is significant that soluble histone can be isolated only from those preparations in which the *p*H of isolation corresponds with the peak at *p*H 3.8 in the curve of proteolysis published by Sarkar and Dounce (1961). It is also interesting to note that a change occurs in the amount of the precipitable histone fraction which is in the opposite direction and of about the same order of magnitude as that observed with the soluble histone fraction.

The decline in the concentration of DNA as well as the changes in the globulin fraction are not well understood. With respect to the globulin fraction it is necessary to suspect at least two different effects, one due to acid denaturation and a second due to proteolytic degradation of globulins themselves as well as of other nuclear proteins.

Protease Activity of Nuclei Isolated at Different pH Values.—If proteolysis is an important cause for the variations in the amounts of the various protein fractions described above, it follows that a marked difference might be found in the proteolytic activity of preparations of nuclei isolated at *p*H 3.6 and 3.8, since the variations in amounts of the different protein fractions are large between these two *p*H values. Quantitative studies were therefore carried out on the proteolytic activity of nuclei isolated at *p*H 3.6 and 3.8 and for purposes of comparison also on nuclei isolated at *p*H

5.8 in 0.44 M sucrose. The modification of the method of Anson already referred to (with hemoglobin as substrate) was used. The specific activity was expressed as change in optical density units per mg of nuclei per minute of incubation. The results of these experiments are shown in Figure 4. In both cases, peaks of proteolytic activity occur at *p*H values close to 3.8, 4.8, and 7.0, and there is also evident a marked elimination of activity at *p*H 5.8. A reduction of over-all proteolytic activity when the *p*H of the preparation is lowered from 3.8 to 3.6 is evident, substantiating our hypothesis that the sudden increase between *p*H 3.6 and 3.8 in the slope of the curve showing the percentage of precipitable histone is mainly due to higher enzymatic degradation of nuclear proteins resulting in a contamination of the histone fraction with products of proteolysis.

The over-all proteolytic activity of the nuclei isolated at *p*H 5.8 is still lower than that of nuclei isolated at *p*H 3.6. These nuclei were prepared in 0.44 M sucrose at *p*H 5.8, with the ball-type homogenizer used to avoid liberation of proteolytic enzymes from disrupted mitochondria (lowest curve in the figure). The curve corresponds to a preparation outstanding for its degree of purity.

Possible Effect of Protease from Lysed Erythrocytes.—Since some contaminating red cells are lysed in the final step of purification of the nuclei isolated at *p*H 5.8, a contamination of the nuclei with erythrocyte proteases appears possible, and such contamination might also conceivably occur when the nuclei are isolated at lower *p*H values (from *p*H 3.0 to 3.8). For this reason, the proteolytic activity of rat erythrocytes was assayed. Several peaks of activity were obtained, but the specific activity was so low and the red cells contaminating the preparation so meager that it was concluded that no measurable contamination by red cell proteolytic enzymes of nuclei isolated in 0.44 M sucrose at *p*H 5.8 could possibly occur, and it is believed that this is also true for nuclei isolated at lower *p*H values.

Effect of Type of Homogenization on Proteolytic Activity of Rat Liver Nuclei.—It is clear that preparations of nuclei made by homogenizing with the Waring Blendor in the absence of sucrose should generally present more proteolytic activity than those with the ball-type homogenizer, owing to liberation of proteolytic enzymes from disrupted mitochondria or lysosomes. The latter would be expected to enter the nuclei during the isolation procedure and cause proteolytic degradation of the nuclear proteins. To confirm this concept, the results listed in Table I may be examined. In the first two rows, the analytical values for several preparations made at *p*H 3.8 are presented, the first corresponding to nuclei isolated in dilute citric acid with the Waring Blendor, and the rest to nuclei isolated in 0.44 M sucrose with the ball-type homogenizer. The only important difference between the first preparation (made by the use of the Waring Blendor) and the second (which was made in 0.44 M sucrose at *p*H 3.8 with the ball-type homogenizer) is the disruption of mitochondria in the first case. The higher values for precipitable histone and globulin obtained in the case of the preparation made with the Waring Blendor can be explained on the basis of contamination of these two fractions with segments of residual protein produced by proteolytic degradation.

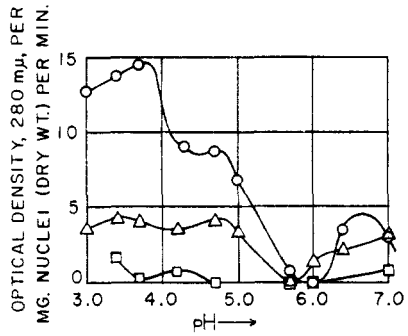

FIG. 4.—Proteolytic activity of rat liver nuclei isolated at various *p*H values in 0.44 M sucrose. ○ = *p*H 3.8; △ = *p*H 3.6; □ = *p*H 5.8.

The effect of the Waring Blendor on the liberation of proteases from the mitochondrial fraction seems less marked at *p*H 3.6, as can be seen from a comparison of the results listed in the third and fourth rows of Table I. It will be noted that in these two preparations the histone content is very similar. It is likely that the effect of the low *p*H (3.6) contributes to the lack of differences in the results of protein analysis.

The last row in Table I represents the analytical values obtained from nuclei isolated at *p*H 5.8 in 0.44 M sucrose with the ball-type homogenizer. Because of the very low activity of proteolytic enzymes at this *p*H, it seems likely that the analysis of this type of nuclei should give a picture very close to the true nuclear composition, except that there is undoubtedly a loss of globulin during the isolation procedure.

Effect of Storage at Low Temperature on Composition of Rat Liver Nuclei.—During the course of these studies, it was noticed that analysis of nuclei isolated from liver stored at −15° for 2 or 3 days gave results that were different from those obtained with nuclei isolated from fresh liver, and therefore some experiments were done with the purpose of studying the effect of the length of time of storage of the livers at −15° on the nuclear composition. In Figure 5 are presented analytical values for the quantities of the various protein fractions of calf liver nuclei prepared in citric acid at *p*H 3.8. Batches of nuclei were isolated from 50-g pooled samples of the same livers before and after standing at −15° for different periods of time. It is evident from this graph that degradation of some sort occurs even at temperatures as low as −15°, and it appears probable that this degradation is due to the action of intracellular proteases. Similar studies with

TABLE I

EFFECT OF *p*H AND TYPE OF HOMOGENIZATION ON LIVER NUCLEI COMPOSITION

Preparation	*p*H	Hp[a]	Hs	Ht	Gl	Rp	Ht/ DNA	% Re- covery	Type of Homogenization
Citric acid	3.8	23.3	4.3	27.6	7.95	—	2.2	—	Waring Blendor
Citric acid–sucrose	3.8	18.6	4.1	22.7	4.6	42	1.9	92	Ball-type homogenizer
Citric acid	3.6	13.6	3.2	16.8	5.2	—	1.4	—	Waring Blendor
Citric acid–sucrose	3.6	13.4	4.6	18.0	2.8	49	1.5	93	Ball-type homogenizer
Citric acid–sucrose	5.8	17.3	3.5	20.8	14.1	40	1.7	97	Ball-type homogenizer

[a] Abbreviations used in the tables: Hp, precipitable histone; Hs, soluble histone; Ht, total histone; Gl, globulins; Rp, residual protein; DNA, deoxyribonucleic acid. Protein content expressed as per cent of the dry weight of the nuclei.

rat liver nuclei were also performed, and in Table II are presented analytical values for rat liver nuclei prepared in dilute citric acid at *p*H 3.4 and 3.6, which had been stored at −15° and analyzed after 18 hours and 36 hours. Note the variation of composition of the protein fractions and the low yield of histone in the samples of nuclei analyzed after the livers had stood at −15° for 36 hours.

Having become aware that degradation of nuclear proteins can take place during the time the livers are stored at −15° and that higher degrees of proteolysis occur at certain regions of *p*H than at others, we decided to study the effect of variations of the conditions for extracting the globulin fraction on the amounts of various protein fractions of the nucleus. Nuclei were isolated in 0.44 M sucrose at *p*H 5.8 and extraction of the globulins was performed at *p*H 7.0, where proteolytic activity has been shown to occur, and at *p*H 5.8, where there is very low proteolytic activity. One preparation was allowed to stand in the deep freeze at −15° for 16 hours and then was extracted at *p*H 7.0. The results of the experiments are presented in Table III. It seems very clear from this table that by choosing conditions for the extraction of globulins under which proteolytic activity is marked or minimal, it is possible to obtain very different values for the amounts of precipitable histone and globulin present in the nuclei. These variations undoubtedly depend on variations in proteolysis.

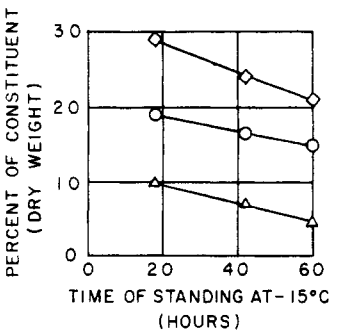

FIG. 5.—Effect of allowing tissue to stand at −15° on histone content of calf liver nuclei isolated at *p*H 3.8 in citric acid. ◇ = total histone; ○ = P-histone; △ = S-histone.

Use of Inhibitors to Minimize Proteolysis During Isolation of Rat Liver Nuclei.—It has been found that the soluble phase of the liver homogenate always presents some proteolytic activity, which might be intrinsic or which might result from slight mitochondrial or lysosomal damage. For this reason, even though the mitochondria and lysosomes be entirely removed, some of these soluble-phase enzymes could enter the nuclei and degrade nuclear proteins to a certain extent, as may be the case even with preparations made at *p*H 5.8. Therefore, the use of inhibitors of proteolysis during isolation of the nuclei would be desirable if possible. From studies carried out by Dannenberg and Smith (1955), Luck *et al.* (1939), Anson (1940), Bergmann *et al.* (1937), and Schales and Hill (1949), it has become evident that many of the intracellular proteinases can be inhibited by substances such as phenylhydrazine, iodoacetic acid, iodoacetamide, and hydroxylamine in very low concentrations. The existence of measurable proteolytic activity in all the preparations of nuclei thus far investigated by us made it seem advisable to try some of the above-mentioned inhibitors in our systems in order to attempt to find conditions for the preparation of cell nuclei in which the nuclear proteins are subject to still less enzymatic degradation than has thus far been possible to attain without the use of inhibitors.

Iodoacetic acid in a concentration of 0.01 M was demonstrated not to have any influence on the activity of the proteases of nuclei prepared in 0.44 M sucrose at *p*H 3.8. Higher concentrations were not tried. Nuclei prepared at *p*H 3.8 in the presence of 2 M urea did not show any degree of reduction of their proteolytic activity. However, phenylhydrazine hydrochloride in a concentration of 0.1 M was demonstrated to inhibit the proteolytic activity of nuclei isolated at *p*H 3.8 to the extent of about 50%, as shown in Figure 6. When the *p*H of the preparation was lowered to *p*H 3.6, a still further decrease of activity could be ob-

TABLE II
EFFECT OF FREEZING TIME ON THE COMPOSITION OF LIVER NUCLEI

Preparation	pH	Freezing Time (hr)	Hp[a]	Hs	Ht	Gl	Ht/DNA
Citric acid	3.4	18	13.3	3.5	16.8	3.7	1.42
Citric acid	3.4	36	10.9	3.2	14.1	5.6	1.17
Citric acid	3.6	18	13.6	3.2	16.8	5.2	1.44
Citric acid	3.6	36	9.1	4.5	13.6	5.9	1.24

[a] See Table I for explanation of abbreviations.

TABLE III
EFFECT OF GLOBULIN EXTRACTION PROCEDURE ON LIVER NUCLEI COMPOSITION

Experimental Conditions	Hp[a]	Hs	Ht	Gl	Ht/DNA	Rp
Globulins extracted pH 5.4	17.3	3.5	20.8	14.1	1.84	40
Nuclei frozen for 18 hours; globulins extracted pH 7.0	16.5	5.8	22.3	21.2	1.78	33
Globulins extracted pH 7.0	11.9	2.6	14.5	8.7	1.14	53

[a] See Table I for explanation of abbreviations.

tained. Unfortunately, it was not possible to obtain usable nuclei in the presence of phenylhydrazine in 0.44 M sucrose at pH 5.8.

Analysis of preparations of nuclei made in the presence of phenylhydrazine at pH 3.8 are presented in Table IV, together with analysis of preparations made at the same pH without phenylhydrazine and those of a preparation made at pH 5.8. It is important to note that the effect of phenylhydrazine is reflected mainly in the histone fraction, which shows a marked decrease in the quantity of soluble histone, to the extent that the precipitable fraction now accounts for nearly all of the histone extracted.

DISCUSSION

Our experiments confirm the existence of proteolytic enzymes in preparations of rat liver nuclei. Proteolytic activity is evident throughout the pH range studied, with reproducible peaks occurring at certain pH values. Such peaks were found at pH 3.0, 3.8, 4.8, 7.0, and 9.0. For some reason, there is a region at about pH 5.8 in which there exists a minimum of proteolytic activity. From other studies performed in this laboratory, it has been found that the mitochondrial fraction, the microsomes, and the soluble phase of the liver homogenate all present measurable proteolytic activities. It has also been shown by the analytical data presented in this paper that whenever methods for isolation of nuclei are employed in which there is mechanical disruption of cytoplasmic organelles (such as mitochondria and lysosomes), the increased proteolytic activity is immediately reflected by large variations in the quantities of the various protein fractions of the nuclei. Therefore, it must be emphasized that chemical or mechanical damage to the cytoplasmic organelles should be avoided insofar as possible in order to obtain protein fractions with a minimal degree of degradation. It is interesting to note that nuclei isolated at pH 3.8 in 0.44 M sucrose, with the ball-type homogenizer used to avoid mechanical trauma, show almost twice as much proteolytic activity as the best preparation of nuclei prepared in the same medium and with the same homogenizer but at pH 5.8. Since the microscopic appearance of mitochondria prepared in this manner is identical to that of mitochondria prepared at pH 5.8, it would appear that some change in the mitochondria must have occurred when the pH was lowered, although this change is not evident under the phase microscope. Such change presumably accounts for the increased proteolytic activity of the preparation.

The fact that it is possible to prepare nuclei which show very low proteolytic activity in the absence of inhibitors and the fact that proteolytic activity increases with the presence of impurities or with the disruption of cytoplasmic organelles leads us to believe that the intrinsic protease content of normal liver cell nuclei is low. When mitochondria are removed with minimal damage

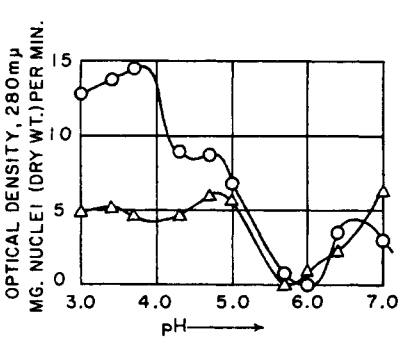

FIG. 6.—Inhibitory effect of phenylhydrazine on proteolytic activity of rat liver nuclei. O = pH of isolation 3.8, no inhibitor; Δ = pH of isolation 3.8, phenylhydrazine present.

TABLE IV
CHEMICAL COMPOSITION OF RAT LIVER NUCLEI ISOLATED UNDER THREE DIFFERENT CONDITIONS OF PROTEOLYTIC ACTIVITY

Separation	Hp[a]	Hs	Ht	Gl	Rp	Ht/DNA
Citric acid–sucrose, *p*H 5.8	17.3	3.5	20.8	14.1	40	1.73
Citric acid–sucrose, *p*H 3.8	18.6	4.1	22.7	4.6	41.7	1.89
Citric acid–sucrose–phenyl hydrazine, *p*H 3.8	19.6	1.6	21.2	4.1	37.7	1.76

[a] See Table I for explanation of abbreviations.

during the isolation of the nuclei, the only proteases appearing in the isolated nuclei are those which occur in solution in the soluble phase of the homogenate, and which presumably could migrate into or out of the nuclei during the isolation procedure. Whether or not the proteases evidenced in the soluble phase of the homogenate represent artifacts resulting from slight mitochondrial or lysosomal disruption or are present in solution in the living cell is a problem that needs further investigation. It is possible that the question of whether liver cell nuclei contain any proteases *in vivo* might be settled subsequently by the use of the Behrens procedure, since Mirsky has already reported that trypsin (Mirsky, 1960) and trypsinogen (Mirsky, 1956) are not present in pancreas nuclei isolated by the Behrens technique although these enzymes can be found in nuclei isolated in aqueous media.

With respect to the possible appearance of protein fractions as artifacts of proteolysis, it is important to note that the soluble histone fraction can be increased or decreased depending upon the conditions of isolation of the nuclei and the conditions for extraction of the different protein components. It is also striking that the use of phenylhydrazine as inhibitor reduces the amount of this histone fraction to a minimum of 1.5% of the dry weight of the nuclei, whereas in preparations in which it is now quite clear that autolytic phenomena do occur, such as, for example, nuclei prepared in dilute citric acid at *p*H 4.0 (Monty and Dounce, 1959), values for soluble histone as high as 13% have been obtained. It appears to follow then that the soluble histone fraction should be regarded as an artifact produced by partial proteolytic degradation of precipitable histone or other proteins present in the cell nucleus.

From a study of different types of preparations of nuclei and their proteolytic activities, it has been concluded that rat liver nuclei prepared in 0.44 M sucrose with the ball-type homogenizer at *p*H 5.8 (Dounce *et al.*, 1955) show a composition closest to the true nuclear composition, although even with these nuclei there has undoubtedly been a loss of some of the globulin fraction as well as of RNA (Dounce, 1955). With the latter type of preparation, the amount of total histone has been found to be slightly less than 20% of the dry weight of the nuclei, with a histone to DNA ratio of about 1.8, while the soluble histone fraction has ranged from 2.5 to 3.5 per cent. As has been stated already, it was possible under other conditions to reduce the content of soluble histone to 1.5%.

Determinations of total histone by the colorimetric method described by Mirsky and Pollister (1946) have yielded values close to those obtained by dry weight determinations when 0.2 N HCl at 0° was used to extract the histones after removal of globulins in 0.9% NaCl, or when the histones and globulins were removed together in 0.2 N HCl. The same results, incidentally, have been obtained when 0.1 N HCl was used instead of 0.2 N. On the other hand, when extractions of the histones from liver cell nuclei were attempted with 1.88 M H_2SO_4 in the presence of 0.33 M $HgSO_4$ as advised by Mirsky and Pollister (1946) or with 0.5 N or 1.0 N HCl, the amount of total histone was only 4 to 8%. In regard to the apparent failure to extract all of the histone in 1.88 M H_2SO_4 in the presence of 0.33 M $HgSO_4$, it may be that this reagent is too acidic. We have noticed that isolated dried histone from rat liver nuclei does not dissolve in the Mirsky reagent (1.88 M H_2SO_4–0.33 M $HgSO_4$) unless a trace of HCl is added first, but, if the isolated histone is first brought into solution in water containing a trace of HCl, addition of the Mirsky reagent produces no precipitate at room temperature or at 60°.

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Studies Involving Sulfur-Containing Azo Dyes Related to Dimethylaminoazobenzene*

R. K. BURKHARD, R. D. BAUER,† AND D. H. KLAASSEN

From the Departments of Biochemistry and Chemistry, Kansas State University, Manhattan, Kansas
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Four sulfur-containing azo dyes related to dimethylaminoazobenzene—the 2-, 3-, and 4(4'-dimethylaminophenylazo)phenyl methyl sulfides and 4-phenylazophenyl methyl sulfide—were synthesized and tested in regard to their ability to induce hepatic tumor formation in the rat. Of these, only two were found to be active although all four were capable of being bound to liver protein. The nature of the bound dye resulting from the administration of one of these was studied in some detail, and it was revealed that the amount, the rate of formation, the intracellular distribution, and the mode of attachment to liver protein were comparable to those of similarly studied bound dyes. Certain of the data, however, suggested the possibility of binding through both halves of the dye molecule. An alternative method for the preparation of rat liver for subsequent bound-dye studies was developed and compared to a previously reported method. It was found that these two methods yielded bound dyes that were qualitatively similar but present in different amounts.

The induction of hepatomas in rats by the administration of azo dyes has been studied extensively for a number of years. These studies have included the relationship between molecular structure and carcinogenicity, cellular changes that accompany dye administration, metabolic fates of dyes, and possible mechanisms of carcinogenesis (Miller and Miller, 1953).

One of the attempts to relate carcinogenic activity and molecular structure has been made by Pullman (1946), who has proposed that this type of activity is associated with dyes having a critical density of electrons at the azo link. Using the Hammett substituent constant (Hammett, 1940) as a measure of this electron density, Badger and Lewis (1952) have examined the validity of this hypothesis by comparing the carcinogenic activities of certain 3'- and 4'-substituted 4-dimethylaminoazobenzenes to the appropriate Hammett substituent constants. Since the Hammett substituent constants for the *m*- and *p*-*S*-CH_3 groups are known (Hammett, 1940; Jaffe, 1953) and they fall in the critical region suggested for carcinogenic activity, it was thought that a further test of this hypothesis could be obtained from a study involving the 3- and 4(4' - dimethylaminophenylazo) phenyl methyl sulfides (abbreviated as *m'*-*S*-Me-DAB and *p'*-*S*-Me-DAB, respectively, to indicate their relationship to dimethylaminoazobenzene, DAB). Furthermore, since slight variations in molecular structure can greatly affect the potency of a dye (Miller and Miller, 1953), and since studies involv-

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† Current address: Department of Chemistry, Long Beach State College, Long Beach, Calif.