STUDIES ON ISOLATED CELL COMPONENTS

VI. THE EFFECTS OF NUCLEASES AND PROTEASES ON RAT LIVER NUCLEI[1,2]

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THE studies to be described, which concern the effects of several enzymes on fresh isolated rat liver nuclei (45) at pH 7.1, were initiated with the following two points in mind. First, it was thought that if nucleases and proteases had characteristic effects on the appearance of isolated nuclei, then the progressive changes observed in nuclei in whole homogenates might be ascribed to specific types of enzymes. Second, it appeared that certain problems of nuclear structure might be attacked through a study of specific enzyme action. Among these are the problems of the origin, structure, and permeability of the nuclear envelope, as well as certain problems concerning the colloidal-chemical properties of the nuclear substance and the nucleolus.

The experiments recorded bear little relation to the use of similar enzymes in histochemical procedures because a) no attempt has been made to remove the enzymatic substrate completely, b) the effects of interest occur in a matter of minutes instead of hours, and c) the reactions involve fresh, unfixed materials, presumably in a native state.

While the effects of nucleases on *Drosophila* salivary gland chromosomes have been studied by a number of workers (9, 26, 32, 34, 35, 36, 38, 42) the results are not comparable to those obtained here, since different pretreatments were used. Studies on the morphological changes induced in whole fresh nuclei by various enzymes do not appear to have been made. Barton (6) showed that a certain fraction of the desoxyribonucleic acid (DNA) of isolated nuclei was not removed by prolonged treatment with desoxyribo-

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nuclease (DNAase) while Laskowski and Engbring (28) used nuclei treated with DNAase in a study of nuclear proteins. In the latter two studies no microscopical observations were reported, however.

METHODS

Rat liver nuclei were isolated in a phosphate-bicarbonate buffer containing sucrose essentially as previously described (45). Both Osborne-Mendel and Sprague-Dawley strains of rats were used. Emphasis was placed on rapidity of preparation rather than purity, and nuclei were rarely observed longer than $2\frac{1}{2}$ hours after killing the rat. Nuclei were prepared and maintained at 0° C but observations and phase contrast photomicrographs were made at room temperature. All solutions were made up in pyrex double distilled water. Crystalline ribonuclease (RNAase), DNAase, chymotrypsin, and twice crystallized salt-free trypsin were obtained from the Worthington Biochemical Company. Enzyme solutions were made up in the isolation medium (Solution I).[1] The effects observed with nucleases were confirmed with enzyme solutions dialyzed against Solution I in the cold to insure complete removal of divalent cations which have been previously shown to have a marked effect on the size and appearance of the isolated rat liver nucleus (4).

RESULTS

Desoxyribonuclease. When DNAase (1 mg/ml Solution I) was introduced under the cover slip the nuclei gradually assumed an empty appearance (Figs. 1, 2), and the nucleoli appeared to condense into irregular masses. The nuclear envelope (karyotheca), much more distinctly seen after this treatment, was rather thick. The clearing process required about 1–4 minutes at room temperatures and appeared to take place evenly throughout the nuclei. Two general types of nuclei could be distinguished at the end of this time in the same preparation. The most predominant type was characterized by free Brownian movement of the nucleoli and by the presence of variable numbers of free granules in very rapid Brownian movement (Fig. 4). A few granules were sometimes seen along the inner edge of the nuclei in this type. The second type showed little if any nucleolar Brownian movement, while the main body of the nucleus was filled with a very fine reticulation (Fig. 3). The difference between the two types may be chiefly one of degree, since the fine reticulation of the first type was often seen to condense gradually into predominantly rod-shaped structures, some of which became attached

[1] The following solutions have been used for isolating nuclei in the present series of studies: *Solution I.* 0.0094 *M* KH_2PO_4, 0.0125 *M* K_2HPO_4, 0.0015 *M* $NaHCO_3$, 0.145 *M* sucrose. *Solution II.* Same salt concentration as Solution I, sucrose 0.218 *M*. *Solution III.* Same salt concentration as Solution I, sucrose 0.272 *M*.

to the inner surface of the nuclear envelope. In some nuclei, however, very few granules were seen and these were too small to determine whether they had a characteristic shape. DNAase treatment also caused the appearance of occasional blebs on the nuclear surface.

The effects of DNAase may be readily demonstrated using lower concentrations of the enzyme. Thus a solution containing 0.01 mg of DNAase/ml (prepared immediately before use) was observed to begin to clear the nuclei in about 10 minutes. Since no divalent cations were added in these experiments, it is evident that the DNAase was not optimally activated.

Nuclei observed with a high, dry, dark contrast phase objective appeared as dark spheres against a grey background. DNAase caused a rapid loss of this apparent optical density (Figs. 5, 6). With oil immersion dark contrast phase objectives, however, the intact nuclei appear very little darker than the background; therefore, the clearing effect of DNAase does not result in as marked a change in appearance (Figs. 12–15).

While DNAase-treated nuclei appear virtually empty in many instances, the addition of basic dyes (Janus green B, methylene blue, pyronin) after DNAse was found to precipitate a considerable amount of material in the nucleus (Figs. 9, 10). No methyl green staining material was evident in the nucleus after DNAase treatment except for a slight staining of the nucleoli.

Distilled water or salt-free sucrose solutions after DNAase treatment caused the nucleoli and the residual nuclear material to swell rapidly and disappear (Figs. 5–7). Little basic staining material was demonstrable within the nuclear envelope after this treatment (Fig. 8). More prolonged treatment with distilled water resulted in extreme bleb formation exceeding that previously observed in 0.001 *M* magnesium chloride (2). Except for the formation of blebs, the nuclear envelope remained dense and intact during the treatment described.

When nuclei previously shrunk with 0.001 *M* magnesium chloride were treated with DNAase made up in the same solution, no change in appearance was noted. However, the nuclei failed to swell and regain a nongranular appearance when replaced in Solution I, indicating that the DNA gel had been depolymerized. (Evidence relating the effect of salts on nuclei to polymerization of the DNA has been presented in a previous paper in this series (4)).

Nuclei isolated by the technique used here have a marked tendency to stick together (45). DNAase was found to decrease this stickiness.

Ribonuclease. Ribonuclease (0.001–1.0 mg/ml in Solution I) caused the nucleoli to condense into dark irregular masses (Figs. 11, 13). Other con-

densations varying in number and density were evident throughout the rest of the nuclear mass. No change in the nuclear envelope was noted, and no general clearing of the nuclear substance such as occurred with DNAase was seen. Nucleoli condensed by RNAase treatment could be dispersed with distilled water, sucrose solutions free of salt, or by heparin in Solution I.

RNAase solutions heated to 100° C for 3 minutes in sealed tubes were found to give the same results as unheated preparations, demonstrating that the effects observed were not due to contamination with thermolabile enzymes.

Desoxyribonuclease followed by ribonuclease. Nuclei treated with DNAase and then exposed to RNAase differ in their response. Many appeared virtually empty except for the nucleoli which often appeared as hollow structures; others exhibited many small granules in rapid Brownian movement. Blebs were also frequently seen.

Ribonuclease followed by desoxyribonuclease. Treatment with DNAase subsequent to RNAase resulted in clearing of the nuclei as seen under the phase microscope, and the appearance of many small granules in rapid Brownian movement (Figs. 12–15) which did not appear to originate in the nucleoli. The number of these granules was very variable, ranging from many and including rods and irregular shapes (Fig. 16) to almost none. These differences were often seen among adjacent nuclei on the same slide. In the photographs shown it should be noted that many small granules present cannot be seen because of Brownian movement during exposure.

Trypsin. Trypsin (0.01–1.0 mg/ml in Solution I) caused a rapid decrease in nuclear density, due evidently to the extrusion of a DNA-containing gel (33), which was observed to push the nuclei apart. With high concentrations of trypsin (1 mg/ml) rupture and partial disintegration of the karyotheca was observed to take place in a few seconds (Figs. 17, 18). The nucleoli disappeared almost at once. Trypsin also digested away the granular contents remaining in nuclei treated with RNAase and DNAase. In preparations from Osborn-Mendel rats the nuclear envelopes remained intact and spherical after this treatment, but were very much thinner (Figs. 19, 20). They resembled the nuclear blebs previously described (2). With Sprague-Dawley liver nuclei, nuclease treatment followed by trypsin left residual envelopes which resembled hollow myelin figures, did not hold their shape, and tended to coalesce to form irregular masses.

Nuclei shrunken with 0.01 *M* magnesium chloride and then treated with trypsin made up in the same solution (1 mg/ml) showed little immediate change. Very gradually the nuclei became less "brilliant" and many oblong

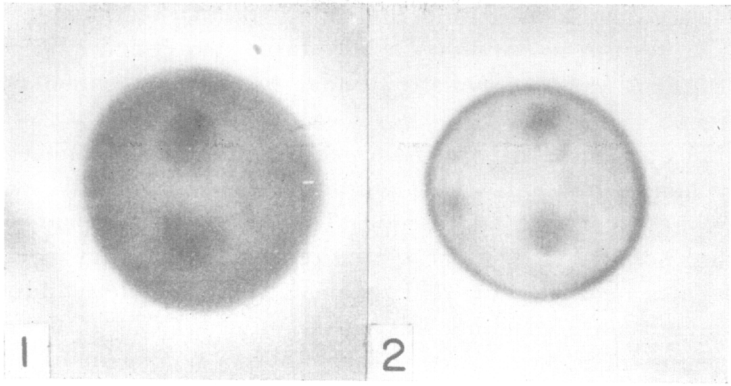
bodies appeared in them. These results are in marked contrast to those obtained with trypsin in Solution I where rapid dissolution took place.

Chymotrypsin. Results essentially similar to those described for trypsin were obtained.

Observations on nuclei in aged homogenates. Nuclei in homogenates (1 g liver/20 ml homogenate) at pH 7.1 gradually lost their density, as observed under the phase contrast microscope. The nucleoli disappeared and the nuclei shrank somewhat. The karyotheca remained intact but much thinner. In homogenates incubated at 37° C changes were first detectable in about 30 minutes and consisted either in clearing or in a slight granulation. Few intact nuclei could be found after 2 hours. When homogenates were left at 0° C, however, little change was observed for several hours. After 18 hours many nuclei were still intact while others were almost empty with no internal structure. In all instances a very considerable difference was evident in the degree of clearing of different nuclei in the same preparation.

DISCUSSION

The effects of DNAase, RNAase, and proteolytic enzymes (trypsin and chymotrypsin) have been shown to be characteristic and distinct. With DNAase the gel-like structure of the nucleus was rapidly broken down with a consequent loss of physical density and image density as observed under the dark contrast phase microscope. (The relation between image density and object refractive index in biological materials with the phase microscope has been discussed by Crawford and Barer (12)). Brownian movement of the nucleoli and granules which generally condensed in the nuclei gave further evidence of loss of gel structure. RNAase, in contrast, caused the nucleoli to condense into small dark masses, but caused no general clearing of the nuclei. The proteolytic enzymes trypsin and chymotrypsin both caused rapid disintegration of the nuclei leaving only shreds of the ruptured nuclear envelopes. These effects are sufficiently distinct to allow tentative identification of the types of enzymes responsible for the alterations observed in aged homogenates. The general clearing of the nucleus at pH 7.1 with a slight thinning of the nuclear envelope appears to be due to both proteolytic activity and DNAase. It is thought that the proteases account for the lack of internal structure (granules and nucleoli), while DNA is lost by a combination of DNAase depolymerization and protease displacement. The proteolytic activity of the homogenate is also thought to account for the thinning of the envelope. It should be noted that no increase in homogenate viscosity has been



Figs. 1 and 2. Isolated rat liver nucleus in Solution I, and 5 minutes after exposure to desoxyribonuclease (1 mg/ml in Solution I). Note general clearing and nucleolar condensation. Photographed with 4-mm dark contrast medium objective.

found with ageing, indicating that the DNA is not merely displaced but is also depolymerized. Marked loss of DNA from nuclei in incubated homogenates has been previously observed (3).

Recently it has been shown that tissue DNAase is almost inactive above pH 7.0 and does not require divalent cations for activation (44). The pancreatic DNAase used here differs from tissue DNAase in having a broad pH optimum between 6 and 7, and in requiring divalent cations for maximal activation (27). While tissue DNAase and pancreatic DNAase differ in these two properties, it is assumed that their effects on nuclear structure are similar. The fact that pancreatic DNAase attacks isolated nuclei readily in solutions free of divalent cations suggests that these ions are present in the isolated nucleus. This view is supported by the finding that nucleohistone isolated from calf thymus contains an appreciable amount of magnesium (21).

Results obtained with various enzyme preparations such as are reported here cannot be evaluated properly unless something is known of their purity

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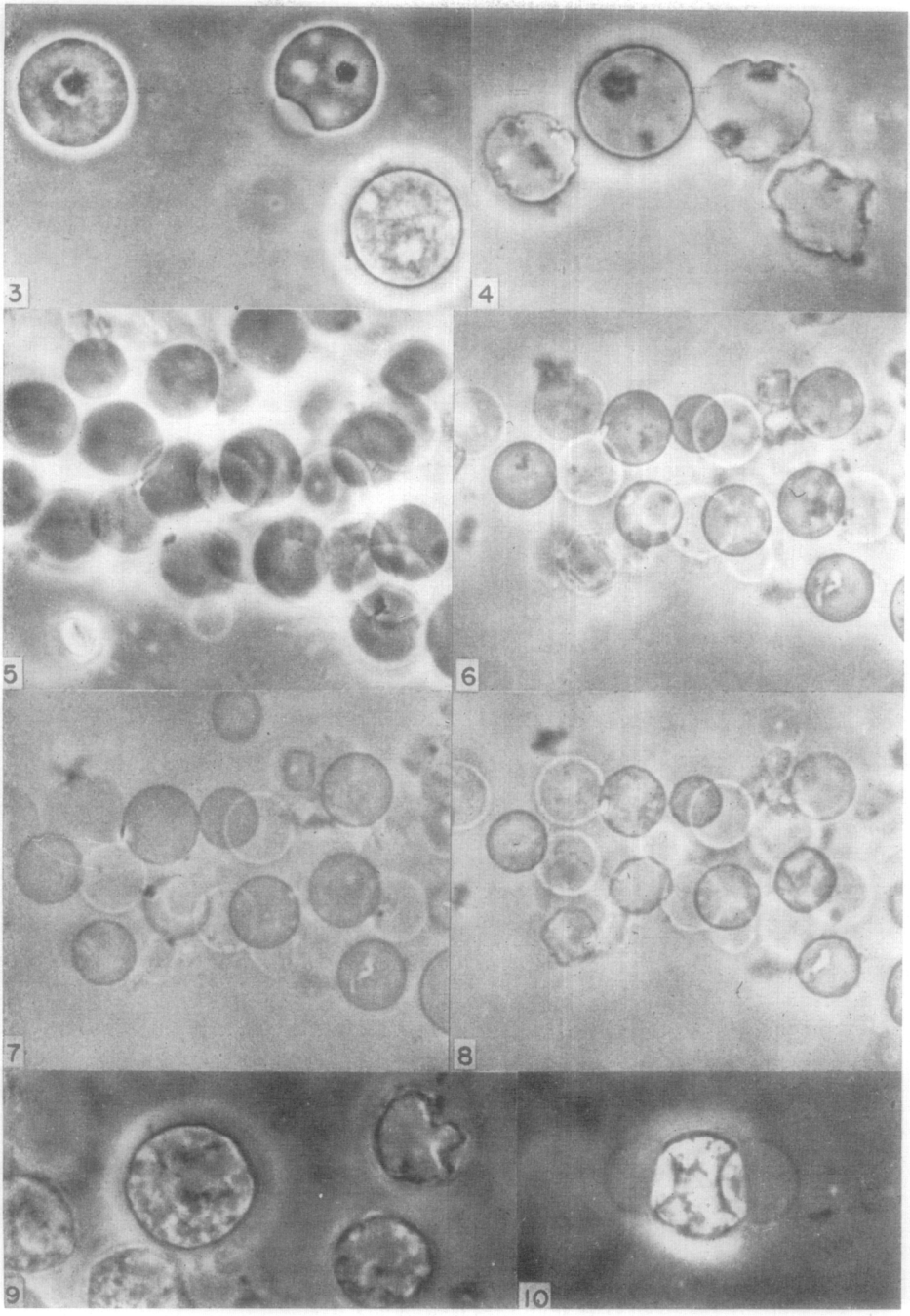
All photographs made with dark contrast medium phase contrast objectives.
Fig. 3. Isolated rat liver nuclei 6 minutes after DNAase (1 mg/ml in Solution I). 4-mm objective.
Fig. 4. Isolated rat liver nuclei from same preparation shown in Fig. 3, 3½ minutes after DNAase (1mg/ml in Solution I). Notice difference in internal granulation and wrinkling of envelopes.
Fig. 5. Isolated rat liver nuclei in Solution I.
Fig. 6. Same nuclei 5 minutes after DNAase (1 mg/ml in Solution I).
Fig. 7. Same nuclei 5 minutes after 0.88 *M* sucrose.
Fig. 8. Same nuclei after pyronin-methyl green in Solution I. Figs. 5–8 photographed with 4-mm objective.
Figs. 9 and 10 Nucleus treated with DNAase and then basic dyes (pyronin and crystal violet in Solution I). Note blebs in Fig. 10.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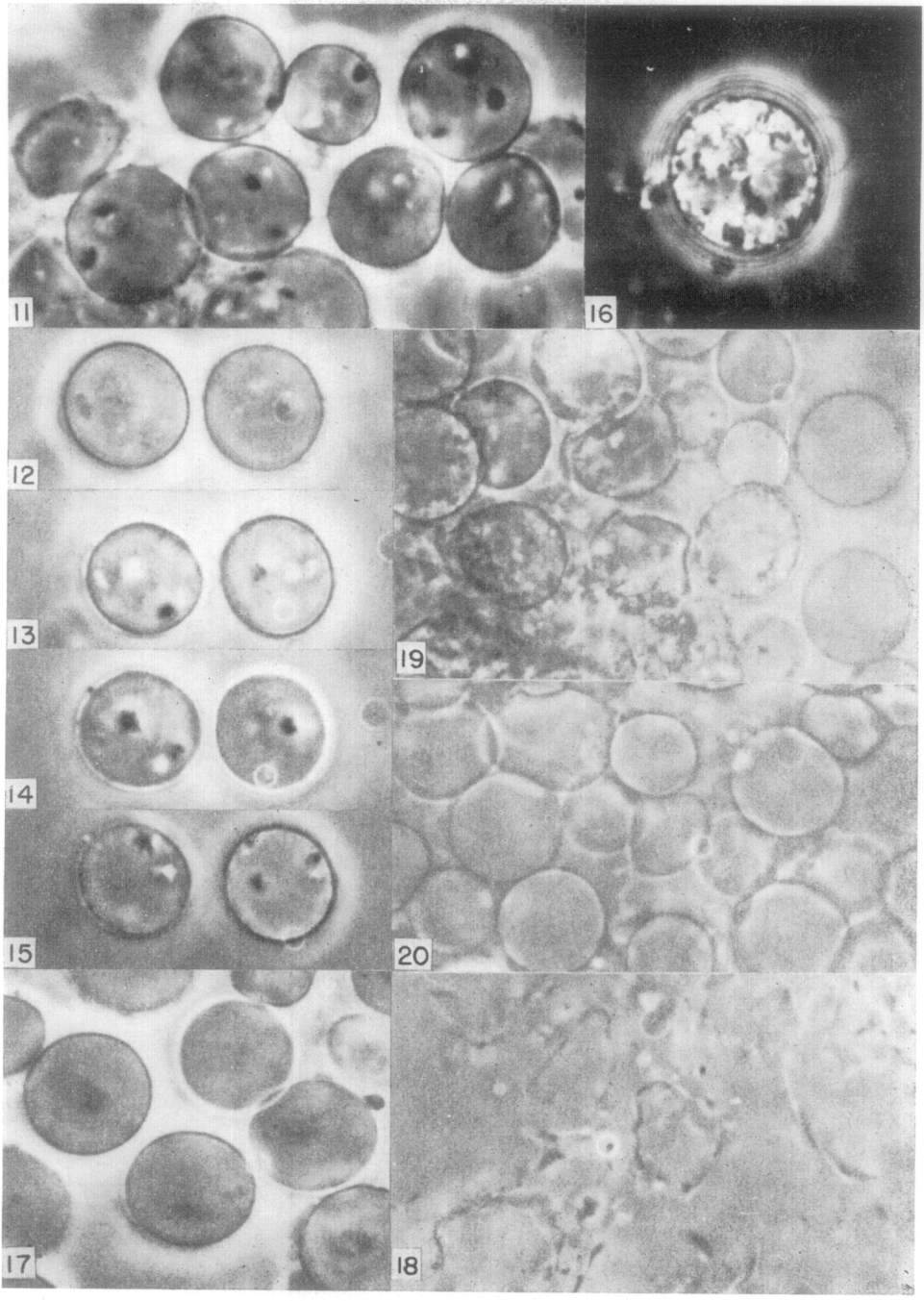
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and specificity. In this regard it should be noted that much attention has been given to proteolytic contaminants found to be present in nucleases used by earlier workers in the field (see (25)). Some doubt also appears to have existed regarding the specificity of the two nucleases generally used. Recent work, however, shows that DNAase and RNAase prepared according to accepted methods are specific for DNA and RNA respectively, and have no measurable nonspecific nuclease activity (16).

The effects of DNAase and RNAase reported here are not believed to have been influenced by possible proteolytic contamination for the following four reasons: 1) The results obtained with RNAase have been confirmed using an enzyme preparation which had been previously heated to 100° C. This destroys the activity of proteolytic enzymes but does not irreversibly inactivate ribonuclease (15, 20, 43). 2) Preparations obtained from the same sources as those used here have generally been found to be free of measurable proteolytic activity (E. Volkin, personal communication). 3) In the type of study where proteolytic contamination has been a matter of some concern, histological sections have been exposed to the enzymes at relatively high temperatures (usually 37° C) for periods of time varying from 1–2 hours up to 12 hours. Enzyme concentrations have usually varied from 0.01–1.0 mg/ml for DNAase and 0.1–6.0 mg/ml for RNAase (see 25). In the present study the effects reported have been observed to occur in 30 seconds with RNAase and 1–4 minutes with DNAase (using concentrations of 1 mg/ml) at room temperature. Proteolytic contaminants exerting slight effect over a prolonged period at 37° C would be expected to have little effect on reactions observed to take place in seconds or minutes at room temperature. 4) In the studies reported here, crystalline trypsin has been shown to produce

Fig. 11. Isolated rat liver nuclei 2 minutes after exposure to ribonuclease (1 mg/ml in Solution I).
Fig. 12. Nuclei in Solution I.
Figs. 13 and 14. Same nuclei 2 and 3 minutes after RNAase (1 mg/ml in Solution I). Focused at different levels. Note nucleolar condensation and slight evidence of structure throughout body of nuclei.
Fig. 15. Same nuclei 4 minutes after DNAase in Solution I. Brownian movement of the nucleoli was evident. Many small granules in rapid Brownian movement are not visible on the photograph. Photographed with oil immersion phase contrast objective. Note difference in appearance of nuclei with this lens and with 4-mm phase objective (Figs. 1 and 5).
Fig. 16. Nucleus treated with RNAase followed by DNAase. Note many internal condensations which were in rapid Brownian movement.
Figs. 17 and 18. Rat liver nuclei before and 2 minutes after trypsin (1 mg/ml in SolutionI). Nucleoli disappear very rapidly, nuclear gel expands, and nuclear envelope ruptures. Fragments of the envelope persist.
Fig. 19. Nuclei treated with DNAase and RNAase in the same solution and then exposed to trypsin (0.1 mg/ml in Solution I). Trypsin solution moving in from right side digests residual nuclear contents and leaves much thinned envelope intact.
Fig. 20. Nuclei treated as in Fig. 19, 10 minutes after introduction of the trypsin.

effects on the isolated nucleus which stand in marked contrast to the results obtained with nucleases. Trypsin digested and removed rapidly and completely the granules and condensed nucleoli which appeared after RNAase and DNAase treatment, while prolonged exposure to either nuclease did not have this effect. Trypsin also digested away most of the nuclear envelope, leaving only a very thin residue. RNAase and DNAase either had no effect on the envelope or made it appear thicker. It is concluded that the effects reported are due to the specific enzymes used and are not affected by contamination with other enzymes or, since dialyzed preparations gave identical results, by extraneous salts.

The possibility must be considered, however, that some of the effects are not enzymatic in the usual meaning of the term. In previous studies (3) it has been shown that DNA may be displaced from the nucleus by the highly charged heparin molecule which combines with the basic nuclear proteins. The result of the formation of a trypsin-protein enzyme-substrate complex may be displacement of DNA even before actual digestion of the protein has begun. The rapidity of the release of DNA by trypsin has been noted by Mazia (33) who used trypsin to prepare highly polymerized DNA.

As previously pointed out by Callan (7), the problem of the permeability of the nuclear envelope is a central one in any consideration of nuclear-cytoplasmic interrelationships. It appears to be assumed generally that it is permeable to rather small molecules only (18) and that the nucleus is capable of exhibiting osmotic properties (10). The changes in nuclear volume which accompany alterations in ionic composition of the suspending medium and which are believed to account for the "pseudo-osmotic" behavior of somatic cell nuclei have been discussed previously (4), and it is not felt that any available data on nuclear volume changes have demonstrated rigorously the existence of a semipermeable nuclear membrane. Goldstein and Harding (17) have found that protein solutions could be devised which would prevent the swelling of isolated frog oöcyte nuclei. Such nuclei, which are not composed chiefly of hydrated chromosomes, as appears to be the case with animal somatic cell nuclei, and which have enlarged considerably by the accumulation of substances within the nucleus, may have very different properties from those of the nuclei studied here. Also, in protein solutions of any considerable concentration, the solution volume of the protein lowers the concentration of water below 55.5 *M*, resulting in a slight dehydrating effect, which may be sufficient to prevent swelling of the nuclear colloids in the absence of a semipermeable membrane.

Nuclei isolated in aqueous media have been shown to have a lower protein

concentration per nucleus than either fixed cell nuclei (39) or nuclei isolated in nonaqueous media (1, 14). Also nuclei isolated by the latter method have been found to lose an appreciable amount of protein when placed in aqueous media. Highly polymerized DNA is extruded through the apparently undamaged envelope by heparin treatment (3), and in the present work, nucleases have been shown to penetrate rapidly. After DNAase and RNAase, distilled water or salt-free sucrose have been shown to disperse the residual intranuclear material, again without destroying the nuclear envelope. These observations show that the envelope of the isolated rat liver nucleus is readily permeable to proteins and other high molecular weight substances.

Two possibilities, however, prevent any final conclusion from being drawn from these observations. First, the nuclear envelope may well be altered by all isolation techniques thus far employed; and second, the substances demonstrated to penetrate the envelope (nucleases, heparin, etc.) may alter it themselves. Thus if the envelope contained patches of DNA and RNA, the nucleases might enter by destroying these substrate areas. At the present time, however, the observations cited, together with the studies of Caspersson (8) on nucleolar activity, and recent observations suggesting the nucleolar synthesis of RNA and its subsequent movement to the cytoplasm (5, 19, 31), all favor the conclusion that the nuclear envelope of the intact somatic cell is a porous, mesh-like structure. The small cytoplasmic particulates are not thought to penetrate it. The substance of the nucleus itself does not appear to impede significantly the free diffusion of dissolved enzymes. This is shown by the fact that DNAase digestion occurs evenly throughout the nuclei and not from the edge inward, and by the rapidity with which RNAase affects the nucleoli.

As previously noted (45) nuclei isolated at pH 7.1 have a marked tendency to stick together. The stickiness may be diminished by (a) lowering the pH (13), (b) by adding small amounts of divalent cations (4, 41), or (c) by treatment with DNAase. This suggested that an acidic substance is involved and that it is DNA. It does not appear unlikely that some strands of DNA may extend through the porous nuclear envelope and account, at least in part, for the clumping observed in the divalent cation-free isolation medium. Whether the stickiness observed is characteristic of the intracellular nucleus is not known. However the observation that nuclei of binucleate cells are generally in contact and tend to remain so (18) suggests that it is.

The nucleolar condensation produced by DNAase admits of no simple explanation. The apparent increase in phase contrast image density may be due to the removal of the surrounding dense DNA. The decrease in size may

be the result of the digestion of the nucleolus-associated chromatin or to condensation of nucleolar RNA with histone freed from its association with DNA.[1]

Observations on the effects of distilled water on the nuclear envelopes remaining after RNAase-DNAase treatment suggest that bleb formation (2) is characteristic of the envelope itself. Further studies on the properties of this structure are in progress.

It has been suggested that the nuclear envelope is composed predominantly of basic proteins (10, 11). Hughes (18) has proposed that the nucleolus and the envelope may have a somewhat similar composition since they both tend to disappear at the same time during prophase. In the present work, however, several differences in the properties of these two structures have been demonstrated. The nucleolus has been shown to condense with nucleases, and to be dispersed by distilled water, salt-free sucrose solutions, heparin, trypsin, and chymotrypsin. The nuclear envelope, in contrast, has been found to show no comparable change after treatment with nucleases or heparin. Distilled water caused marked bleb formation but did not appear to remove substances from the envelope. Proteases digest the karyotheca rather slowly but cause almost immediate dissolution of the nucleoli. Further, in incubated homogenates the nucleoli were observed to disappear while the nuclear envelope was still intact and apparently unchanged. These observations suggest that the karyotheca is a rather stable structure, while the constituent substances of the nucleoli appear to be loosely bound.

It has been proposed that the nuclear envelope is composed of the peripheral sections of the chromosomal sheaths or vesicles (22, 23, 29, 30, 40). No evidence supporting this view has been obtained here. A number of procedures have been used which leave intact nuclear envelopes with no indication of internal septation. While the envelope may well originate from the chromosomal sheaths, it is evident that it differentiates in the interphase rat liver nucleus into a separate and distinct organelle.

The differences observed in nuclei in the same preparation after nuclease treatment or ageing may well reflect differences in the properties of nuclei at different times during interphase or in the physiological state of the individual cells.

The intranuclear granules observed after nuclease digestion may be comparable to the so called "residual chromosomes" (37). However, considering the variable size and number of these particles, this conclusion is not war-

[1] The effects of basic proteins on nuclear structures have been investigated and will be discussed in a subsequent publication.

ranted at the present time. Also it does not appear profitable to attempt to relate in detail the results of the present investigation to previous studies on the effects of enzymes on giant chromosomes (9, 24, 26, 32, 35, 36, 38, 42), since fixatives or solutions of markedly different ionic composition were used.

A more complete interpretation of the intranuclear structures produced by the treatments described will undoubtedly require the use of traditional cytological techniques.

SUMMARY

The effects of two nucleases and two proteases on isolated rat liver cell nuclei in a phosphate-bicarbonate-sucrose medium at pH 7.1 have been studied.

Desoxyribonuclease caused 1) rapid loss of density and a virtually empty appearance under the phase contrast microscope, 2) condensation of the nucleoli into granular masses which often exhibited Brownian movement, 3) a slight decrease in nuclear diameter, and 4) a sharper definition of the nuclear envelope. Brownian movement of intranuclear particles is taken as an indication of loss of gel structure (depolymerization). The effects were observed 1–4 minutes after adding the enzyme solution (1 mg/ml).

Ribonuclease (0.001–1.0 mg/ml) caused the nucleoli to condense into small dense masses within 30 seconds. A variable number of other very small condensations were also seen distributed throughout the body of the nucleus. No general clearing of the nuclear mass was detected and no Brownian movement of intranuclear particles was seen.

Treatment with both nucleases resulted in the formation of numerous intranuclear particles which, together with the condensed nucleoli, showed rapid Brownian movement.

Trypsin and chymotrypsin caused a rapid expansion of the nuclear gel and rupture of the nuclear envelope, which appeared much more resistant to digestion than nucleoli or intranuclear granules produced by nucleases.

The gradual clearing observed in incubated homogenates is believed to be due to the combined action of proteases and desoxyribonuclease.

The observations presented, together with the results of previous studies, are taken to indicate that the envelope of the isolated liver cell nucleus is a porous structure which allows free passage of a number of high molecular weight substances. The results do not support the theory that the nuclear envelope is composed solely of the chromosomal sheaths of swollen or hydrated chromosomes.

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