

CELL DIVISION

III. THE EFFECTS OF AMINES ON THE STRUCTURE OF ISOLATED NUCLEI

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THE volume of many interphase nuclei and of condensed chromosomes has been considered to reflect the degree of condensation of a nucleoprotein gelwork [9]. Factors that may produce volume changes in isolated nuclei have therefore been explored in the hope of finding mechanisms that may be active in chromosomal condensation during cell division.

At least three factors control nuclear volume in the cell. For a given somatic cell type, the volume of the nucleus appears to be a reflection of the amount of deoxyribonucleoprotein (DNP) present. Thus liver cell nuclei fall into discrete volume classes [10-12, 19, 23, 29, 33, 40] whose volumes are proportional to the ploidy of the nuclei and to their DNA content [1, 2, 12, 14]. The second factor is a tissue-specific one illustrated by the large differences in nuclear volume seen in different somatic tissues having the same DNA content, in oocytes, and in sperm heads. The third factor is the ionic environment of the nucleus. The isolated rat liver cell nucleus swells and shrinks in response to changes in pH, ionic strength, and ionic composition in a manner that parallels the volume changes (or coiling) of DNA in solution [10]. Thus liver cell nuclei and cerebral cortex nuclei swell in distilled water, shrink in very dilute calcium chloride or in acid, but still maintain their tissue-specific volume differences [9].

Since the volume changes produced experimentally appear to reflect properties of the DNP, it has been suggested [9, 32] that chromosomal contraction at prophase is produced by a specific DNP-condensing agent possessing positively charged groups. Basic polypeptides, protamine, histone, or a polyamine such as agmatine, spermidine, or spermine were proposed as

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possible natural condensing agents. Although divalent cations such as calcium and magnesium have been thought to be important in maintaining chromosomal structure [3, 24, 25, 28, 31, 35–39, 46] and, when present in low concentration, have been shown to cause a marked decrease in the volume of isolated nuclei [10, 45], they were not considered as physiological condensing agents because their effects were readily reversible either by distilled water or by simple monovalent salt solutions. In the studies described here, the effects of a number of diamines, agmatine, lysine, arginine and betaine, *n*-propylamine, spermine, and spermidine on the volume and structure of isolated rat liver nuclei were examined. We considered it desirable to determine whether the number and spacing of basic groups were important for producing condensation reversible under conditions in which metaphase chromosomes might expand before proceeding with attempts to isolate possible physiological condensants.

The original suggestion, made purely on theoretical grounds—that polyamines may function as condensing or cross-linking agents—has received strong support from the finding that putrescine, spermine, and spermidine are associated with viral DNA [4, 5, 17, 20], are important in preserving DNA structure [17], and prevent the intracellular breakdown of RNA [13].

MATERIALS AND METHODS

Male adult Sprague-Dawley rats were perfused as previously described [8], and the livers were homogenized in a weakly buffered sucrose solution (Solution I [44] to give 10 per cent breis (w/v)). Nuclei were concentrated by brief centrifugation (15 minutes at max. 51, min. 39 × *g*), resuspended in Solution I, and used. This procedure was adopted to avoid the delay incident to more-elegant isolation methods. *Amphiuma* red blood cells were obtained by removing a small portion of the tail of etherized animals. Suspensions of nuclei or red blood cells were mounted under coverslips retained in place by thin vaseline ridges along two sides. Solutions studied were introduced at one edge of the coverslip and gently withdrawn along the opposite side with a small strip of tissue. Measurements of nuclear diameters were made either on the screen of a closed circuit television set or on projections of high contrast negatives.

The amines studied were used in the chloride form except for agmatine, which was used as the sulfate. The hydrochlorides were generally acid when first dissolved but were adjusted to pH 7.1 by adding Dowex-50 ion-exchange resin in the hydroxyl form to remove chloride ions. Approximately 0.5 *M* stock solutions prepared in this manner were diluted with double pyrex-distilled water to give the series of concentrations used. An average of 38 nuclei were measured at each concentration, and preparations from at least two different animals were examined with each agent. All observations and photographs were made with dark medium phase contrast.

RESULTS

The simple amines studied fall into three general groups on the basis of their effects on isolated nuclei. The first group includes substances having only one positive charge, the second those with two, and the third those having three or more positive charges. The effects observed include bleb formation by the nuclear envelope, changes in nuclear volume with and without extraction of most of the nucleoprotein, and effects on the nucleoli. The last include condensation in the form of small granules or as discrete nucleoli. Added groups that may attach to nucleoprotein by other than ionic bonds may be expected to have properties quite different from those of simple amines. Betaine (carboxymethyltrimethylammonium hydroxide anhydride) is an example of a substance that produces effects not attributable to simple charge effects alone.

n-Propylamine was chosen for study as an example of compounds of the first group (Fig. 1). In low concentrations (0.001 to 0.01 *M*), marked swelling

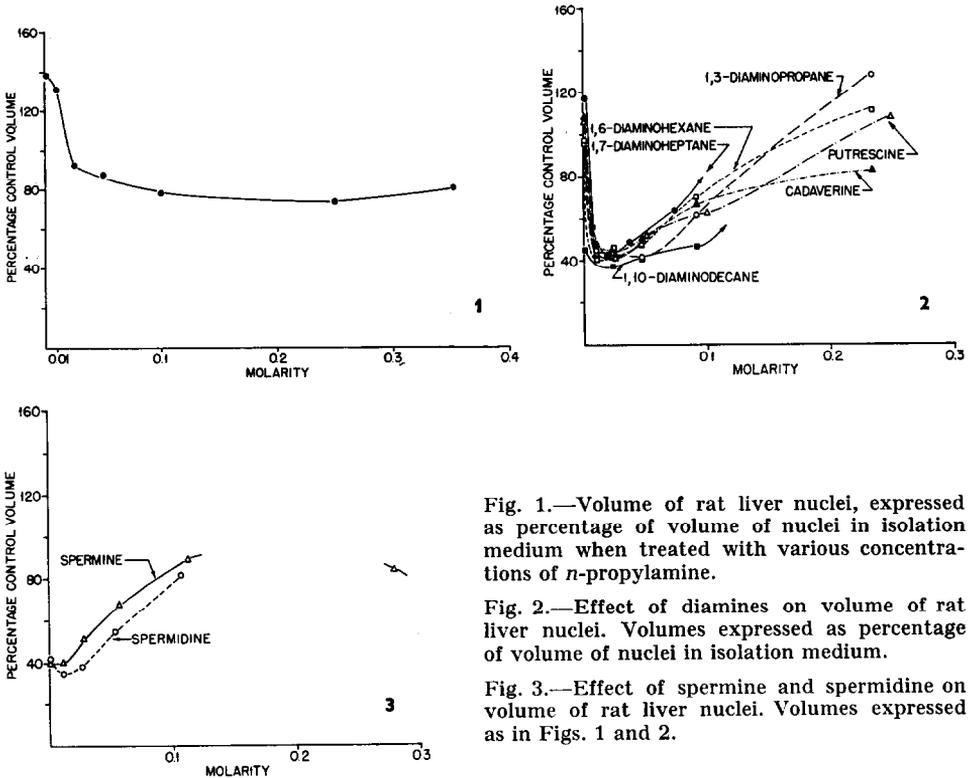


Fig. 1.—Volume of rat liver nuclei, expressed as percentage of volume of nuclei in isolation medium when treated with various concentrations of *n*-propylamine.

Fig. 2.—Effect of diamines on volume of rat liver nuclei. Volumes expressed as percentage of volume of nuclei in isolation medium.

Fig. 3.—Effect of spermine and spermidine on volume of rat liver nuclei. Volumes expressed as in Figs. 1 and 2.

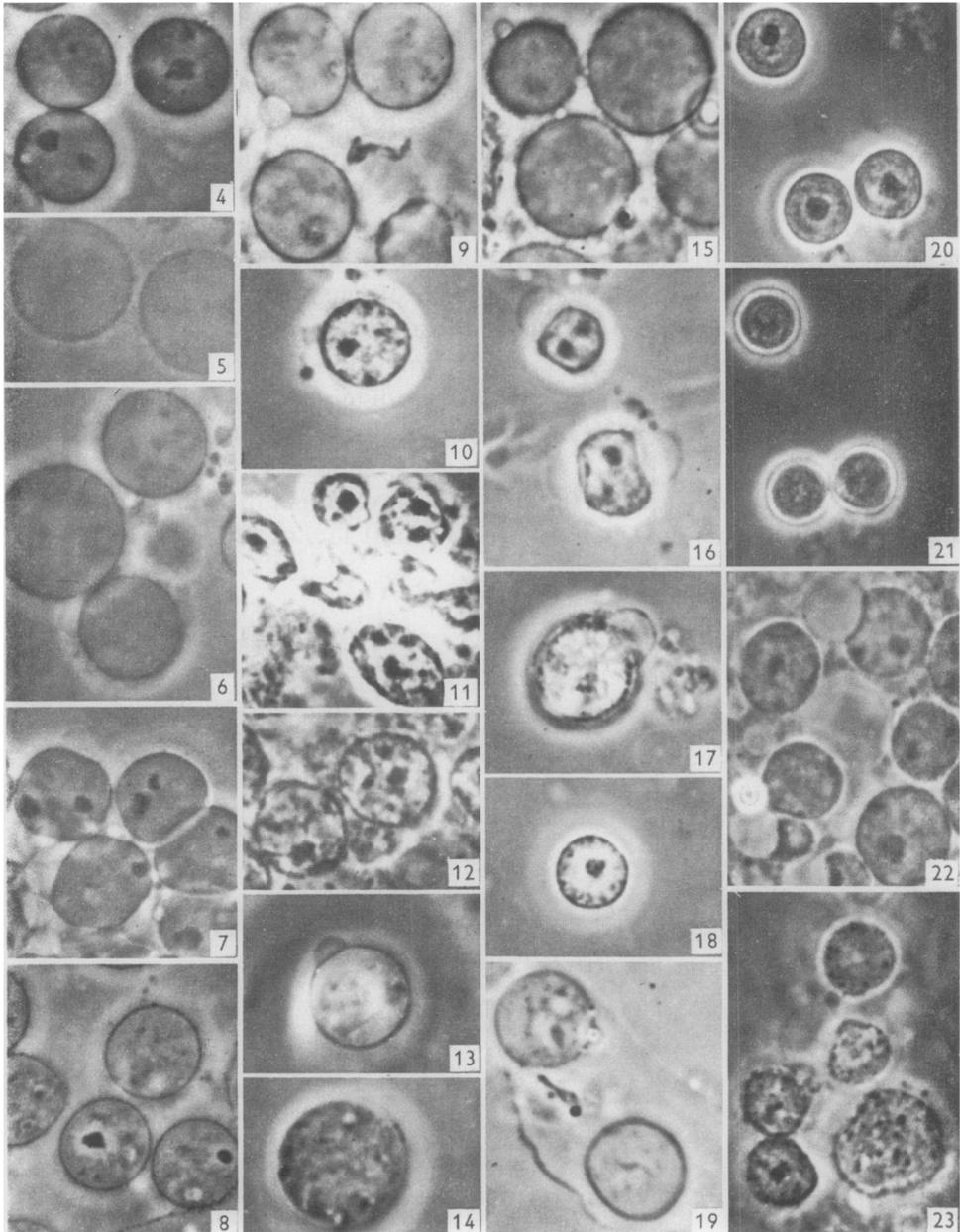


Fig. 4.—Rat liver nuclei in sucrose-phosphate isolation medium. All photographs enlarged to the same magnification.

Fig. 5.—Rat liver nuclei in 0.001 *M*_n-propylamine. Nucleoli not readily visible.

was observed (Figs. 5, 6). From about 0.025 to 0.35 *M* the volume was only slightly less than that observed in the isolation medium (Figs. 7, 8). At the highest concentration some evidence of extraction of part of the nuclear mass was observed (Fig. 8). Blebs were observed in most concentrations studied.

The second group of substances includes those having two positively charged groups: diaminopropane, putrescine, cadaverine, diaminoheptane, diaminoheptane, diaminodecane, lysine, arginine, and agmatine. With these compounds, swelling was observed in 0.001 *M* (Figs. 2, 9, 15) and in 0.5 *M* solutions (Figs. 13, 14, 19) with marked shrinkage and granulation in intermediate concentrations (Figs. 10–12, 16–18). Bleb formation was observed

Fig. 6.—Rat liver nuclei in 0.01 *Mn*-propylamine. Nucleoli barely visible. Note swelling and bleb formation.

Fig. 7.—Rat liver nuclei in 0.05 *Mn*-propylamine. Nucleoli dense and slightly shrunken. Nuclei nearly normal in volume but with large blebs.

Fig. 8.—Rat liver nuclei in 0.357 *Mn*-propylamine. Nucleoli dense and shrunken. Part of the nuclear mass has been extracted leaving behind fine granular structures.

Fig. 9.—Rat liver nuclei in 0.001 *M* cadaverine. Nucleoli visible as vesiculate structures. Note bleb on nuclear surface.

Fig. 10.—Nucleus in 0.01 *M* cadaverine. Nucleolus shrunken, chromatin material condensed in threadlike formations.

Fig. 11.—Nuclei in 0.05 *M* cadaverine. Nucleoli dense and shrunken, nuclei very condensed and irregular.

Fig. 12.—Nuclei in 0.10 *M* cadaverine. Similar to 0.05 *M* but slightly less condensed.

Fig. 13.—Nuclei in 0.25 *M* cadaverine. Nuclei less condensed than in lower cadaverine concentrations. Many fine granules visible in nuclear mass.

Fig. 14.—Nucleus in 0.5 *M* cadaverine. Nucleus swollen with many small granules in rapid Brownian motion. Evidence of extraction of nuclear substance or rupture seen in many nuclei.

Fig. 15.—Nuclei in 0.001 *M* 1,7-diaminoheptane. Nucleoli slightly swollen, many small blebs on nuclear envelope.

Fig. 16.—0.01 *M* diaminoheptane. Nuclei and nucleoli shrunken, with large blebs on most nuclei.

Fig. 17.—Nucleus in 0.025 *M* diaminoheptane. Note numerous small granules scattered throughout nucleus.

Fig. 18.—Nucleus in 0.05 *M* diaminoheptane. Nucleolus dense and shrunken. Note many small elongated bodies.

Fig. 19.—Nuclei in 0.25 *M* diaminoheptane. Note extracted material moving away from upper nucleus. Fine granulation left after treatment.

Fig. 20.—Nuclei in 0.5 *M* betaine. Note dense shrunken nucleoli.

Fig. 21.—Same nuclei shown in Fig. 20 after treatment with distilled water. Nuclei are more condensed. Material appears to be extracted from the nucleoli.

Fig. 22.—Nuclei in 0.001 *M* agmatine. Nucleoli slightly condensed.

Fig. 23.—Nuclei in 0.1 *M* agmatine. Note large number of small granules seen in all nuclei.

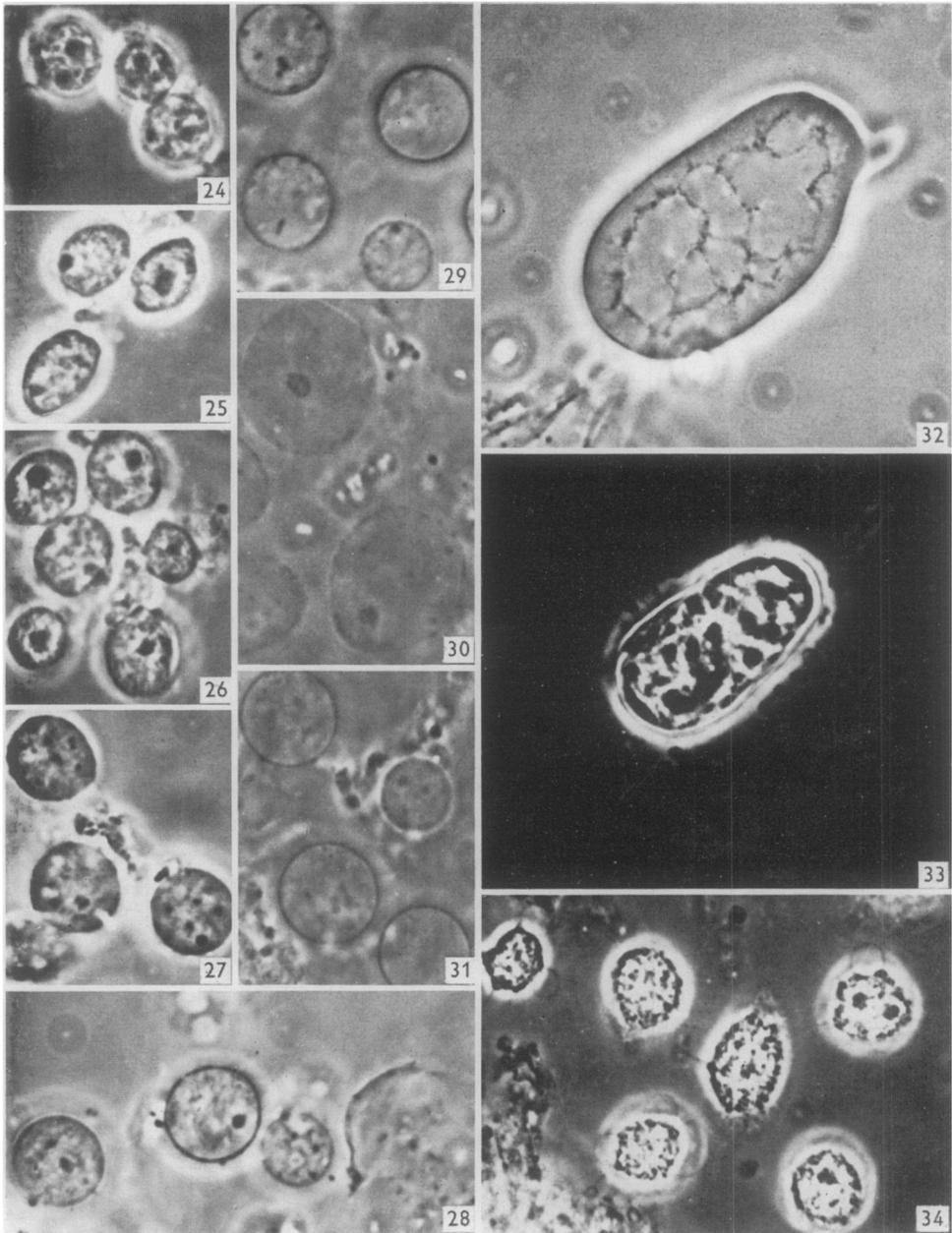


Fig. 24.—Rat liver nuclei in 0.001 *M* spermine. Nuclei and nucleoli dark and shrunken.

Fig. 25.—Nuclei in 0.01 *M* spermine. Very similar to nuclei in 0.001 *M* spermine.

in all but the highest concentrations. In the most dilute (0.001 *M*) solutions the nucleoli appeared as aggregates of small granules (Figs. 9, 15), in intermediate concentrations as irregular dense bodies (Figs. 10–12, 16–18), and at high (0.25 to 0.5 *M*) concentrations they appeared as small, poorly defined entities (Figs. 13, 14, 19). Similar condensing effects were seen with arginine and lysine. All condensing effects were reversed by either distilled water or the isolation medium. Agmatine differed slightly in that shrinkage was seen in 0.001 *M* solutions (Fig. 22) and complete reversibility in distilled water did not occur. Instead the nuclei returned to approximately normal size but did not then swell greatly as in other dibasic solutions. In 0.1 *M* agmatine a large number of fine granules were visible in the nuclei (Fig. 23).

With spermine and spermidine (class three), shrinkage with bleb formation and nucleolar condensation was observed in even the most dilute (0.001 *M*) solutions (Figs. 3, 24), and in all concentrations up through 0.025 *M* (Figs. 25, 26). Shrinkage was not reversed with distilled water, but was slightly accentuated. As the concentration of the polyamines was increased further, marked swelling and some lysis was observed (Figs. 27–31). However, remnants of the nucleoli persisted. The effect was partially attributable to the rate at which the concentration around the nuclei changed. When 0.25 *M* spermine was applied quickly marked swelling was observed (Fig. 30), but less swelling accompanied by some extraction of nuclear mass was seen

Fig. 26.—Nuclei in 0.025 *M* spermine. Note threadlike condensations throughout nuclei in Figs. 24 to 26.

Fig. 27.—Nuclei in 0.05 *M* spermine. Nuclei not as condensed as in more dilute spermine solutions. Nucleoli appear to have lost mass.

Fig. 28.—Nuclei in 0.1 *M* spermine. Part of nuclear substance appears to have been extracted. Note many small granules, especially those released by ruptured nucleus at right.

Fig. 29.—Nuclei in 0.25 *M* spermine. More material appears to have been extracted than was in 0.1 *M*.

Fig. 30.—Nuclei in 0.25 *M* spermine, showing marked swelling often seen when spermine solution was abruptly added to nuclei. Many nuclei lyse after this treatment, others such as those shown in Figs. 29 and 31 appear to be slowly extracted.

Fig. 31.—Nuclei in 0.5 *M* spermine showing marked evidence of extraction of nuclear substance.

Fig. 32.—*Amphiuma* erythrocyte nucleus in 0.1 *M* spermine. Vesiculate appearance is thought to be attributable to presence of particles between swollen chromosomes.

Fig. 33.—Same nucleus as in Fig. 32 after treatment with 0.001 *M* spermine. Substance in "vesicles" condenses into dark masses.

Fig. 34.—Rat liver nuclei treated with 0.1 *M* spermine and then distilled water. Note large number of particles formed after this treatment.

when the concentration was changed more slowly (Fig. 29). In some instances when the highest concentration was reached (0.5 *M*) the nuclei swelled only slightly but appeared, on the basis of their phase contrast density, to have lost most of their internal mass, leaving behind many small granules and nucleolar remnants (Fig. 31). No accurate measurements of nuclear volumes at 0.25 *M* concentrations could be made. Nuclei treated with 0.1 *M* spermine and subsequently washed with distilled water showed many chromosome-like bodies (Fig. 34). As many as 50 could be counted but accurate enumeration was not possible. All condensing effects were reversed by the isolation medium. In contrast to the results obtained with class I and II compounds, the reversal observed here required an interval of several minutes to be completed.

Betaine (glycocolbetaine) has been taken as an example of substances having only one positive charge but whose effects are not easily explained in terms of charge alone. In 0.00078 *M* solution nuclear swelling and bleb formation were observed followed by return to much less than normal size, with fine internal granulation and slight condensation of the nucleoli. Betaine in the first part of the solution flowing across the slide may have been removed by other nuclei before the solution reached those under observation, causing the initial swelling. Higher concentrations also caused internal granulation, which was not reversed by distilled water. From 0.00078 to 0.4 *M* (Fig. 20) a gradually increasing concentration of nucleolar material was observed. Distilled water produced shrinkage when applied after all concentrations of betaine (Fig. 21), and partial extraction of nucleolar mass, whereas the original isolation medium slowly reversed all effects observed.

Although many chromosome-like bodies condensed in liver nuclei, the number expected in the average tetraploid rat liver nucleus [26] is so high (84) that accurate counts could not be expected. Comparison was therefore made with the large nuclei of *Amphiuma* erythrocytes to see if the condensed granules were much larger as might be expected from the greater size of these nuclei and their smaller chromosome number. When treated with 0.1 *M* spermine, *Amphiuma* RBC nuclei swell and show an internal compartmentalization that seems to be caused by particles trapped between individual swollen chromosomes (Fig. 32). Accurate counts of these compartments were not possible, but there appear to be about 20. The diploid chromosome number for this animal is 24 [30]. When subsequently condensed in 0.001 *M* spermine solution, large discrete masses of chromatin appeared (Fig. 33) that stand in marked contrast to the much smaller structures observed in rat liver nuclei. Accurate counts again were not possible, but the numbers obtained were of the right order of magnitude.

DISCUSSION

In the studies reported here the effects of a variety of organic cations on nuclear structure were examined. The effects observed varied greatly with concentration and with the number of charged groups on the molecule. Reversibility with distilled water and buffered salt solutions was also examined. The condensing effects of calcium, magnesium, simple aliphatic diamines, and dibasic amino acids are easily reversed by distilled water, or the isolation medium, suggesting that these substances may not be the physiological condensing agents active during cell division. Spermine, spermidine, or agmatine—which falls between group two and three—are much more probable agents since they produce effects not so easily reversed. Little evidence exists at present that either compound is actually involved in normal division, but they provide model substances and suggest certain properties of the physiological condensing agent.

If simple charge effects are critical, then it appears that more than two charged groups may be necessary. If the negative charge is attached to groups that may be bound to nucleoprotein for other reasons, as seems to be the case with betaine one basic group may be enough.

A wide variety of organic amines are found in cells [18] but the most promising are spermine, spermidine, agmatine, or a basic polypeptide. Evidence that these polyamines preserve nucleic acid structure *in vivo* [13] and *in vitro* [17] has been presented. In cell-free extracts of *Escherichia coli*, it has been shown that putrescine and methionine supply the four- and three-carbon moieties, respectively, of spermine and spermidine [42]. The thiomethyl residue could possibly be involved in other aspects of mitosis. This system provides a ready means for producing triply and quadruply charged amines.

Spermine and spermidine are found in many different cells and tissues [18, 34], in bacteria [41], and associated with DNA in several viruses [4, 5]. Although agmatine increases the mitotic rate in the grasshopper neuroblast (see paper IV of this series), information available on the reversibility of prophase chromosomal condensation is insufficient to allow the properties of the hypothetical condensing agent to be more precisely defined.

Prophase liver nuclei [32] may be made homogeneous with pyrophosphate treatment, which would complex with basic substances. Recondensation could then be achieved with histone. Chromosomes do not appear to swell when hypotonic solutions are used to spread them for cytological study [21,

22, 43], although as a rule only the concentration of sodium chloride is reduced, leaving the calcium concentration unaltered. It is essential that the effects of distilled water and phosphate-containing isolation media on metaphase chromosomes be studied.

The effects of a simple monoamine (*n*-propylamine) parallel closely the results obtained with NaCl and KCl [10]. The aliphatic diamines in low concentrations shrink nuclei; higher concentrations cause swelling, lysis, and a loss of nucleoprotein. Similar concentration-dependent effects were previously described for calcium and magnesium chlorides [10]. The shrinkage is believed to be caused by neutralization of the phosphate groups on DNA and the swelling to be the result of charge reversal. Charge reversal would not occur with a singly charged cation, and accordingly no swelling at higher concentrations is observed with *n*-propylamine or with betaine.

It was originally thought that greater differences would be seen between different diamines, depending on whether their intercharge distances matched the 7.65 Å distance between the phosphate bond oxygens of DNA [27]. The diamines studied here are extremely flexible and would be able to bridge distances much shorter than their maximum N–N distance. Reasonable values for these distances are: for diaminopropane, 5.3 Å; putrescine, 6.1 Å; cadaverine, 8.0 Å; and for diaminohexane, 14.2 Å. Considerably less swelling caused by charge reversal for diaminopropane and putrescine would have been expected than for the shorter or longer compounds. Experimentally, greater swelling was observed in 0.25 *M* 1,3-diaminopropane, 1,7-diaminoheptane, and 1,10-diaminodecane than in putrescine or cadaverine. Cadaverine, whose intercharge distance most nearly matches that of the DNA phosphate groups, gave the least charge-reversal swelling of any diamine. Diaminoheptane and diaminodecane produce extreme swelling and lysis at 0.25 *M* concentration.

Triply and quadruply charged amines would be expected to produce shrinkage at lower concentrations than doubly charged counterparts, to show less reversal with distilled water, but to show charge reversal when sufficiently high concentrations are reached. Spermidine and spermine fulfill these expectations. Less shrinkage at intermediate concentrations is seen with spermine than with spermidine. The maximum distance between the terminal primary amines in spermine is approximately 16.5 Å. However, the average molecule in solution is generally not maximally extended, and the terminal groups could easily attach to two phosphate oxygens spaced 15.5 Å apart, which is the distance between every other group on the DNA. The secondary amine groups then lie on either side of the middle phosphate

oxygen. Thus the phosphates are essentially masked and residual positive charges remain along the molecule producing swelling. With spermidine no interchange distance matches the spacings on DNA. By bending, the two terminal charges can associate with adjacent phosphates, leaving the middle secondary amine group to reverse the charge. A higher concentration of spermidine than spermine, however, would be required to produce swelling, as is observed experimentally. The reversibility of spermine and spermidine condensation by the isolation medium appears to be caused by complexing of these compounds with the phosphate buffer.

One difficulty with consideration of nuclear volume as a reflection of nucleoprotein condensation is that quite obviously the nucleoprotein volume may decrease more than the nuclear volume. This is seen in the many instances in which internal granulation and free intranuclear spaces occur.

In many solutions structures resembling chromosomes are observed. Using phase contrast microscopy, we have been unable to make accurate counts of structures. In more favorable instances, however, it has been possible to show that more than 50 particles are present in rat liver nuclei ($4n = 84$), but many fewer are seen in *Amphiuma* nuclei ($2n = 24$). It is not unlikely, therefore, that the particles observed are chromosomes or heterochromatic regions of chromosomes.

The question arises: why do not all agents that precipitate or fix chromatin cause chromosomes to appear? If a group of tenuous gel masses are caused to swell greatly, only very gentle deswelling will establish the original structure. Drastic shrinkage may cause breaks throughout the gelwork destroying the original organization. If the chromosomes in their expanded or interphase form are uncoiled and more loosely cross-linked internally, then a nonspecific condensation, especially by acid, may be expected to yield only an amorphous precipitate. Furthermore, acid would be expected to extract many of the naturally cross-linking histones and precipitate DNA isoelectrically. Additional difficulties arise because coiling and shrinking apparently occur together in the cell. If the precipitation occurs too quickly or does not allow those forces responsible for coiling to act, then little evidence of discrete chromosomal units may be seen. The most interesting point about the present work is that, although fine granulation or overprecipitation have been observed under certain conditions, with others, discrete, chromosome-like particles are observed (Fig. 34).

Effects on the nucleolus of the various organic cations studied deserve comment. In low concentrations, these cations cause marked condensation even when little effect was noted on the rest of the nucleus. In higher con-

centrations, when the rest of the nucleus begins to swell, the nucleoli often remain condensed and swell only when the nuclear volume is very large indeed. The nucleoli therefore seem to be slightly more sensitive to condensing agents than the rest of the nucleus. In high amine concentrations the nucleoli appeared as many tiny dots; with distilled water after diamine treatment the nucleoli appeared as a group of swollen vesicles.

Previous studies showed that nuclear blebs were formed by low concentrations of calcium or magnesium in the absence of sucrose [6], and that free blebs were impermeable to protein. Here a variety of organic bases have been shown to produce blebs—suggesting that many polycations may be effective. Nuclear shrinkage is not essential to bleb formation, as is shown by bleb formation on swollen nuclei in low concentrations of diamines (compare Figs. 9, 15). However, larger blebs are observed on shrunken nuclei. In the more-concentrated polyamine solutions some evidence of nuclear envelope dissolution was observed. The polyamines also produced bleb or vesicle formation by cytoplasmic masses. Bleb formation by the cell surface during cell division is well known and may be attributable to the release of polycationic material in the cell. It seems likely that polyvalent cations may cause a decrease in the binding of lipids to proteins.

As previously suggested, the bleb-forming material may be distributed in the nuclear envelope structure in such a manner as to leave this structure permeable to large molecules under some conditions and may rearrange itself under other conditions to form a less permeable surface [6, 7]. Changes in permeability could account for the great swelling observed in some solutions (0.25 *M* spermidine or spermine) and the slight swelling with loss of nuclear mass in others such as 1 *M* NaCl [10]. The results may also be explained in terms of stepwise changes in a highly organized nucleoprotein gelwork. A gel swelling without disruption of all the cross links may not be able to penetrate the envelope and would cause an increase in nuclear volume. However, if cross links between long single DNA-protein threads are broken, the threads may be able to traverse the envelope and dissolve in the surrounding medium. Evidence for extrusion of viscous material is shown in Fig. 19. Evidence for the existence of such linear protein-DNA strands in 1 *M* NaCl extracts, where nuclei lose mass without swelling [10] has been presented [15, 16]. Excessive nuclear swelling therefore may involve either partial breakage of cross links or breakage of some end-to-end links without breakage of cross links.

The observations reported here provide many clues to the selective removal of various constituents from isolated nuclei.

SUMMARY

Information on the condensation of chromatin by organic substances is of interest in relation to attempts to formulate the properties of the chromosomal condensant active during cell division. The relation of nuclear volume to concentration has therefore been studied. *n*-Propylamine, an example of an aliphatic monoamine, produced swelling in low concentrations (0.001 to 0.01 *M*) but little volume change in the range 0.025 to 0.36 *M*, closely paralleling results obtained with NaCl and KCl. Diamines (diaminopropane, -butane, -pentane, -hexane, -heptane, and -decane) produced shrinkage and granulation and nucleolar condensation in ~ 0.025 *M* solutions and less shrinkage as the concentration of the diamines was increased. Condensation was readily reversed by either distilled water or the isolation medium. The effects of diamines resemble those of $MgCl_2$ and $CaCl_2$.

Spermidine and spermine, which possess three and four positive charges, respectively, produce condensation at very low (0.001 *M*) concentrations, with marked swelling at approximately 0.2 *M*. Condensation was not reversed by distilled water and was slowly reversed by the phosphate-containing isolation medium. Chromosome-like structures were observed in a number of solutions but accurate counts were not possible. In a comparison of *Amphiuma* erythrocyte nuclei and rat liver nuclei, relatively few granules were seen in the former ($2n = 24$) whereas many were seen in liver nuclei ($4n = 84$), suggesting that the structures observed may be chromosomal in origin. Available evidence suggests that the prophase condensing agent contains more than two charges if condensation is caused only by charge effects.

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