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STUDIES ON ISOLATED CELL COMPONENTS

II. The Release of a Nuclear Gel by Heparin*

By NORMAN G. ANDERSON‡ and KARL M. WILBUR

(From the Department of Zoology, Duke University, Durham)

PLATE 3

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Heparin, a mucoitin polysulfuric acid derivative, is considered to be the most strongly acidic substance produced in the animal body (1). It combines with protamines (2) and with a variety of blood proteins and enzymes (3, 4, 1). That heparin may also combine more generally with intracellular proteins, particularly histones, appears likely, though such reactions have apparently not been studied.

Our attention was directed to the possibility of such an interaction between heparin and cell components during the development of a procedure for the isolation of rat liver nuclei (5). In an attempt to prevent agglutination of components of the liver homogenate, heparin was added because of its reported inhibition of protoplasmic clotting (6). This produced a dramatic increase in the viscosity of the homogenate caused by the release of a nuclear gel containing desoxyribonucleic acid (DNA). The effect is interpreted as the result of the combination of heparin with protein in the nucleus with a consequent displacement of nucleic acid. This study is concerned with an examination of certain details of the reaction of heparin with nuclei from rat tissues.

EXPERIMENTAL

Effect of Heparin on the Viscosity of Homogenates

The sequence of changes occurring as a result of the release of a nuclear gel by heparin has been followed in two ways: by microscopic study of isolated nuclei, and by following in a viscosimeter the changes in viscosity of finely divided homogenates and purified suspensions of nuclei. The viscosity changes will be considered first.[1]

Perfused rat liver was homogenized in a cold salt-sucrose solution[2] buffered at

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‡ Atomic Energy Commission Fellow.

[1] The term viscosity is used here in the restricted sense stated in the Discussion.

[2] The solution used in these studies is the medium used for homogenization (solution I) in the nuclear isolation technique described (5) and contains KH_2PO_4 0.0094 M, K_2HPO_4 0.0125 M, $NaHCO_3$ 0.0015 M, and sucrose 0.145 M. pH 7.1. Liver perfused with 0.9 per cent NaCl.

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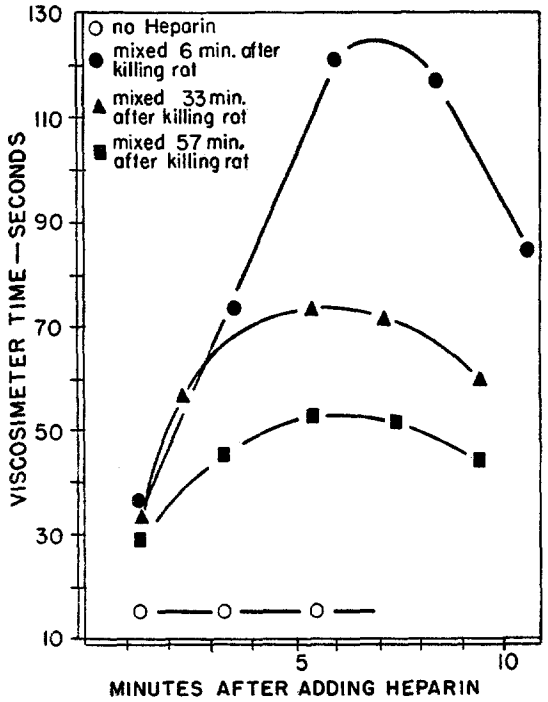
pH 7.1 (5) using 1 gm. of liver for each 5 ml. of solution. The homogenate was strained through bolting silk (10XX) and kept at 0°. The largest particles in the homogenate were groups of 2 or 3 whole cells. For each series of viscosity determinations 2 ml. of homogenate were rapidly mixed with 4 ml. of the salt-sucrose solution containing the sodium salt of heparin and placed in an Ostwald-Cannon-Fenske (7) viscosimeter



TEXT-FIG. 1. Viscosity changes following the addition of heparin to a rat liver homogenate. The curves represent aliquots of a single homogenate tested at various times after killing rat. 6 mg. of heparin per 100 mg. fresh liver added to each aliquot immediately prior to viscosity determinations. Temperature 23°, pH 7.1. See text for other details.

(size 200) maintained at 23°. Sodium heparinate did not alter the pH of the solution. Brain and kidney homogenates were prepared and studied in a similar manner.

Aging of Homogenates.—The viscosity changes following the addition of heparin to a rat liver homogenate are illustrated in Text-fig. 1. The viscosity reaches a maximum in about 6 or 8 minutes and then falls but does not reach the control level within an hour. The maximum viscosity increase which may be obtained with a given concentration of heparin is a function of the age of the homogenate, the viscosity curve immediately after preparation showing the highest peak (Text-fig. 1 and solid line in Text-fig. 2). The decline in peak

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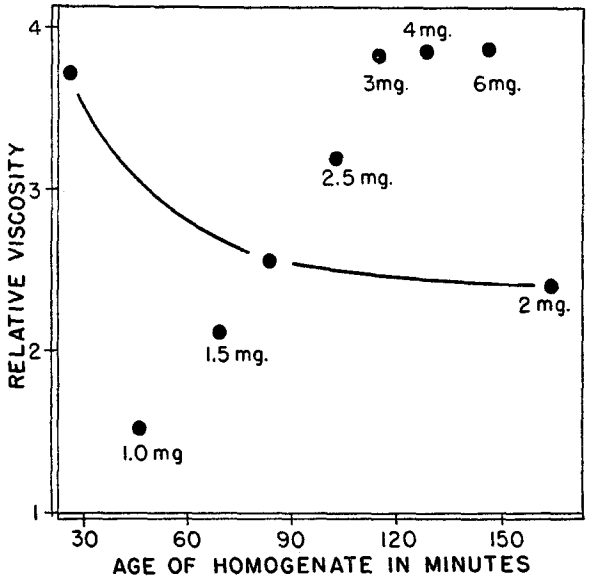
values with time is observed even though the homogenate is maintained at 0°. The possible causes of the viscosity changes will be considered later.

Since the response to heparin declines with the age of the homogenate, a correction must be made for the decline in studying the influence of any variable. This may be accomplished by alternating the experimental determinations with a series of control determinations; all peak values are then plotted as in



TEXT-FIG. 2. Solid line indicates rate of change of viscosity response to heparin with age of rat liver homogenate. Solid line plotted from peak values such as shown in Text-fig. 1. Other points represent peak viscosity values obtained with varying concentrations of heparin. Viscosity is given relative to that of the control homogenate without heparin.

Text-fig. 2. Any experimental peak value (points without connecting lines) can then be referred to the value which the control would have had at the time the experimental determination was carried out (curve connecting control peaks). This method was used in studying the influence of heparin concentration.

Effect of Heparin Concentration.—The viscosity of a liver homogenate was found to vary directly with the heparin concentration until a maximum was reached (Text-fig. 2, points without connecting curve). With further increase in concentration the viscosity was relatively unchanged. The threshold was slightly less than 0.1 mg. of heparin per 100 mg. of fresh liver for the most active heparin preparations used. Quantitatively the viscosity effect varied from rat to rat; but within the range of heparin concentrations indicated in

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Text-fig. 2 a viscosity increase was the invariable finding in more than 300 viscosity curves run on preparations from more than 40 rats.

Brain and kidney homogenates also showed viscosity changes of a similar nature but of a much smaller magnitude.

Relative Effectiveness of Heparin Preparations.—The effectiveness of different heparin preparations in increasing the viscosity of rat liver homogenates

TABLE I

Relative Effectiveness of Heparin Preparations on the Viscosity of Rat Liver Homogenates

Relative effectiveness is expressed as the ratio of the number of milligrams of a reference preparation (Connaught C 11492) to the number of milligrams of the experimental sample giving the same viscosity effect. Sulfur and nitrogen analyses were furnished by the manufacturers. The anticoagulant activities of all samples determined by Hoffman-LaRoche and expressed in Toronto units.

Preparation	Relative effectiveness on viscosity	Anti-coagulant activity	Sulfur	Nitrogen
			per cent	*per cent*
Connaught C 11492	1.0	117.4	—	1.9
Connaught C 11481	1.8	121.8	—	2.0
Hoffman-LaRoche 45-49	2.1	106.8	12.8	2.3
Upjohn T-9872	2.1	116.4	13.5	2.05
Upjohn 257-LLC-2	1.9	112.0	12.8	2.54
Abbott pork liver*	1.2	91.8	9.5	—
Abbott pork lung*	2.1	80.6	10.5	—
Abbott beef lung*	2.7	118.0	12.3	—
Abbott dog liver*	1.2	137.6	10.47	—

* Special non-commercial preparations.

Four other preparations from Lederle; Hynson, Wescott, and Dunning; and Connaught also gave the typical liver homogenate viscosity increase but are not included in this table since they were not extensively studied.

was examined in view of differences among commercial preparations, reported differences in the anticoagulant activity of heparin preparations of similar sulfur content prepared from different species (8), and particularly because of the suggestion that impurities sometimes present in heparin may have effects on protoplasmic viscosity (6). The preparations examined differed in their effect on viscosity by a factor of 2.7 (Table I, column 1). No correlation was found between the viscosity and anticoagulant activity (column 2), and only a slight positive correlation between viscosity and heparin sulfur content (column 3).

The highly acidic sulfate groups on the heparin molecule are essential for the effect reported here since desulfated heparin (9) has no effect on the viscosity of liver homogenates.

Heparin Effects on Liver Homogenate Fractions.—In order to ascertain the

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component or components of the homogenate responsible for the viscosity increase the homogenate was fractionated centrifugally and the effect of heparin on the viscosity of the fractions was studied. A pure nuclear fraction (5), a pure mitochondrial suspension, and a supernatant fraction containing material which did not sediment in 20 minutes at 21,000 x *g*, were used.

The mitochondrial and supernatant preparations in concentrations comparable to those present in the whole homogenate exhibited no increase in viscosity on the addition of heparin. A highly purified nuclear preparation, on the other hand, gave the high viscosity typical of the whole homogenate. It is therefore concluded that the viscosity increase observed in the whole homogenate is due presumably to the effect of heparin on the nuclei.

The increase in viscosity observed on the addition of heparin to a suspension containing nuclei can be shown to be due to the extrusion of a nuclear gel. The reaction as seen under the microscope is very striking. Untreated nuclei of a homogenate of rat liver, brain, or kidney in buffered phosphate-sucrose solution appear as smooth non-granular hyaline spheres (Fig. 2). With the addition of heparin (2 mg./ml.) a gel is extruded from the nucleus forming a thick layer, pushing back mitochondria and neighboring nuclei (Fig. 3). The reaction takes place within a few seconds. The diameter of the gel may be two and a half times that of the nucleus. The nucleus itself increases in size very slightly when the gel is extruded and enlarges more later. The gel then softens and becomes dispersed in a very few minutes in the case of kidney or in about 25 minutes or longer with liver or brain. Small granules appear under the nuclear membrane and the nuclei finally disintegrate (Fig. 4). Control nuclei remain unaltered during the same period.

The effect of heparin here reported is not dependent on the phosphate-sucrose buffer used since the gel is released and the viscosity increase is also observed in homogenates prepared and studied in distilled water and in 0.14 M NaCl. Histone nucleates prepared by the method of Mirsky and Pollister (10) lose their viscosity and precipitate in 0.14 M NaCl and differ in this respect from the gel described here.

Desulfated heparin did not bring about the extrusion of a gel but caused nuclear shrinkage.

In many of the whole cells of a heparin-treated homogenate a clear perinuclear space is observed (Fig. 1) which is taken as indication of the extrusion of a gel as described for isolated nuclei. Since this reaction is not observed in all the whole cells present, and since the gel-releasing reaction is invariably obtained with isolated nuclei, it seems probable that those cells which react are injured, permitting heparin to enter.[3]

[3] Heparin is without effect on the nucleus of the intact immature oocyte of *Rana pipiens*, but in the isolated nucleus treated with heparin all the nucleoli decreased in size and disappeared, leaving only fine granules.

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Analysis of Material Released from Nuclei by Heparin.—The extranuclear gel released by heparin clings tenaciously to the nuclei at 0° and cannot be separated for analysis even with prolonged centrifugation at 21,000 × *g*. As an initial approach to the identification of the material released the fluid surrounding the gel has been analyzed for soluble DNA by the following method. Aliquots of fresh homogenates to which heparin had been added were kept at 23° for 10 to 45 minutes to allow the viscosity to decline, and were then centrifuged at high speed to sediment all nuclei and nuclear and gel fragments. Supernatants and sediments were analyzed for DNA using the diphenylamine reaction (11, 12).

TABLE II

Soluble DNA in Rat Kidney and Brain Homogenates Containing Heparin

Heparin added to fresh rat tissue homogenates and incubated at 23°. All preparations centrifuged for 20 minutes at 0° at 21,000 × *g* except as indicated. Supernatants and sediments analyzed for DNA using the diphenylamine reaction. It should be noted that DNA which would otherwise be considered as soluble is probably sedimented by the centrifugal forces used here. Total volume 13 ml.

	Heparin	Incubation time	Control		Heparin-treated	
			Total DNA present	Supernatant	Total DNA present	Supernatant
	mg.	*min.*	*mg.*	*per cent*	*mg.*	*per cent*
Kidney	9.0	10	2.03	5.9	2.10	20.9
	9.0	10	0.76	1.7	0.69	54.9
	9.0	20	0.60	6.7	0.67	76.1
	12.0*	45	0.56	33.9	0.53	81.1
Brain	9.0	10	1.14	6.1	1.03	13.6
	9.0	10	0.67	0.6	0.65	14.2
	9.0	20	0.83	2.4	0.76	65.8

* Centrifuged at 8930 × *g*.

Table II shows the results of analyses on kidney and brain. Three points may be noted. (1) The amount of soluble DNA is increased by heparin treatment and in some instances more than half the total DNA present may be soluble. The values are probably minimal since the high centrifugal forces used to sediment the gel may also remove a portion of the soluble DNA from the supernatant. (2) The amounts of soluble DNA released from homogenate nuclei may well reflect the extent of depolymerization as influenced by DNAase and DNAase inhibitor activity (13, 14). (3) Some DNA may be released from nuclei on incubation without heparin treatment.

The supernatants of purified preparations of rat liver nuclei (5) treated with heparin for 20 minutes at 23° and centrifuged at 21,000 × *g* for 60 minutes invariably contained DNA while none was found without heparin.

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Similar studies were made with rat liver homogenates and an increase in soluble DNA was demonstrated. The results are not included in Table II, however, since the viscosity of the heparin-treated liver preparations remained high even after incubation. This viscosity difference between heparin-treated and control homogenates would prevent comparable DNA sedimentation.

The composition of the extranuclear gel was studied using the following histochemical techniques: the Feulgen reaction for DNA (15); methyl green staining for polymerized DNA (16); the Thomas method for arginine-rich proteins (17); the Serra and Quieroz Lopes modification of the Berg ninhydrin test for α-amino acid groups (18); and the perchloric histochemical method for DNA (19).

The Feulgen reaction, methyl green staining, and the perchloric-DNA test all demonstrated the presence of DNA in the extranuclear gel following heparin treatment. That histone is present in the gel with DNA is indicated by a positive test for arginine-rich proteins. However the expected test for α-amino acid was not obtained either inside or outside the nucleus.

DISCUSSION

The principal finding of the present study is the release of a gel from mammalian nuclei by heparin. The presence of highly polymerized desoxyribonucleic acid in the gel is indicated by its viscosity in solution and its affinity for methyl green. Extranuclear DNA has also been shown by histochemical tests and by chemical analysis. The gel has been found to contain protein as well.

The displacement of DNA from histones found in the nucleus (20) seems likely in view of the high negative charge on the heparin molecule and its known combination with basic proteins (2, 4). Once DNA is displaced from histone either by strong saline as in the Mirsky and Pollister method or with heparin as reported here, the gel swells rapidly. Histone and heparin, presumably in the form of a complex, are believed to be carried out of the nucleus in the expanding gel since the gel contains an arginine-rich protein and since microscopically visible material (presumably protein) remains after removal of all DNA from the gel by hot perchloric acid. It should be noted that while the histone nucleate preparation of Mirsky and Pollister (10) precipitates in 0.14 M NaCl, histone, heparin, and DNA together do not precipitate but form a highly viscous solution at this salt concentration.

The ease with which a gel is extruded from the nucleus without marked change in nuclear membrane diameter (Figs. 2–4) suggests that either the membrane of the isolated nucleus is extremely porous or that it is altered by heparin. However, possible heparin effects on the nuclear membrane are not thought to explain the extrusion of the gel.

The inhibitory action of heparin on the division of marine eggs (6), mammalian somatic cells (21), and bacteria (22), and the mutagenic action of

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heparin on certain bacteria (22) may find a partial interpretation in the alteration of the state of the nucleoprotein—and probably also the metabolism—of the nucleus. The failure of heparin-treated *Chaetopterus* eggs to show the normal sequence of viscosity changes during the latter half of division may involve earlier changes analogous to those described here for the liver cell nucleus. However, the possibility of a direct action of the highly charged heparin on the protoplasmic colloids and the inhibition of enzymes by heparin (1, 23, 24) must also be taken into account.

The extrusion of a gel containing DNA and protein has been shown here to be the cause of the marked decrease in the rate of flow of the homogenate through the viscosimeter. Since the gel has been shown to cling to the nuclei it is apparent that the term viscosity as applied to the homogenate is used in a somewhat restricted sense, and that the measurements merely provide an arbitrary means for following certain aspects of the reaction. Whether the observed decrease in viscosity following the initial rise (Text-fig. 1) represents a depolymerization of the gel resulting from enzyme action or other causes remains to be investigated.

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SUMMARY

1. The addition of heparin to rat liver, kidney, or brain nuclei has been found to bring about the release of a gel. Chemical analysis and histochemical studies on whole homogenates and isolated nuclei demonstrated that the material released by heparin contained desoxyribonucleic acid (DNA) and protein. The action of heparin on nuclei is interpreted as the result of a combination with the basic proteins of the nucleus with a consequent displacement of DNA.

2. The addition of heparin to a finely divided dilute liver homogenate prepared in a phosphate-sucrose solution at pH 7.1 brings about a marked increase in viscosity which reaches a maximum in 6 to 8 minutes at 23° and then declines.

3. The concentration threshold for the viscosity effect was 0.1 mg. per 100 mg. fresh rat liver, with further increases in viscosity at higher heparin concentrations. Over a period of several hours a marked decrease in response to heparin was observed in homogenates stored at 0°.

4. Fractionation of the homogenate demonstrated that the viscosity increase was due to the presence of the nuclei alone, other components showing no

effect. Microscopic observation showed that the increase in viscosity was associated with the appearance of a clear gel around nuclei treated with heparin.

5. Heparin brought about the release of DNA from the nuclei of incubated rat liver, kidney, and brain homogenates. In some instances over half the DNA is found in the supernatant after high speed centrifugation (20 minutes, 21,000 × *g*).

6. No correlation was found between anticoagulant activity of heparin preparations and their effectiveness in causing an increase in the viscosity of liver homogenates. Desulfated heparin produced none of the results described here for heparin.

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EXPLANATION OF PLATE 3

FIG. 1. Whole cell from rat liver isolated in salt-sucrose after treatment with heparin (2 mg./ml.). Note clear space around nucleus where the nuclear gel has pushed back cytoplasmic components. All photographs made at room temperature. Unfixed, unstained. × 980.

FIG. 2. Rat liver nuclei from homogenate prepared in salt-sucrose solution before treatment with heparin. Unfixed, unstained. Phase contrast. × 1150.

FIG. 3. Same nuclei shown in Fig. 2 3 minutes after treatment with heparin (2 mg./ml.). Note gel extruded from nuclei pushing them apart. Mitochondria show outer limits of gel. Streaming of mitochondria seen at right. × 1150.

FIG. 4. Same nuclei shown in Figs. 2 and 3 49 minutes after heparin treatment. Gel has begun to disperse. Nuclei show characteristic irregularities along the inner boundary of the nuclear membrane. Rupture of several nuclei is shown. × 1150.

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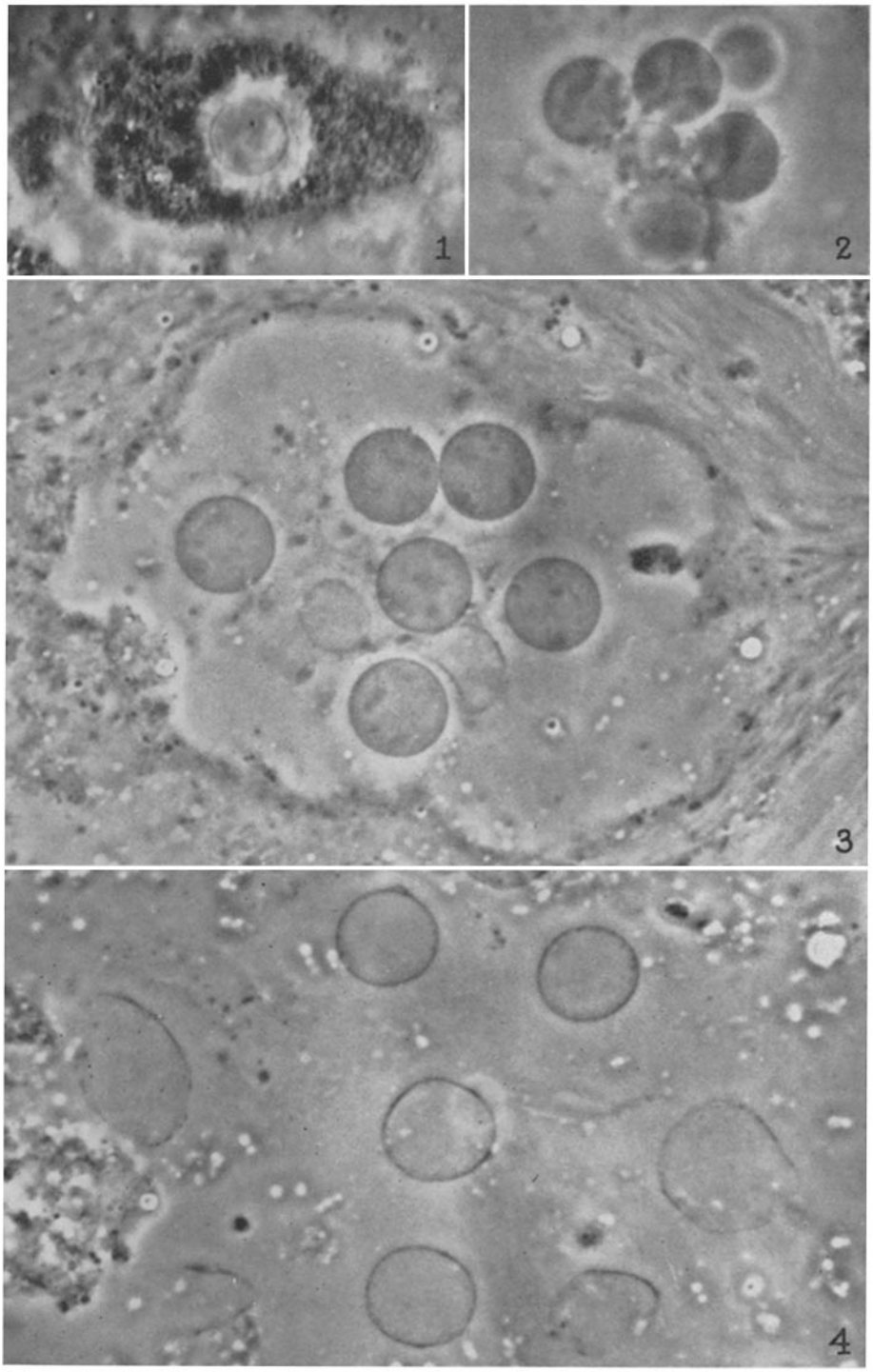

(Anderson and Wilbur: Nuclear gel released by heparin)