ON THE ORIGIN OF HEPARIN

An Examination of the Heparin Content and the Specific Cytoplasmic Particles of Neoplastic Mast Cells

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PLATES 6 TO 9

Tumors arising from a tissue that normally produces substances which affect generally the biological activity of the organism present a double interest to the investigator. Not only can they be studied from the viewpoint of the neoplastic process, but there remains the concomitant problem of the disturbances produced by this process in the formation of the biologically active substances and the consequent widespread effects that may result from these alterations. The latter, as with tumors of the parathyroid or the gonads, are usually plainly evident, but the changes, whether quantitative or qualitative, in the nature and formation of the active substances remain obscure.

In the present investigation a tumor is examined in which alterations in a biologically potent substance can be examined by the techniques of morphology, biochemistry, and histochemistry. A correlation of these varied data with the functional activity of the tumor tissue will be attempted.

Mast cell tumors, first described by Bloom (1), are a not uncommon neoplasm in dogs and so present an exceptional opportunity for the study of these cells of obscure origin and uncertain function. This is true, not only because great numbers of mast cells are available in the tumor for study in almost "pure culture," but also, since the tumors vary greatly in those growth characteristics which may be summarized as "malignancy," because they present a correspondingly varying degree of anaplasia in their constituent cells. The opportunity is thus afforded for the examination of mast cells of every degree of maturity from primitive forms with sparse fine granulations to mature cells crowded with their coarse metachromatic granules. It is in particular this cellular variation that is to be used in our study as a means to examine one aspect of their function, namely their rôle in heparin formation.

Following Jorpes's (2) demonstrations of the metachromatic reaction of heparin and of its chemical constitution as a member of the group of mucoitin-sulfuric acid esters, Wilander (3) showed the correlation that exists between the variation in frequency of mast cells in the liver capsule and the liver parenchyma of the ox and the varying content of the two tissues in heparin. The latter was

prepared by the method of Charles and Scott (4) and the amount of heparin found expressed in the conventional unit content that measures its ability to prevent coagulation of blood. Ten times as much heparin was found in the liver capsule where mast cells are frequent as in the parenchyma where they are present in moderate numbers. No heparin was obtained from the liver of rats which contains no mast cells, while in the subcutaneous tissue of the rat, with its moderate mast cell content, as much was found as in the ox liver.

Our problem is in its first part similar to that of the investigators just mentioned; to determine the heparin content of the tissues of mast cell tumors. Since the quantity of mast cells greatly surpasses that in any normal tissue, the heparin content of the tumor tissue should greatly exceed that obtainable from any normal tissue. This conclusion presumes that the tumor mast cells maintain their usual function, a presupposition supported by what is known of tumors of other analogous secretory tissues, such as the parathyroid or the pancreatic islets and gonads. As our findings will show, not only is this supposition confirmed, but by the examination of two mast cell tumors of varying degrees of cellular maturity an interesting correlation between function and structure of the tumor cell becomes apparent that casts further light on the ultimate origin of heparin.

The Tumor Material

Since the original description (1) of the mast cell tumor of the skin of dogs, 15 additional cases have been observed. These neoplasms occur most commonly in older animals and males are more often affected. The tumors may be either solitary, in which case they are usually benign, or multiple and malignant. The gross appearances of the tumors in different animals vary considerably as concerns their size and number and the extent of superficial ulceration that ultimately occurs. In the skin, the tumors are subepithelial and occur principally on the trunk, with less common involvement of the extremities. Metastatic lesions, either microscopic or macroscopic, commonly occur in the spleen, liver, and regional lymph nodes. The general health of the dogs is usually little affected except in the terminal stages of the malignant forms when anemia, cachexia, and other symptoms manifest themselves. No abnormalities in the clotting time of the blood have been observed. Repeated attempts to transmit the tumors to other dogs have been unsuccessful.

The tumors consist essentially of atypical tissue mast cells whose cytoplasm contains metachromatic basophilic granules. The granule structure is best demonstrated by staining tumor imprints with Wright's stain. As has been mentioned, the morphologic characteristics of the mast cells in the various tumors show all gradations from mature cells that resemble the normal tissue mast cells to immature cells that manifest various degrees of anaplasia. While it is generally true that the mature cells have large coarse granules and that the immature cells have finer granules, exceptions occur inasmuch as cells with

atypical nuclei may have coarse granules and cells otherwise quite mature in appearance may have fine granules. All combinations of cellular maturity and immaturity with fine and coarse granules have been found in the series of tumors examined.

The general structure of the tumor consists of mast cells heavily infiltrating the corium and subcutaneous tissue. The cells may be grouped in nodular collections or form diffuse sheet-like infiltrations. The metastatic lesions resemble in their cellular content the primary tumor. Invasion of blood vessels is frequently seen and the sinuses of the spleen may be filled with tumor cells.

I. The Heparin Content of a Mast Cell Tumor of Mature Cell Type

A 10 year old male Pointer had for 1 year a spherical nodule 2.7 cm. in diameter in the skin of the scrotum. The nodule was elevated 6 mm. above the surrounding skin and the surface was superficially ulcerated. Under local anesthesia, the entire scrotum and testes were removed. Ten weeks following the operation, examination revealed a round firm swelling that measured 10 cm. in diameter in the region of the left peripenile lymph node. The skin over the enlargement and for an area of 15 cm. in diameter in the left inguinal region and the inner surface of the thigh was diffusely thickened to a depth of 6 to 12 mm. The involved skin was corrugated and pale tan in color. In consideration of the extent of the tumor process, the dog was killed with a lethal dose of soluble pentobarbital given intravenously. Necropsy showed no outstanding gross lesions with the exception of enlargement of the right peripenile lymph node which measured 2.6 by 3.7 cm. and replacement of the sublumbar lymph nodes with tumor tissue. The latter nodes were 3 in number and were greatly increased in size. The largest measured 6 X 9 cm. and the smallest measured 3.5 X 5 cm.

For histological examination tissues from the subcutaneous tumor and also from regional lymph nodes and viscera were fixed in Zenker's fluid and sections were stained with the Giemsa method and toluidine blue. Imprints were also made of all the tissues and stained immediately with Wright stain. The detailed cytological studies were made on these latter preparations as all danger of solution of granules was thus avoided.

Cellular Structure of the Tumor

Sections showed the epidermis either intact and thinly stretched overmasses of tumor or ulcerated by its extension to the surface. In the corium and extending deep into the subcutaneous tissue were solid infiltrations of mast cells (Fig. 1). These cells were of mature type, resembling in all details the normal tissue mast cell except perhaps for their somewhat larger size. They were so filled with metachromatic granules that in most instances all cellular detail was obscured, even the nucleus being covered by the gross granules (Fig. 2). When visible the nucleus was of moderate size, oval, with finely divided chromatin and obscure nucleoli. The mast cells lay in a stroma of collagen fibrils which varied inversely in density with the concentration of the tumor cells; in no areas was there extensive fibrosis. In spite of the lack of cellular anaplasia, the tumor was definitely malignant as was evident not only from local invasion but by the occurrence of metastases in lymph glands, spleen, and liver. The sinuses of the pulp of the spleen and branches of the portal vein were filled with solid masses of similar mature tumor mast cells.

In the imprints of the tumor the mast cells,—since they were freed of tissue pressure and lay in tissue fluid while the slide was being pressed upon the fresh cut surface,—had lost their polygonal or irregular shape and assumed spherical contours (Fig. 4). Except for this difference in shape the cells showed the same structural characteristics as in sections. The granules, however, were better preserved and the details of their shape and size were more apparent. The cytoplasm of all the cells was filled with them, some being so crowded that no detail of cell structure could be seen. The granules varied in shape; some were spherical, others ellipsoid, and a few presented the appearance of short rods. Many lay free in the tissue fluid between the cells. They were strongly metachromatic when stained with toluidine blue or with Wright's stain, whereas the nuclei stained a contrasting blue with the latter method.

In summary, the tumor consisted of a mass of mast cells that morphologically appeared identical with the coarsely granular tissue mast cells of the normal subcutaneous tissues.

The Extraction of Heparin

330 gm. of subcutaneous tumor tissue were emulsified in a Waring blendor and made up to 3300 cc. in phosphate buffer at a pH of 6.8. Toluol was added to the mixture and it was then autolyzed at 37°C. for 24 hours. The alkalinity was raised with NaOH to pH 9.0 and extraction continued for 30 minutes at 70°C. The emulsion was then made acid with glacial acetic to a pH 4.5 and heated for 10 minutes at 85°C. A heavy coagulation of proteins occurred which was removed by filtration. A clear opalescent filtrate resulted. After standing 12 hours in the ice box a further slight precipitate occurred which was removed by filtration, and to the clear filtrate 3 volumes of absolute alcohol were added. A heavy white precipitate resulted which after 12 hours in the ice box was removed by centrifugation. The precipitate was washed twice in alcohol, dried in a desiccator, and found to weigh 20.1 gm.

Although a preparation procured in the way described represents an extremely crude "heparin" and must contain a large component of proteins and glycoproteins other than heparin (5) it was decided to assay its biological activity at this point rather than to risk the losses that would accompany its purification. The method devised by one of us was used (6). The assay showed, with an optimum dosage of 60 γ per cc., an activity of 41 A.C.U. (8.2 I.U.)[1] per mg. With increasing dosage the titre fell; 100 γ per cc. equaling 5.5 A.C.U. (1 I.U.) per mg. This decrease in titre when an excessive amount of the preparation was used in the titration was considered to be the effect of contaminating protein and thrombokinase. Jaques and Charles (7) have discussed these difficulties in the assay of crude preparations and have pointed out that when the anticoagulant activity is less than 5 I.U. per mg. it may become necessary to decide whether the activity is due to heparin or other substances. Our preparation lay just above this minimum figure so that it was decided to attempt some purification to raise the activity per milligram to a figure that would

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[1] A.C.U., anticoagulating unit; I. U., international unit.

be incompatible with any other assumption than that it was due to heparin. A modification of the first step in the method of purification of Charles and Scott (4) was used. The product of their complete procedure is still considered by these authors to be "crude heparin."

2.5 gm. of the preparation was dissolved in 100 cc. of H_2O at a pH of 8.5 and digested with 2.0 gm. of pancreatin for 36 hours with xylene at 37°C. 2 volumes of 95 per cent alcohol and 0.5 cc. of HCl were then added. A light precipitate occurred and after 12 hours in the ice box was removed by centrifugation. It was dissolved in 50 cc. of H_2O at pH 8 and heated to 75°C. A small amount of dark material was removed with the centrifuge and to the clear supernatant 2 volumes of acetone and 0.3 cc. HCl were added. The floculent precipitate was removed, washed in alcohol, and dried. Only 0.080 gm. was recovered, but this product on assay showed an activity of 147 A.C.U. (29.4 I.U.) per mg. This is an increase in potency of $3\frac{1}{2}$ times over the original preparation and is compatible with the conclusion that the anticoagulant effect is due to heparin.

In estimating the total heparin content of the tumor tissue the assay of the first sample was used, since much had been lost in the attempt at purification. Since 1 mg. of this preparation contained 41 A.C.U. and 20 gm. of preparation was obtained from 330 gm. of tumor, a kilo of tumor would contain 2,460,000 A.C.U. or 492,000 I.U. Dog's liver contains about 10,000 I.U. per kilo so it follows that the mast cell tumor contained almost 50 times as much heparin as has been obtained from the richest normal source of the most active form of heparin.

II. The Heparin Content of a Mast Cell Tumor of Immature (Anaplastic) Cell Type

The tumor was situated on the right chest of an 8 year old male fox-terrier. Two previous attempts at surgical removal had been unsuccessful for the tumor recurred in each instance several months following operation. On examination, the tumor was 18 cm. in diameter and elevated 4.5 cm. above the surface of the skin. The covering epithelium was intact with the exception of an ulcerated area 3.6 cm. in diameter on the upper edge of the tumor. In the immediate vicinity of the main tumor mass were 5 small subepithelial nodules that varied from 0.8 to 1.7 cm. in diameter. Under general anesthesia, the entire tumor tissue including the small nodules was surgically removed. On section, the tissue was a deep grey color and firm to palpation. This material was used for heparin determination. The operative wound healed uneventfully and the animal was observed at frequent intervals. No evidence of recurrence was noticed at these examinations. Three months following the last operation, the animal died of a strangulated perineal hernia. Necropsy, both macroscopic and microscopic, showed no lesions pertinent to the history of the tumor.

The Cellular Nature of the Tumor

Histological sections of the subcutaneous tumor showed masses of tumor cells lying beneath the epidermis and invading the deeper tissue. Even with low magnification a striking difference from the first specimen could be noticed in the appearance of the tumor. The tumor cells were less clearly seen, for their abundant cytoplasm appeared relatively pale as compared to the densely

stained granulations of the first tumor (Fig. 3). This contrast in cytological detail was most apparent, however, in the imprint preparations (Fig. 5). In these the cells varied greatly in size, some having twice the average diameter of those of the previous tumor. The abundant cytoplasm in every case contained granules, but these varied from dust-like particles to the typical coarse metachromatic granules of the mature cell. The latter were at best, however, few in number and widely scattered and in no instance was the nucleus obscured by them. The nucleus was spherical or oval, vesicular with fine scattered chromatin, and one or two large nucleoli were usually present. With Wright's stain it took on the definite reddish tone of metachromasia appearing very unlike the blue nucleus of the more mature cells of the first tumor. These contrasts between the morphological maturity of the heavily granular cells of the first tumor and that of the lightly staining anaplastic cells of the second tumor will be best appreciated in the comparison of Figs. 4 and 5.

To summarize, the tumor consists of anaplastic mast cells of immature type, the more primitive forms resembling very closely, with their large nuclei and fine granulation, the early stages in the development of tissue mast cells described by Downey (8). In occasional cells the typical coarse metachromatic granules of the adult form were sparsely present.

The Extraction of Heparin

168 gm. of subcutaneous tumor was ground in a Waring blendor and submitted to the same procedure as the previous material: autolysis at 37°C. at pH 6.8, extraction at 70°C. at a pH of 9 in phosphate buffer, filtration, acidification with acetic acid to a pH of 4.5, coagulation of proteins at 65°C., filtration, and final precipitation with 3 volumes of alcohol. A dried precipitate of 9.05 gm. was obtained.

As it was suspected that the heparin yield in this tumor might not be so abundant as in the previous experiment, it was decided to repeat, at a higher degree of alkalinity, the extraction of the usually discarded first precipitation of proteins. This precipitate was therefore resuspended in 250 cc. of 0.5 NaOH and heated to 70°C. for 30 minutes. It was then acidified to pH 4.5 with acetic acid and reheated to 70°C. The protein precipitate was removed by filtration and to the clear supernatant 3 volumes of alcohol were added. The resulting precipitate, after washing in alcohol and drying, weighed 1.50 gm.

Assays of the two preparations obtained as described showed that the first sample extracted with the relatively weakly alkaline phosphate buffer had no determinable anticoagulant effect. The second sample extracted by stronger alkali, showed an anticoagulant activity of 9.6 A.C.U. (1.9 I.U.) per mg.

If one accepts this weakly anticoagulant effect as due to the presence of heparin the content per kilo of the latter would be 86,400 A.C.U. or 17,280 I.U., since 1.50 gm. was obtained from 168 gm. of tumor. On this assumption the immature tumor therefore contained only about one twenty-ninth as much heparin as the mature tumor but nevertheless 1.7 times as much as does normal dog liver (7).

The Particulate Content of Living Neoplastic Mast Cells

In preparations fixed and stained with appropriate methods the mature cells of the tumor rich in heparin were seen to be filled with coarse metachromatic granules, while in the immature cells of the tumor of moderate heparin content the granules were present as fine dust-like particles. Whether there were as many of these fine granulations in the cell protoplasm as there were of the coarse granules in the mature cells is impossible to determine, since their minuteness faded into invisibility in the stained preparation.

Phase contrast microscopy allows the examination of living cells unaltered by reagents so this method was applied to the examination of the cytoplasmic particulate material of the tumor mast cells.[2]

The cells were prepared for examination by scraping the surface of the fresh tumor and mounting the resulting turbid fluid in the usual manner of a fresh preparation beneath coverslip and slide. One of the tumors selected resembled the mature tumor of rich heparin content while in the other the anaplastic cells of an immature tumor contained a mixture of fine and moderately coarse particles.

Figs. 6 to 11 show living unstained cells from an immature tumor. Detail is best seen in Fig. 6, as in this specimen gentle pressure on the coverglass had flattened the cell without disrupting its content; in the remaining figures the cells were floating in fluid and therefore their thickness disturbed somewhat the clarity of the phase contrasts.

In Fig. 6 three optical effects are produced by the particulate matter of the cytoplasm; this appears to be composed of densely black granules, similar dark granules with optically clear centers, and fine, ill-defined grey particulate material. The dark granules appear in irregular clusters scattered on a diffuse background of the grey material. In the interpretation of these optical effects it is to be remembered that with phase contrast microscopy the examination is made with diaphragms widely open and that contrasts are at a maximum only when the objects are in exact focus, so that the differences in the optical appearance of the various granules in these figures are not due to the diffraction artifacts that result from "cutting down the light" and "out of focus" examination that are so commonly used in conventional microscopy to emphasize a detail which is largely spurious. We can therefore be certain that the photographed differences in the particulate matter are at least optically real.

Fig. 12 shows a cell from a mature tumor the cells of which are filled with coarse metachromatic granules similar in stained preparations to those shown in the cell in the upper left hand corner of Fig. 4. It will be seen that the cell appears distended almost to bursting with dark granules of even greater size than those of the anaplastic cells and that scattered among them are a few of

the indefinite grey particulate bodies. The picture is therefore the exact converse of the immature cell of Fig. 6. Figs. 7 to 11 show cells with varying amounts of the two forms of particulate matter.

<div align="center">DISCUSSION</div>

If the mast cell seemed proven to be the normal source of heparin by Wilander's (3) correlation of the concentration of these cells in normal tissues with their heparin content, then this conclusion is not merely confirmed but is pushed close to its logical limit of certainty by our demonstration of the massive heparin content of a tumor composed of well differentiated mast cells. It is also evident from our findings that the heparin content of mast cell tumors may vary with the granule content of the tumor cells in not only a quantitative but also a qualitative sense. In the anaplastic mast cells of an immature tumor granules were present and numerous, but their fine dust-like character in stained preparations contrasted strikingly with the coarse granule of the mature cell. In such a tumor the heparin content was relatively low.

Since the granule of the typical mast cell is strongly metachromatic, a property also of the heparin which can be extracted from it, the assumption has been general that the coarse granule is heparin in particulate form. Our demonstration of the variation in the heparin content of the tumor with the variation in the structure as well as number of the granules reopens this question and allows an examination of an earlier phase of the functional activity of the cells. For Downey (8) in his studies of the histogenesis of the normal tissue mast cell has traced back the coarse metachromatic granules of the adult cell to earlier fine dust-like particles which are not metachromatic. This dust-like material he derives ultimately from the nucleus. Our findings with anaplastic mast cells afford no conclusive evidence on this point though the contrast between the reddish metachromasia of the nuclei of the immature cells and the blue nuclei of the mature might be considered indicative of a passage of metachromatic material from nucleus to cytoplasm.

The examination by means of phase contrast microscopy of living tumor mast cells unaffected by fixative or stain clarifies considerably the uncertain data of the stained preparations. Under living conditions the cytoplasmic particulate matter appears in two forms; as an indefinite grey granular material and as densely black discrete granules. The former is the dust-like material of the stained immature cell and the latter are the coarse metachromatic granules of the stained adult cell. A comparison of Fig. 6 showing an immature cell with scanty dark granules and rich content of grey granular material with the mature cell of Fig. 12 in which the greyish particulate matter is virtually absent but dense black granules are present instead leaves little doubt that the morphological expression of the maturation of these tumor cells involves either a replacement of the greyish particulate matter by granules which appear dense

and black or a transformation of the former material into a substance having the latter character.   Our biochemical determinations have shows that the heparin content of the immature tumor cell is comparatively small.   It is therefore possible in a series of mast cell tumors to correlate the formation of heparin with the maturing of the primitive granular material of the anaplastic cell and its replacement by, or transformation into, the strongly metachromatic coarse granules of the more mature tumor type, with its abundant heparin content. There would seem to be, therefore, a primitive particulate material in the mast cell in which "heparin" formation, or accumulation, has not yet occurred.

These conclusions concerning the structure and function of the tumor mast cells are supported by the results of tissue culture, which are reported in an accompanying paper (9).   Briefly, pure cultures of granular mast cells grew from explants made of tissue from the immature second tumor of the present study.   In the growing preparations gradation of granule size was not apparent, but variation in the metachromasia of the granules in the proliferating mast cells was clearly evident.   In some cells metachromasia was intense and in others it was only feebly present, while in still others both metachromatic and unstained granules were present in mixture (reference 9, fig. 3).   These experimental findings, along with our demonstrations of the varied heparin content of mast cell tumors, also direct attention to the possible existence of a pre-heparin primitive particulate matter as a structural precursor in the genesis of the functionally active product of the cells.

A final question concerns the qualitative nature of the heparin of the mast cell tumors.   Jaques, Waters, and Charles (10) have shown that crystalline barium salts of heparin from various species show variation in their biological activity, though no chemical difference was noted between the different samples. Dog heparin for example is 10 times as potent per mg. as that derived from sheep tissue.   One would like to know whether the heparin from a dog tumor is identical with that derived from normal dog tissue.   In our calculations of "heparin content" only biological activity was measured; if tumor heparin is more potent than heparin of normal tissue then the actual heparin content of the tumors we examined was somewhat less than 50 times that of dog liver, whereas if the tumor heparin is less potent, the actual content must have exceeded the figure given.

Of even greater ultimate interest is the possibility that the heparin from neoplasms may differ in its chemical constitution from that derived from mature normal tissues, representing perhaps a precursor in the formation of the normal substance or a variant on its constitution.   In the case of the mature tumor, from which active "heparin" was obtained in such large amount by chemical manipulation, great numbers of mast cells with mature granulations were present in the portal blood stream and in the sinuses of the spleen, yet no anticoagulative activity was observed *in vivo*.   Such a paradox empha-

sizes the present uncertainty not only as to the exact chemical structure of isolated heparin but in particular as to the form in which it occurs in nature. A more detailed biochemical examination of the heparin of tumors is therefore indicated.

SUMMARY AND CONCLUSIONS

1. The spontaneous mast cell tumor of the dog contains heparin.

2. The cytoplasmic particulate content of the tumor mast cells varies with their anaplasia. This conclusion is based on the following findings: (a) in the immature cell of the more malignant tumor the particulate matter appeared in the living cells by phase microscopy to be composed of greyish ill-defined particles or as a fine, weakly metachromatic granulation in the fixed and stained preparation; (b) in the mature cells of a relatively benign mast cell tumor, both in the living cell and in stained preparations, the particulate matter occurred in the form of discrete, dense, and strongly metachromatic granules, resembling those of the normal mast cell.

3. The heparin content was large (fifty times that of dog liver) in the growth with mature cells and only moderate (1.7 times) in that with immature cells.

4. Since there may be a great amount of greyish particulate matter (or fine stained granules) in a tumor of relatively low heparin content, it is suggested that this material represents an early or precursor phase in the development of heparin.

5. This possibility and the fact that the blood stream may be invaded by mature tumor mast cells of large heparin content without evident disturbance in the coagulability of the blood suggest the value of a comprehensive biochemical study of the heparin of mast cell tumors.

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EXPLANATION OF PLATES

PLATE 6

FIG. 1. Mast cell tumor of mature cell type described in the text. Beneath the epidermis and invading the deep fibrous subcutaneous tissues are seen dense infiltrations of tumor mast cells. Dominici stain. × 70.
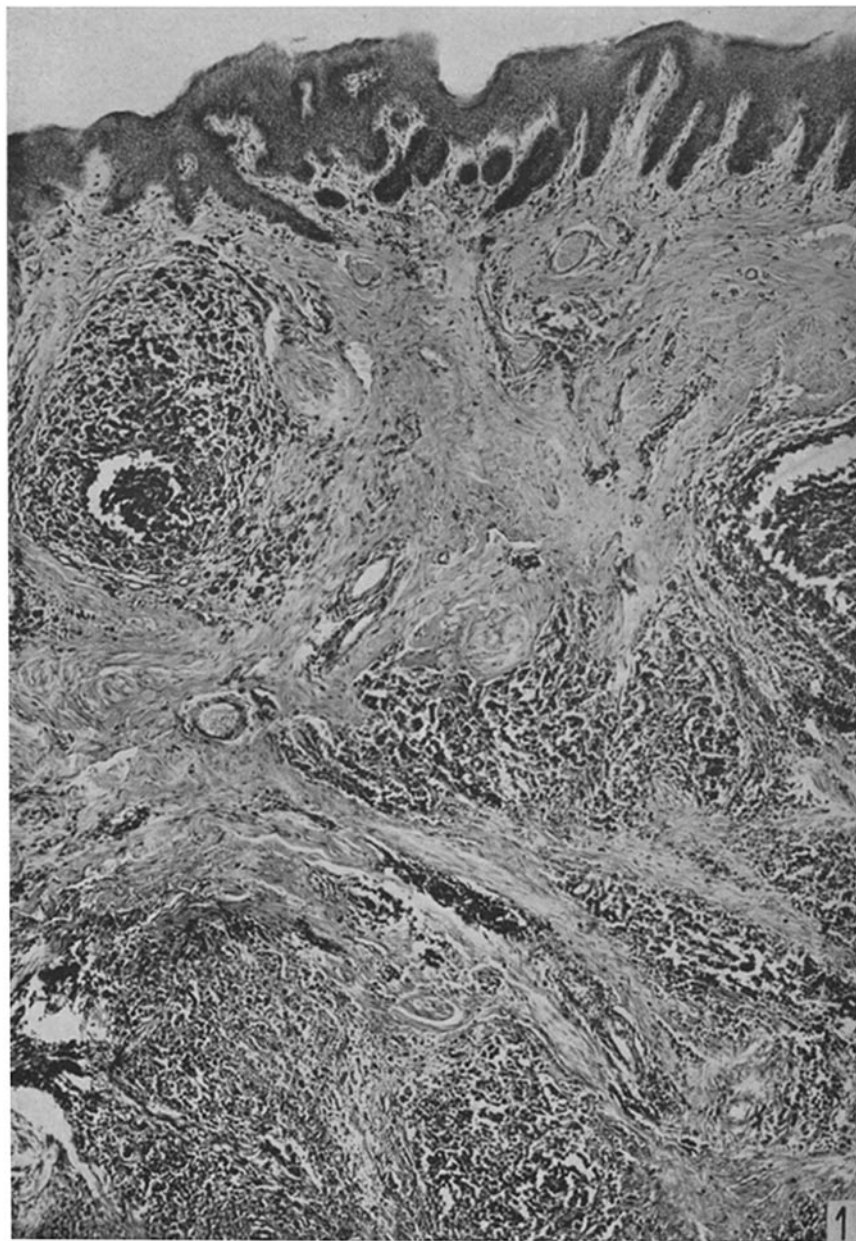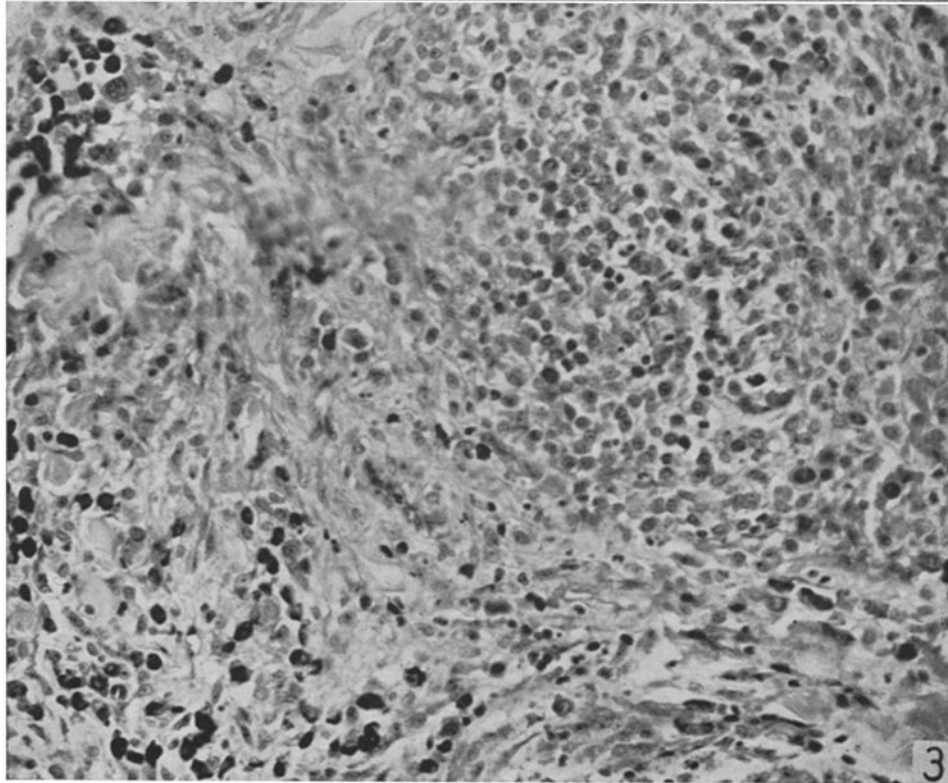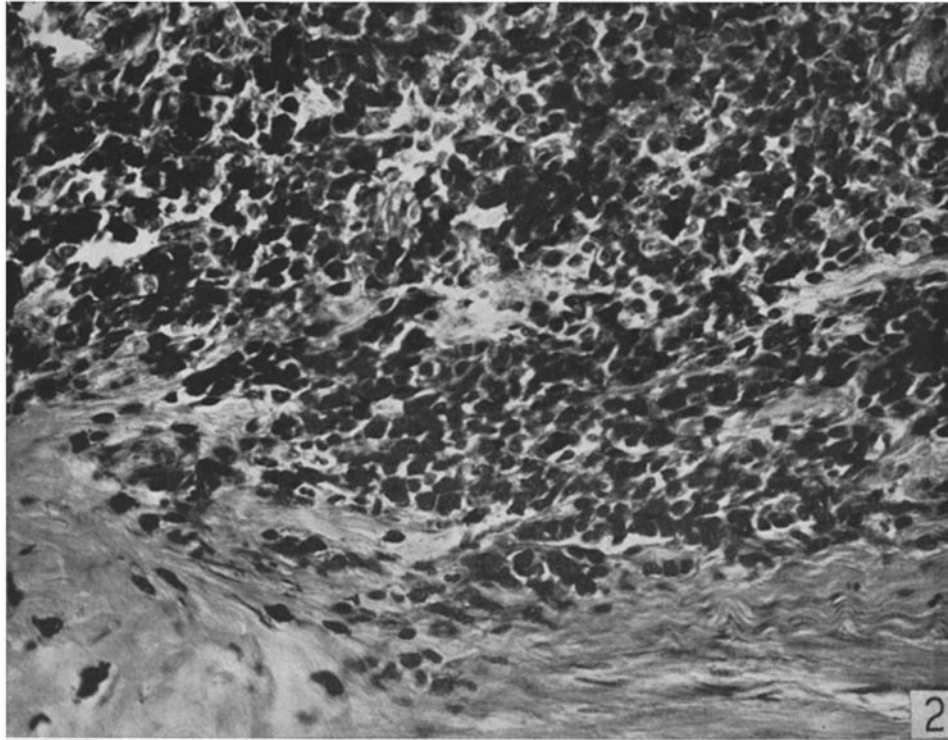
(Oliver *et al.*: Origin of heparin)

FIG. 2. The edge of the invading tumor of the previous section. The mast cells are so filled with metachromatic granules, appearing black in the photograph, that no detail in their cellular structure can be seen. This tumor contained fifty times as much heparin as normal dog liver. Dominici stain. × 300.

FIG. 3. Detail of the tumor of immature cell type from which the second preparation of heparin was made. Scattered through the section, particularly along its left and lower margin, is seen a sprinkling of black-stained mature mast cells similar to those of the preceding specimen. In the right half of the figure the tumor cells contain fine granulations which are invisible in the photograph so that they lack the appearance of mast cells. As is shown in Fig. 5, an imprint from this same tumor, these apparently clear cells contained a fine dust-like granulation. The growth yielded about one-thirtieth as much heparin as the mature tumor of Fig. 1. Dominici stain. × 300.
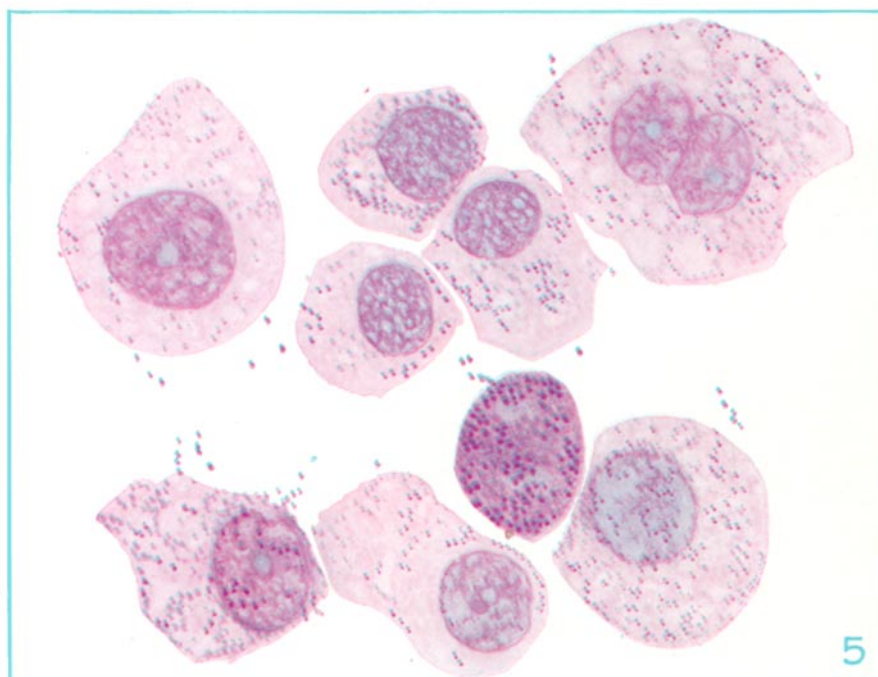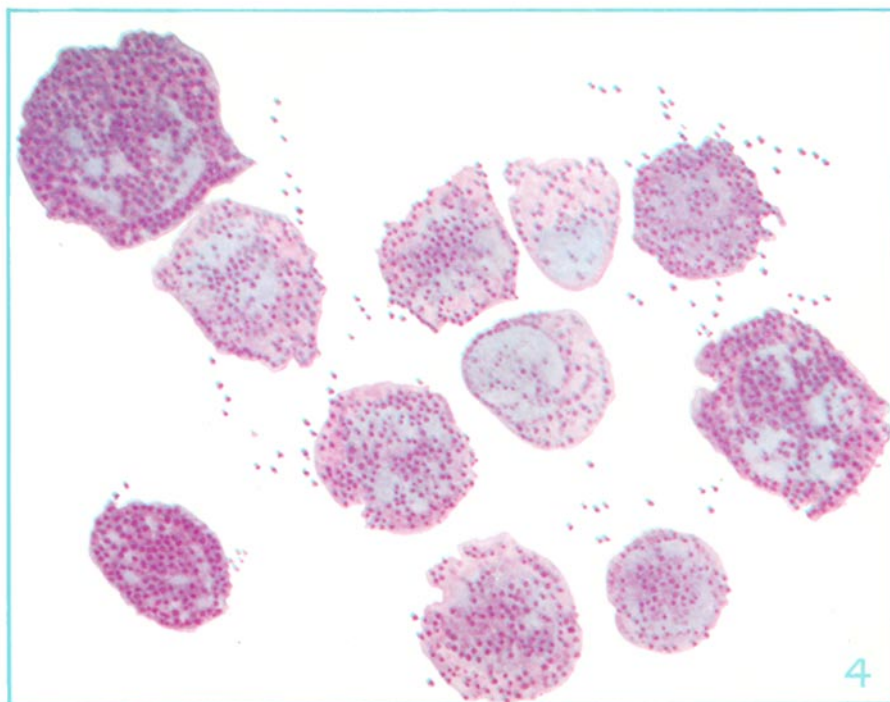
(Oliver *et al.*: Origin of heparin)

FIG. 4. Imprint preparation from the mature mast cell tumor of Figs. 1 and 2, stained by Wright's method. The cells are filled to a greater or less extent with large metachromatic granules which in most cases obscure the light blue-stained nucleus. × 1800.

FIG. 5. Similar preparation of the immature mast cell tumor. The anaplastic cells of the immature tumor have an ample vacuolated cytoplasm which contains many fine dust-like granulations. The nuclei are large and deeply metachromatic as compared with the light blue nuclei of the more mature cells in Fig. 4. In one cell nuclear division has occurred. In the lower center is seen one of the occasional mature mast cells scattered sparsely throughout the immature tumor. × 1800.
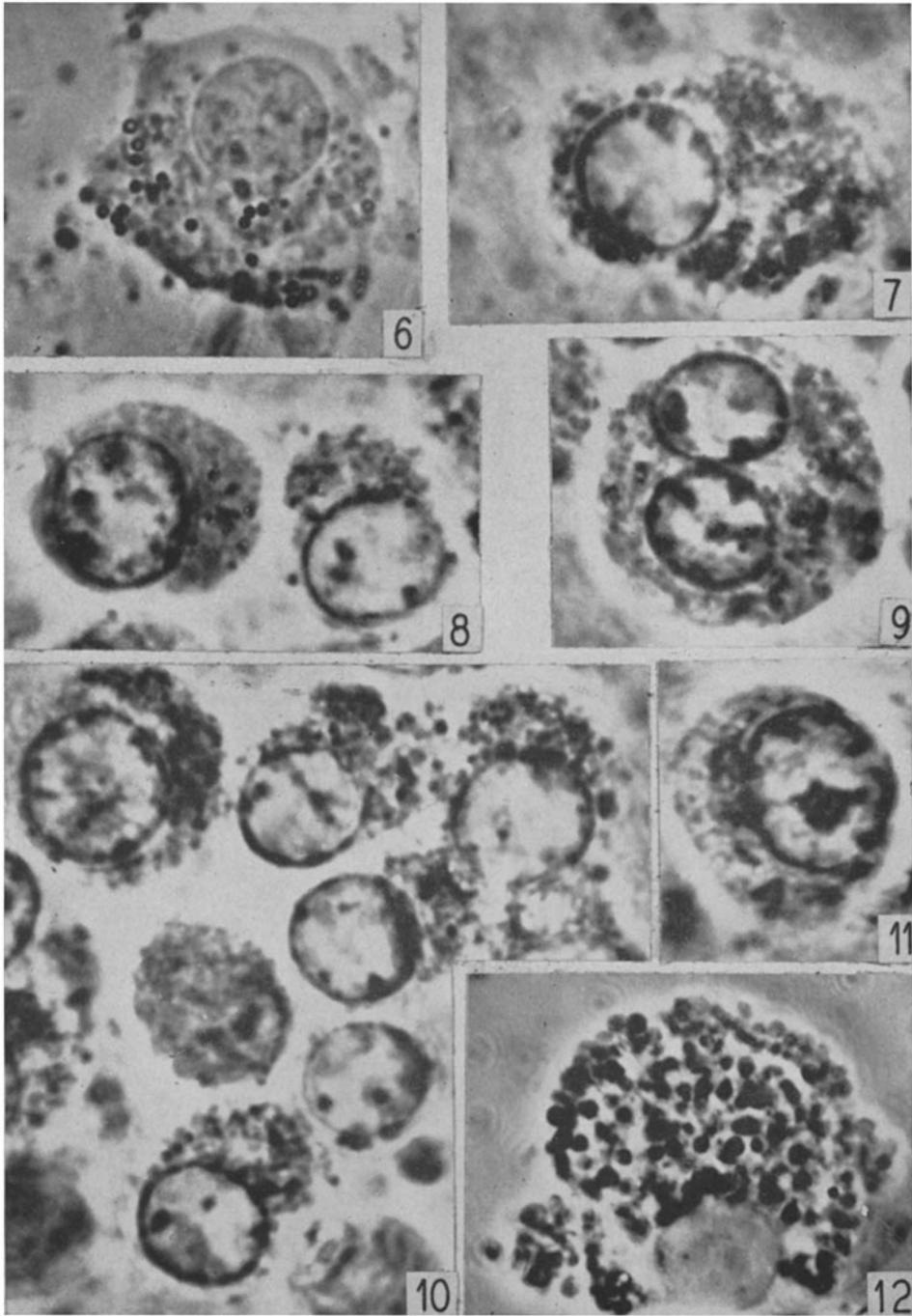
PLATE 9

FIGS. 6 to 12. Photographs of living unstained tumor mast cells taken by phase contrast microscopy. Under such circumstances the diaphragm is fully open and the maximum contrast occurs only at the exact focus. Objects out of focus appear with diffraction rings (*vide* floating extracellular granules in Fig. 12). This means that the variations in the density of the intracellular particulate material are not the result of the common optical artifacts produced by cut-down illumination and out of focus examination, but that the considerable differences in appearance are optically real. × 2860.

FIG. 6. Living unstained cell of the immature type. The cell has been slightly flattened by pressure. The cytoplasm is filled with particulate material presenting three optical appearances; densely black discrete granules, similar black granules with optically clear centers, and diffusely scattered greyish material in granular form. This last is in the same optical plane of focus as the sharply defined black granules.

FIGS. 7 to 11. Living mast tumor cells from the same specimen. In these preparations the cells were floating in fluid, so the cytological detail is less clear because of their relatively great thickness. All show diffusely distributed greyish particulate matter among which the dense black granules are scattered in irregular clumps.

FIG. 12. A living mast cell from a mature tumor. The cytoplasm is crowded almost to bursting with dense black granules and there is very little greyish particulate matter.

(Oliver *et al.*: Origin of heparin)