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# HEPARIN, 5-HYDROXYTRYPTAMINE, AND HISTAMINE IN NEOPLASTIC MAST CELLS*

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**Abstract**—During culture *in vitro* of an ascitic mast-cell tumor of the mouse, P-815, three strains have been developed, two of which were obtained by a cloning-technique. One of these pure strains is near diploid, the other tetraploid. All strains maintain their intracellular levels of heparin, histamine, and 5-hydroxytryptamine (5-HT) in culture. The levels of these three substances and the incorporation of $S^{35}$-sulfate, glucosamine-1-$C^{14}$, and glucose-U-$C^{14}$ into heparin have been compared in the cells both in the mouse and in culture. Strains vary in their contents of these compounds and in their capacity to incorporate $S^{35}$-sulfate and glucosamine-1-$C^{14}$ into heparin, but in all cases the amounts of heparin, 5-HT, and histamine are higher in culture than when the same strain of cells are grown in the mouse; similarly, the incorporation of $S^{35}$-sulfate into heparin is higher in culture. The amounts of the amines, especially of 5-HT, are proportional to the amount of heparin. The three substances are found in the same intracellular fraction. By paper chromatography mast-cell heparin has been resolved into three fractions. The concentration of one of these is proportional both to the amount of heparin, as determined by bioassay, and to the levels of amines, especially 5-HT. This component of heparin is not present in bovine heparin. It was also noted that sulfate and glucosamine in heparin turn over at the same rate. The half-life of heparin in the mast-cells is approximately two and one half days.

## INTRODUCTION

A TRANSPLANTABLE mouse mastocytoma, P-815,[2] containing 5-hydroxytryptamine (5-HT), histamine,[3,4] and heparin[5,6,7] maintains its levels of these compounds after continuous growth in culture.[4,8] The development of a cell line, termed the T-line, having higher levels of 5-HT and histamine than the original line (0-line) in culture, has already been described.[4] From the T-cells, two clonal sub-lines X-1 and X-2, were obtained[4] by two consecutive cloning procedures.[9] In this paper are recorded some cytological characteristics of these cells, their capacity to take up $S^{35}$-sulfate, glucose-U-$C^{14}$, and glucosamine-1-$C^{14}$, and to incorporate them into heparin, and the turnover-rate of sulfate and glucosamine in heparin. In addition, some aspects of the relationships among 5-HT, histamine, and heparin have been explored.

## METHODS

ALL the mast cell lines were maintained in (AKR × DBA/2) F1 hybrid mice or in DBA/2 mice, the tumor being routinely carried in the ascitic form. The culture techniques and the medium in which the cells were grown have already been described in

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detail;[4] the medium differed only in the addition of Neomycin (0·1 mg/ml) and Mycostatin (Squibb, 50 units/ml). An inoculum of 1–3 $\times$ $10^4$ cells/ml could be used provided that 30 ml of 2 per cent tris(hydroxymethyl)-aminomethane in 0·15 N-HCl were added to every liter of medium to give a final of pH of 7·4. This inoculum was used routinely in the later experiments. Under these conditions, a mean generation-time of 10 to 12 hr was obtained. In experiments in which the cells were incubated with radioactive isotopes, a cell-inoculum of 3 $\times$ $10^5$ cells/ml was used. Magnesium chloride was substituted for magnesium sulfate in the medium when the uptake of $S^{35}$-sulfate was studied, and when glucose-U-$C^{14}$ was added to the medium, the concentration of the glucose of the medium was reduced to one-half that normally used (i.e., to 0·5 g/l.). The levels and sources of radioactive isotopes used in culture were 0·5 mc (0·5 mmoles) of $S^{35}$-sulfate (obtained from the Oak Ridge National Laboratory); 0·001 mc (0·001 mmole) of glucosamine-1-$C^{14}$, kindly donated by Charles Pfizer and Co. through the courtesy of Dr Domenic G. Iezzoni; 0·005 mc (0·02 mmole) of glucose-U-$C^{14}$, and 0·005 mc (0·005 mmole) of glucuronate-6-$C^{14}$ (the last two substances were obtained from Nuclear Chicago). For studies of the uptake of $S^{35}$-sulfate by mast cells in the mouse, 1·0 mc of $S^{35}$-sulfate was injected intraperitoneally.

Cells were removed from the mouse by aspiration of the peritoneal fluid and, like those obtained from culture, collected by centrifugation, and washed three times with ice-cold 0·9 per cent sodium chloride.

To count chromosomes, cells were removed from the mice four days after inoculation and five hr after injecting 1·25 $\mu$g of colchicine per g body weight. Cells in culture were incubated for 15 hr with 3 $\mu$g colchicine/ml of medium.

5-HT-levels were determined on the isolated heart of *Venus mercenaria* and histamine was assayed on the isolated ileum of the guinea pig, as described previously.[4]

To extract heparin, the mast cells were sonicated, placed in a boiling water-bath to coagulate protein and, after cooling, placed in cellophane membranes which had been heated for three days at 80°.[5] Pancreatin or crystalline trypsin (10 mg/5 $\times$ $10^8$ cells) was added to each bag, and the proteolysis-dialysis carried out against 0·02 M-Tris buffer, pH 8·6, for 24 hr. A further 24-hr dialysis was routinely carried out against running tap-water; in experiments in which the dialysates were collected, dialysis was carried out against distilled water, and the dialysates were concentrated *in vacuo*. The non-dialyzable material was heated, centrifuged, and five volumes of acetone were added to the supernatant fluid. After refrigeration over-night, the precipitate was dissolved in a small volume of water. The solution was shaken with three volumes of trichlorotrifluoroethane[10] ($CF(Cl_2)$–$CCl(F_2)$; "Genetron 113", Allied Chemical and Dye Corp.) to yield, after centrifugation, an upper aqueous phase containing all the heparin, a gel-like intermediate layer of protein, and the heavy solvent layer devoid of heparin. The aqueous phase was collected and residual Genetron 113 removed by gentle heating.

In the assays, heparin was compared with a standard preparation (1 unit = 10 $\mu$g; Nutritional Biochemicals Corp.) either by metachromasia or by anticlotting activity, or both; hereafter, the anticlotting activity is referred to as "bioassay". Metachromasia was determined with Azure A by the method of Jaques *et al*,[11] (absorption at 490 m$\mu$ in a Beckman DU-spectrophotometer was employed). For bioassay of heparin, blood was taken from a dog by venepuncture, mixed with nine volumes of 4 per cent

sodium citrate, and the plasma was collected. The clotting system consisted of 0·2 ml of plasma; 0·2 ml (12 mg) of fibrinogen (Armour) in 0·005 M-Tris Buffer, pH 7·3; 0·2 ml (0·2 mg) of thrombin (Upjohn); and 0·1 ml of the unknown solution or of a standard solution of heparin. With some samples of plasma, it was necessary to reduce the concentration of thrombin by one-half to obtain an anticoagulant effect with the standard heparin. Thrombin was added last, at which point timing was begun. The tubes were tilted gently every 15 sec until a clot was formed. For each assay, at least two concentrations of heparin were employed. The unknown was diluted and its heparin content estimated by comparison with the standard solution, which usually contained 0·03 units of heparin. This method proved more sensitive than the method described in the U.S. Pharmacopeia, but the values obtained by the two methods were in good agreement.

Heparin was chromatographed on Whatman No. 3 paper in *iso*propyl alcohol-0·04 M-Ammonium Formate (35 : 65). Metachromasia on paper was detected by dipping the dried paper into 0·1 per cent Azure A in ethanol—water—1 N-acetic acid (5 : 4 : 1) and then into the same solvent mixture without Azure A. Papers were also dipped into an alkaline solution of silver nitrate[12] after preliminary experiments had shown that several hours after being dipped and dried, heparin formed a violet color on paper. The radioactivity of all samples was measured in a liquid scintillation counter, and radioactivity on paper was detected with a Geiger–Mueller tube. Hydrolysis of heparin was carried out in a sealed tube for 48 hr in 3 N-HCl, and the hydrolysate, together with a glucose-marker, was chromatographed in 1-propanol—ethyl acetate—water (70 : 10 : 20).[12] After the paper dried, it was dipped in an alkaline solution of silver nitrate. Cells were fractionated in 0·25 M-sucrose by the method of Schneider and Hogeboom.[13]

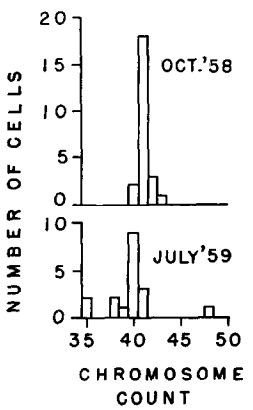



FIG. 1. Chromosome counts on X-2 cells maintained in the mouse.

## RESULTS

*Cytology.* Chromosome counts on X-1 and X-2 cells were made in October, 1958, four months after the development of the two clones, by Dr. T. S. Hauschka of the Roswell Park Memorial Institute. In July, 1959, comparable counts were made by Dr. Julian J. Jaffe of this department. The X-2 line in the mouse retained its near diploid chromosome number after repeated transfers during this nine-month period, as shown in Fig. 1. On the other hand, the polyploid X-1 cells (Fig. 2), after successive

transfers in mice during the same period, showed marked instability, as revealed by a widened distribution of chromosome counts in the population. However, the X-1 cells which had been kept in culture during the nine-month period remained stable (Fig. 2).

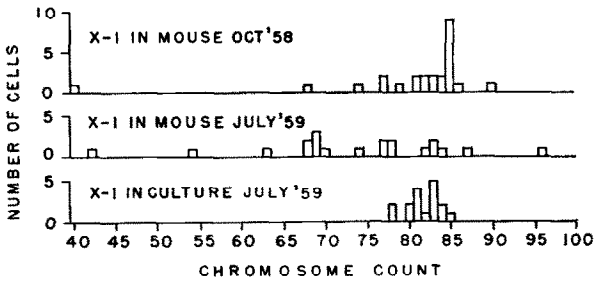



FIG. 2. Chromosome counts on X-1 cells maintained in the mouse and in culture.

*Cell size.* As shown in Figs. 3a and 3b, the polyploid X-1 cells are larger than the near-diploid X-2. They are also less uniform in size.

*Neoplastic virulence.* After twenty generations in culture, the cloned mast cell lines, X-1 and X-2, retained their neoplastic virulence when injected into mice; this is shown in Fig. 4. During culture, the large polyploid (X-1) cells, exhibited slightly greater infectivity than did the original parent strain of cells (O-cells)[4], whereas X-2 cells showed the same infectivity as the O-cells. The "T-line", from which both X-1 and X-2 were developed, had the same infectivity as the original tumor. Necropsies on all animals showed no grossly different anatomical effects among the cell lines.

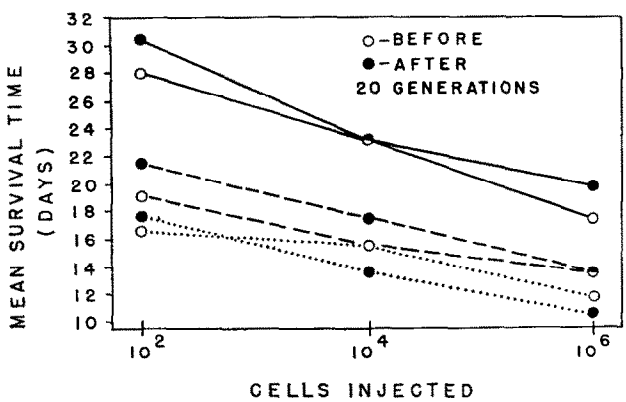



FIG. 4. The virulence of different cell-lines before and after culture. X-1 cells ———; original cells (O-cells) – – –; X-2 cells · · · · ·.

*Incorporation of glucose-U-$C^{14}$ into heparin by* X-1 *and* X-2 *cells in culture.* Both clones incorporated glucose-U-$C^{14}$ into heparin at the same rate (Fig. 5). That the non-dialyzable material was indeed heparin was shown in several ways: (a) it had anticoagulant activity; (b) it was metachromatic, both in solution and on paper after chromatography; (c) all the radioactivity of the non-dialyzable material

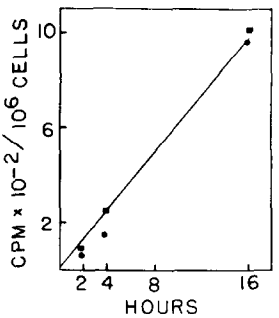



FIG. 5. Incorporation of glucose-U-$C^{14}$ into heparin by X-1 (■——■) and X-2 (●——●) cells, in culture.

was precipitated by the addition in aqueous solution of each of several quaternary salts (cetyltrimethylammonium bromide, cetylpyridinium chloride, and cetyldimethylbenzylammonium chloride) known to precipitate acidic and sulfated polysaccharides, and also by 4-amino-4'-chloro-diphenyl, a precipitant of only highly sulfated polysaccharides.[14]

The acid-hydrolysate of the heparin showed a radioactive spot at $R_f$ 0·83 coincident with a brown spot which formed after dipping in an alkaline solution of silver nitrate. Authentic glucosamine, chromatographed separately and in admixture with the hydrolysate, gave the same $R_f$ value. In this solvent system, galactosamine had an $R_f$ of 0·45.

*Failure of* X-1 *and* X-2 *cells to take up glucuronate*-6-$C^{14}$. During an incubation period of 24 hr, neither strain of cells took up any glucuronate-6-C.[14]

*Incorporation of glucosamine*-1-$C^{14}$ *into heparin by* X-1 *and* X-2 *cells in culture.* Glucosamine-1-$C^{14}$ was incorporated into heparin by both strains of cells, X-1 showing the more rapid uptake (Fig. 6). Paper chromatography of the acid-hydrolysates of the heparin (as above) showed a single radioactive spot corresponding to glucosamine.

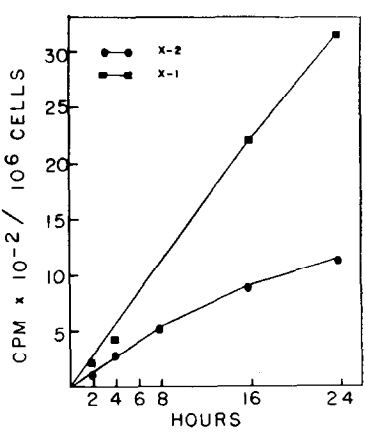
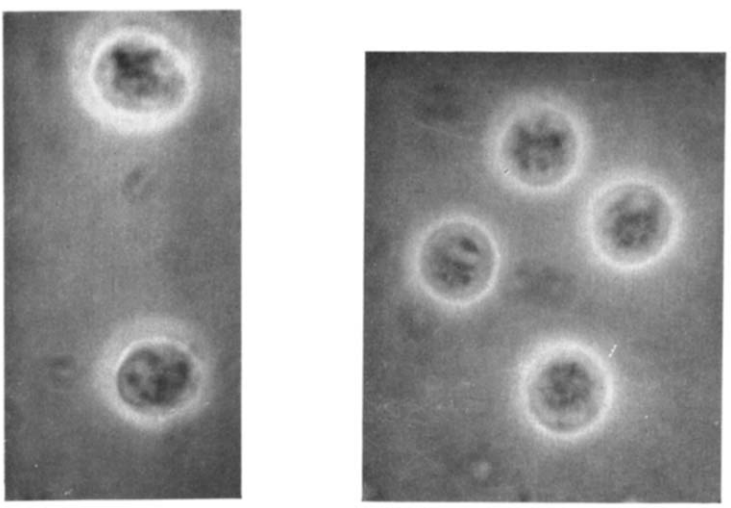



FIG. 6. Incorporation of glucosamine-1-$C^{14}$ into heparin by X-1 cells (■——■) and X-2 (●——●) cells, in culture.



(a) (b)

FIG. 3. (a) The polyploid X-1 cells and (b) the near-diploid X-2 cells, as seen with the phase microscope.

*Uptake of* $S^{35}$*-sulfate by mast cells* in vitro *and in culture and its incorporation into heparin.* The uptake of $S^{35}$-sulfate and its incorporation into heparin by X-1 and X-2 cells, both in the mouse and in culture, was measured. Fig. 7 indicates that the isotope was rapidly taken up by the X-1 cells *in vivo*. As early as two hr after its injection, the major portion of the $S^{35}$-sulfate was in the form of heparin; by 16 hr, 95 per cent of the isotope was accountable as heparin. The amount of dialyzable $S^{35}$-sulfate in the cell reached a peak at four hr and then fell. The dialyzable material had different $R_f$ values from those of the non-dialyzable material, and metachromasia was not exhibited either in solution or on paper after chromatography.

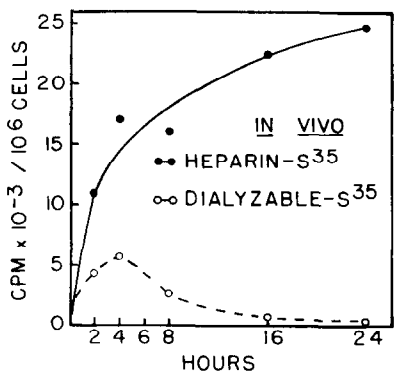



FIG. 7. The uptake of $S^{35}$-sulfate by X-1 mast cells in the mouse.

Fig. 8 shows the uptake of $S^{35}$-sulfate by X-2 cells in culture and the incorporation of the isotope into heparin. Unlike the same strain of cells in the mouse, the cells in culture continued to take up the radioactive isotope, so that at 24 hr more than 50 per cent of the radioactivity was still dialyzable. The simplest interpretation of the difference between the experiments *in vivo* (Fig. 7) and *in vitro* (Fig. 8) is that the cells incubated in culture are exposed to a persistently high level of $S^{35}$-sulfate in the medium, whereas *in vivo* the inorganic $S^{35}$-sulfate is being rapidly excreted.[15]

*Failure of* X-2 *cells in culture to secrete heparin.* After the growth of the X-2 cells in culture, the nutrient medium containing the $S^{35}$-sulfate was collected and concentrated *in vacuo*. All radioactive material was dialyzable, non-metachromatic, and when chromatographed on paper had the $R_f$ value of inorganic sulfate. Radioactive heparin could not be detected.

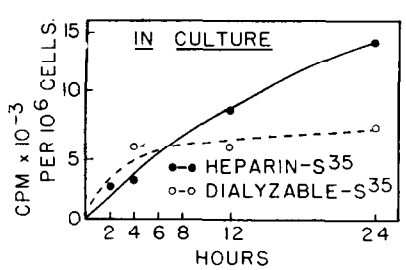



FIG. 8. The uptake of $S^{35}$-sulfate by X-2 mast cells, in culture.

*Absence of heparin in the horse-serum used in the culture medium.* Horse serum (100 ml) was digested with trypsin (100 mg) and extracted for heparin after dialysis and concentration *in vacuo* of the non-dialyzable material. None was detectable either by metachromasia in solution or after paper chromatography. The amount of heparin in this sample of horse serum was less than 0·01 units/ml. The concentration of heparin in the culture medium was thus less than 0·002/units per $10^6$ cells; this was too low to account for the levels of heparin in the cell, even if the cells could concentrate preformed heparin.

*Failure of* X-1 *cells to take up heparin-*$S^{35}$ *or heparin-*$C^{14}$ *in culture.* To determine whether the cells could take up heparin from the medium, radioactive heparin was prepared from X-1 cells that had been incubated with either $S^{35}$-sulfate or glucosamine-1-$C^{14}$. The labeled heparin was added to two separate cultures of X-1 cells. After 4, 6, and 24 hr, cells were harvested, washed in the usual way, and their radioactivity was measured. In no instance was there any detectable radioactivity in the cells. Under these conditions, therefore, the mast cells were unable to take up heparin.

*Half-lives of heparin-*$S^{35}O_4$ *and of heparin-glucosamine-*$C^{14}$. Twenty-four hr after the addition of $S^{35}$-sulfate or of glucosamine-1-$C^{14}$ to the X-1 cells in culture, and every 24 hr thereafter for one week, the cells were harvested and a cell-count was taken. The cell-population was then divided into two portions. One aliquot was extracted for heparin and the radioactivity was measured; the other was placed in an isotope-free medium. The loss of radioactivity with time, corrected for cell-multiplication, is shown in Fig. 9. The turnover-rate of glucosamine and of sulfate in heparin were the same. The half-life of heparin was two and one-half days.

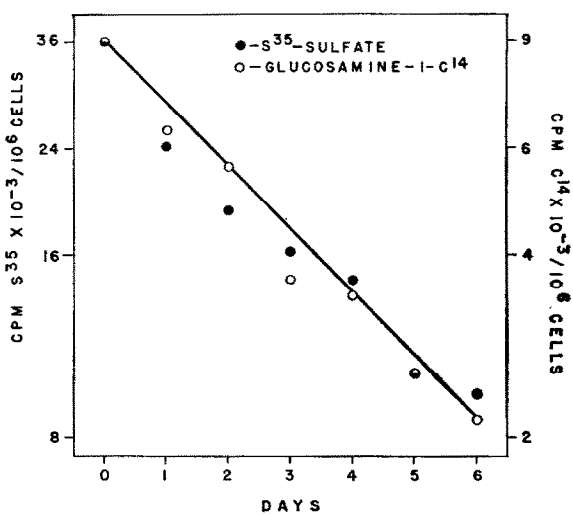



FIG. 9. Turnover of $S^{35}$-sulfate (○——○) and glucosamine-1-$C^{14}$ (●——●) in heparin by X-1 cells.

*Levels of* 5-HT *and histamine.* Over a six-month period, both 5-HT and histamine were simultaneously determined in three cell lines (T, X-1, and X-2) grown in the mouse as well as in tissue culture (Table 1). Consistently, in each cell-line, cells grown

in culture had greater amounts of the amines than the same cells grown in the mouse. The levels of the two amines varied widely (as is evident from the standard deviation), even in the genetically stable X-2 cells. These cells, in comparative experiments, usually, though not always, showed lower levels of amines than the larger X-1 cells, as suggested by the data in Table 1.

TABLE 1. CONTENT OF 5-HT AND HISTAMINE OF THREE STRAINS OF MAST CELL TUMORS IN THE MOUSE AND IN CULTURE

| Cell Line | In Mouse | | In Culture | |
|---|---|---|---|---|
| | 5-HT | Histamine | 5-HT | Histamine |
| T | 0·17 ± 0·05(7) | 0·25 ± 0·08(6) | 0·26 ± 0·10(15) | 0·40 ± 0·36(12) |
| X-1 | 0·32 ± 0·14(12) | 0·47 ± 0·26(13) | 0·89 ± 0·37(10) | 0·66 ± 0·31(11) |
| X-2 | 0·16 ± 0·15(4) | 0·28 ± 0·14(3) | 0·64 ± 0·25(6) | 0·42 ± 0·20(5) |

The numbers within parentheses denote the number of samples.

*Relative levels of amines and heparin.* As the levels of amines varied in any one cell-line widely between experiments (Table 1) a comparison of these levels among groups of cells was carried out at the same time. In Table 2 are recorded the values of 5-HT, histamine, and heparin, the last measured by two methods which are seen to agree well. In every instance, cells grown in culture had greater amounts of all three compounds than cells grown in the mouse, an increase especially remarkable with the X-1 cells. These polyploid X-1 cells, it should be added, usually have greater amounts of the three substances than the smaller, diploid X-2 cells, but in this experiment the X-1 cells in the mouse had significantly lower levels than the X-2 cells. In general, levels of 5-HT and histamine paralleled each other; and the levels of the amines, especially 5-HT, varied as the levels of heparin varied.

TABLE 2. LEVELS OF 5-HT, HISTAMINE AND HEPARIN IN FOUR DIFFERENT STRAINS OF MAST CELLS, IN THE MOUSE AND IN CULTURE

| Cell Strain | In Mouse | | | | In Culture | | | |
|---|---|---|---|---|---|---|---|---|
| | 5-HT μg | Histamine μg | Heparin Bioassay units | Heparin Azure A units | 5-HT μg | Histamine μg | Heparin Bioassay units | Heparin Azure A units |
| O | 0·01 | 0·19 | 0·01 | 0·02 | — | — | — | — |
| T | 0·10 | 0·12 | 0·03 | 0·04 | 0·20 | 0·40 | 0·11 | 0·13 |
| X-1 | 0·05 | 0·08 | 0·03 | 0·02 | 1·05 | 0·31 | 0·13 | 0·15 |
| X-2 | 0·39 | 0·35 | 0·09 | 0·09 | 0·70 | 0·50 | 0·18 | — |

All values are for $10^6$ cells.

The lack of a more precise relationship between and among these compounds can be explained, at least in part, by the variability of the levels of these amines in a single cell-line at any one time, especially if grown in the mouse. In Table 3 are shown the levels of the compounds in X-1 cells which had been inoculated into mice at the same

TABLE 3. LEVELS OF 5-HT, HISTAMINE AND HEPARIN IN X-1 CELLS COLLECTED FROM MICE ON THE SAME DAY

| Sample | 5-HT μg | Histamine μg | Heparin-$S^{35}O_4$ | | |
|---|---|---|---|---|---|
| | | | units* | cpm | cpm/μg |
| 1 | 0·32 | 0·66 | 0·091 | 12,400 | 134,000 |
| 2 | 0·48 | 0·46 | 0·101 | 15,800 | 156,000 |
| 3 | 0·56 | 0·71 | 0·106 | 15,000 | 142,000 |
| 4 | 0·80 | 1·15 | 0·121 | 17,400 | 143,000 |
| 5 | 0·90 | 0·92 | 0·124 | 17,300 | 139,000 |

All values are for $10^6$ cells.
* Determined by metachromasia with Azure A.

time and harvested one week later. Sixteen hr before collecting the cells, each animal was given $S^{35}$-sulfate intraperitoneally. The amounts of histamine detected were more variable than those of 5-HT, although the latter also showed a range greater than one would expect from experimental error. Least variable were the levels of heparin. Here again the amount of heparin appeared more closely correlated with the amount of 5-HT than with that of histamine. Also, the amount of heparin in X-1 cells was nicely reflected in their capacity to incorporate $S^{35}$-sulfate into heparin.

The parallel in X-1 cells between their heparin-content and the labeling by $S^{35}$-sulfate of the heparin suggested that a similar relationship may exist within the other cell lines. Accordingly, the amounts of heparin (and of 5-HT and histamine), and the labeling of heparin by $S^{35}$-sulfate, were measured in three other strains in the mouse (Table 4). Again the amount of heparin was correlated with the amount of amines,

TABLE 4. LEVELS OF 5-HT, HISTAMINE AND HEPARIN AND ITS CHROMATOGRAPHIC DISTRIBUTION IN DIFFERENT STRAINS OF MAST CELLS

| Cell Strain | 5-HT μg | Histamine μg | Heparin | | Chromatographed | | Heparin-$S^{35}O_4$ |
|---|---|---|---|---|---|---|---|
| | | | units* | cpm† | $R_f$ 0·00‡ | $R_f$ 0·76‡ | $R_f$ 0·94‡ |
| O | 0·03 | 0·11 | 0·04 | 3900 | 70 | 140 | 25 |
| T | 0·12 | 0·35 | 0·14 | 4200 | 250 | 50 | 3 |
| X-2 | 0·18 | 0·38 | 0·20 | 14,200 | 300 | 300 | 15 |

The values given are for $10^6$ cells.
* Determined by bioassay.
† Counted in liquid scintillation counter.
‡ Paper chromatograms counted directly with probe.

especially 5-HT, but the $S^{35}$-labeling in the heparin-extract was not correlated with the amount of heparin in the O and T cells: O cells had only 0·04 units of heparin/$10^6$ cells but showed almost as much $S^{35}$-label in the heparin extract as the T cells, which contained 0·14 units of heparin. The T cells, on the other hand, when compared with the X-2 cells, showed much less labeling than the heparin-value would lead one to expect. Ostensibly, then, there was no clear relationship between the amounts of heparin in a cell and its capacity to incorporate $S^{35}$-sulfate into heparin.

When however, the radioactive heparin extract was chromatographed, a different picture emerged. The heparin was resolved into three radioactive spots: $R_f$ 0·00, 0·76 and 0·94. The amount of radioactivity at $R_f$ 0·00 was proportional to the amount of heparin in the sample and to the amounts of amines, especially 5-HT. Bovine heparin, chromatographed concomitantly, exhibited two mobile spots, none at $R_f$ 0·00.

The relative proportions of the three heparins were the same in X-1 and X-2 cells; the O cells had preponderance of the $R_f$ 0·76—heparin, and the T cells a relatively large amount of the immobile heparin. Since the relative amounts of these heparins remained the same within each cell line, measurements of total $S^{35}$-heparin within each cell line reflect the amount of heparin, a conclusion supported by the data of Table 3.

Metachromasia on the paper was shown by all three components, especially the immobile heparin. The amount of metachromasia at $R_f$ 0·00 paralleled the amount of radioactivity there, and hence the total amount of heparin as measured by bioassay.

The heparins at $R_f$ 0·76 and 0·94 were eluted with water and the heparin activity was measured by bioassay. The fast-moving component was inactive, but the $R_f$ 0·76-heparin had slight anticoagulant activity. The immobile heparin could not be eluted with water and was eluted only partially with 0·1 M-$Na_2HPO_4$.

*Intracellular distribution of 5-HT, histamine, and heparin in the solid mastocytoma.* The apparent parallel between the levels of amines and the concentration of heparin suggested that the amines may be associated with heparin within the cell. X-1 cells were grown in the solid form (subcutaneously) in the mouse. Sixteen hours before removal and fractionation of the tumors, 1 mc of $S^{35}$-sulfate was injected. Histological examination of this tumor by Drs. A. Coulombre and R. Barrnett showed mast cells with variable amounts of granules, small cells with dense nuclei and no granules, connective tissue, fat, and blood vessels. The extracted heparin was determined by metachromasia with Azure A, and its $S^{35}$-labeling was measured. The bulk of the 5-HT, histamine and heparin was found in the particulate material, i.e., the fraction containing cell debris, nuclei and mitochondria (Table 5; column headed endogenous).

TABLE 5. PERCENTAGE DISTRIBUTION OF 5-HT, HISTAMINE AND HEPARIN IN THE SOLID X-1 MASTOCYTOMA

| Fraction | Endogenous | | | | Exogenous | | |
|---|---|---|---|---|---|---|---|
| | 5-HT | Histamine | Heparin Azure A | Heparin $S^{35}O_4$ | 5-HT | Histamine | Heparin Azure A |
| Nucleis and debris | 24 | 33 | 50 | 51 | 23 | 10 | 16 |
| Mitochondria | 30 | 36 | 23 | 32 | 14 | 12 | 17 |
| Microsomes | 16 | 15 | 11 | 5 | 3 | 16 | 12 |
| Supernatant | 30 | 16 | 16 | 12 | 60 | 62 | 55 |

To rule out the possibility that these compounds are only adventitiously bound to particulate material following cell-disruption, histamine (1 μg/g of tissue), 5-HT (1 μg/g of tissue) and heparin (2·0 units/g of tissue) were added to the homogenate and the cells fractionated. It may be seen (Table 5; column headed exogenous) that the

major portion of these compounds, when added to the homogenate, remained in the supernatant fraction.

*The increase in* 5-HT, *histamine and heparin in culture.* To show more clearly the increase in 5-HT, histamine, and heparin when cells are grown in culture, the experiment presented in Fig. 10 was carried out. When taken from culture, X-1 cells had

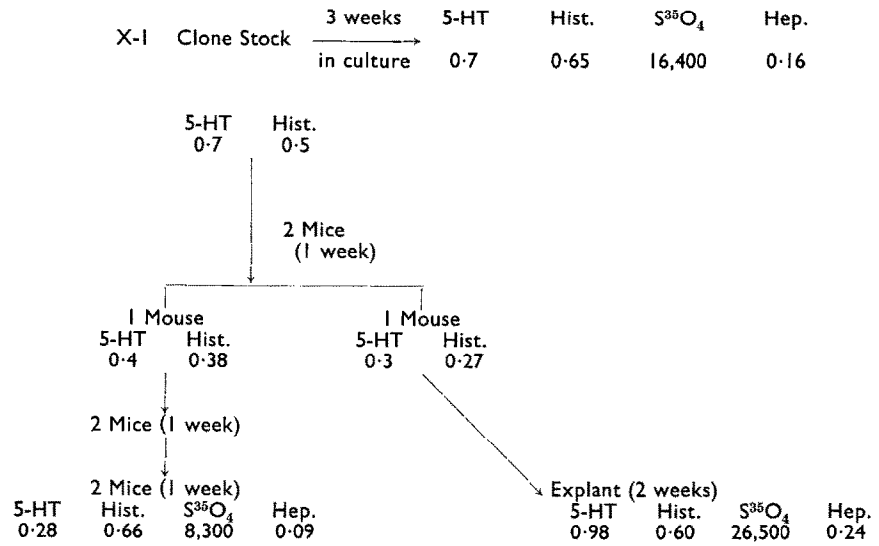



FIG. 10. Changes in the levels of 5-HT, Histamine, and Heparin produced in culture. All values are expressed as $\mu$g, units or counts/min. per $10^6$ cells.

0·7 and 0·5 $\mu$g of 5-HT and histamine, respectively, per million cells. One aliquot of these cells, maintained in culture for three weeks, showed no significant change in the amount of 5-HT or histamine. Another aliquot of these cells was inoculated into mice and one week later, the cells were removed; 5-HT and histamine values in one of these mice had fallen to 0·4 $\mu$g and 0·38 $\mu$g, in another to 0·3 $\mu$g and 0·27 $\mu$g, respectively. When an explant of the tumor cells from one of the mice was made and allowed to grow in culture for two weeks, the 5-HT and histamine levels increased markedly (to 0·98 and 0·60 $\mu$g, respectively). When the same cells were passed through two pairs of mice for one-week intervals, the levels of 5-HT stayed low (0·28 $\mu$g); inexplicably, however, the histamine levels rose to 0·66 $\mu$g. With this single exception, the experiments showed that the intracellular levels of 5-HT and histamine increase in culture and decrease when the same cells are grown in the mouse.

Where possible, measurements of heparin were made by metachromasia and by determining the amount of $S^{35}$-sulfate incorporated into the heparin. It can be seen that the levels of heparin, by both methods, paralleled the amine levels, particularly that of 5-HT: the high concentration in the cells grown in culture fell after passage in the mouse and was restored when the cells were again placed in culture.

Nutritional and hormonal effects are two factors that may explain the increased concentration of these substances in culture. It appeared possible that in culture, the

concentration of the precursors and co-factors were more favorable for the synthesis of 5-HT, histamine, and heparin, not only because the levels of tryptophane and histidine may be higher in the culture medium than in plasma, but also because the number of metabolic pathways available for these amino acids and co-factors are fewer in isolated mast cells than in the whole animal. Accordingly, mice bearing X-1 cells were given seven daily intraperitoneal injections of one of the following: uridine (50 mg per day); glucuronate, glucosamine, tryptophane, or histidine, each 8 mg per day, and also the last three compounds in combination. Sixteen hr before harvesting the cells, $S^{35}$-sulfate was injected. None of the treated animals showed an increase in 5-HT, histamine or heparin or in $S^{35}$-sulfate incorporation. Similarly, X-2 cells grown for two weeks in a culture medium fortified by the addition to 1 l of medium of uridine (2·5 mg), glucosamine (2·5 mg), pyridoxal (0·05 mg), serine (5·0 mg), histidine (4·5 mg), tryptophane (1·5 mg), and 5-hydroxytryptophane (15 mg) showed no change in levels of 5-HT and histamine. That the size of the cell-inoculum did not influence the concentration of the amines in the cells was shown by the observation that 5-HT and histamine levels were the same whether cells were grown from a cell-inoculum of $3 \times 10^4$ cells/ml or $3 \times 10^5$ cells/ml. To test the possibility that dialysates of the horse serum needed for cell growth in culture might contain one or more factors that enhance the synthesis of the three amines, dialysates of 100 ml of horse serum were collected, lyophilized, restored to a volume of 25 ml, and 1 ml was injected daily for one week. There was no detectable increase in 5-HT, histamine, heparin, or $S^{35}$-sulfate-incorporation into heparin. Finally, it seemed possible that in the mouse certain hormones may inhibit the synthesis of the three substances. As cortisone has been shown to inhibit the formation of histamine[16] and the incorporation of $S^{35}$-sulfate into mast cells,[17] cortisone (1·25 mg) was injected daily for seven days into mice, some bearing X-1 cells, others X-2. Although cortisone reduced cell-growth to one-third of the normal rate, it did not alter the levels of 5-HT, histamine or heparin or the incorporation of $S^{35}$-sulfate into heparin, in either cell-line. A much more potent steroid, the acetonide of triamcinolone (i.e., 9-$\gamma$-fluoro-16-$\gamma$-hydroxyprednisolone; Kenalog.®), a gift of the Squibb Institute for Medical Research in a dose of 0·2 mg/day was administered for two days to another group of mice bearing X-1 cells; this therapy result in a 50 per cent reduction in cell growth, but had no effect on the four parameters mentioned.

## DISCUSSION

It is clear that these neoplastic mast cells, like normal mast cells of the mouse and rat, not only store but produce 5-HT, histamine, and heparin. Normal rat mast cells *in vitro* decarboxylate 5-hydroxytryptophane[18] and histidine[19] while these neoplastic mast cells produce 5-HT from tryptophane when growing in culture and also as an homogenate of the mastocytoma.[8] Although direct evidence for the formation of histamine from histidine by these neoplastic cells is lacking, the presence of histamine in cells grown in a culture medium devoid of measurable amounts of histamine[4] strongly implies that they produce histamine from histidine, as has been shown in a canine mastocytoma.[20] While evidence that normal mast cells produce heparin is inferential, resting on the incorporation of sulfate[21, 22] and of glucose[23] into heparin in the intact animal, there is abundant evidence that neoplastic mast cells form heparin. As shown in this study the quantity of heparin in the culture medium is less

than 0·1 units and, thus it is far too low to account for the heparin-content of the cells. Moreover, the cell is unable to take up pre-formed heparin, though precursors of heparin viz., glucose, glucosamine, and sulfate, are incorporated into the sulfomucopolysaccharide by the cell in culture. Furthermore, slices of the tumor incorporate sulfate and glucose into heparin,[25] and homogenates[6, 24, 25] form 3′-phosphoadenosine-5′-phosphosulfate[26] and transfer its sulfate to heparin. Finally, the sulfate and glucosamine of heparin exchange at the same rate (Fig. 9), suggesting that the entire molecule of heparin is turning over, as in chondroitin sulfate,[27] rather than simply exchanging its sulfate and hexosamine. It follows from this that measurements of the incorporation into heparin of sulfate or glucosamine actually measure the synthesis of heparin, a conclusion supported by concomitant determinations of sulfate-uptake and heparin-concentration (e.g., Table 3). The turnover time of heparin of two-and-a-half days in these mast cells compares with three and one half days found in a dog liver[23] and contrasts, interestingly, with the ten-day turnover-time of chondroitin sulfate in rat skin.[27]

Failure to show the presence of heparin in the culture medium of actively growing and metabolizing cells suggests that mast cells, under these conditions, do not secrete heparin. There is no evidence that normal mast cells secrete heparin. [28]

Unlike glucose, glucosamine, and sulfate, glucuronate was not taken up by the mast cells. Its presence in heparin (see Walton[29]) is readily explained by oxidation of glucose to glucuronate.[30] Glucose was readily incorporated into heparin by both X-1 and X-2 cells at the same rate (Fig. 5), whereas X-1 cells in parallel experiments incorporated glucosamine (Fig. 6) more rapidly than X-2. The dilution of glucose-U-$C^{14}$ by the high levels of non-labeled glucose in the medium may explain this discrepancy.

Compared with the near-diploid X-2 cells, the polyploid X-1 cells are less virulent. They usually, though not always, contain greater amounts of 5-HT, heparin, and histamine and have a greater capacity to take up and to incorporate into heparin both $S^{35}$-sulfate and glucosamine-1-$C^{14}$. The fact that X-1 cells are larger may appear to be an explanation for their having higher levels of these compounds, but this is difficult to reconcile with the observation that X-2 cells occasionally show the same or greater amounts of these compounds than X-1 cells (e.g., Table 1). It is likely that factors other than cell size influence these differences. A possible clue to some of the factors controlling the levels of these compounds may lie in the observation that higher levels are obtained in culture than in the mouse.

Attempts to increase the levels of heparin, 5-HT, and histamine in the mast cells by injecting their known precursors into mice bearing the tumor were unsuccessful. Similarly, increasing the levels of these precursors in the culture medium did not increase the levels of the amines. It would appear, then, that nutritional factors are not limiting the synthesis of these compounds. Another factor that could explain the lowered levels of these compounds in mast cells grown *in vivo* is the presence of hormones inhibiting synthesis or storage or accelerating loss. Thus, administration of cortisone to rats inhibits both sulfate-uptake by mast cells[17] and histamine-binding by tissues,[16] but in the mouse, histamine-binding by tissues was not influenced by cortisone.[17] Experiments presented here show that the administration of cortisone (and triamcinolone acetonide), in amounts that inhibited cell growth did not affect the concentration of heparin, histamine or 5-HT in neoplastic mast cells in the mouse. Other hormones are known to inhibit the synthesis of sulfomucopolysaccharides.[17]

One of these hormonal factors could explain the higher levels found in culture and perhaps also the wide variation of the three substances found from one experiment to another. Whatever these factors may be, they need not necessarily influence directly the synthesis of 5-HT and histamine, but instead may control the levels of heparin which, in turn, may determine the limits of histamine and 5-HT which can be bound by the cell.

This suggestion arises in part from the observation that in every experiment in which levels of heparin and the amines were measured concomitantly, the concentration of amines, especially that of 5-HT, was found to be proportional to the concentration of heparin (Tables 2, 3, 4). Moreover, the three compounds were found to be associated intracellularly: in the experiments reported here, with the large granule fractions (Table 5) and in the experiments of Hagen *et al.*[31] with a specific granule fraction. The fact that the solid mastocytoma in the experiments described here contained cells other than mast cells (as contrasted with a population purely of mast cells when cells are grown in the ascitic form or in culture) does not invalidate the conclusion that the three compounds are associated in the same cellular fraction.

Riley and West[32] first noted the association of histamine and heparin in mast cells. This association may not be restricted to mast cells, for basophils contain histamine[33] and heparin (or a heparin-like compound),[34, 35] as do platelets of some species.[36, 37] The idea that histamine may be linked to heparin stems from (a) the proportionality of the two compounds in mast cells,[28, 38] (b) studies on histamine-release,[28, 38, 43] and (c) the observation that heparin and histamine can, *in vitro*, form a complex.[44–46] Although the possibility remains that the association of histamine with heparin is fortuitous or attributable to yet a third unknown factor, the data presented here are consistent with the idea that histamine may be linked to heparin in the mast cell.

In the test tube, histamine has an affinity for heparin which is about four times greater than that of 5-HT for heparin.[45] Yet, the amount of 5-HT in the mast cell is more closely proportional to the amount of heparin than is the amount of histamine. This relationship did not obtain in human platelets.[47] Instead, in these cells, the amount of 5-HT is proportional to the amount of adenosine triphosphate (ATP).[48] It is interesting to note that in buffered solutions, phosphate ions (and ATP) increased the affinity of histamine for heparin;[46] thus, it is possible that ATP may also be concerned in binding of amines in the mast cells. On the other hand, the mechanism of binding of amines may differ in different cells. Indeed, Mota and his associates[49] have noted that histamine is found in organs that lack mast cells and, although the amine may be bound to a sulfomucopolysaccharide like heparin, the fact that it is not released by compound 48/80 may indicate a different kind of binding. Similarly, reserpine releases histamine as well as 5-HT from both mast cells and rabbit platelets but has little or no effect on the histamine content of organs.[50] Moreover, Schayer[51] has shown that the capacity of a variety of organs to bind exogenous histamine differs from their content of histamine. Analogously, the platelet may bind 5-HT to a compound different from that by which it is held in the mast cell (or in the rabbit platelet).

The ease of extractability of histamine[41] and of 5-HT from tissues suggests that the amines are loosely bound, perhaps in an ionic type of linkage. Heparin has free sulfate and carboxyl groups as sites for such binding. If the sulfur-content of the heparin of mast cells is assumed to be 10 per cent, as in bovine heparin,[29] the number of sulfate groups in the mast cells are insufficient to account for the binding of amines (there are

about twice as many moles of amine as sulfate). There are several possible explanations for this discrepancy: (a) the carboxyl groups of heparin also bind amines; (b) the amines are held non-ionically; (c) only one of the amines is linked to heparin; (d) the heparin in the mastocytoma has a larger number of sulfate groups than bovine heparin or differs in some other way that would increase its affinity for amines.

That the mast-cell heparin is unusual is suggested by paper chromatography. The heparin-extract was resolved into three compounds only one of which was correlated with the cellular levels of the amines, especially of 5-HT (Table 4). This component was not present in chromatograms of bovine heparin. Thus, a unique heparin in the mast cells of the mouse may account for the capacity of these cells to bind large amounts of amines. If also present in the mast cells of the rat, it may explain the apparently anomalous presence of 5-HT in the mast cells of this species[18] as well as of the mouse.

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## REFERENCES

1. S. M. DAY and J. P. GREEN, *Fed. Proc.* **18**, 381 (1959).
2. T. B. DUNN and M. POTTER, *J. Nat. Cancer Inst.* **18**, 587 (1957).
3. A. SJOERDSMA, T. P. WAALKES and H. WEISSBACH, *Science* **125**, 1202 (1957).
4. R. SCHINDLER, M. DAY and G. A. FISCHER, *Cancer Res.* **19**, 47 (1959).
5. E. D. KORN, *J. Biol. Chem.* **234**, 1321 (1959).
6. L. SPOLTER and W. MARX, *Biochem. Biophys. Acta* **32**, 291 (1959).
7. J. P. GREEN, *Nature*, in press, (1960).
8. R. SCHINDLER, *Biochem. Pharmacol.* **1**, 323 (1958).
9. G. A. FISCHER, *Cancer Res.* **19**, 327 (1959).
10. A. S. MARKOWITZ and J. R. HENDERSON, *Nature* **181**, 771 (1958).
11. L. B. JAQUES, F. C. MONKHOUSE and M. J. STEWART, *J. Physiol.* **109**, 41 (1949).
12. I. SMITH, *Chromatographic Techniques*, p. 169. Heinemann and Co., London (1958).
13. W. C. SCHNEIDER and G. H. HOGEBOOM, *J. Biol. Chem.* **183**, 123 (1950).
14. B. C. BERA, A. B. FOSTER and M. STACEY, *J. Chem. Soc.* 3788, (1955).
15. D. D. DZIEWIATKOWSKI, *J. Exp. Med.* **98**, 119 (1953).
16. R. W. SHAYER, R. L. SMILEY and K. J. DAVIS, *Proc. Soc. Exp. Biol.* N.Y. **87**, 590 (1954).
17. G. ASBOE-HANSEN, *Physiol. Rev.* **38**, 446 (1958).
18. D. LAGUNOFF and E. P. BENDITT, *Amer. J. Physiol.* **196**, 993 (1959).
19. A. M. ROTHSCHILD and R. W. SCHAYER, *Biochem. Biophys. Acta*, London, **34**, 392 (1959).
20. S. E. LINDELL, H. RORSMAN and H. WESTLING, *Experientia* **15**, 31 (1959).
21. S. MAGNUSSON and B. LARSSON, *Acta. Chem. Scand.* **9**, 534 (1955).
22. H. B. EIBER and I. DANISHEFSKY, *J. Biol. Chem.* **226**, 721 (1957).
23. H. B. EIBER and I. DANISHEFSKY, *Arch. Int. Med.* **102**, 189 (1958).
24. E. D. KORN, *J. Biol. Chem.* **234**, 1647 (1959).
25. C. A. PASTERNAK, *J. Biol. Chem.* 235, 438 (1960).
26. F. LIPMANN, *Science* **128**, 575 (1958).
27. S. SCHILLER, M. B. MATHEWS, J. A. CIFONELLI and A. DORFMAN, *J. Biol. Chem.* **218**, 139 (1956).
28. G. B. WEST, *J. Pharm., Lond.* **11**, 313 (1959).
29. K. W. WALTON, *Brit. Med. Bull.* **11**, 62 (1955).
30. J. L. STROMINGER, E. S. MAXWELL, J. AXELROD and H. M. KALCKAR, *J. Biol. Chem.* **224**, 79 (1957).
31. P. HAGEN, R. J. BARRNETT and F. LEE, *J. Pharmacol.* **126**, 91 (1959).
32. J. F. RILEY and G. B. WEST, *J. Physiol.* **120**, 528 (1953).

33. H. T. Graham, M. A. Lenz, O. H. Lowry, H. H. Parish and F. Wheelwright, *Blood* **10**, 467 (1955).
34. H. Martin and L. Roka, *Acta Haemat.* **10**, 26 (1953).
35. A. W. A. Boseila and G. Asboe-Hansen, *Proc. Soc. Exp. Biol.* N.Y. **101**, 198 (1959).
36. J. H. Humphrey and R. Jaques, *J. Physiol.* **124**, 305 (1954).
37. T. T. Odell and B. Anderson, *Proc. Soc. Exp. Biol.* N.Y., **94**, 151 (1957).
38. J. F. Riley, The Mast Cells, p. 85, 93, E. and S. Livingstone, London (1959).
39. W. D. M. Paton, *Pharmacol. Rev.* **9**, 269 (1957).
40. F. C. MacIntosh, in *Ciba Symposium on Histamine*, p. 20. Little, Brown and Co., New York (1956).
41. F. C. McIntyre, In *Ciba Symposium on Histamine*, p. 170. Little, Brown and Co., New York (1956).
42. R. D. Higginbotham, *Ann. N.Y. Acad. Sci.* **73**, 186 (1958).
43. F. J. McIlreath, *Canad. J. Biochem.* **37**, 151 (1959).
44. E. Werle and R. Amann, *Klin. Wschr.* **34**, 624 (1956).
45. R. Keller, *Arzneitmitt. Forsch.* **8**, 390 (1958).
46. R. K. Sanyal and G. B. West, *J. Pharm.* **11**, 548 (1959).
47. G. P. Kerby and S. M. Taylor, *J. Clin. Invest.* **38**, 1059 (1959).
48. G. V. R. Born, G. I. C. Ingram and R. S. Stacey, *Brit. J. Pharmacol.* **13**, 63 (1958).
49. I. Mota, W. T. Beraldo, A. G. Ferri and L. C. U. Junquiera, in *Ciba Symposium on Histamine* p. 47. Little, Brown and Co., New York (1956).
50. T. P. Waalkes, H. Coburn and L. T. Terry, *J. Allergy* **30**, 408 (1959).
51. R. W. Schayer, *Amer. J. Physiol.* **187**, 63 (1956).