ACTA CHEMICA SCANDINAVICA 14 (1960) 312–320

Acid Polysaccharides of two Mast Cell Tumors in Mice

NILS R. RINGERTZ

Department of Cell Research and Genetics, Karolinska Institutet, Stockholm 60, Sweden

Polysaccharides prepared from two mast cell tumors in mice were fractionated by chromatography on ECTEOLA cellulose. Both tumors contained heparin and also large quantities of other polysaccharides. These were present in both solid and ascites sublines of the tumors. Polysaccharide fractions from one of the tumors were analyzed and found to differ from each other in sulfate content, anticoagulant activity, and type of amino sugar present.

The first indication of the chemical nature of the metachromatic component of the mast cell granules came from the findings of Jorpes [1] that heparin contained sulfate groups and gave a strong metachromatic reaction with toluidine blue. Subsequent chemical and histological studies by Jorpes, Holmgren and Wilander [2] disclosed a close relationship between the heparin content of various tissues and the number of mast cells found therein indicating that heparin was present in the mast cells.

The histochemical significance of the metachromatic staining reaction was also pointed out by Lison [3], who showed that high molecular polysulfuric acid esters gave such a reaction.

The observations made by Jorpes, Holmgren and Wilander [2] received further support by the finding of Oliver *et al.*[4] in 1947 that a dog mast cell tumor of dermal origin contained large quantities of heparin. During the last decade the heparin content of mast cells has been further substantiated by the work of a number of authors [5,6].

In spite of the fact that several authors [7–9], have advanced theories about the participation of mast cells in the production of the metachromatic ground substance of connective tissue, little is known if other polysaccharides are present in mast cells.

Data obtained by Magnusson and Larsson [10] on a dog mastocytoma and by Korn [11] and Rodén [12] on mast cell tumors in mice, indicate that besides heparin these neoplastic mast cells also contain other polysaccharides with lower sulfate content, similar to heparin monosulfuric acid [13] and chondroitin sulfuric acids.

The aim of the present investigation was to study the total polysaccharide content of two types of mast cell tumors in order to obtain a basis for further studies on the biosynthesis of heparin in the tumor mast cells.

MATERIAL AND METHODS

Tumors. Two mast cell tumors of independent origin were used in the present investigation. One tumor was obtained in July 1957 in its 23rd transplant generation from Dr. Thelma Dunn [14], National Cancer Institute, Bethesda, Maryland, USA. It has been maintained in this laboratory by routine transplantation every 14—20 days to inbred dba, dba F_1, or dba back cross mice as a subcutaneous tumor (DMS). The tumor yield was 1—2 g (wet weight)/mouse. The tumor was easily transformed to the ascites form (DMA) upon intraperitoneal inoculation. A maximum of 1.5 ml ascites was obtained from each mouse. In these fluids 85 % of the cell population were tumor cells.

The second tumor was obtained in March 1958 from Dr. J. Furth [15], Children's Cancer Research Foundation, Harvard Medical School, Boston, Mass., USA. It has been kept in our laboratory as a solid, subcutaneous tumor in LAF_1 mice (FMS). When grown as a subcutaneous tumor a maximum of 8—17 g (wet weight) of tumor/mouse was obtained 4—8 months after inoculation. Often, however, these large tumors showed central necrotic areas and the tumors were therefore usually taken at the size of 4—6 g (*i. e.* 2—4 months after inoculation). When grown intraperitoneally 0.5—1.5 ml of ascites containing 60—80 % tumor cells was obtained from each mouse (FMA). This tumor also produced large solid infiltrates in the peritoneum and in the mesenteries. The intraperitoneal solid tumor mass thus obtained could to a large degree be dissected free from other tissues. The histology of the tumors was studied by routine examination of toluidine blue stained sections and smears.

Preparation of polysaccharide. The tumors were cut into small pieces and homogenized in 2—3 volumes of 0.1 M phosphate buffer, pH 7.8. The homogenate was boiled for 5 min and then digested with a pancreatic extract [16] (Pankreatin, Merck, Darmstadt, Germany). The digestion was performed with 10 mg of enzyme/g wet tissue for 24—48 h.

In some experiments, where a higher degree of purity of the polysaccharide material was desired, the homogenate was first digested with a crude papain preparation (10—30 mg/g wet tissue) for 16 h at 65° in 0.1 M phosphate buffer pH 6.2 containing 0.3 M NaCl, 0.005 M cysteine hydrochloride and 0.0005 M versene [17]. After digestion the preparation was precipitated with 3—4 volumes of ethanol. The precipitate was washed twice with 75 % ethanol and then dried at 50°C in a vacuum. After this the material was digested over night with a pancreatic extract as described above.

After digestion the material was boiled for 5—10 min and centrifuged. The precipitate was washed with distilled water and the combined supernatants were precipitated with 3—4 volumes of ethanol. The precipitate was washed with 75 % ethanol, redissolved in water and acidified at 0°C by the addition of 4 N perchloric acid. The precipitate was washed repeatedly with cold 0.1 N perchloric acid. The combined supernatants were neutralized with KOH and after standing for 1 h the precipitate was removed by centrifugation. The supernatant was then made 0.05 M with respect to Cl^- by the addition of HCl—NaCl (1:1).

Chromatography on ECTEOLA cellulose [18,19]. For the separation of 400 mg of polysaccharide, an ECTEOLA column 4 × 7 cm was used. For smaller quantities a correspondingly thinner column was used. Step-wise chromatography was performed with 250—300 ml of eluent for each step with a 4 × 7 cm column. Fractions were collected at 50 ml/30 min. For elution of the columns, chloride buffers containing equimolar amounts of HCl and NaCl were used. The chloride concentrations in the different elution steps were 0.1, 1.1, 1.4, 1.7 and 2.5 M, respectively. The columns were finally washed with 0.5 M NaOH. This sometimes eluted materials giving a positive carbazol reaction. The absorption spectra for these materials in the carbazol reaction, however, were not those expected for uronic acid containing compounds. These fractions were not studied further.

The chloride concentrations in the different elution steps were chosen on the basis of previous model experiments with known polysaccharides. The fractions were analyzed by the carbazol reaction [20]. Salt was removed from the effluent either by dialysis or by gel filtration on Sephadex G-25 (Pharmacia, Uppsala, Sweden) [21].

Analytical methods. Total amino sugars were determined by the Elson-Morgan reaction [22] after hydrolysis for 8 h in 6 N HCl at 100°C. Glucosamine and galactosamine were determined separately by the method of Gardell [23]. The identity of the amino sugars was checked by paper chromatography according to Gardell *et al.* [24] as modified by Stoffyn and Jeanloz [25]. Uronic acid was estimated by the carbazol reaction [20] using glucuronic acid

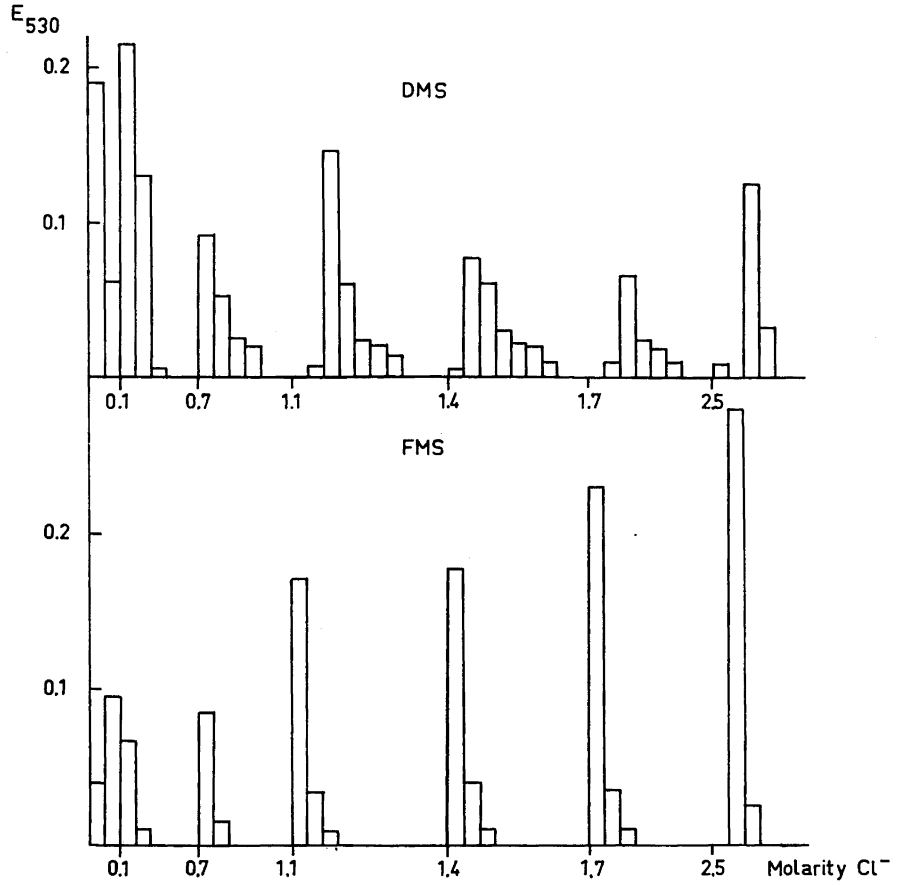

Fig. 1. Stepwise chromatography on ECTEOLA columns of polysaccharides from "pankreatin"-digested DMS and FMS subcutaneous tumors.

as standard. Nitrogen was determined according to Kjeldahl and sulfate by a benzidine method [26,27]. Optical rotation was measured on 1 % solutions in a Zeiss poalrimeter using 10 cm cuvettes.

RESULTS AND DISCUSSION

Chromatographic patterns. Fig. 1 shows the chromatograms obtained with polysaccharides from subcutaneous DMS and FMS tumors. It can be seen, that when the chloride concentration of the eluent was changed, carbazol reacting material was eluted in both chromatograms. The elution steps were chosen so that hyaluronic acid was expected to be completely eluted with the 0.1 M fraction, chondroitin sulfuric acid A and B mainly with the 1.1 M fraction, and heparin mainly with the 2.5 M fraction. From both tumors, carbazol

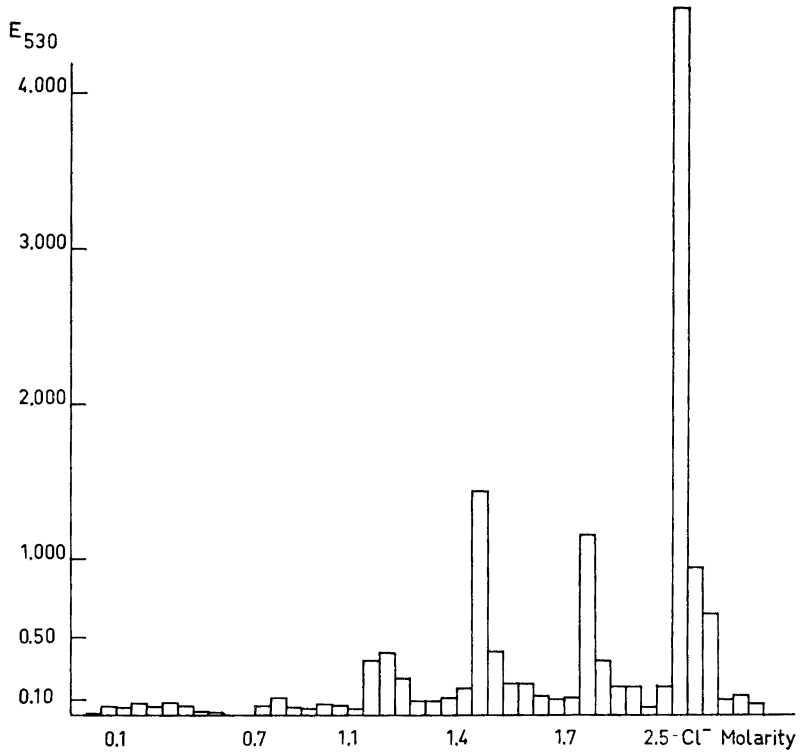

Fig. 2. Stepwise chromatography on an ECTEOLA column (4 × 8 cm) of 450 mg of polysaccharide from "pankreatin" digested solid intraperitoneal FMS tumors. Fractions: 50 ml/30 min.

reacting materials were eluted at the position of heparin, but it was also obvious that both tumors contained large quantities of other polysaccharides. The maximum total quantity of polysaccharides obtained from the FMS tumor was 3—4 mg/g wet weight of tumor tissue. The corresponding value for the DMS tumor was only about 0.5—1.0 mg.

The chromatographic patterns obtained with polysaccharide from the DMS and FMS tumors differed somewhat as to the relative quantity of carbazol reacting material eluted in the different fractions but otherwise showed great similarity. With the FMS tumor the chromatographic patterns varied with the site of growth. With intraperitoneal solid tumors relatively more polysaccharide was eluted at higher chloride concentrations than with the subcutaneous tumors (*cf.* Figs. 1 and 2).

In order to investigate whether the main part of the polysaccharides found in the solid tumors originated from the tumor cells and not from blood vessels and connective tissue, the polysaccharide content of ascites sublines of the two tumors was studied. The chromatogram obtained during the separa-

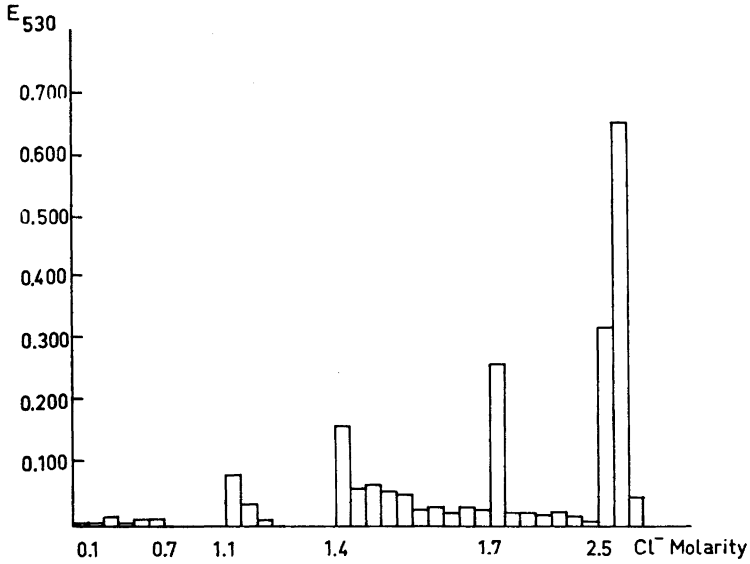

Fig. 3. Stepwise chromatography on an ECTEOLA column (2 × 8 cm) of 70 mg of polysaccharide from "pankreatin" digested FMA ascites tumor. Fractions: 8 ml/30 min.

tion of polysaccharide from the ascites line of the FMS tumor, (FMA), (Fig. 3) was closely similar to that obtained from the solid intraperitoneal tumors in the same animals (Fig. 2).

The same results were obtained with the DMS and DMA tumors except for the 1.1 M fraction, which in the DMA tumor was practically free from carbazol reacting material.

Analyses of the amino sugar type present in the unfractionated FMS and DMS polysaccharides showed that 20—40 % and 24 %, respectively, of the total amino sugars was galactosamine, the remainder being glucosamine. In the unfractionated FMA polysaccharides < 10 % of the amino sugars was galactosamine.

The results indicate that a major part of the non-heparin polysaccharides come from the tumor mast cells while the polysaccharide quantity contributed by the blood vessels and connective tissue in the solid tumors is small.

In order to obtain some information on the chemical nature of the polysaccharides prepared from the mast cell tumors the fractions obtained from the FMS tumor were analyzed. It was found difficult to obtain enough material from the DMS line because of the small size of the tumors and their low polysaccharide content.

Analysis of the 0.1 M fraction. This fraction showed a strong ultraviolet absorption at 260 and 280 mμ and gave an absorption spectrum in the carbazol reaction which was not typical of uronic acids (Fig. 4).

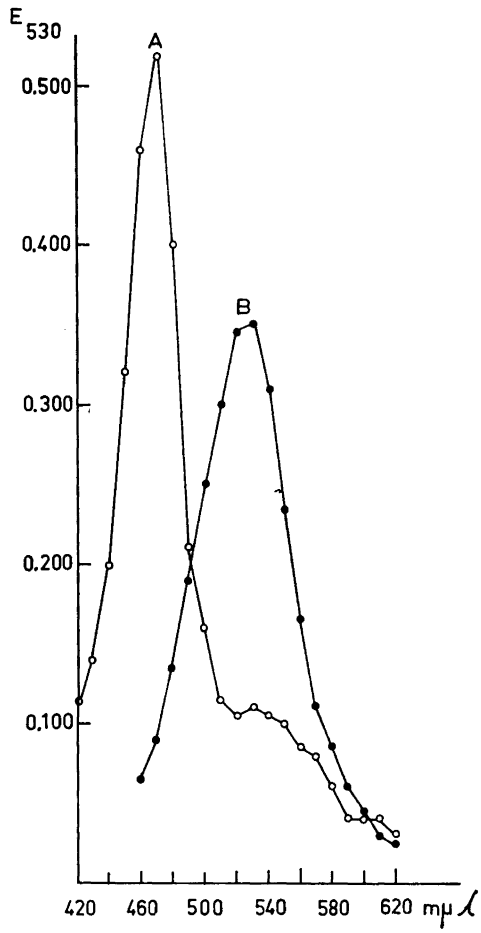

Fig. 4. Carbazol spectra for the 0.1 M fraction (A) and for the 1.1 M fraction (B) obtained in an ECTEOLA chromatogram of the type shown in Fig. 1 (FMS). Curve B is the spectrum obtained for hexuronic acid-containing polysaccharides.

In order to obtain further information about the carbazol reacting material eluted in the 0.1 M fraction, the material obtained from a chromatogram of about 400 mg of FMS tumor polysaccharide (120 g wet tumor tissue) was neutralized to pH 7 with 1 N NaOH and concentrated to 58 ml. From this material, 2 ml were added to a 2 × 14 cm Sephadex column [21]. The column was eluted with distilled water, 1.5 ml fractions being collected at 30 sec intervals. Two components giving carbazol reactions were found (Fig. 5). The main component was eluted after the void volume of the column and gave a typical uronic acid spectrum in the carbazol reaction. A smaller component was eluted close to the salt peak. In its last fractions it contained material giving a maximum at 470 mμ in the carbazol reaction. The main component contained amino sugar and traces of sulfate. The smaller component was not studied further.

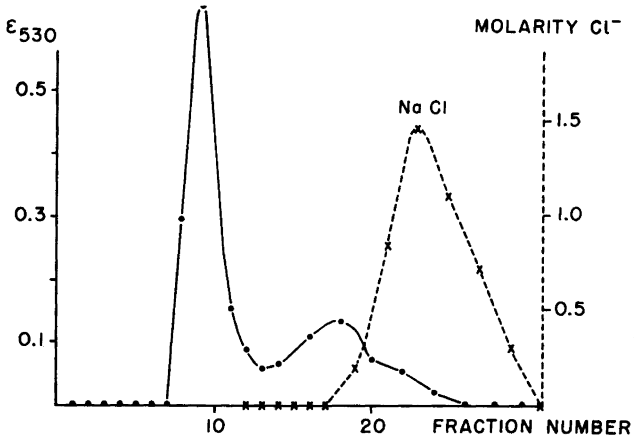

Fig. 5. Fractionation on a Sephadex gel column (2 × 14 cm) of material (= 0.7 mg of glucuronic acid) from the 0.1 M ECTEOLA fraction. The main component is eluted after the void volume of the column indicating a high molecular weight.

A similar separation into two components was obtained also by gradient chromatography on ECTEOLA at pH 5.0 [19]. The major component was eluted between 0.20 and 0.33 M Cl^- and the smaller component between 0.40 and 0.55 M Cl^-. In this chromatographic system, hyaluronic acid, prepared by different methods from cock's comb, bovine vitreus and a human mesothelioma was eluted as sharp peaks between 0.40 and 0.50 M Cl^-.

The results indicate the presence in the 0.1 M fraction of a high molecular polysaccharide with little or no sulfate and with a chromatographic behaviour different from that of high molecular hyaluronic acid.

Analysis of the 0.7—2.5 M fractions. Table 1 shows some analytical data for FMS polysaccharides eluted at higher salt concentrations. The results show that all fractions contained uronic acid, amino sugar and varying quantities of sulfate. The analytical data indicate that the fractions were contaminated with non-polysaccharide material. The nitrogen analyses are in excess of what can be expected from the amino sugar determinations suggesting that in the 1.1—2.5 M fractions 1—5 %, and in the 0.7 M fraction about 17 % of the dry weight may be due to contaminating protein.

The method used for the estimation of uronic acid is known to give values deviating from those obtained by decarboxylation methods. Heparin is thus known to give values about 50 % higher than theory [20]. As the nature of the uronic acid moiety of the different polysaccharide fractions is not known, exact calculations of the ratio of uronic acid to amino sugar is impossible. The values in Table 1, calculated on the basis of glucuronic acid standards indicate, however, that the 1.1—2.5 M fractions contained equimolar quantities of amino sugar and uronic acid.

The amino sugars in the different fractions were isolated by ion exchange chromatography according to Gardell [23]. Glucosamine and galactosamine were identified by their effluent volumes in ion exchange chromatography

Table 1. Analytical data for FMS tumor polysaccharide fractions. The values are for dry polysaccharides.

	Fraction				
	0.7 M	1.1 M	1.4 M	1.7 M	2.5 M
Amino sugar %	15.7	27.6	23.5	37.3	27.6
Galactosamine, % of total amino sugar	*	73	56	20	14
Glucosamine, % of total amino sugar	*	27	44	80	86
Uronic acid	9.8	20.0	20.1	36.5	36.5
SO_4/amino sugar, molar ratio	—	0.98	1.02	1.50	2.20
Nitrogen, %	3.91	2.36	2.64	3.28	2.34
Anticoagulant activity, U/mg	0	0	6	55	100
$[\alpha]_D^{22}$	—	+ 1°	+ 1°	+ 39°	+ 35°

and by paper chromatography after degradation with ninhydrin to arabinose and lyxose, respectively [25].

The 1.1 and 1.4 M fractions contained glucosamine and galactosamine in about equal quantities. In the 1.7 M and 2.5 M fractions glucosamine dominated over galactosamine. In the 0.7 M fraction paper chromatography indicated the presence of both amino sugars.

The fractions eluted at lower salt concentration in the chromatogram contained less sulfate than the fractions eluted later in the chromatogram. The 1.1 and 1.4 M fractions contained about 1 mole of sulfate per amino sugar molecule. The 2.5 M fraction regularly contained more than 2 sulfate groups per molecule of amino sugar. The 1.7 M fraction gave intermediate values varying between 1.4 and 1.6 sulfate groups per amino sugar.

The 1.7 and 2.5 M fractions showed marked anticoagulant activity when tested according to Studer and Winterstein [28]. These fractions also showed a strong positive optical rotation while the 1.1 and 1.4 M fractions showed no optical activity.

The analytical data show that the 2.5 and 1.7 M fractions contain heparin. The absolute value for the anticoagulant activity must, however, be interpreted with great caution as the *in vitro* assay system is based on bovine plasma and the polysaccharides tested derive from another species.

The nature of the polysaccharides eluted in the 0.7—1.4 M fractions is not altogether clear, but the results strongly suggest that these fractions may contain mixtures of heparin-like polysaccharides with a low sulfate content, and chondroitin sulfuric acid.

The finding of other polysaccharides than heparin in mast cell tumors raises the question whether these polysaccharides are present in normal mast cells. The results obtained by Schiller and Dorfman [29] indicate that heparin is the only major polysaccharide present in peritoneal mast cells in the rat. Using a micromodification of the ECTEOLA method, we [30] have obtained data on the same material which support the results of Schiller and Dorfman. The polysaccharide content of mouse mast cells is being studied.

* Identified by paper chromatography only.

Acknowledgements. The author is indebted to Drs. E. A. Balazs, T. Dunn, J. Furth, T. Laurent and J. Scott for generous gifts of tumors and of hyaluronic acid samples and to Dr. H. Danielsson for help with the determinations of optical activity.

REFERENCES

1. Jorpes, J. E. *Acta Med. Scand.* **88** (1936) 427.
2. Jorpes, J. E., Holmgren, H. and Wilander, O. *Z. mikroskop. anat. Forsch.* **42** (1937) 279.
3. Lison, L. *Arch. biol. Liége* **46** (1935) 599.
4. Oliver, J., Bloom, F. and Mangieri, C. *J. Exptl. Med.* **86** (1947) 107.
5. Riley, J. F. *The Mast Cells*, E. and F. Livingstone Ltd. Edinburgh 1959.
6. *A guide to the Literature on Heparin and Anticoagulant Therapy in Thrombosis.* Ed. Vitrum, Stockholm 1959.
7. Staemmler, M. *Frankfurt. Z. Pathol.* **25** (1921) 391.
8. Sylvén, B. *Acta Chir. Scand.* **87** (1941) Suppl. 66.
9. Asboe-Hansen, G. *Intern. Rev. Cytol.* **33** (1954) 399.
10. Magnusson, S. and Larsson, B. *Acta Chem. Scand.* **9** (1955) 534.
11. Korn, E. D. *J. Am. Chem. Soc.* **80** (1958) 1520.
12. Rodén, L. and Dorfman, A. *To be published.*
13. Jorpes, J. E. and Gardell, S. *J. Biol. Chem.* **176** (1948) 267.
14. Dunn, T. B. and Potter, M. *J. Natl. Cancer Inst.* **18** (1957) 587.
15. Furth, J., Hagen, P. and Hirsch, E. I. *Proc. Soc. Exptl. Biol. Med.* **95** (1957) 824.
16. Korn, E. D. *J. Biol. Chem.* **234** (1959) 1325.
17. Scott, J. E. in Glick, D., Ed. *Methods of Biochemical Analysis*, Interscience Publishers Inc. New York. *To be published.*
18. Ringertz, N. R and Reichard, P. *Acta Chem. Scand.* **13** (1959) 1467.
19. Ringertz, N. R. and Reichard, P. *Acta Chem. Scand. In print.*
20. Dische, Z. *J. Biol. Chem.* **167** (1947) 189.
21. Porath, J. and Flodin, P. *Nature* **183** (1959) 1657.
22. Blix, G. *Acta Chem. Scand.* **2** (1948) 467.
23. Gardell, S. *Acta Chem. Scand.* **7** (1953) 207.
24. Gardell, S., Heijkenskjöld, F. and Roch-Nordlund, A. *Acta Chem. Scand.* **4** (1950) 970.
25. Stoffyn, P. J. and Jeanloz, R. W. *Arch. Biochem. Biophys.* **52** (1954) 373.
26. Andersson, L. *Acta Chem. Scand.* **7** (1953) 689.
27. Balazs, E. A. *Unpublished.*
28. Studer, A. and Winterstein, A. *Helv. Physiol. et Pharmacol. Acta* **9** (1951) 6.
29. Schiller, S. and Dorfman, A. *Biochem. et Biophys. Acta* **31** (1959) 278.
30. Bloom, G. and Ringertz, N. R. *Arkiv Kemi. In press.*

Received December 23, 1959.