A STUDY OF ACID MUCOPOLYSACCHARIDES OF BOVINE AORTA WITH THE AID OF A CHROMATOGRAPHIC PROCEDURE FOR SEPARATING SULFATED MUCOPOLYSACCHARIDES*

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Interest in the ground substance of cardiovascular structures has been stimulated over the past few years by the advancement of biochemical techniques. These techniques offer a good opportunity to study the acid mucopolysaccharides (MPS) from connective tissue and to investigate the nature of the substances in cardiovascular structures. Since there is limited information concerning the characteristics of the MPS in the aorta, a restudy of aortic tissue seemed warranted.

The presence of sulfated MPS in the aorta has been known for some time[1]. Earlier observations by MEYER AND RAPPORT[2] indicated the presence of two sulfated MPS, chondroitin sulfuric acid (CSA) C and B, with the absence of hyaluronic acid (HA); however, more recent studies[3] which have appeared during the course of these investigations indicate the presence of a family of CSA compounds and of HA. Another recent study[4] further suggested the presence of MPS in the aorta containing both glucosamine and galactosamine, but did not completely characterize the compounds isolated.

The following studies describe the isolation of a mixture of acid MPS from bovine aorta which was resolved into three compounds with the aid of zone electrophoresis and column chromatography. These studies also give evidence of another compound not yet isolated in a pure state. The chromatographic procedure, which was developed for the purpose of separating the CSA compounds, was found in addition to fractionate a mixture that occurs in a commercial preparation of α-heparin. One of the compounds isolated from the α-heparin, β-heparin, was similar to or identical with a fraction obtained from aorta.

EXPERIMENTAL

Preparation of MPS mixture from bovine aorta

Bovine aortas were obtained fresh from a local abattoir. The aortas, dissected free of excess fat and connective tissue, were ground, defatted with acetone, air dried, and were stored at —20° until used for extraction. The procedure for isolation of the crude MPS was similar to the method used to isolate MPS from rabbit skin[5]. A graphic summary of the procedure for isolating the crude MPS fraction from the aorta and resolution into component compounds is presented in Table I. Briefly, the procedure for isolating the MPS mixture consisted of the following: the dried-defatted aortic tissue was extracted with 2% NaOH (15 ml/g) by vigorous shaking overnight and reextracted by approximately 1/3 original volume of NaOH. The alkali-extracts were pooled,

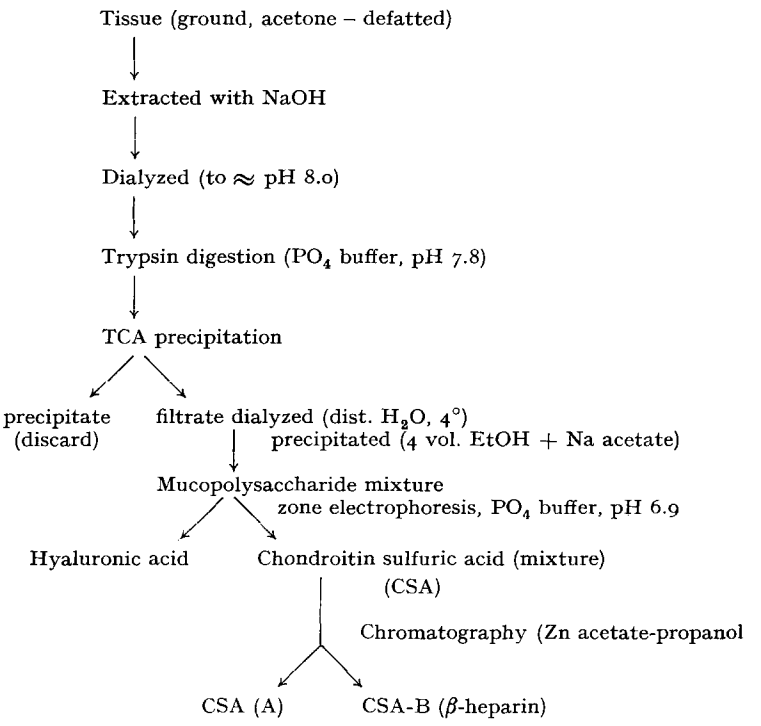
* These studies were aided by grants from the American Heart Association and the National Heart Institute of the Public Health Service (H-2942).

References p. 183.

dialyzed against tap water and digested with trypsin (2.5 mg/g of protein) in PO_4 buffer, pH 7.8 at 38° for 5 days. A 10% concentration of trichloracetic acid was made and the solution filtered clear. This solution after dialysis against distilled H_2O at 4° was refiltered and concentrated. Na acetate was added to make a 1% solution and 4 volumes of alcohol were used to precipitate the polysaccharide. The precipitate, crude MPS mixture, was centrifuged, washed with alcohol, ether and dried under *vacuo*. The yield was approximately 1.54 g/100 g of dried aortic tissue and analyzed 4.1% N, 13.0% hexosamine and 12.4% uronic acid.

TABLE I

METHOD OF ISOLATION OF ACID MUCOPOLYSACCHARIDES FROM AORTA



Resolution of MPS into two components by electrophoresis

The crude MPS was redissolved in water and was separated into two components by zone electrophoresis on celite (Johns-Manville, Analytical Filter Aid). The separated fractions were recovered by displacement from the celite with water and analyzed for uronic acid[6]. Selected fractions were pooled, dialyzed and the polysaccharide precipitated with alcohol and sodium acetate as described above. An illustration of electrophoresis of MPS from aorta is shown in Fig. 1. Two groups of compounds were separated with mobilities resembling HA and CSA, the CSA compounds accounting for approximately 65% of the material isolated.

Chromatography of the sulfated MPS

The analyses of material isolated as CSA by electrophoresis suggested it to be a mixture. Since other studies[2,3] showed the presence of more than one CSA compound in aorta, the development of a column-chromatographic procedure to fractionate this mixture was attempted. The isolation of β-heparin[7], a substance that may be identical to CSA-B[3,5], with zinc acetate-*n*-propanol fractionation suggested a solvent system for a chromatographic procedure.

Preliminary experiments. A series of preliminary experiments was performed to determine conditions for making the column and achieving fractionation of the sulfated MPS. Alkali-extracted CSA-A of bovine cartilage, which was prepared by a method previously described[8], served as polysaccharide material to determine conditions for the chromatography. Columns of

References p. 183.

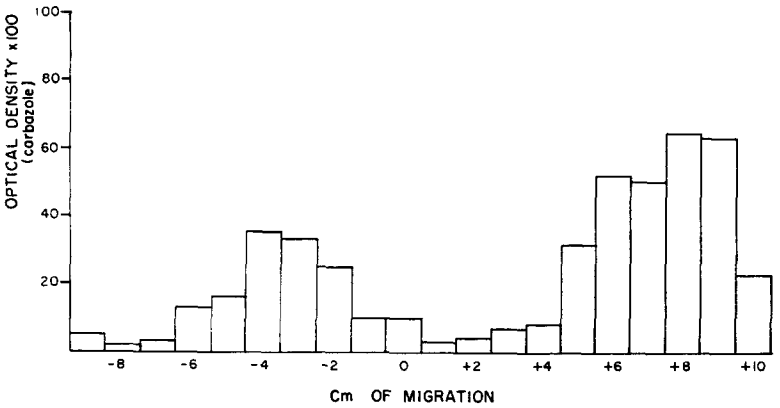


Fig. 1. An example of zone electrophoresis on celite of the mixture of MPS extracted from bovine aorta. The bars represent approximate concentrations of MPS in eluents from cm sections of the celite slab. The direction of migration represented a resultant movement due to both mobility of the compounds and electroosmosis of the buffer. Selected fractions were pooled and the MPS isolated for further study. Electrophoretically two fractions were isolated, each fraction containing a mixture of compounds. Refer to text for further details.

celite (Johns-Manville No. 545) were found to retain MPS whereas cellulose powder alone failed to do so; however, mixtures of celite and cellulose were used since there was a decreased tendency of channeling during elution. Variations in concentration of zinc acetate (0.01 and 0.1 *M*) produced similar fractionation, but the higher concentrations seemed to enhance retention of polysaccharide by the column.

Preparation and elution of columns. 35 g of celite and 20 g of a coarse grade of Whatman ashless cellulose powder were mixed into a slurry with a solution of 0.1 *M* zinc acetate–*n*-propanol in a ratio of 45/55 (v/v). These materials were then poured to form a column 4.7 cm in diameter and 11.0 cm in length. When the column was allowed to remain overnight, a more uniform flow rate seemed to result, and a flow rate of approximately 3 ml/min was maintained. Approximately 200 mg of the bovine aorta-CSA mixture obtained by isolation after electrophoresis was placed on the column as a water–propanol solution. Fractionation was accomplished by elution with 500 to 750 ml of 0.1 *M* zinc acetate–*n*-propanol by stepwise increase in the proportion of zinc acetate solution. The solutions of zinc acetate–*n*-propanol were filtered before use on the column.

Isolation of MPS following chromatography. Aliquots of 1 ml of the eluent fluids were analyzed for the presence of MPS by addition of 1 ml of acidified bovine albumin solution (0.1 % in 0.1 *M* acetate buffer at pH 3.75), and the resulting turbidities were read in a colorimeter after 5 min at 620 mμ[9]. These determinations were related to a standard curve, obtained with cartilage CSA-A and served as an approximation of the concentration of the different acid MPS and as a simple and rapid means of their detection.

The MPS were isolated by pooling selected fractions from the column. The pooled solutions were dialyzed against distilled water at 4°, after which they were concentrated and the MPS precipitated. Traces of celite or other insoluble material were eliminated by redissolving in water, centrifuging and recovering the MPS from the supernatant.

Since the chromatographic procedure was found to separate the sulfated material of the bovine aorta, fractionation of a commercially available α-heparin* was also attempted. The α-heparin was precipitated with sodium acetate and alcohol and recovered in a dry state. 300 to 400 mg of the heparin was placed on the column as a water–propanol solution and chromatographed as described.

Analyses of isolated compounds

The following analyses were obtained and calculated on a dry-weight basis by allowing a sample to dry to a constant weight at 105°: (1) optical rotation, (2) nitrogen by micro-Kjeldahl digestion and nesslerization, (3) sulfate[10], (4) uronic acid by DISCHE carbazole procedure[6], (5) hexosamine content[11], and (6) assay of anticoagulant properties[12,13]. Enzymic hydrolysis of approximately 0.2 mg of MPS by 48 turbidity-reducing units of testicular hyaluronidase** was

* Heparin, sodium – Lederle Laboratories, Division American Cyanamid Company.

** Kindly supplied by Dr. J. SEIFTER, Wyeth Institute for Medical Research.

References p. 183.

performed by incubation for 45 min, 1 h and 4 h at 38° and assayed for hydrolysis by a turbidimetric procedure[8,9]. Differentiation of glucosamine and galactosamine in the isolated fractions was performed by paper chromatography[14] and by a modification of the resin method of GARDELL[15], with comparison to known standards. The uronic acid of the sample isolated as CSA-B was compared to a known sample of CSA-B* by a method of paper chromatography suggested by FISHER AND DÖRFEL[16].

RESULTS

The results are summarized in Figs. 2 and 3 and in Tables II and III.

A representative example of a chromatographic column to separate the sulfated MPS from bovine aorta is shown in Fig. 2. CSA-A is eluted with zinc acetate–*n*-propanol solutions at a ratio of 60/40 (v/v) and is almost completely eluted from the column at approximately 70/30 (v/v). CSA-B is eluted with solutions at ratios greater than 75/25 (v/v). The preparative chromatography illustrated in Fig. 2 demonstrates on overlap during elution of the two compounds but other experiments, particularly with smaller quantities of MPS, suggested better resolution. CSA-A composed approximately 75% of the sulfated mixture. Chemical analyses of the two sulfated components obtained by chromatography are shown in Table II and agree with results previously published[3,5,7]. The assays for antithrombin activity demonstrated activity for CSA-B from the aorta comparable to those of a known CSA-B from urine. The material was resistant to enzymic hydrolysis by testicular hyaluronidase and L-iduronic acid was demonstrated.

The material obtained by electrophoresis which migrated toward the cathode (resultant direction due to electroosmosis of the buffer) was principally HA. Refer to Table II. Analyses of this material even after reelectrophoresis consistently demon-

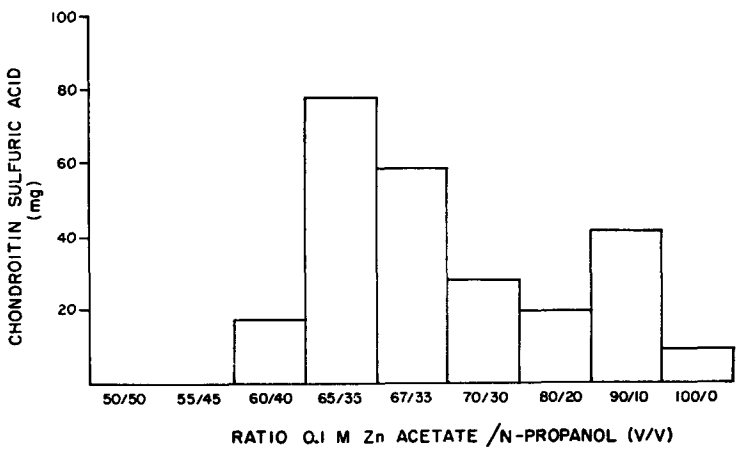


Fig. 2. Results of chromatographic separation of chondroitin sulfate A and B from a mixture obtained from bovine aorta. The concentration of MPS eluted from the column was determined by a turbidimetric method with the use of an albumin solution at pH 3.75 and cartilage CSA-A as a reference. The values shown indicate total amount in each change of zinc acetate–*n*-propanol solutions of approximately 500 ml. In this experiment CSA-A was isolated from solutions containing 60/40 to 67/33 (v/v) and CSA-B from solutions containing 80/20 to 100/0 (v/v) of 0.1 *M* zinc acetate–*n*-propanol. The analyses of the compounds isolated are shown in Table II.

* Kindly supplied by Dr. J. A. CIFONELLI, University of Chicago.

References p. 183.

strated a low optical rotation, —30 to —40° $[\alpha]_D^{23}$ but contained only glucosamine and less than 0.5% S as sulfate. After passing over charcoal, Darco G 60[17], a purification was achieved to obtain a rotation of —64°. The data suggest that the major material thus isolated from this group of substances was HA but the need for continued purification by charcoal even after reelectrophoresis suggested the presence of another compound. Studies by MEYER *et al.*[3] suggesting the presence of a heparin-

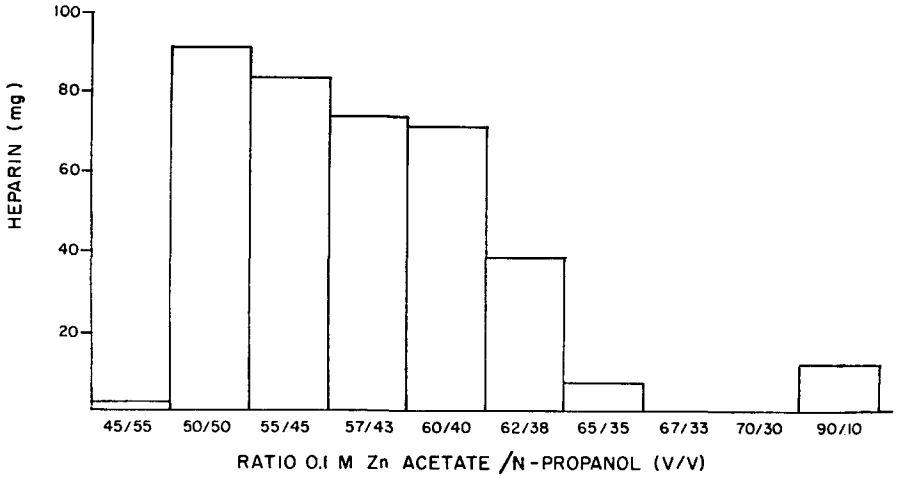


Fig. 3. Results of chromatographic fractionation of a commercially available preparation of α-heparin. The conditions are similar to those for fractionation of CSA described in the text and indicated for Fig. 2. Fraction I was isolated from solutions of 50/50 to 57/33 zinc acetate–*n*-propanol, fraction II from 62/38 to 65/35, and fraction III from 90/10. Analyses of the MPS isolated as the various fractions are described in the text and are shown in Table III.

TABLE II

CHEMICAL ANALYSES OF MUCOPOLYSACCHARIDES ISOLATED FROM BOVINE AORTA

Preparation	*Optical rotation* $[\alpha]_D^{23}$	*N* %	*S* %	*Hexosamine** %	*Hexuronic acid* %	*Hyaluronidase*
Hyaluronic acid	—64**	3.78	< 0.5	34.5 (glucosamine)	44.9	hydrolyzed
Theory	—70	3.48	0	44.5 (glucosamine)	48.0	hydrolyzed
Chondroitin sulfuric acid-A	—30	3.05	6.4	23.6 (galactosamine)	30.0	hydrolyzed
Theory	—30	2.78	6.4	35.5 (galactosamine)	38.5	hydrolyzed
Chondroitin sulfuric acid-B***	—55	3.27	5.4	23.0 (galactosamine)	20.9§	resistant
Theory	—60	2.78	6.4	35.5 (galactosamine)	38.5	resistant

* Values were uniformly low in all of the analyses of hexosamine.

** Obtained after passing over charcoal[17].

*** Antithrombin activity comparable to a known sample of CSA-B from urine was demonstrated.

§ Analyses by the carbazole colorimetric procedure are low[3,5] and the uronic acid was demonstrated to be L-iduronic acid by paper chromatography[16].

References p. 183.

TABLE III

CHEMICAL ANALYSES OF MUCOPOLYSACCHARIDE FRACTIONS ISOLATED AFTER CHROMATOGRAPHY OF A COMMERCIAL PREPARATION OF α-HEPARIN

Preparation	*Optical rotation* $[\alpha]_D^{23}$	*N* %	*S* %	*Hexosamine** %	*Hexuronic acid* %
Commercial heparin**	48	3.1	11.1	24.2 (glucosamine and galactosamine)	34.5
Fraction I	44	2.4	12.1	19.4 (glucosamine)	34.0
Fraction II	40	2.3	10.6	18.6 (glucosamine and galactosamine)	28.2
Fraction III	—34	2.6	6.6	20.0 (galactosamine)	18.2***
Theory:					
α-Heparin	49	2.2	12.4	27.8 (glucosamine)	30.2
β-Heparin	—60	2.8	6.4	35.5 (galactosamine)	38.5

* Results of hexosamine determinations were uniformly low in all of the analyses.

** Precipitated and recovered from a solution of commercial heparin. Anticoagulant activity was demonstrated with equivalent activity observed for Fr. I and II but less for III.

*** Low values are found with the carbazole method. The uronic acid was demonstrated to be L-iduronic acid by paper chromatography[16].

like material in the aorta agree with these findings but this substance remains to be isolated and identified. Preliminary studies suggest that a compound might be separated from the HA by the chromatographic procedure described above and by means of charcoal.

Since the chromatographic technique described above separated sulfated MPS, a similar effort was made to fractionate a commercial α-heparin. The results are reflected by Fig. 3. This study demonstrates fractionation into at least two components, fraction I at 50/50 (v/v) and fraction III at 90/10 (v/v). There is a possibility of an additional component, a mixture designated as fraction II, and isolated at 62/38 (v/v). Analyses of these fractions and, for comparison, of the commercial heparin are shown in Table III. Since only small quantities of fraction III were present, this material was pooled from several columns. Fraction III had a slight yellow discoloration that suggested impurity; unfortunately, the limited amount isolated precluded further identification than is shown in Table III. The relative amounts of fractions I, II and III obtained from the columns were 23, 6.5 and 1, respectively. The analyses performed on the MPS isolated from the heparin columns compared to known values seem to indicate that fraction I is α-heparin[18,19]; that fraction II is principally α-heparin with the presence of trace amounts of a substance containing galactosamine, possibly CSA-A; that fraction III is probably β-heparin, which has been isolated from lung in other preparations of heparin[7]. All of these samples were resistant to testicular hyaluronidase, and studies of anticoagulant activities of fractions I and II

References p. 183.

were consistent with those for α-heparin and fraction III for β-heparin. Such results indicate that the chromatography may aid in purification of α-heparin without degradation of the polysaccharide.

DISCUSSION

In these experiments three acid MPS were isolated from bovine aorta, HA and CSA-A and B. The data suggest another compound present in the material isolated electrophoretically with the HA but without further investigation little comment can be made. The available information only indicates a glucosamine-containing compound, possibly with a low sulfate content and positive optical rotation. However, that there is a heterogeneous mixture of compounds with characteristics of acid MPS present in the connective tissue of the aorta is clear from the general findings of these studies.

The CSA compounds were separated by a column-chromatographic procedure which appears to be of value in supplementing other methods of isolating MPS. Since these substances were indistinguishable by zone electrophoresis, it was necessary to resort to another method of separation. In other experiments[20], separation of sulfated MPS by paper chromatography had been attempted but the need remained for a method that would permit isolation of larger quantities for analytical characterization. By incorporating the technique of chromatography, this method may result in better separation of the MPS than can be accomplished by alcohol fractionation alone. In other studies[2] using alcohol fractionation CSA-C has been found but these studies did not demonstrate the presence of this compound, and no further attempts were made to duplicate the methods used previously.

With the accumulation of information about CSA-B, the presence of this compound in the aorta becomes of increasing interest. Since it has anticoagulant activity, its location in a blood vessel may have considerable significance. The uronic acid of the CSA-B (β-heparin) has been demonstrated to be L-iduronic acid[21,22], which is a material in the body thus far unique to this MPS. Furthermore, studies of compounds from various sites: skin[23], lungs[7], urine[24] and gastric mucosa[25], indicate the presence of a similar compound and on the basis of those observations CSA-B and β-heparin have been suggested to be identical. These experiments were not designed to confirm the similarity or identity of these substances but the chromatography of MPS mixture from two different sources, aorta and lung, demonstrated elution of the material at approximately the same region, which with the same chemical analyses may be taken as further evidence of identity. Antithrombin activity, characteristic of β-heparin, was demonstrated for the compound isolated from the aorta.

ACKNOWLEDGEMENTS

The assistance and suggestions of Dr. RICHARD REEVES are gratefully acknowledged. RONALD F. REINISCH and LEE ROY MORGAN, JR. participated in these experiments during student summer trainee ips sponsored by the National Heart Institute.

References p. 183.

SUMMARY

These studies describe a method for isolating a heterogeneous mixture of acid mucopolysaccharides from bovine aorta. The mixture was resolved into three compounds, hyaluronic acid and chondroitin sulfuric acids A and B by zone electrophoresis and column chromatography. Evidence is presented for the presence of another substance which could be separated from hyaluronic acid by charcoal. The chondroitin sulfuric acid B isolated from the aorta was chemically similar to samples obtained from lung and urine and demonstrated antithrombin activity. The experiments offer further evidence that CSA-B and β-heparin are the same compound.

REFERENCES

1 P. A. LEVENE, *Monographs of the Rockefeller Institute for Medical Research*, No. 18 (1922) 85–6.
2 K. MEYER AND M. M. RAPPORT, *Science*, 113 (1951) 596.
3 K. MEYER, E. DAVIDSON, A. LINKER AND P. HOFFMAN, *Biochim. Biophys. Acta*, 21 (1956) 506.
4 J. E. KIRK AND M. DYRBYE, *J. Gerontol.*, 12 (1957) 23.
5 S. SCHILLER, M. B. MATHEWS, H. JEFFERSON, J. LUDOWEIG AND A. DORFMAN, *J. Biol. Chem.*, 211 (1954) 717.
6 Z. DISCHE, *J. Biol. Chem.*, 167 (1947) 189.
7 R. MARBET AND A. WINTERSTEIN, *Helv. Chim. Acta*, 34 (1951) 2311.
8 M. B. MATHEWS, S. ROSEMAN AND A. DORFMAN, *J. Biol. Chem.*, 188 (1951) 327.
9 A. DORFMAN AND M. L. OTT, *J. Biol. Chem.*, 172 (1948) 367.
10 G. TOENNIES AND B. BAKAY, *Anal. Chem.*, 25 (1953) 160.
11 L. A. ELSON AND W. T. J. MORGAN, *Biochem. J.*, 27 (1933) 1824.
12 VON A. STUDER AND A. WINTERSTEIN, *Helv. Physiol. Acta*, 9 (1951) 6.
13 B. J. GROSSMAN AND A. DORFMAN, personal communication.
14 P. J. STOFFYN AND R. W. JEANLOZ, *Arch. Biochem. Biophys.*, 52 (1954) 373.
15 S. GARDELL, *Acta Chem. Scand.*, 7 (1953) 207.
16 F. G. FISCHER AND H. DÖRFEL, *Z. physiol. Chem.*, 301 (1955) 224.
17 J. A. CIFONELLI AND M. MAYEDA, *Biochim. Biophys. Acta*, 24 (1957) 397.
18 M. L. WOLFROM, R. MONTGOMERY, J. V. KARABINOS, P. ROTHGEB, *J. Am. Chem. Soc.*, 72 (1950) 5796.
19 R. MARBET AND A. WINTERSTEIN, *Experientia*, 8 (1952) 41.
20 G. P. KERBY, *Proc. Soc. Exptl. Biol. Med.*, 83 (1953) 263.
21 P. HOFFMAN, K. MEYER AND A. LINKER, *Science*, 124 (1956) 1252.
22 J. A. CIFONELLI, J. LUDOWEIG AND A. DORFMAN, *Federation Proc.*, 16 (1957) 165.
23 K. MEYER AND E. CHAFFEE, *J. Biol. Chem.*, 138 (1941) 491.
24 A. E. LORINCZ AND A. DORFMAN, *Abstracts Society for Pediatric Research*, 27th Annual Meeting (1957) 73.
25 H. SMITH AND R. C. GALLOP, *Biochem. J.*, 53 (1953) 666.

Received October 25th, 1957