ANALYSIS OF THE NATIVE HEPARIN-LIPOPROTEIN COMPLEX INCLUDING THE IDENTIFICATION OF A HEPARIN COMPLEMENT (HEPARIN CO-FACTOR) OBTAINED FROM EXTRACTS OF TISSUE MAST CELLS

by

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INTRODUCTION

It is generally conceived that the serum albumin fraction contains a labile protein which acts as heparin complement, and which is necessary for the antithrombic activity of heparin (cf. reviews by CHARGAFF, ASTRUP AND QUICK). This particular co-factor is considered to be different from another plasma antithrombin (VOLKERT, 1942), also regarded as an albumin component and which acts without heparin. The possibility that some as yet poorly defined lipid constituents may enter these complex antithrombin reactions is still not decided. Lipids suggested to interfere with thrombin activity have been extracted from brain, spinal cord, and red blood cells (CHARGAFF, 1945). Unclassified plasma lipids have, moreover, repeatedly been suggested to participate in the normal antithrombin reaction and/or in the heparin-complement-thrombin reaction (HOWELL AND HOLT, 1918; BRINKHOUS, 1939; QUICK, 1938; WÖHLISCH AND KÖHLER, 1942; GRÜNING, 1943).

Previous reports on the occurrence and alledged characteristics of this heparin complement in serum and plasma are contained in a variety of papers (QUICK, 1938; BRINKHOUS, SMITH, WARNER, AND SEEGERS, 1939; JAQUES AND MUSTARD, 1940; ZIFF AND CHARGAFF, 1940; WÖHLISCH, 1940; SEEGERS, 1940; FERGUSON AND GLAZKO, 1941; ASTRUP AND DARLING, 1941; VOLKERT, 1942; SEEGERS, 1942; SEEGERS AND SMITH, 1942; GRÜNING, 1943; ASTRUP AND DARLING, 1943; ASTRUP, 1944; and FEISSLY AND ENOWICZ, 1946). The most comprehensive study on the electrophoretic behaviour of plasma albumin fractions associated with heparin was presented by CHARGAFF, ZIFF AND MOORE (1941). It seems thus established that the heparin complement is part of the albumin fraction of blood, serum, and plasma, but is not found in crystalline serum albumin. The real nature of the heparin complement is still obscure, and the possible rôle of suggested lipid co-factor(s) mentioned above cannot as yet be evaluated.

As a sequel to other cytochemical work on the cytoplasmic constituents of tissue mast cells (JULÉN, SNELLMAN, AND SYLVÉN, 1950) this paper deals with the study of

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a native heparin-lipoprotein complex compound obtained from ox liver capsules and rat skin. By very mild fractionation procedures this complex was split into an inhomogeneous carbohydrate part to be further investigated and a lipo-protein part. The present study of the lipo-protein material includes chemical, bio-assay, and physico-chemical data. Although simultaneous studies on the serum complement have not yet been performed, part of this tissue lipo-protein complex presents characteristics very similar to those of the previously reported serum complement.

In order to prevent confusion of terminology it should be mentioned that the name "heparin complement" previously signified a fraction of the serum albumin group of proteins. According to the evidence to be presented the same or similar complement obtained from tissue mast cells is actually a lipo-protein compound in which the polypeptide part plays a dominant rôle for the linkage between heparin and the lipid component. In this paper the term heparin complement will thus be used to denote this tissue lipo-protein.

MATERIAL AND METHODS

Fresh ox liver capsules mechanically freed from liver cells, and fresh frozen rat skin were extracted according to previously described differential centrifugation method (JULÉN, SNELLMAN, AND SYLVÉN, 1950). The final supernatant S_3 obtained from liver capsules had a reddish colour and the solutions were covered by a thin lipid pellicle. The corresponding supernatants from rat skin had a yellowish colour and a thicker lipid top layer. For technical reasons the preparation could not be performed on perfused tissues. The possible admixture of serum proteins will be discussed below.

The physico-chemical determinations were performed according to current methods as indicated in the text. The antithrombic activities were assayed by means of the thrombin method of JAQUES AND CHARLES (1941). For the biological clotting assays purified solutions of fibrinogen and "Thrombin Topical" were used. The fibrinogen was prepared by Dr MACFARLANE according to the phosphate buffer method of JAQUES (1943). In these preparations prothrombin had previously been removed by adsorption on $Al(OH)_3$. A rough estimate indicates that at least 96% of the protein is removed by clotting with thrombin. "Thrombin Topical" was obtained from Parke, Davis & Co. and also contains inert protein, thromboplastin, calcium chloride, phemerol and sucrose. The amount of accelerator globulin is probably zero (SEEGERS). For metachromatic staining reactions we used alcoholic solutions of Azure A (National Aniline Co.).

EXPERIMENTAL

As was previously described (JULÉN, SNELLMAN AND SYLVÉN), the metachromatic material extracted from a suspension of ox liver capsule mast cells could by differential centrifugation be recovered in the final supernatant S_3 and was not sedimentable at 60,000 × g. This supernatant also contained a small admixture of free lipid material. The main constituent of S_3 presented metachromatic and anticoagulant activities and had a sedimentation constant of about 5 S. This constituent appeared to be a complex compound of polysaccharides (mainly heparin) and proteins. Available data indicated that this native heparin complex should be regarded as a readily water-soluble microsome material.

The native complex had a greater anticoagulant activity than the corresponding amount of purified heparin when assayed by the thrombin method of JAQUES AND CHARLES (1941). It therefore seemed possible that the supernatant S_3 might also contain the heparin complement either associated with the heparin complex or else included in the supernatant in another state. Interest was consequently focused on the native protein and lipid components present in the supernatant S_3.

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I. *Analysis of* S_3

To the freshly prepared supernatants S_3 in phosphate buffer solutions at p_H 7 and at 4°C a sufficient amount of phosphate was added until complete saturation, when the protein and lipid material precipitated together and the polysaccharide residue was left in solution. This precipitate was further treated by shaking with 2 to 3 volumes of a mixture of ethanol + ether (equal parts) at 4°C for 48 hours. The dissolved lipid material was then recovered by drying in vacuum.

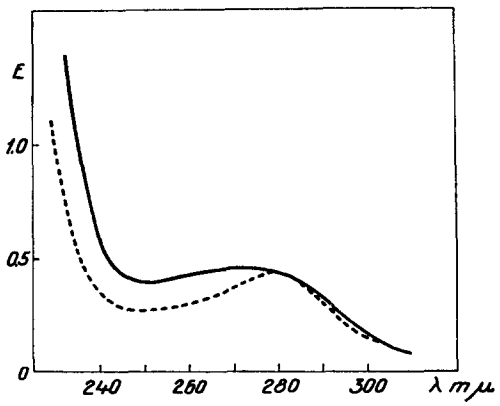

Fig. 1. UV-spectrophotometry for the demonstration of nucleic acids. The fully drawn line represents the whole supernatant S_3; the dotted line represents fraction u_6 isolated by salting out with KH_2PO_4 + K_2HPO_4. No correction made for unspecific scattering.

500 g fresh ox liver capsules yielded a phosphate precipitate of 150 g w/w. From this the following materials were obtained in dry weight: proteins and salts 50 g, lipid material 10 g, heparin 2 mg as assayed by the thrombin method of JAQUES AND CHARLES. The impure protein fraction mentioned above could so far not be freed from salts.

The lipid fraction obtained from liver capsules presented a dark yellow colour whereas that obtained from rat skin was light yellow. The lipids were easily soluble in ethanol. The P content was determined according to the method of KING (1932), and the N content was determined by KJELDAHL analysis. Cholesterol was determined by the method of SCHOENHEIMER AND SPERRY (1935). The P/N ratio was found to be almost equal to 1. Solubility tests indicate that all P was present as lecithin. Calculations on that basis with the usual factor P × 25 gave the lecithin content (Table I).

TABLE I

ANALYSIS OF LIPID FRACTION OF S_3

Source	P in %	N in %	P:N	Lecithin in %	Cholesterol in %	Calculated Residue (mostly neutral fats) in %
Liver capsule	2.20	1.0	1:1	55	8	37
Rat skin	0.45	0.20	1:1	11	1	88

The precipitated protein component was easily resoluble in $^1/_6$ *M* Na_2HPO_4. Upon acidification the protein remained in solution down to p_H 1.9, where only a small amount precipitated. The protein rapidly passed a cellophane membrane and could not be obtained in a salt-free state. Ultracentrifugation studies showed a certain polydispersity and the mean sedimentation constant S_{20} was found to be about 1.8. The molecular weight must be low (about 8,000) and consequently the protein was considered to be essentially a polypeptide. For amino acid content the readers are referred to page 103.

The carbohydrate residue largely remained in solution following the first precipitation

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of S_3 with phosphate. Adhering to the protein part, however, a small amount of heparin was found (about 1% of the total weight of heparin). More detailed studies on the composition of the heterogeneous carbohydrate (heparin) component in S_3 will be published elsewhere (cf. JENSEN, SNELLMAN, AND SYLVÉN, 1948).

II. *Electrophoretic separation of* S_3

Previously published electrophoretic data (JULÉN *et al.*, 1950) indicate that the metachromatic and anticoagulant constituent contained in S_3 could be segregated into two different components. Further electrophoretic experiments on freshly prepared S_3 in isotonic buffer solutions (phosphate buffer at p_H 7.0) have resulted in the separation of S_3 into four components with the following mobilities: $u_6 = 5.9 \cdot 10^{-5}$ sq. cm per volt per second, $u_4 = 3.6 \cdot 10^{-5}$, $u_3 = 3 \cdot 10^{-5}$ and a fourth inconstant and very insignificant component u_8 = about $8 \cdot 10^{-5}$ (possibly nucleic acid)/cf. Table II; Exp. A and Fig. 2.

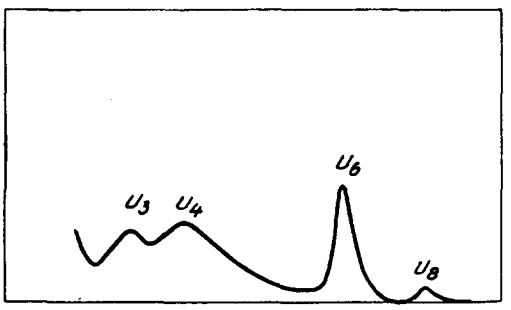

Fig. 2. Electrophoretic diagram of whole supernatant S_3

TABLE II

ELECTROPHORETIC DATA

All experiments performed in phosphate buffer with ionic strength 0.1

Experiment	pH	Substance	Mobilities $u \times 10^5$ sq.cm per volt per second			
A	7.0	S_3	(7.8)*	5.9	3.6	2.7
B	7.0	S_3 + small amount of Heparin	(7.8)*	5.8	4.4	2.8
C	7.0	S_3 + larger addition of Heparin	(18.4)**	6.4***	3.0	1.6
D	7.0	Fraction u_6 precipitated from S_3 by 65–80% phosphate saturation		6.6	—§	—§
E	6.8	Residue following precipitation of S_3 with clupein sulphate		5.4	3.1	—§

* Inconstant and insignificant fraction
** Heparin peak (cf. JENSEN, SNELLMAN, AND SYLVEN, 1948)
*** Increase in breadth compared with fraction $u = 5.8$
§ In this region previously present component had disappeared

No component could be detected with the mobility of isolated heparin. If the p_H is dropped from 7.0 to about 4, the greater part of the first component u_6 precipitated and at the same time 90% of the solution's antithrombic activity disappeared.

Following precipitation with phosphate and subsequent resuspension of the material contained in S_3 these electrophoresis data had changed. The effect of freeze-drying cannot as yet be stated.

In order to determine whether the native heparin complex was "saturated" with heparin electrophoretic experiments were performed after the addition of various

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amounts of purified heparin (Vitrum) to native S_3 (Table II). Following the addition of small amounts of heparin, no new electrophoretic component with the mobility of heparin was found. However, when a larger amount was added a free heparin component appeared. We have therefore concluded that the native complex is not saturated with respect to heparin.

III. *Identification of the complement-active lipo-protein*

A quantitative electrophoretic separation of the above mentioned components contained in the whole S_3 fraction and a further investigation of their properties showed that only component u_6 possessed antithrombic activity and was very strongly stained metachromatically. The two slower components u_4 and u_3 were inactive in repeated tests, and showed little or no metachromatic staining. It is thus clear that heparin must occur in fraction u_6 as part of a complex compound. Under the same conditions purified heparin has a mobility of $u = 18 - 22 \cdot 10^{-5}$ sq.cm per volt per second.

Salting out studies. Salting out studies on freshly prepared S_3 showed that component u_6 could be precipitated with $KH_2PO_4 + K_2HPO_4$ at p_H 7.2 within a range of 65–80% saturation at 20°C. Only insignificant amounts of the other components were precipitated with u_6. Component u_6 could be purified by reprecipitation, but it underwent some change during still higher salt concentrations.

The salted out and resuspended u_6 had a sedimentation constant of $S_{20} = 4.4$ and showed a good homogeneity in the ultracentrifuge (Fig. 3). This sedimentation constant lies near or agrees with that of serum albumin. Resuspended heparin-containing u_6 had a higher mobility than serum albumin at p_H 7. However, if part of the heparin was removed with protamine sulphate, u_6 showed a lower mobility ($u = 5.4$) which more nearly approaches that of serum albumin ($u = 4.8$).

Spectroscopic observations. The precipitated and purified fraction u_6 was largely devoid of nucleic acid (Fig. 1).

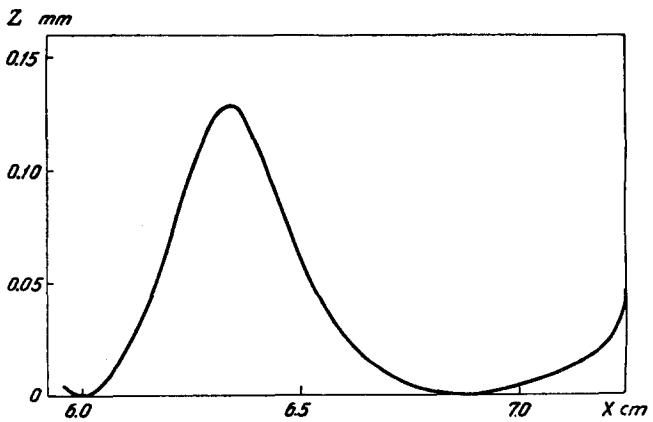

Fig. 3. Ultracentrifugal sedimentation diagram of the salted out and resuspended fraction u_6. Ultracentrifuge run at 59,000 r.p.m. Buffer solution 0.4 *M* NaCl, 0.05 *M* Na-phosphates. p_H 7.0. Time 80 min.

Relative amounts of fractions. Attempts were made to ascertain the relative amounts of the different components contained in fraction S_3 from the areas under the electrophoretic curves. The following values were obtained after correction for the high refractive indices. Fraction u_6 obtained from ox liver capsules constituted 8% (corr.) of the entire mass of S_3. The ratio between $u_6/u_4 + u_3$ was calculated to be 8:92. The corresponding figures for fraction u_6 derived from rat skin were 12% (corr.) and 12:88. Electrophoretic data not reported in detail suggest that most of fraction u_6 was made up of heparin complement, but the exact figures cannot be stated.

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Paper chromatography. Two-dimensional paper chromatography with phenol-butanol was performed on hydrolysates of electrophoretically isolated u_6. The following amino acids were identified: *cysteine* (cystine), *threonine, tyrosine, glycine* and *leucine.* No other amino acids could be demonstrated. The tyrosine spot was very marked. Spectrophotometry showed an additional content of *tryptophan,* which was destroyed during acid hydrolysis, and the tyrosine: tryptophan ratio was found to be 5.5:1 (HOLIDAY, 1936). Paper chromatography of the whole fraction S_3 demonstrated the presence of the same amino acids and in addition considerable amounts of arginine but others could not be identified.

The available data mentioned above are sufficient for the provisional identification of the lipid and protein components contained in fraction u_6.

IV. *Biological Clotting Assays*

For localizing the heparin complement a series ot clotting experiments were performed using a system of purified fibrinogen and "Thrombin Topical" at p_H 7.0 and ionic strength 0.07–0.1. The qualitative results are compiled in Table III.

TABLE III

BIOLOGICAL CLOTTING ASSAYS

Clotting assay on the system purified fibrinogen + thrombin following the addition of the substances mentioned below:	Antithrombic effect
S_3, native condition, obtained either from liver capsules or rat skin	+
Fraction u_6, isolated by electrophoresis from liver capsules	+
Fractions $u_4 + u_3$, isolated by electrophoresis from liver capsules	—
Fractions $u_4 + u_3$ (as mentioned above) + heparin (Vitrum)	—
Heparin (Vitrum) added alone	—
Heparin (Vitrum) + polypeptide residue alone, obtained from native S_3 (ox liver capsules and/or rat skin) (cf. page 100)	—
Heparin (Vitrum) + lipid residue alone, obtained from native S_3 (ox liver capsules and/or rat skin)	(+)*
Heparin (Vitrum) + polypeptide and lipid residues mentioned above	+
Residue of S_3, following treatment with clupein sulphate (cf. text)	—

* Some preparations negative, others slightly positive, probably due to admixture of small amounts of polypeptide residue.

It seems evident from Table III that (1) the so-called heparin complement necessary for the anticoagulant effect of heparin was *only* contained in fraction u_6, and that (2) the lipid fraction is essential for the antithrombic effect of heparin and the polypeptide part of the complement. It should further be emphasized that a mixture of the three isolated components, namely purified heparin + purified polypeptide residue + native lipid residue showed a marked anticoagulant effect similar to that of S_3 and fraction u_6. It was consequently possible to reconstruct a complex compound exerting complement activity towards heparin. The composition and behaviour of this compound has not as yet been further investigated by other means.

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In this connection it may be mentioned that the composition of the lipid part seems to be non-specific. When using the same polypeptide preparatien the same effects were obtained with liver capsule lipid containing 55% lecithin as with skin lipid containing 11% lecithin (cf. Table I). Other lipids (ordinary lecithin) have not been tested so far.

When native S_3 is treated with protamine (clupein sulphate) the heparin moiety is slowly precipitated together with part of the protein contained in S_3 over a period of 24 hours. During the precipitation reaction there was a gradual loss in antithrombic activity of S_3 until after 24 hours all heparin had been precipitated. The surprisingly low rate of the protamine-heparin reaction may perhaps explain the statement by TOCANTINS (1949) that protamine would prolong the clotting time.

Available data indicate that heparin + its complement forms an addition compound which inactivates thrombin. This thrombin reaction was further studied in some detail:

a. In a solution of Thrombin Topical only one component was found presenting an electrophoretic mobility of $u = 4 \cdot 10^{-5}$ sq. cm per volt per second at p_H 7. This mobility agrees very well with the data on thrombin reported by SEEGERS, MCCLAUGHRY AND FAHEY (1950). This was moreover the only component in Thrombin Topical reacting with our fraction u_6. It was consequently justified to consider this material as thrombin *per se* contained in Thrombin Topical;

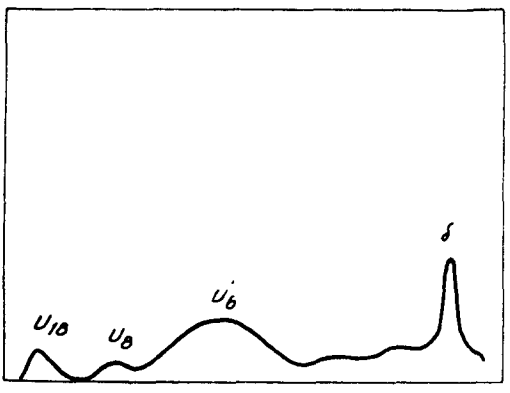

Fig. 4. Electrophoretic diagram of thrombin (Topical) + isolated heparin (Vitrum) + purified heparin complement. Heparin peak u_{18}; thrombin has reacted with heparin + complement and consequently no free thrombin peak has appeared. δ is a salt peak.

b. Isolated heparin + thrombin do not react, as evidenced by the electrophoresis data;

c. Thrombin + fraction u_6 obtained by electrophoresis and saturated with heparin gives a reaction as demonstrated in the electrophoresis diagram (Fig. 4). No free thrombin with the above mentioned mobility could be detected. Fraction u_6 had instead increased in size and had become broader (Fig. 4). This indicates that thrombin reacts with the heparin lipo-protein complex (u_6);

d. In another series of experiments the antithrombic effect of different fractions was studied, and it was found that in the absence of added heparin fraction u_6 exhibits a high antithrombic effect. This indicates that the native fraction u_6 also reacts with thrombin.

Since only small amounts of fraction u_6 were obtainable by electrophoretic separation a complete physico-chemical investigation cannot be presented. More data will be added latei using material obtained by salting out, and more purified thrombin. As mentioned before no direct comparison has been made between our tissue complement and that of serum albumin.

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DISCUSSION

Differential centrifugation of extracts of fresh ox liver capsules and/or rat skin containing mast cells yields a final non-sedimentable material containing a polysaccharide associated with a lipoprotein. The polysaccharide part was previously shown to contain mainly heparin (JULÉN *et al.*, 1950). The lipid part is now reported to contain lecithin (11–55%), cholesterol (8%), and a residue of neutral fats (about 37%). The protein component is largely made up of a polypeptide of low molecular weight containing only 6 amino acids. In addition, other protein components and a small amount of nucleic acid were demonstrated to be present in the supernatants. By means of electrophoresis of the whole complex (S_3) it was shown that the polysaccharide moiety was linked to a special protein fraction (u_6) with an electrophoretic mobility at p_H 7 of about 5.9, which is slightly more than that of serum albumin (cf. CHARGAFF, ZIFF, AND MOORE, 1941). The clotting assay showed that this fraction (u_6) was solely responsible for *both the heparin and complement* (heparin co-factor) *activities* contained in the original supernatant S_3. Two other protein fractions (u_4 and u_3) not further investigated were devoid both of heparin and complement activities. It was further electrophoretically demonstrated that fraction u_6 reacts with purified thrombin in the presence of heparin.

Available data justify the conclusion that the heparin complement must be located in the above mentioned native fraction u_6 linked to the carbohydrate residue. The isolated lipoprotein described in this paper thus presents characteristics very similar to the previously investigated heparin complement in serum, and these substances seem to be identical.

The clotting assays further indicate that the isolated polypeptide residue under question has no biological activity in presence of heparin without the simultaneous presence of the lipid co-factor.

The internal linkages. The amino acids identified in the polypeptide residue provide no marked basic or acid residual groups. The amino acids mentioned contain either dipolar groups or hydrocarbon chains. It therefore seems possible that a complex of such a character may be held together by VAN DER WAALS' forces. This opinion is supported by the observation mentioned above that the complex is easily split and reorganized. The phospholipid part of the complex and the polar groups of the polypeptide should in principle be directed outwards towards the water phase. Such a theoretical structure for lipoproteins has previously been discussed (MACHEBOEUF, 1932; MCFARLANE, 1949; ONCLEY *et al.*, 1950) and seems to be applicable to the common reactions and properties of the heparin complement.

The external linkage of heparin. Tentative suggestions may be made as to the mode of linkage between heparin and its complement, and further between the thrombin inhibitor and the thrombin molecule.

As mentioned above, the heparin co-factor should have polar groups directed towards the water phase. In addition, the phospholipids should also be directed towards the water phase. These phospholipids presumably play the dominant rôle in the electrophoretic mobility of the lipoprotein.

Besides heparin other high polymer carbohydrate ester sulphates give with the heparin complement a similar antithrombic effect as heparin, but to a lesser degree. None of these compounds has hydrophobic groups. The addition of the esters to the

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heparin complement must therefore take place on the surface of the molecule. Further, the electrophoretic mobility of these thrombin inhibitors is greater than that of the heparin co-factor and this implies that only some of the acid groups can be involved in the linkage between the substances. It seems impossible that the carbohydrate could be connected with the phospholipid part so we have to assume that the linkage is achieved by the polypeptide. The linkage must further be of dipolar type since the heparin complement can be salted out while most of the carbohydrate residue remains in solution. The polypeptide part devoid of lecithin will not be precipitated by heparin at any p_H. The conditions are thus different from the reaction between heparin and the basic protamines, in which case salt linkages are involved.

Linkage with thrombin. When considering the reaction between the thrombin inhibitor and thrombin, attention should be called to the possibility of a co-operation between the acid groups both of heparin and of the phospholipids. This opinion is supported by CHARGAFF'S study on the lipid thrombin inhibitors. He showed that sulphonated phospholipids had antithrombic activity. This also suggests that the polypeptide residue forms a link between heparin and the lipid residue. Due to the present lack of information on the structure of thrombin, no suggestions are justified as to the possible linkages between the thrombin molecule and its inhibitor(s).

Biological implications. Present results point to the fact that heparin, either obtained from ox liver capsules or rat skin, is associated with a special lipoprotein. This favours the assumption that the whole complex is probably synthesized and produced in the intergranular cytoplasm of tissue mast cells (cf. JULÉN, SNELLMAN, AND SYLVÉN). Direct evidence to this effect has, however, not been obtained by the present methods. Another explanation might be that only the polysaccharide part could be synthesized in the cells and that the lipoprotein linkage would occur in the tissue fluid after the release of the polysaccharide. The first-mentioned theory of a polysaccharide-lipoprotein complex already present in the mast cell cytoplasm is in agreement with the present conceptions of the composition of microsome material containing about 20–40% of lipids. When considering the storage of heparin in the cell cytoplasm it seems most likely to assume that the polysaccharide will not be in a free state (cf. WILANDER, 1938; and JORPES, 1946).

The amounts of heparin complement obtained by extraction indicate that this material cannot be derived from the blood, particularly since liver capsules have few blood vessels.

Other biological phenomena involving the release of strongly metachromatic material from living mast cell cytoplasm during experimentally induced local detoxication reactions (SYLVÉN AND LARSSON, 1948) indicate, that the native heparin compound is neither reacting with the native connective tissue proteins nor with the native epithelial proteins surrounding the mast cells. Heparin will, on the other hand, be firmly bound to denaturated proteins (cf. SYLVÉN, 1949). The former phenomena may now be interpreted as evidence for the theory that heparin is already bound to protein at the time of its release from the mast cells.

The present findings may possibly have a bearing on the origin of those small amounts of heparin demonstrated to be present in normal blood serum (MONKHOUSE, STEWART, AND JAQUES, 1949). This question will form the subject of future investigations.

Another line of research deals with the suggested inhibiting activity of isolated heparin on different proteolytic enzymes such as trypsin, pepsin and papain (HORWITT,

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1940; GLAZKO AND FERGUSON, 1940; WELLS *et al.*, 1945). With reference to the regulation of growth and protein synthesis (SYLVÉN, 1949) it would be of considerable biological importance to investigate the reactions between the native heparin complex and the proteolytic enzymes.

ACKNOWLEDGMENTS

We are indebted to Drs R. G. MACFARLANE AND ROSEMARY BIGGS of the Radcliffe Infirmary, Oxford, for the kind supply of purified fibrinogen, and further to Parke, Davis & Co., Detroit, Mich., for the supply of "Thrombin Topical". Our thanks are also due to Dr RICHARD J. WINZLER of the University of Southern California, Los Angeles, and to Dr W. H. SEEGERS of the Wayne University, Detroit, for consultations and revisal of the manuscript. The costs have been defrayed by grants from an anonymous donor, from the Swedish Medical Association (Bohman's Fund), and from Swedish Anti-Cancer Funds.

SUMMARY

The native heparin-containing compound present in tissue mast cell cytoplasm has been extracted and purified by means of previously described differential centrifugation methods. The lipoprotein part associated with the carbohydrate moiety (heparin) is further analysed as to its chemical composition and physiological function.

The non-sedimentable and water-soluble material obtained in the final supernatant was thus segregated into three distinct components with different electrophoretic mobilities. One of these with a mobility of $u = 5.9$ was the only one containing heparin and also exerting antithrombic activity. The two slower moving components were inert and not further analysed.

This native antithrombic material (fraction u_6) contained heparin associated with a polypeptide and a lipid residue. This fraction could be isolated both by electrophoresis and by a salting out method. The polypeptide part was of low molecular weight and contained only six amino acids. The lipid part contained lecithin, cholesterol and neutral fats, and presented no structural characteristics.

The results indicate that the carbohydrate moiety was not in a free state. It is suggested that heparin forms a loose linkage with the non-denaturated lipoprotein part of the molecule.

This heparin complex (u_6) has a potent antithrombic effect when tested on a system of purified fibrinogen + thrombin. Neither heparin alone nor the lipoprotein part alone gave this effect. The clotting assays show that all three parts of the complex must be present simultaneously in order to produce the antithrombic effect. If the three components have been segregated and subsequently are added together again full biological activity is re-established.

The lipoprotein part of the complex was thus identified as a heparin complement or heparin co-factor necessary for the antithrombic effect of heparin. The electrophoretic data and our clotting assays suggest that this tissue complement is very similar or identical with the previously known heparin complement of blood serum.

Electrophoresis data show that purified thrombin forms a complex compound with the native heparin-lipoprotein molecule (fraction u_6).

The structure of the complex and its lipoprotein component are discussed. It is assumed that the carbohydrate (heparin) part is linked to the surface of the molecule, and further that both the phospholipid and the acidic groups of heparin may partake in the reaction with thrombin.

From a cytochemical point of view the results suggest that the whole heparin complex is probably produced in the intergranular cytoplasm of the tissue mast cells.

RÉSUMÉ

Nous avons extrait et purifié le composé natif contenant de l'héparine qui se trouve dans le cytoplasme des mastzellen, à l'aide des méthodes de centrifugation différentielle précédemment décrites. Nous avons analysé plus à fond la part lipoprotéique associée à l'hydrate de carbone (héparine) quant à sa composition chimique et à sa fonction physiologique.

Ainsi nous avons séparé le matériel non sédimentable et soluble dans l'eau, contenu dans le

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dernier liquide surnageant, en trois fractions distinctes de mobilité électrophorétique différente. La fraction de mobilité $u = 5.9$ était la seule qui contenait de l'héparine et qui possédait également de l'activité antithrombique. Les deux fractions de mobilité plus petite étaient inertes et ne furent pas analysées davantage.

Ce matériel natif à activité antithrombique (fraction u_6) contenait de l'héparine associée à un polypeptide et un reste lipoïde. Cette fraction pouvait être isolée par électrophorèse et par une méthode de relargage. Le polypeptide avait un poids moléculaire peu élevé et contenait seulement six acides aminés. La partie lipoïde contenait de la lécithine, du cholestérol et des corps gras neutres et ne présentait pas de particularités structurales.

Les résultats indiquent que l'hydrate de carbone n'était pas à l'état libre. L'héparine pourrait être faiblement liée à la partie lipoprotéique non dénaturée de la molécule.

Ce complexe d'héparine (u_6) possède une activité antithrombique puissante vis à vis du système fibrinogène purifié + thrombine. Ni l'héparine seule ni la partie lipoprotéique seule n'exerçait cet effet. Les expériences de coagulation montrent que les trois composantes doivent être présentes simultanément pour produire l'effet antithrombique. Lorsqu'on sépare les trois composantes et qu'on les réunit à nouveau, l'activité biologique est rétablie intégralement.

Nous avons ainsi identifié la part lipoprotéique du complexe comme complément ou co-facteur d'héparine nécessaire à l'effet antithrombique de l'héparine. Les données obtenues par électrophorèse et par nos essais de coagulation suggèrent l'idée que ce complément tissulaire soit très semblable ou identique au complément d'héparine déjà connu du sérum sanguin.

Les données obtenues par électrophorèse montrent que la thrombine purifiée forme un complexe avec la molécule native héparine-lipoprotéine (fraction u_6).

Nous avons discuté la structure du complexe et de son constituent lipoprotéique. Nous admettons que l'hydrate de carbone (héparine) est fixé à la surface de la molécule et que le phospholipoïde aussi bien que les groupes acides de l'héparine pourraient prendre part à la réaction avec la thrombine.

Du point de vue cytochimique les résultats semblent indiquer que tout le complexe héparinique soit probablement élaboré dans le cytoplasme intergranulaire des mastzellen.

ZUSAMMENFASSUNG

Die native heparinhaltige Verbindung, welche im Zytoplasma der Gewebe-Mastzellen vorkommt, wurde mit Hilfe der früher beschriebenen Differential-Zentrifugiermethoden extrahiert und gereinigt. Der mit dem Kohlenhydrat (Heparin) assoziierte Lipoproteinanteil wurde im Hinblick auf seine chemische Zusammensetzung und physiologische Funktion weiter analysiert.

Das nicht sedimentierbare und wasserlösliche Material, das sich in der letzten überstehenden Flüssigkeit vorfand, wurde so in drei deutlich verschiedene Komponenten mit verschiedener elektrophoretischer Mobilität geteilt. Die Komponente, deren Mobilität $u = 5.9$ betrug, war die einzige, die Heparin enthielt und auch Antithrombinwirkung besass. Die beiden langsameren Komponenten waren inert und wurden nicht weiter analysiert.

Dieses native Material mit Antithrombinwirkung (Fraktion u_6) enthielt Heparin, assoziiert mit einem Polypeptid und einem Lipoidrest. Diese Fraktion konnte sowohl durch Elektrophorese als durch Aussalzen isoliert werden. Der Polypeptidanteil hatte ein niedriges Molekulargewicht und enthielt nur sechs Aminosäuren. Der Lipoidanteil enthielt Lecithin, Cholesterol und neutrale Fette und zeigte keine charakteristischen strukturellen Merkmale.

Die Ergebnisse weisen darauf hin, dass der Kohlenhydratanteil nicht frei vorlag. Das Lipoprotein könnte lose an den nicht denaturierten Lipoproteinanteil des Moleküls gebunden sein.

Dieser Heparinkomplex (u_6) zeigte eine starke Antithrombinwirkung gegenüber einem System von gereinigtem Fibrinogen + Thrombin. Weder Heparin allein, noch der Lipoprotein-anteil allein riefen diesen Effekt hervor. Die Gerinnungsversuche zeigen, dass alle drei Teile des Komplexes gleichzeitig anwesend sein müssen, um den Antithrombineffekt zu bewirken. Werden die drei Komponenten getrennt und dann wieder zusammen gefügt, so wird die volle biologische Aktivität wiederhergestellt.

Der Lipoproteinanteil des Komplexes wurde so als ein, für den Antithrombineffekt des Heparins notwendiges, Heparin-Komplement oder Heparin-Cofaktor identifiziert. Die Ergebnisse der Elektrophorese und unsere Gerinnungsversuche lassen vermuten, dass dieses Gewebe-Komplement sehr ähnlich oder identisch mit dem schon früher bekannten Heparinkomplement des Blutserums ist.

Die Ergebnisse der Elektrophorese zeigen, dass gereinigtes Thrombin eine Komplexverbindung mit dem nativen Heparin-Lipoproteinmolekül (Fraktion u_6) formt.

Die Struktur des Komplexes und Lipoproteins wurde erörtert. Es wird angenommen, dass das Heparin an der Oberfläche des Moleküls adsorbiert ist und dass sowohl das Phospholipoid als die sauren Gruppen des Heparins an der Reaktion des Komplexes mit Thrombin teilnehmen könnten.

Vom zytochemischen Standpunkt deuten die Ergebnisse darauf hin, dass der ganze Heparinkomplex wahrscheinlich in dem intergranulären Zytoplasma der Gewebe-Mastzellen gebildet wird.

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Received November 21st, 1950