

# THE THROMBOPLASTIC ACTION OF CEPHALIN

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In 1912 Howell (1) reported the results of a study of the thromboplastic action of the tissues in which he showed that the active substance is a phosphatid having the general properties of cephalin. The effect of solutions of this phosphatid upon coagulation is very striking and it has been shown that lecithin prepared according to the customary methods is lacking in this property. The identification of the phosphatid rested mainly on its insolubility in cold alcohol and its slight solubility in hot alcohol and the possibility was recognized that the thromboplastic action might be due to some adherent impurity rather than to the phosphatid itself. At the suggestion of Dr. Howell I have undertaken a re-examination of this subject to determine if possible whether the thromboplastic effect may be attributed to an impurity, or is a property of the cephalin itself, and also to determine in how far a similar property is exhibited by other related phosphatids. The phosphatids which have been examined in regard to their thromboplastic action are cephalin, lecithin, sphingomyelin, cuorin and heparphosphatid.

## THE METHOD OF TESTING THROMBOPLASTIC ACTIVITY

To determine thromboplastic activity a method was employed which has been in use in this laboratory for some time in connection with other researches on coagulation. The method rests upon the fact that in fresh serum the amount of actual or effective thrombin is small, but the amount of ineffective thrombin is relatively large. This metathrombin may be converted to active thrombin by Morawitz's (2) method of adding alkali with subsequent neutralization. But it can also be activated to effective thrombin by the addition of thromboplastic extracts, especially solutions of cephalin, provided the serum is perfectly fresh. As the serum stands the activating effect of the cephalin becomes less marked and finally disappears entirely after a certain

variable time. Long after cephalin activation has ceased entirely the alkali activation is still effective. This activating effect of cephalin on fresh serum was used in the following way. Fresh serum and fresh oxalated and centrifugalized plasma were prepared from the same animal. By trial the amount of serum was determined which would cause clotting of a definite amount of plasma within a time convenient for observation—say thirty minutes to two hours. The thromboplastic activity of cephalin or any other solution under observation was determined by adding it to the serum and noting the acceleration in the clotting time. Ordinarily the serum of dogs and cats was used and the proportions taken were two or three drops of serum to eight drops of plasma. With these proportions clotting occurs usually in from 30 minutes to 2 hours, but if to the mixture one adds a solution of cephalin or a saline extract of fresh tissue clotting occurs promptly in from 30 seconds to 2 or 3 minutes. The tests were carried out according to the following example:

*Control*—Oxalated plasma, 8 drops; serum, 3 drops; water, 3 drops. Clot forms in from 30 minutes to 2 hours and usually the clot is imperfect.

*Solution to be tested*; e.g., solution of cephalin—Oxalated plasma, 8 drops; serum, 3 drops; cephalin solution, 3 drops. A solid clot forms in one minute plus or minus.

In all of the experiments the solutions of the purified phosphatids were compared not only with a control of plasma, serum and water, but also with a control made up with an incompletely purified but very active preparation of cephalin made from the pig's brain. This form of cephalin is now used constantly in the laboratory for experimental and therapeutical purposes and for brevity's sake is designated in the following experiments as "laboratory cephalin." Its mode of preparation is as follows:

#### PREPARATION OF LABORATORY CEPHALIN

A fresh brain obtained from the slaughter house is freed as far as possible from membranes and blood and is then ground to a pulp in a mortar. The pulp is spread as thin as possible on a glass plate and dried in a current of warm air. When thoroughly dry the material is ground to a powder and is extracted for 4 or 5 hours with an excess of ether. The ether solution is filtered off in a closed space and is passed through the filter paper two or more times until the filtrate comes through clear. The ether filtrate is allowed to evaporate in

a current of cool air to a moist residue. The residue is extracted twice with an excess of acetone for 15 to 20 minutes and then twice with an excess of 95 per cent alcohol. Finally the residue is treated again with acetone, the acetone drained off, and the material dried in a desiccator. In using the cephalin for thromboplastic experiments a 0.1 per cent solution is made up in distilled water.

#### CEPHALIN PREPARED BY THE METHOD OF RENALL

Cephalin was prepared according to the modification of Parnas's (3) method proposed by Renall (4), and by which he states that a yield of very pure cephalin may be obtained. Fresh pig's brains obtained from a slaughter house are carefully freed from membranes and blood; then macerated in a mortar to a fine pulp. This pulp is placed directly into three times its volume of acetone for a day, with occasional shaking. The acetone extract is centrifugalized off and the same amount of fresh acetone added to the residue. After remaining a day in this acetone and being subjected to occasional shaking, the insoluble portion is centrifugalized away from the acetone solution. The residue is shaken for an hour with cold absolute alcohol and the insoluble portion, after removal of the alcohol, is treated twice with three times its volume of petroleum ether of low boiling point (used instead of ethyl oxide to prevent a certain amount of oxidation which is liable to occur with the use of ordinary ether). The petroleum ether extracts, which are colorless or of a lemon yellow tinge, are concentrated in an atmosphere of carbon dioxide and precipitated by pouring into absolute alcohol. The cephalin falls as a white flocculent precipitate, which when dry has a white waxy appearance and forms with water an opalescent solution resembling that of laboratory cephalin. It possesses very active thromboplastic power as is shown in the following experiment.

#### *Dog's oxalated plasma and dog's serum*

*Using*—Plasma, 8 drops; cephalin, 2 drops; serum, 2 drops.

*Control*—Plasma, 8 drops; water, 2 drops; serum, 2 drops.

|                          |            |         |
|--------------------------|------------|---------|
| Renall's cephalin.....   | good clot  | 1½ min. |
| Laboratory cephalin..... | solid clot | 1 min.  |
| Control.....             | no clot in | 30 min. |

To the precipitate obtained above cerebrosides are adherent as impurities. They are removed by redissolving the precipitate in petroleum ether and allowing the solution to stand over night in the cold. The cerebrosides settle out and by centrifuging are separated from the

solution. They are washed with ether and dried. When tested in aqueous solution for thromboplastic activity they were found to be wholly negative. The petroleum ether solution is concentrated and poured into absolute alcohol—the cephalin is precipitated as a white amorphous and somewhat waxy mass, which after drying is made up into a thin emulsion with water and precipitated with normal hydrochloric acid. The acid is added to the emulsion until a good cheesy precipitate is obtained, which settles to the bottom of the container. It is collected by centrifugalization, taken up in a little ether and precipitated with acetone and dried. It forms an opalescent solution in water which upon trial shows marked thromboplastic activity as is indicated in the following typical experiments:

*Dog's oxalated plasma and serum*

*Series I. Using*—Plasma, 8 drops; cephalin, 3 drops; serum, 3 drops.

*Control*—Plasma, 8 drops; water, 3 drops; serum, 3 drops.

Renall's cephalin.....good clot 2 min., 25 sec.

Laboratory cephalin.....solid clot 2 min., 45 sec.

Control.....poor clot 2 hours, 15 min.

*Series II. Using*—Plasma, 8 drops; cephalin, 2 drops; serum, 2 drops.

*Control*—Plasma, 8 drops; water, 2 drops; serum, 2 drops.

Renall's cephalin.....good clot 3 min.

Laboratory cephalin.....solid clot 1 min.

Control.....minute clot in 5 min. not solid in 30 min.

Thus purified cephalin prepared with a view to the prevention of oxidation and to the removal of adherent impurities possesses thromboplastic activity to a marked degree.

CEPHALIN PREPARED BY THE METHOD OF LEVENE AND WEST

Through the kindness of Dr. Levene a specimen of this cephalin was furnished in order to test its thromboplastic action. The mode of preparation of the specimen is described by the authors (5) and it will be remembered that in the quantitative hydrolysis made by them only 90 per cent of the original weight of material was obtained in the hydrolytic products. The authors therefore consider the possibility that the material contained some impurity. The sample furnished had been precipitated by hydrochloric acid from aqueous solution and after treatment with an organic solvent was finally obtained in the form of a dry powder. When this powder was dissolved in water the solution exhibited marked thromboplastic activity as is shown in the following typical experiment.

*Cat's oxalated plasma and cat's serum**Using*—Plasma, 8 drops; cephalin, 3 drops; serum, 3 drops.*Control*—Plasma, 8 drops; water, 3 drops; serum, 3 drops.

Cephalin—Levene and West.....solid clot 75 sec.

Laboratory cephalin.....solid clot 40 sec.

Control.....poor clot 1 hour, 30 sec.

## CEPHALIN AND CUORIN PREPARED FROM THE HEART

Erlandsen (6) as a result of an extended research upon the lipoids of the heart came to the conclusion that there is no cephalin in heart muscle, but in its place he discovered a monoamino-diphosphatid related to cephalin which he called cuorin. To determine if this substance had any thromboplastic effect a quantity was prepared for trial according to the following method as given by Erlandsen:

Ox heart is freed from fat, blood vessels, peri- and endocardium and valves, ground in a meat grinder and spread upon glass plates to dry, then ground to a powder and desiccated over sulphuric acid or calcium chloride. The powdered mass is extracted with ether at room temperature, with a frequent change of the ether until all of the ether soluble material is removed. These extracts are united and concentrated to a syrup, then taken up in a little ether and allowed to stand. An insoluble fraction settles out; it is centrifugalized off and discarded. The ether solution is repeatedly precipitated with dry acetone and then with cold absolute alcohol (zero to minus 2°). This last precipitate is a dark brown sticky mass which upon dissolving in ether and precipitating with alcohol is divisible into two substances—one insoluble in alcohol at 60°, the cuorin a light brown powder, and the other appreciably soluble in alcohol at 60°, a substance having the general properties of cephalin. This cephalin was recovered from the united alcoholic extracts, which were concentrated, precipitated with acetone, taken up in a little ether and precipitated by pouring into alcohol to free from lecithin. This precipitate resembles laboratory cephalin, having the same fish-like odor, and is shown to be as active thromboplastically as the laboratory cephalin.

*Cat's oxalated plasma and cat's serum**Using*—Plasma, 8 drops; cephalin, 3 drops; serum, 3 drops.*Control*—Plasma, 8 drops; water, 3 drops; serum, 3 drops.

Heart cephalin.....solid clot 37 sec.

Laboratory cephalin.....solid clot 37 sec.

Control—not clotted in 1 hour.....clotted over night

The cuorin, on the contrary when purified by repeated precipitation in alcohol at 60°, has no thromboplastic effect—indeed it possesses an anticoagulating power as may be illustrated by the following experiment.

*Dog's oxalated plasma and dog's serum*

*Using*—Plasma, 8 drops; phosphatid, 3 drops; serum, 3 drops.

*Control*—Plasma, 8 drops; water, 3 drops; serum, 3 drops.

Heart cephalin.....solid clot 3 min.

Cuorin.....not clotted in 6 hours

Control.....sliding clot in 9 min.

Cuorin added to blood fresh from the artery will delay its coagulation remarkably. These two phosphatids from the heart have practically the same solubilities, they pass through repeated processes of extraction together and in the final stages are only separated from each other by the solution of the cephalin in alcohol at 60°. It is unlikely that an impurity would adhere to one of these phosphatids and not to the other.

CEPHALIN AND HEPARPHOSPHATID PREPARED FROM THE LIVER

Baskoff (7), in his work on the phosphatids of the liver, undertaken primarily for the study of the jecorin described by Drechsel (8), succeeded in isolating a phosphatid which resembles cuorin, except that analysis shows a N:P ratio of 1:1.5 instead of 1:2. To this phosphatid he gave the name of heparphosphatid. In my experiments the liver was worked up according to Baskoff's method for obtaining heparphosphatid, which is practically the same treatment to which Erlandsen subjects the heart muscle in order to secure his cuorin. The final subst nce insoluble in cold alcohol was further divided into a fraction insoluble in alcohol at 60° which resembles cuorin and constitutes Baskoff's heparphosphatid, and a portion soluble in alcohol at 60° which is apparently identical with cephalin. When the latter was recovered from the alcoholic solutions as in the case of the cephalin of the heart, it had all of the general properties of cephalin including the power to hasten the coagulation of blood as is shown in this experiment.

*Using*—Plasma, 8 drops; cephalin, 3 drops; serum, 3 drops.

*Control*—Plasma, 8 drops, water, 3 drops; serum, 3 drops.

Liver cephalin.....solid clot 1½ min.

Laboratory cephalin.....solid clot 1½ min.

Control.....not clotted in 1 hour and 45 min.

The heparphosphatid on the other hand when purified by many precipitations in alcohol at 60° has no thromboplastic action and in fact shows a marked power to inhibit the coagulation. The anticoagulating action of this phosphatid is being studied and will be reported upon later. Cuorin and heparphosphatid when dry have no odor, but when moist with warm alcohol have a characteristic odor common to both. It is possible that on further purification the heparphosphatid may be shown to be identical with cuorin.

#### THE ACTION OF SPHINGOMYELIN

Sphingomyelin is assumed to be a diamino-monophosphatid; it is insoluble in ether and alcohol. In the process of extracting organs for phosphatids some may be carried along with the soluble phosphatids but separates out later from the ethereal solutions together with their cerebrosides. The impure cerebrosides as previously mentioned were found to possess no thromboplastic action. By the kindness of Dr. Levene a specimen of sphingomyelin was obtained. When tested by the usual method it was found to have no thromboplastic activity.

#### THE ACTION OF LECITHIN PREPARED BY THE METHOD OF MacLEAN

Howell (1) showed clearly that lecithin as customarily prepared from the brain and from the yolk of egg exhibits no thromboplastic action. At that time there was no satisfactory method for the absolute separation of lecithin from cephalin, for cephalin though relatively insoluble in alcohol is quite soluble in an alcoholic solution of lecithin. Therefore in precipitating an ether solution of lecithin and cephalin a certain amount of the cephalin remains in solution with the lecithin. Or if the original extract of the tissue is made with alcohol much cephalin will go into solution with the lecithin. MacLean (9) has shown that "lecithin" as customarily prepared can always be separated into true lecithin, which has all of its base as choline and into cephalin with its amino ethyl alcohol base, together with other impurities. He has devised a method fully described in his article by which he has obtained lecithin with all of its base as choline. The method involves the formation of the cadmium compounds of lecithin and cephalin from the alcoholic solution of these phosphatids as obtained from the egg yolk. The separation is made on the basis of the insolubility of the lecithin cadmium chloride compound in ordinary

ether, the cephalin cadmium compound being soluble. Pure lecithin is recovered from the cadmium compound by boiling with ammonium carbonate after the manner recommended by Bergell (10). Lecithin prepared by this method when tested in the usual manner exhibited no thromboplastic activity. Attempts made to recover the cephalin from its cadmium compound were unsuccessful.

#### CONCLUSIONS

1. Cephalin when prepared as pure as possible exhibits marked thromboplastic activity, as indicated by its effect in increasing the thrombic action of fresh serum.
2. Cephalin exhibiting this reaction has been prepared from the liver, heart and brain.
3. The other phosphatids that have been described, lecithin, cuorin, heparphosphatid and sphingomyelin have no thromboplastic action.
4. Evidence is presented to show that this property of cephalin is not due to adherent impurities, but is a characteristic property of the cephalin itself.

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