15134

Horse Brain Thromboplastin: II. Effect of Various Factors on Activity of Horse Brain Extracts.*†

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It has been suggested[1] that horse brain thromboplastin might be used as a reagent for the quantitative determination of prothrombin by the one-stage method of Quick.[2] The optimal conditions necessary for the preparation of the reagent solution from acetone-dried horse brain powder have not previously been studied; heretofore, the concentration, temperature, and period of activation employed were the same as those used by Quick for rabbit brain thromboplastin. The influence of these factors on the activity of extracts prepared from desiccated horse brain has now been investigated. In addition, a modified technic for the preparation of the acetone-dried horse brain powder has been evolved.

Experimental Results and Discussion. A. Preparation and activity of acetone-dried horse brain powder. The desiccated horse brain powder used in these experiments was prepared by acetone dehydration of fresh brains.‡ The basic principle of this method does not differ from that previously used for horse brains[1] nor from the method of Quick for rabbit brain thromboplastin.[2] However, the technic employed is different. In the present procedure a stainless steel press was employed for the removal of the pia mater and the major portion of the blood vessels, and a colloid mill was used for the rapid acetone desiccation of the remainder of the brain tissue. Approximately 11% of the tissue failed to pass through the press and was discarded. This procedure permitted the handling of whole horse brains with a minimum of time and effort. It was possible to suspend each horse brain in acetone within 20 to 30 minutes from the time it was obtained from the animal. With this rapid and convenient means for desiccating brain tissue, relatively large quantities of material, *i.e.*, 300 g or more, could be prepared in a single day. Furthermore, the new method has the advantage of producing a product with a relatively constant thromboplastic activity, which was not true of the previous method. The yields and the activities of several batches of brain powder are reported in Table I.

In determining thromboplastic activity, 6% suspensions of brain powder were prepared in 0.85% sodium chloride solution that had been heated previously to 50°C, and the suspensions were held at this temperature for 15 minutes, while stirring at intervals with a glass rod to break up as many as possible of the large particles of powder. The suspensions then were centrifuged at approximately 3000 r.p.m. for 30 seconds, just long enough to remove the coarse particles. (The concentration of solids remaining in the supernatant was approximately 2%.)

The activities of the saline extracts and solutions prepared from these extracts were determined by the one-stage method of Quick,[2] using citrated human plasma and 0.184% calcium chloride solution. A concentration of 0.184% calcium chloride was optimal for our standard plasma.[1] Except where indicated, all determinations were made on one sample of pooled (10 bleedings) citrated human plasma (prepared from blood containing 50 cc of 4% sodium citrate in each 500 cc). Ten

* Kazal, L. A., and Arnow, L. E., *Arch. Biochem.*, 1944, **4**, 185, is No. I in this series.

† Preliminary report appeared in *Am. Chem. Soc. Abstracts*, Sept. 11, 1944.

1 Kazal, L. A., and Arnow, L. E., *Arch. Biochem.*, 1944, **4**, 183.

2 Quick, A. J., *J. Am. Med. Assn.*, 1938, **110**, 1658; 1938, **111**, 1775.

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TABLE I.
Horse Brain Thromboplastin Preparations.

Preparation No.	No. of brains	Wet wt whole brain, g	Wet wt pressed brain, g	Acetone-dried brain powder, g	Activity* clotting time, (sec)
1	4	2025	—	324	16.1
2	4	2190	1940	382	16.1
3	5	2820	2500	455	15.8
4	6	—	2690	490	16.2
5	6	—	2960	538	15.4
6	5	—	2490	464	15.8

* Clotting time of 0.1 cc of a standard citrated human plasma recalcified with 0.1 cc of 0.184% calcium chloride solution in the presence of 0.1 cc of thromboplastic extract that had been diluted with an equal volume of distilled water.

cc portions of this plasma were frozen rapidly in small glass vials in a methyl "cellosolve"-dry ice mixture, and were stored at −20°C. Samples were thawed at 37°C in a water bath just before use. The clotting time of this plasma remained constant throughout the period of investigation. All clotting time determinations reported are the average of 3 to 5 individual readings.

In order to determine whether or not optimal conditions were being employed for the preparation of these extracts, a study was made of 4 variables in the procedure, namely, concentration, method of extraction, temperature of extraction, and the duration of heat treatment.

B. *Effect of concentration of brain powder on thromboplastic activity.* Brain powder suspensions ranging in concentration from 1% to 20% were prepared in physiological saline solution at 50°C and centrifuged as described above. The supernatants were diluted with distilled water, except in the case of the 20% suspension of which only enough could be obtained for a few tests. The activities of each extract and its dilutions are presented in Table II (non-homogenized preparations). The most active extract was obtained from a 6% suspension of brain powder. Dilution of this extract with an equal volume of water did not significantly change its activity. Very often, however, such dilution will effect an increase of thromboplastic activity.[1] An example of this may be found in Table III, preparation No. 2.

Attempts to enhance the thromboplastic activity of brain powder suspension by increasing the amount of solids in suspension by homogenization were unsuccessful. Suspensions (1% to 20%) were prepared as above with the exception that, after the 50°C incubation, they were homogenized in a water-cooled colloid mill for 1 to 2 minutes instead of being centrifuged. These emulsions were tested for activity in the same manner as the non-homogenized preparations. The results,

TABLE II.
Effect of Concentration on Thromboplastic Activity of Homogenized and Non-homogenized Saline Extracts of Acetone-dried Horse Brain Powder.

Preparation	Conc. of brain powder susp., %	Dilutions with distilled water: 100% (undiluted)	50%	25%	10%	1%
		Plasma clotting times (sec.)				
Non-homogenized	3	18.2	23.8	33.4		
	6	**16.1**	16.7	19.7		
	10	17.8	18.0	22.4		
	20	32.5				
Homogenized	1	32.0	28.5	30.6	34.4	
	3	36.6	28.0	28.2	28.5	51.0
	6	66.6	39.0	29.8	**27.7**	29.5
	20	152.5	87.0	43.7	29.5	37.0

Numbers in heavy type are the minimal clotting times for each type of preparation.

TABLE III.
Influence of Method of Extraction on Thromboplastic Activity of Saline Extracts of Acetone-Dried Brain Powder.

Methods of extraction (6% susp. of brain powder)	Dilutions with distilled water		
	100% (undiluted)	50%	25%
	Plasma clotting times (sec.)		
1. No stirring	17.6	19.0	
2. Mild stirring (manually)	19.2	16.8	19.4
3. Vigorous stirring (air-driven stirrer)	22.1	21.6	23.2
4. Homogenization (colloid mill)	36.6	28.0	28.2

which are presented in the second section of Table II, show that all such preparations exhibited a lower order of thromboplastic activity.

C. *Influence of method of extraction.* The experiments on homogenization suggested that the activity of thromboplastic extracts could be influenced by the manner in which the extraction was made. Accordingly, saline extracts were prepared from 6% suspensions as described, except that during the period of incubation at 50°C, one extract (No. 1) was not stirred after the brain powder had been moistened, another (No. 2) was stirred with a glass rod as usual, and a third (No. 3) was stirred vigorously with an air-driven stirrer. All extracts were centrifuged, and the supernatants tested for activity at various dilutions. The results in Table III, which include those of homogenized 6% suspensions for the sake of comparison, indicate that the method of mild stirring produced the most active extract. Vigorous stirring and homogenization proved to be detrimental. When stirring was not employed, the resulting extracts were fairly active, but lacked the advantage of increased activity upon dilution which the mildly stirred extracts possessed. The ex-

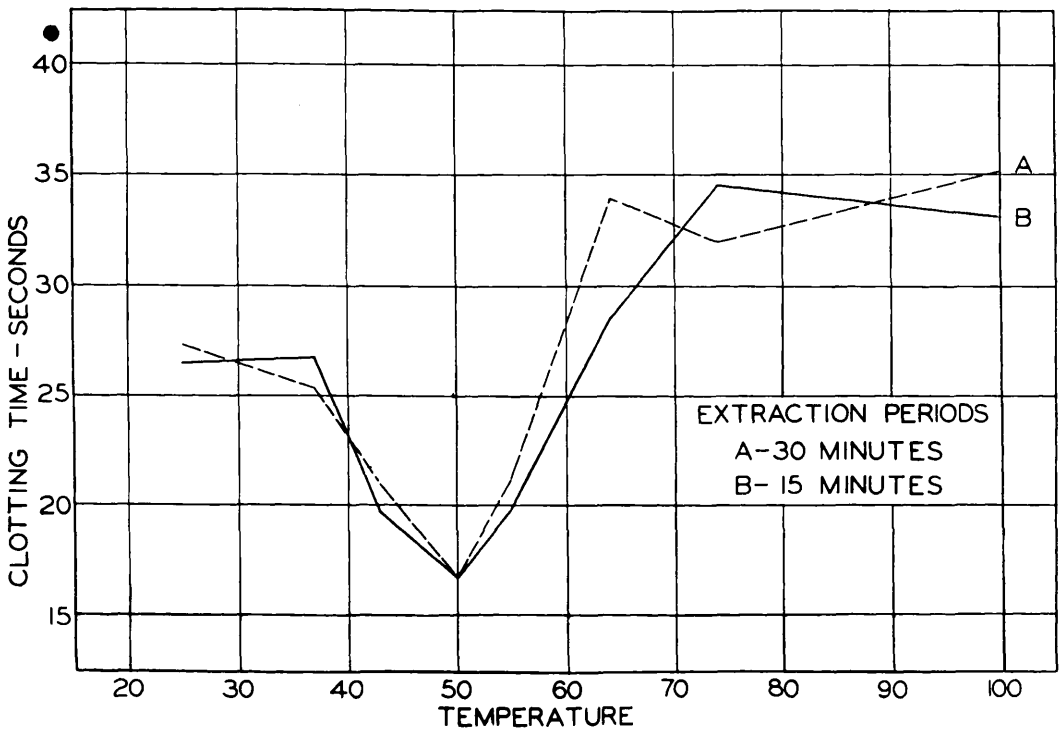

FIG. 1.
Effect of temperature on thromboplastic activity of suspensions of a horse brain powder.

periments on the effect of concentration and methods of extraction suggest the presence of a blood clotting inhibitor in horse brain, which is released under certain conditions.

D. *Effect of temperature and duration of extraction.* Temperatures ranging from 25°C to 100°C for periods of 15 to 30 minutes were substituted for the standard temperature of 50°C, and temperatures of 25°C and 50°C were investigated further over a period of 1 to 300 minutes. Except for these changes the technics of preparation and testing of the extracts were the same as those described in section A. The testing, however, was limited to the 50% dilutions of the supernatant. Fig. 1 illustrates the effects observed. A temperature of 50°C for a period or 15 to 30 minutes proved to be optimal. It appears that in the temperature range of 25° to 50°C an activation of the thromboplastic substance occurs. Also, at 50°C activation took place very rapidly; extracts exposed to this temperature for only a few minutes were considerably more active than similar extracts maintained at 25°C. In fact, at the latter temperature the thromboplastic activity at no time approached that of the extracts heated at 50°C although at both temperatures individual maximal activity was attained in 15 minutes.

In the Quick technic, a temperature of 50°C apparently was employed for the destruction of any prothrombin that might be present in the thromboplastic extract of rabbit brain.[3] Although destruction of prothrombin undoubtedly takes place under these conditions, it is believed that, at least for horse brain, the increased activity obtained after incubation at 50°C may be due to other factors as well. For example a centrifuged suspension of brain powder prepared at 30°C is less active than one prepared at 50°C. Significantly, the activity of the suspension prepared at the lower temperature cannot be increased by subsequently heating it at 50°C for as long as 25 minutes. On the other hand, when this suspension is heated at 50°C for 15-20 minutes in the presence of the insoluble brain residue previously removed by centrifugation, its activity partially is restored. It would appear from such experiments that thromboplastin is not completely extracted by saline from the brain powder at temperatures below 50°C. Whether this temperature causes an increase in the dispersibility of the active principle, or perhaps minimizes the extraction of an inhibitor, can only be conjectured at the present time. These observations are in agreement with the experiments of Astrup and Darling[4] who observed a 300% increase in the potency of thromboplastic extracts of lung heated at 40°C for 1 hour. In their opinion the increased potency was due "to a greater solubility of the active substance, probably by obtaining a greater degree of dispersion."

The preparation of Astrup and Darling was inactivated at 56°C. Partial inactivation of horse brain thromboplastin under the conditions of our experiments occurred at temperatures above 50°C. Between 75° and 100°C there was no further decrease of thromboplastic activity. Total loss of activity was not observed at these temperatures.

Summary. A modified procedure for the preparation of relatively large batches of acetone-desiccated horse brain powder has been developed. The thromboplastic activity was influenced by the concentration of brain powder used for the preparation of the saline extracts. The optimal mixture was a 6% suspension, whose supernatant quite often had optimal activity after dilution with distilled water. The optimal temperature for the preparation of saline extracts was 5°C; the optimal period of exposure to this temperature was 15 to 30 minutes. Exposure to temperatures between 50°C and 100°C for 15 to 30 minutes produced only a partial destruction of thromboplastic activity.

[3] Quick, A. J., *Am. J. Physiol.*, 1935, **114**, 282.

[4] Astrup, T., and Darling, S., *Acta. Physiol. Scand.*, 1942, **3**, 168.