I. THE ACTION OF SODIUM HYDROXIDE UPON COAGULATION OF FIBRINOGEN.

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(Received October 28th, 1920.)

THE well-known action of sodium hydroxide in retarding coagulation of fibrinogen in presence of thrombin has been variously interpreted. J. Mellanby [1909] regards this retardation as due to the solution of the newly formed fibrin by the alkali and to a destruction of the ferment. According to W. H. Howell [1916] the effect of addition of alkali is to prevent the formation of fibrin fibrils, though the formation of fibrin aggregates, to which this author regards gelatinisation of fibrinogen as due, may still occur.

With a view of further investigating the action of alkali upon the coagulation of liquids containing fibrinogen and thrombin the present work was undertaken.

It is obvious that if the action of alkali is exerted exclusively upon fibrinogen or is directed solely to thrombin, it would be easy to determine the nature of this action by a comparison of (1) the effect upon coagulation of adding increasing amounts of alkali to plasma or fibrinogen solution with (2) the course of coagulation in a series of dilutions of (a) fibrinogen, to each of which the same amount of thrombin has been added, and (b) thrombin, to each of which the same amount of plasma has been added.

The effect of dilution of fibrinogen is shown in Table I, that of dilution of thrombin in Table II and the action of alkali in Table III. In these experiments fibrinogen, which cannot at present be prepared in even an approximately pure condition, was represented by citrated human plasma: it is well known that when fibrinogen is precipitated from plasma by dilution and acidification relatively considerable amounts of albumin and globulin are carried down in the precipitate [J. Mellanby, 1905]. As source of thrombin the venom of *Echis carinatus* [Barratt, 1913], dissolved in citrated 0.85 % sodium chloride solution, was employed¹. The volume of citrated plasma used (undiluted or diluted) was in each experiment 0.050 cc.: to this was added 0.0026 cc. of citrated sodium hydroxide solution of suitable concentration (Table III) or of citrated 0.85 % sodium chloride solution (Tables I

¹ For a supply of this venom I am indebted to the kindness of Dr C. J. Martin, Director of the Lister Institute.

Table I.

To citrated human plasma, undiluted and in the dilutions given below, venom was added in amount required to produce a dilution of 1 in 3,000,000. The time of first appearance of fibrils is calculated from the equation on p. 6.

Plasma %	Time of first appearance of fibrils (min.)		Dimensions (approximate) and aspect of fibrils
	observed	calculated	
100	24	24	$\cdot 25 \mu$ thick; 50μ long; numerous; meshes wide
90	24	24	
80	23	24	$\cdot 25 \mu$ thick; 50μ long; numerous; meshes wide
70	50	55	
60	110	99	$\cdot 25 \mu$ thick; 50μ long; numerous; meshes wide
50	140	151	$\cdot 25 \mu$ thick; 50μ long; less numerous; meshes fairly wide
40	191	215	$\cdot 2 \mu$ thick; 40μ long; less numerous; meshes less wide
30	360	297	$\cdot 1 \mu$ thick; 7μ long; scanty; barely visible
20	no fibrils seen	414	

and II); finally 0.0026 cc. of citrated venom was added, of such concentration as would furnish, after admixture, the titre indicated in the tables. Immediately after mixing, a few cubic millimetres were placed on a microscope slide within an area ringed with vaseline and examined on a dark ground, illuminated by an arc light. The temperature of experiment was 16–18°.

On comparing Tables I and II it will be observed that the effect of dilution of fibrinogen (the amount of thrombin added being the same in each experiment) or of dilution of thrombin (the amount of fibrinogen present being the same in each experiment) is to cause (1) delay in the appearance of fibrin fibrils and (2) diminution in their number. Here, however, the resemblance

Table II.

To citrated human plasma (undiluted) venom was added in amount required to produce the dilutions given below. The times of first appearance of fibrils are calculated on the assumption that concentration of venom $\times$ time of first appearance of fibrils = 8.3×10^{-6} .

Venom	Time of first appearance of fibrils (min.)		Dimensions (approximate) and aspect of fibrils
	observed	calculated	
1: 600,000	5	5	$\cdot 11 \mu$ thick; 5μ long; fine; very numerous; meshes fine
1: 1,000,000	8	8	
1: 1,300,000	11.5	11	$\cdot 2 \mu$ thick; 15μ long; numerous; meshes less fine
1: 1,500,000	10.5	12.5	
1: 2,000,000	19	16.5	$\cdot 25 \mu$ thick; 40μ long; numerous; meshes wide
1: 3,000,000	26	25	$\cdot 25 \mu$ thick; 50μ long; numerous; meshes wide
1: 6,000,000	47	50	$\cdot 3 \mu$ thick; 60μ long; fairly numerous; meshes larger
1: 12,000,000	175	100	$\cdot 35 \mu$ thick; 80μ long; less numerous; meshes very wide
1: 24,000,000	350	200	$\cdot 5 \mu$ thick; 150μ long; scanty; coarse

in the two cases ceases: with increasing dilution of fibrinogen the fibrils become short and fine, and ultimately are barely visible (Table I); with increasing dilution of thrombin, on the other hand, the fibrils become long

and coarse (Table II). The effect of adding alkali (Table III) is seen to be identical with that of diluting fibrinogen: this is very strikingly seen when the penultimate experiment in Table III is compared with the penultimate experiment in Table I and with the last experiment in Table II. It follows, therefore, that the action of alkali is exerted upon fibrinogen: no recognisable effect upon thrombin is observable.

The experiments in Table I, when plotted with concentration of plasma as ordinates and time of first appearance of fibrin fibrils as abscissae are found to lie at first upon a vertical line and then upon a curve, as is shown in Fig. 1. Fibrinogen in solution has been shown to be diphasic [Barratt, 1920], the time of first appearance of visible fibrils (made up almost entirely of the period occupied by the growth of the primitive fibrils, which thereby pass from invisibility into the range of ultramicroscopic vision, this growth occurring at the expense of fibrinogen derived from the continuous or dilute phase) being unaltered by dilution until the disperse (concentrated) phase has nearly completely passed into solution (dilute phase), after which further dilution causes a lowering of the concentration of the dilute phase and as a

Table III.

To citrated human plasma (undiluted) sodium hydroxide was added in quantities corresponding to the concentrations given below. Venom was then added in amount required to produce a dilution of 1 in 3,000,000.

Exp.	NaOH	Fibrils first seen at end of	Dimensions (approximate) and aspect of fibrils
1	0	24 min.	$\cdot 25\mu$ thick; 50μ long; numerous; meshes wide
2	1/120 <i>N</i>	23 "	
3	1.3/120 <i>N</i>	23 "	$\cdot 2\mu$ thick; 20μ long; numerous; meshes small
4	1.6/120 <i>N</i>	24 "	
5	1.9/120 <i>N</i>	50 "	$\cdot 2\mu$ thick; 20μ long; very numerous; meshes very small
6	2.2/120 <i>N</i>	54 "	
7	2.5/120 <i>N</i>	63 "	$\cdot 15\mu$ thick; 10μ long; very numerous; meshes very small
8	2.8/120 <i>N</i>	150 "	
9	3.1/120 <i>N</i>	173 "	$\cdot 1\mu$ thick; 5μ long; very numerous; barely visible
10	3.4/120 <i>N</i>	285 "	
11	3.7/120 <i>N</i>	345 "	$\cdot 1\mu$ thick; 5μ long; less numerous; barely visible
12	4/120 <i>N</i>	no fibrils seen	

result retardation of the growth of primitive fibrils (exhibited by a lengthening of the period of first appearance of visible fibrils). The curve, it may be observed, can be calculated from the equation

$$-lc = kt + a$$

where c = concentration of fibrinogen, $t + 24$ = time of first appearance of fibrils and k and a are constants, represented by 0.0035 and -4.357 respectively.

On reference to Tables I and III it will be seen that in the earlier experiments of each series the period in which visible fibrils first appear is the same,

namely 23 to 24 minutes: only in the later experiments is a retardation observable. This behaviour indicates that the result of adding alkali to plasma is to cause disappearance of fibrinogen, the disperse (concentrated) phase in consequence becoming reduced in amount but the continuous (dilute) phase not being affected until the former phase has nearly completely disappeared, and is therefore no longer able on further dilution to maintain the saturation concentration of the continuous phase, the process occurring being in this respect comparable in its effect to simple dilution of fibrinogen. The concentration of fibrinogen corresponding to the periods given in Table III,

Fig. 1. Graphic record of experiments given in Tables I and III. Ordinates indicate (1) on the left concentration of fibrinogen, that present in undiluted citrated human plasma being represented by 100, and (2) on the right titre of sodium hydroxide; abscissae show time of first appearance of fibrin fibrils. On the curve, which represents the experiments in Table I, the estimated disappearance of fibrinogen on addition of alkali in Tables III and IV is exhibited.

col. 3, may be determined graphically from the curve in Fig. 1 (or by calculation with the aid of the equation on p. 6). The figures so obtained are shown in Table IV, col. 2. If the average amount of fibrinogen disappearing for each increment of alkali is determined (namely that contained in approxi-

mately 7.5 % of plasma for each increment represented by 0.3/120 *N* NaOH) the percentages of fibrinogen given in the third column of Table IV are obtained: these are indicated in Fig. 2, the amount of fibrinogen present in undiluted plasma being represented by 100. (It will be seen from Fig. 2 that no action of alkali on fibrinogen is observable until an amount represented by 0.74/120 *N* has been added.)

Table IV.

Disappearance of fibrinogen on addition of sodium hydroxide in experiments recorded in Table III.

Exp.	Fibrinogen	
	observed	calculated
1	100	100
2	<100	94.5
3	<100	87
4	<100	79.5
5	72	72
6	71	64.5
7	68	57
8	50	49.5
9	47	42
10	32	34.5
11	27	27
12	—	20

Fig. 2. The relation between amounts of sodium hydroxide added in the experiments given in Table III and corresponding estimated diminution of fibrinogen (Table IV).

The variations from a simple proportionality between addition of alkali and disappearance of fibrinogen in the individual experiments recorded in Tables III and IV are most conveniently exhibited graphically, as in Fig. 2. These variations lie within the error of experiment inseparable from the circumstance that on the one hand the volume in which the thrombin and alkali added is contained must be kept very small relatively to that of the plasma (fibrinogen) employed, in order that the concentration of the latter may not be lowered thereby in any marked degree (in these experiments the respective volumes of (1) plasma and (2) thrombin or alkali solutions employed were 19 : 1), while on the other hand owing to the necessity of rapid working the amounts of fluid used must be small. Although the fibrinogen used for experiment was contained in plasma and the experiments given in Tables III and IV indicate, as already noted, the occurrence of a subsidiary reaction, nevertheless the results obtained suggest that the relation between alkali and fibrinogen is stoichiometric. As is well known fibrinogen is not destroyed by alkali: on addition of carbonic acid to alkali plasma the fibrinogen which has disappeared reappears and clotting in presence of thrombin is now readily obtained. The action of fibrinogen in respect of alkali thus appears to be comparable to that of an extremely weak organic acid.

With the increasing dilution of fibrinogen exhibited in Table I, the fibrin fibrils become gradually finer until ultimately they can no longer be recognised. When this stage is reached there is, however, still evidence of the existence of fibrils, though no longer capable of demonstration by ultramicroscopic illumination, for soft clot continues to be formed *in vitro*. It must therefore be inferred that in such apparently structureless clot amicroscopic fibrils are present, though, owing to the dilution of the continuous phase, no longer able to increase sufficiently in size, by further addition of fibrinogen from this phase, to become visible. In support of this inference, reference may be made to the circumstance that under suitable conditions of experiment the formation of structureless clot precedes visible fibril formation: thus when the dilution of fibrinogen is just insufficient to lead to the non-appearance of fibrin fibrils, "setting" *in vitro* may be observed to precede the appearance of visible fibrils on the microscope slide. It has already been shown [Barratt, 1920] that the formation of primitive (amicroscopic) fibrils precedes by a relatively considerable time the appearance of visible fibrils.

When alkali is added to citrated human plasma the same phenomenon is observable with much greater facility, owing to the circumstance that the volume of solution of alkali added may be made very small compared with that of the plasma employed, and thus the diminution in number of fibrils dependent upon simple dilution avoided, with the result that greater firmness of clot is secured. If sufficient alkali is added to plasma to cause disappearance of visible fibrils, a structureless gel of soft consistence is still obtainable: as more alkali is added gel formation becomes uncertain and ultimately ceases. Thus in a series of experiments of the type shown in Table III, in which the

concentration of venom was 1 in 600,000, no visible fibrils were observed after the fifth experiment: in experiments 6 to 9 "setting" *in vitro* occurred in periods ranging from 30 minutes to 3 hours; in the remaining experiments "setting" was imperfect or did not take place.

The appearance of structureless gel after addition of alkali was noted by Howell [1916], who moreover obtained structureless gel from plasma on dilution and on drying.

Addition of alkali to plasma in amount sufficient to delay fibril formation does not at first affect phenolphthalein. No marked red coloration could be observed until fibrils were no longer recognisable and gel formation absent: in other words, until the amount of sodium hydroxide added was as much as would, if added to a volume of distilled water equal to that of the fibrinogen solution employed, give to the latter a concentration of alkali represented by $1/30 N$.

SUMMARY.

(1) Sodium hydroxide, added in increasing amounts to citrated plasma, causes the gradual disappearance of fibrinogen from the plasma: the fibrin fibrils observable in the gel forming on addition of thrombin diminish in size and number, and ultimately pass out of the range of ultramicroscopic visibility; when this stage is reached gel formation may be still observable, but the gel obtained is structureless under microscopic illumination, being made up of amicroscopic fibrils; with still further disappearance of fibrinogen gel formation ceases.

(2) No evidence of any action of alkali upon thrombin was obtained.

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