STAPHYLOCOAGULASE ACTIVITY *IN VIVO*

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THE ability of certain staphylococci to clot plasma was demonstrated as early as 1908 (Much), but it was many years before it became recognised that coagulase production was peculiar to *Staph. aureus* and therefore could be used as a criterion of pathogenicity (Cruickshank, 1937). At the present time the *in vitro* production of coagulase is the commonest and most important test used to differentiate pathogenic and non-pathogenic staphylococci.

Menkin and Walston (1935) made the first attempt to determine whether coagulase production was merely an *in vitro* character or whether it also played a part in the mechanism of disease. Unlike streptococcal lesions which tend to spread, those due to *Staph. aureus* are characteristically localised. Menkin and Walston suggested that this localisation might be due to a fibrin barrier resulting from coagulase action. They failed, however, to show *in vivo* clotting by coagulase and concluded that although the barrier existed, it was due to toxic action on the surrounding tissues. Subsequently Fisher (1936) also failed to find evidence of coagulase action *in vivo*.

Strong presumptive evidence for the *in vivo* action of coagulase came from the work of Wilson Smith and his colleagues (Hale and Smith, 1945 ; Smith, Hale and Smith, 1947), who found that plasma clotted by coagulase inhibits phagocytosis of staphylococci. They also demonstrated that clotting of plasma *in vivo* might be a pre-requisite for the establishment of staphylococcal infection. In their experiments strains of staphylococci capable of clotting human but not guinea-pig plasma did not cause more than a transient lesion in guinea-pigs, but when the organisms were injected at a site into which human plasma had been previously injected, multiple abscesses developed. Lominski and Roberts (1946) showed that the serum of many healthy people contained a substance which prevented coagulase clotting *in vitro*: this inhibitory substance was absent from patients who had recently suffered from a major staphylococcal infection such as acute osteomyelitis. Sera with high anti-coagulase titres protected rabbits against experimental infection (Lominski, 1949). Coagulase inhibition by human serum was subsequently demonstrated by a number of other workers (Kaplan and Spink, 1948 ; Rammelkamp, Badger, Dingle, Feller and Hodges, 1949 ; Tager and Hales, 1948*a*). Finally it was found that animals actively immunised against coagulase were also protected from infection (Boake, 1956).

In the face of all this evidence there can be little doubt that coagulase plays a part in staphylococcal infection, but there is no direct evidence that it does so by exerting its clotting action. The nearest approach to this is possibly the work

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of Tager (1954) who found that after the injection of massive doses of coagulase rabbits died with lowered fibrinogen levels in the blood.

We believe that the present work briefly reported earlier (Smith and Johnstone, 1956) provides direct evidence that coagulase may produce clotting *in vivo*. This clotting action was judged both by the estimation of rabbit blood fibrinogen levels before and after intravenous coagulase injections, and by the histological demonstration of intravascular fibrin deposits in rabbits after these injections.

MATERIALS AND METHODS

Coagulase

Three preparations of coagulase were used. Coagulases 1 and 2 were dried, purified preparations obtained from the well-known coagulase-producing strain Newman: they contained no alpha toxin, being non-haemolytic for rabbit red cells and producing no dermonecrosis in rabbits. Both were very potent: coagulase 1 at a dilution of 1 in 500,000 clotted human plasma diluted 1 in 10 in 1 hr.; coagulase 2 was approximately four times more active. Although highly purified these preparations were not pure and contained only approximately 5 per cent of coagulase (Duthie, personal communication).

Coagulase 3 was a crude preparation from our own high coagulase-producing strain 5R. It was the supernatant of 4-day broth cultures and clotted human plasma at a dilution of 1 in 2000 in 1 hr. This preparation was used for *in vitro* titrations only.

Each of these coagulase preparations clotted not only human but also mouse and rabbit plasma. As measured by clotting times the potency against rabbit and human plasma was the same, but against mouse plasma it was about 16 times lower.

Coagulase injection

Each dose of purified coagulase was dissolved in 5 ml. of saline and slowly injected (10–20 min.) into the ear vein of rabbits. In mice the coagulase, dissolved in 0·5 ml. of saline, was injected into the tail vein.

Animals

Chinchilla rabbits weighing 2–4 kg. and the Porton Swiss White strain of mice were used.

Rabbits immunized against coagulase

Animals were immunised with a coagulase similar to coagulase 3; it was the supernatant of 4-day fluid cultures of strain 5R. In addition to coagulase it also contained gamma lysin. Initially 0·2 ml. of this preparation was injected intracutaneously, followed at weekly intervals by 2 subcutaneous, and 3 intravenous injections, each of about 0·75 ml. The animals were bled 10 days after the last injection and the serum examined for its anti-coagulase titre.

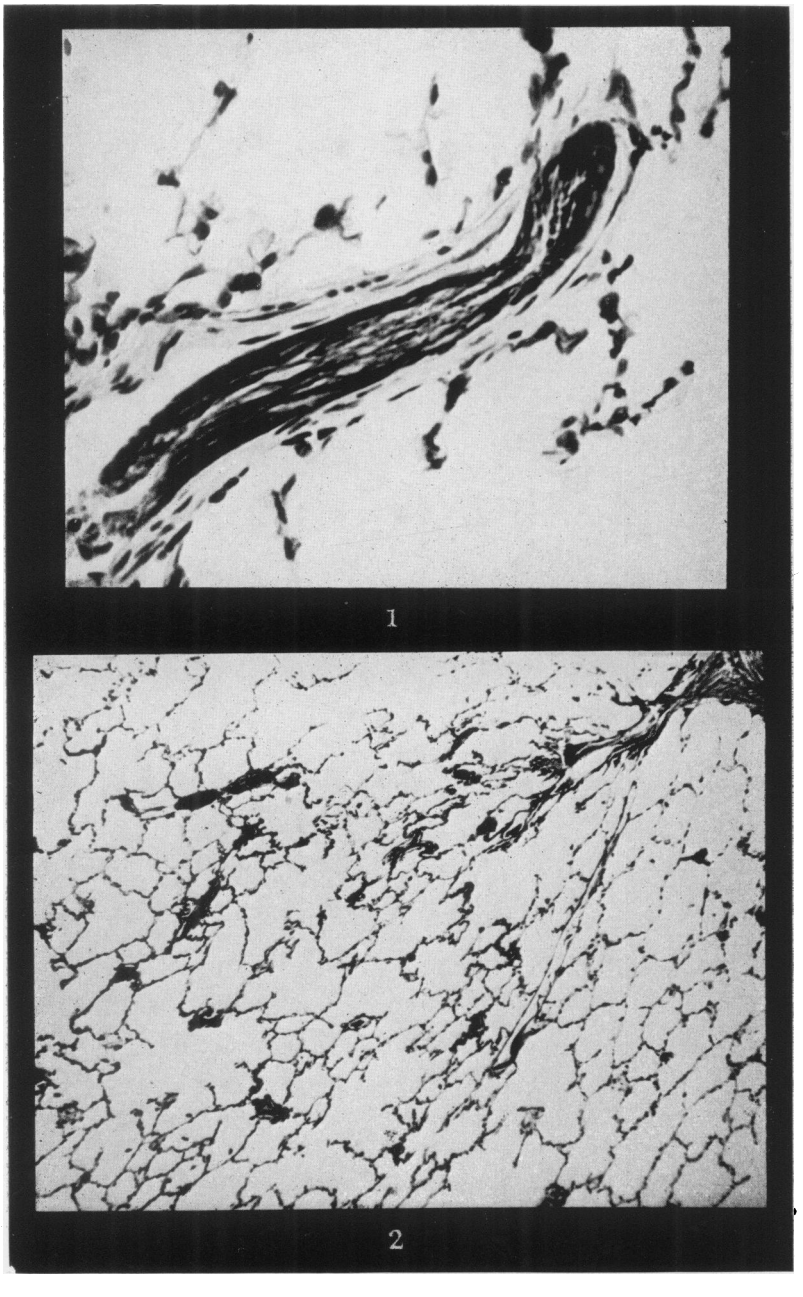
Titration of coagulase inhibition of rabbit sera

The titration was that devised by Lominski and Roberts (1946). Constant amounts of coagulase together with doubling dilutions of the serum being tested were incubated at 37° for 90 min.; thereafter human plasma was added to a final dilution of 1 in 10 and the highest inhibiting dilution of serum was recorded. Readings were taken at the time the control clotted.

EXPLANATION OF PLATE

FIG. 1.—After intravenous coagulase injection the resulting fibrin deposits are formed of multiple strands, ranged parallel to one another and to the axis of the occluded vessel. Gram. × 480.

FIG. 2.—This representative lung field from a rabbit killed by intravenous injection of coagulase shows occlusion of the majority of capillaries and vessels by dark fibrin deposits. Masson's trichrome. × 100.



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Smith and Johnstone.

Estimation of blood proteins

Fibrinogen was estimated by the biuret method described by Barry, Feeney and Geoghegan (1955): heparin was found to be preferable to oxalate as anticoagulant. The method of Gornall, Bardawill and David (1949) was used for the estimation of albumin and globulin.

Histological demonstration of fibrin

Rabbits died within 30 min. of the intravenous injection of large doses of coagulase (*i.e.* 10 mg. of coagulase 1 or 3 mg. of coagulase 2), or were killed at 30 min. after the injection of smaller doses. After preliminary fixation in 10 per cent formal saline the organs were fixed for 24 hr. in corrosive formol. Paraffin sections were prepared and stained by haemalum and eosin, Gram's method, Masson's trichrome, Picro-Mallory, eosin phloxine and tartrazine and Liebs' phosphotungstic acid haematoxylin.

RESULTS

The intravenous injection of coagulase in rabbits resulted in a marked and very rapid fall in fibrinogen with widespread intravascular clotting affecting particularly the lungs.

Effect of coagulase on blood fibrinogen levels

Blood fibrinogen levels before and after the intravenous injection of coagulase 1 are shown in Table I. The slow administration of small amounts had little general effect on the animals, but, if given quickly, they became very agitated and developed leg weaknesses for a short period. After the administration of a large amount the animals rapidly became extremely excited before collapsing with violent spasmodic leg movements and gasping respirations preceding death. In all animals the puncture wounds in the ears bled profusely and bleeding was difficult to control.

TABLE I.—*Effect of Coagulase I on Rabbit Blood Fibrinogen Levels when Injected Intravenously*

Rabbit No.	Coagulase 1 (mg.)	Fibrinogen level (mg./100 ml.) Zero	30 min.	1 day	2 days	3 days	4 days	6 days	Remarks
F2	2	270	95	400	460	360	370	265	Mild reaction.
F6*	4·5	270 (7200)	40 (6300)	320 (5800)	500 —	410 (5800)	435 (5800)	330 (6000)	No apparent effect.
F4	5	300	60	410	—	550	—	—	,, ,,
D30	5	270	110	410	670	525	—	290	Severe reaction.
D31	10	185	25	—	—	—	—	—	Death (½ hr.).
W6	10	480	18	—	—	—	—	—	,, ,,
E54	6 autoclaved	330	305	—	—	—	—	—	No effect: autoclaved coagulase 1.

* Figures in brackets record total protein values in mg./100 ml.

After injection the blood fibrinogen level fell very rapidly, reaching its lowest point at about 30 min., then it quickly rose until by 48 hr. it had reached approximately twice the original value. The level slowly returned to normal in 6 to 8 days (Table I).

Blood samples were also examined electrophoretically and by this means the fibrinogen values obtained chemically were confirmed. It was also shown that fibrinogen alone was being estimated. Further, electrophoresis of the plasma after chemical removal of fibrinogen, confirmed that the fibrinogen was being estimated completely.

A sample of coagulase 1 inactivated by autoclaving for 60 min. was found to have no *in vitro* clotting power. Intravenous injection of 6 mg. had no apparent effect on the rabbit and its fibrinogen level was not altered significantly (Table I).

Effect of coagulase on plasma protein

In 4 animals the total plasma proteins were estimated after injection of coagulase daily for 8 days. The total protein value fell rapidly with the fibrinogen after intravenous coagulase 1 injection, but the fall was much greater (0·8 to 1·4 g./100 ml.) than could be accounted for by removal of fibrinogen alone. Thereafter the total protein value slowly returned towards the original level (*e.g.*, Table I, rabbit F6). The albumin values remained constant and the fall was largely accounted for by a general decrease in globulins, particularly in the gamma globulin fraction.

Inhibition of coagulase in vitro

The coagulase inhibitory titre of the serum of 5 normal rabbits ranged from 1 in 16 to 1 in 64, but in immune rabbits it was 1 in 1000 or more. This inhibition refers to titrations against both coagulase 2 and the homologous coagulase 3.

Inhibition of coagulase in vivo

Graded doses of coagulase 2 were injected intravenously into rabbits and it was found that the fall in fibrinogen was roughly proportional to the amount of coagulase injected (Table II). With 0·125 mg. of this preparation in normal rabbits approximately 60–70 per cent of fibrinogen was removed from the blood in 30 min. When the same amount was injected into actively immunised rabbits

TABLE II.—*Percentage of Fibrinogen Removed from Blood in Coagulase Immune and Non-immune Rabbits at 30 min. after Intravenous Injection of Coagulase 2*

Rabbits E43, C8, and F7 were actively immunised against coagulase.

Rabbit No.	Amount of coagulase 2 (mg.)	Fibrinogen level (mg./100 ml.)		Percentage of fibrinogen removed	Anti-coagulase titre (reciprocal) of serum
		Zero	30 min.		
F28	1·0	210	30	86	64
F20	0·5	170	40	77	32
F19	0·25	310	92	70	—
F36	0·125	270	104	61	—
F17	0·125	338	120	66	16
F27	0·125	300	87	71	64
F23	0·125	435	120	72	32
E43	0·125	255	217	15	1000
C8	0·125	265	190	48	1000
F7	0·125	450	320	29	2000

whose sera inhibited coagulase, the quantity of fibrinogen removed was very much less (15, 29 and 48 per cent respectively).

Histological evidence of intravascular clotting

No macroscopic abnormality was seen in the organs of rabbits killed by, or after, the intravenous injection of coagulase. Microscopically, however, numerous and widespread intravascular lesions were evident. The lungs were the most severely affected organs and when the injections were given very rapidly and death occurred within 5 or 10 min., few lesions were seen in other viscera. If the coagulase was administered more slowly (15–20 min.) the intravascular lesions were readily seen in other organs such as the kidneys and liver but were never as numerous as in the lungs.

The lesions consisted of intravascular deposits giving the typical staining reactions of fibrin. These deposits occluded capillaries and arterioles mainly, and, to a lesser extent small and moderate sized arteries. The deposits were formed of multiple strands of fibrin arranged parallel to one another and to the long axis of the vessel which they occluded (Fig. 1). They were entirely intravascular, occasionally enmeshing small numbers of red cells. No evidence of damage to the vessel walls was seen.

In the lungs an enormous number of fibrin deposits occluded the majority of the vessels of capillary size (Fig. 2) and death appeared to be due to acute right heart failure following mechanical blockage of the pulmonary vasculature.

Effect of coagulase on mice

Doses of up to 2 mg. of the very potent coagulase 2 were injected intravenously into pairs of mice. After one hour one of each pair was killed. The remaining mice survived apparently unaffected by the injection and no evidence of undue bleeding was noted. Microscopical examination of the organs of the mice which were killed failed to show any evidence of intravascular clotting, such as was seen in the rabbit, or any other abnormality.

DISCUSSION

The present experiments give direct evidence that coagulase causes clotting *in vivo*. The failure of some of the previous attempts to show this action may be related to the route by which coagulase was introduced, *i.e.*, subcutaneous, intracutaneous and intrapleural (Menkin and Walston, 1935; Fisher, 1936) and to the low potency of the coagulase used. Although Fisher used the intravenous route the coagulase injected was comparatively weak. Lack and Wailling (1954) injected intrapleurally into rabbits plasma along with a potent purified coagulase similar to that used in the present work. They attributed their failure to show clotting *in vivo* to the action of staphylokinase present in the preparation of coagulase itself.

Since coagulase will clot heparinised or oxalated blood *in vitro*, it might have been expected that blood drawn shortly after the injection of massive doses of coagulase would clot despite the presence of an anticoagulant. The fact that it did not do so suggests the absence of active coagulase; its dilution, neutralisation by antibodies, and fixation in the tissues may account for this.

In mice no histological changes, such as were found in rabbits, followed the intravenous injection of coagulase ; the explanation of this lies probably in the relative deficiency of coagulase-reacting factor of mouse blood, approximately one hundredth that of rabbit blood (Tager and Hales, 1948*b*).

The coagulase preparations used, although purified, were not pure. The possibility, however, that some fraction other than coagulase was responsible for the *in vivo* clotting action is remote. Coagulase has a known *in vitro* clotting activity and therefore it seems reasonable to assume that both the fall in fibrinogen level and the intravascular fibrin deposits are the results of the action of coagulase itself.

SUMMARY

Potent purified coagulase injected intravenously into rabbits produced fibrinogen depletion and intravascular clotting.

The fibrinogen level fell precipitously after injection, then rose rapidly, reaching twice the original level at 48 hr., and returned to normal in a week. The fall in fibrinogen was much less in rabbits immunised against coagulase.

No significant fall in fibrinogen was observed when coagulase, inactivated by autoclaving, was injected into normal rabbits.

Following intravenous coagulase injection into rabbits multiple fibrin deposits occluded many capillaries and small vessels : they were most marked in the lungs but occurred also in the liver and kidneys.

In mice no changes were observed after the intravenous injection of coagulase.

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