3. Caffeic acid, *p*-coumaric acid and an unidentified fluorescent compound of R_F 0·28 are present in many cacao tissues, but ferulic and sinapic acids are of limited occurrence.

4. The major anthocyanin of the young leaf and young pod wall was shown to be 3-α-L-arabinosidylcyanidin, and in the flower 3-α-L-arabinosidylcyanidin and 3-β-D-galactosidylcyanidin were present in approximately equal amounts.

5. Cyanidin hydrochloride was the only anthocyanidin detected in tissue hydrolysates of *T. cacao*.

6. *p*-Coumarylquinic acid was detected in the cotyledons of the bean and in young-leaf tissue. Chlorogenic acid was also shown to occur in young-leaf tissue.

7. A fluorescent phenolic compound releasing caffeic acid on hydrolysis, but not identical with chlorogenic or isochlorogenic acid, was detected in pod-wall tissue.

8. Leucocyanidin material is present in large amounts in young-leaf tissue but the content falls to a very low level at maturity. Similar developmental changes were observed in pod-wall tissue.

The author wishes to thank Mr D. B. Murray for advice on physiological aspects of the current investigation and to express his gratitude to Dr J. Friend, Dr A. E. Flood, Dr V. C. Quesnel, Dr E. A. H. Roberts, Mr N. W. Simmonds and Dr A. H. Williams, for generous gifts of authentic specimens of various polyphenols.

REFERENCES

Alston, R. E. & Hagan, C. W. (1955). *Nature, Lond.*, **175**, 990.
Bate-Smith, E. C. (1954). *Biochem. J.* **58**, 122.
Cartwright, R. A. & Roberts, E. A. H. (1955). *Chem. & Ind.* p. 230.
Forsyth, W. G. C. (1952). *Biochem. J.* **51**, 511.
Forsyth, W. G. C. (1955). *Biochem. J.* **60**, 108.
Forsyth, W. G. C. & Quesnel, V. C. (1957). *Biochem. J.* **65**, 177.
Griffiths, L. A. (1957*a*). *Nature, Lond.*, **180**, 286.
Griffiths, L. A. (1957*b*). *Nature, Lond.*, **180**, 1373.
Hillis, W. E. (1956). *Aust. J. biol. Sci.* **9**, 263.
Hillis, W. E. & Swain, T. (1957). *Nature, Lond.*, **179**, 586.
Lindner, R. C., Fitzpatrick, H. C. & Weeks, T. E. (1950). *Science*, **112**, 119.
Robinson, R. & Robinson, G. M. (1939). *J. Amer. chem. Soc.* **61**, 1605.
Roux, D. G. (1957). *Nature, Lond.*, **180**, 973.
Simmonds, N. W. (1954). *Nature, Lond.*, **173**, 402.
Swain, T. (1953). *Biochem. J.* **53**, 200.
Underhill, E. W., Watkin, J. E. & Neish, A. C. (1957). *Canad. J. Biochem. Physiol.* **35**, 219.

Purification of Free Staphylococcal Coagulase

By E. S. DUTHIE and G. HAUGHTON
Royal South Hants Hospital, Southampton, and the Department of Physiology, Southampton University

(*Received* 24 *February* 1958)

Two types of material capable of interacting with plasma or fibrinogen are formed by pathogenic staphylococci. One of these, termed bound coagulase, is apparently part of the cell wall and is released only by disintegration or autolysis, whereas the second, known as free coagulase, is liberated into the medium during growth (Duthie, 1954*a*). Free coagulase has been shown by numerous workers to interact with a so-called coagulase activator found in the plasma of certain animals to give a thrombin-like material capable of clotting fibrinogen (Smith & Hale, 1944). The nature of the coagulase activator is disputed, but recently the work of Tager (1956) suggests that both prothrombin and another factor, possibly a prothrombin-breakdown product, can function in this respect. The nature of their interaction is unknown.

Partial purification of free coagulase from cultures in digest broth was described by Duthie & Lorenz (1952). They found that coagulase was easily precipitated by cadmium salts, which could be removed by dialysis at an acid pH, giving a coagulase which was relatively stable in solution at pH 2·0. Jacherts (1956) has recently submitted material prepared in this way to further purification by column chromatography on aluminium hydroxide, followed by paper electrophoresis. A single active component staining with ninhydrin was obtained, but no details regarding its specific activity are given.

In the present purification studies, use was made of the finding by Duthie (1954*b*) that if 1 volume of a fully grown shaken culture of staphylococci in digest broth is inoculated into 9 vol. of fresh warm broth, i.e. to give a concentration of about 40 μg. of bacterial nitrogen/ml., and is then shaken at 37°, there is a maximal release of free coagulase in less than 2 hr. By inoculating into a casein-hydrolysate medium instead of digest broth and growing for exactly 1 hr. 20 min. a maximal ratio of free

coagulase to extracellular protein was obtained. This relatively pure starting material was then concentrated by the cadmium-precipitation method already described. The material was stable only if precipitated by ammonium sulphate and stored as a dry powder. By fractionation with ammonium sulphate at low pH values, it was finally possible to obtain coagulase in an electrophoretically pure form. The stages in this purification are the subject of this paper.

MATERIALS AND METHODS

Culture medium. (*a*) The digest broth was Hartley's broth prepared from ox heart (Mackie & McCartney, 1956). Equally good results were obtained with Bacto Brain–Heart Infusion Broth (Difco Laboratories, Detroit, Mich., U.S.A.). (*b*) The casamino acid medium used was similar to that given by Rogers (1945) as medium C, with the following modifications: (1) the casamino acid (Oxoid; Oxo Ltd., London) content was 2% (w/v); (2) hydrated sodium β-glycerophosphate was 1·25% (w/v); (3) the final pH after seeding was 7·4.

The final medium was made as follows and it is referred to as CAA medium. Casein acid hydrolysate (Oxoid), 500 g., anhydrous Na_2HPO_4, 70 g. and KH_2PO_4, 100 g., were dissolved in 3 l. of water. The pH was adjusted to 9·0 with 10N-NaOH. The solution was boiled for 30 min., cooled and filtered. The filtrate was made approximately neutral with 10N-HCl. Tryptophan, 0·5 g., cystine, 2·5 g., and tyrosine, 2·5 g., were added in solution. A stock salt solution containing anhydrous $CuSO_4$, $ZnSO_4$ and $FeSO_4$, each at 0·5% dissolved in N-HCl, was prepared and 0·5 ml. of this was added.

The volume was adjusted to 5 l. with water, and the pH to 6·8 with 10N-HCl. This concentrated medium was dispensed in 200 ml., bottles and autoclaved at 20 lb./in.^{2} pressure for 20 min. Solutions containing sodium β-glycerophosphate, 50% (w/v), and glucose, 5% (w/v), were dispensed in 100 ml. volumes and steamed for 15 min.

When required, 200 ml. of the concentrated acid hydrolysate as above, 25 ml. of the sodium β-glycerophosphate–glucose solution, 0·2 ml. of 0·1% thiamine and 2 ml. of 0·1% nicotinamide were made up to 1 l. with non-sterile water immediately before use. The final pH was 7·6.

Shaking. Penicillin flasks containing 550 ml. of culture were shaken on a horizontal table made to rotate eccentrically about 56 times/min. with a horizontal throw of 12 cm.

Organism used. The Newman strain of *Staphylococcus aureus* (Duthie & Lorenz, 1952), N.C.T.C. 8178, was used.

Filters. Hyflo Super-Cel (Johns Manville, 22 East 40th Street, New York 16, U.S.A.), 16 g., was boiled for 1–2 min. with 200 ml. of 2N-NaOH. This was cooled and filtered through two Whatman no. 1 filter papers in a 15 cm. Büchner funnel. A third filter paper was placed over the earth and the whole pad was washed with 4–5 l. of water. A filter prepared in this way removed bacteria from 20 l. of lightly centrifuged culture supernatant with minimal loss of coagulase.

Assay of coagulase. Fresh human plasma, diluted 1 in 5 with saline, was added in 0·2 ml. amounts to tubes measuring 5·0 cm. × 0·8 cm. in a water bath at 37°. Immediately before use one drop (0·02 ml.) of 6% (w/v) Filter-Cel (Johns Manville) was added to each tube. A volume (0·2 ml.) of coagulase, diluted if necessary in 0·067M-Na_2HPO_4–KH_2PO_4 Sørensen buffer, pH 6·8 (Clark, 1928), was added to a tube and viewed by indirect light against a black background, the tube being slowly tapped with one finger. The end-point was recognized by a rapid agglutination of the Filter-Cel particles. A curve was constructed for dilutions of coagulase, giving clotting times between 15 and 40 sec. A solution which, when diluted one part in two in buffer and tested as above, gave a clotting time of 20 sec. was defined as containing 1 unit/ml. Approximate values for concentrations giving longer clotting times than 40 sec. were obtained by extrapolating the straight line obtained when clotting time was plotted against the reciprocal of the coagulase concentration. Fig. 1 shows that the curve for culture supernatants differed from that for purified material. One unit as above equalled approx. 3500 minimal clotting doses as defined previously (Duthie & Lorenz, 1952). All preparations of coagulase were spun free of precipitates or bacteria, and were neutralized if necessary before testing.

Fibrinogen plates. These contained approx. 75 mg. of human or bovine fibrinogen/100 ml. and 0·2% (v/v) of human plasma (Klemperer & Haughton, 1957). Human fibrinogen was obtained from the Blood Products Laboratory, Lister Institute, Elstree, Herts, and bovine fibrinogen was bovine-plasma fraction I obtained from Armour Ltd., Hampden Park, Eastbourne, Sussex.

Culture storage. Strains were stored after drying by the method of Stamp (1947).

Growth measurement. This was expressed as μg. of bacterial nitrogen/ml. and measured by the indirect method of Rogers (1954) with a Spekker photo-electric colorimeter and spectrum-red filters. Triplicate samples were taken from a shaken culture of *S. aureus* strain Newman growing in

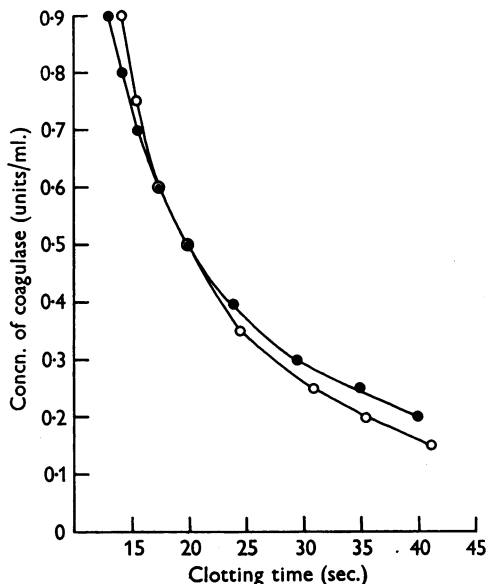

Fig. 1. Relation between clotting time and concentration of free coagulase; in culture supernatants (○) and after extraction from culture supernatant (●).

CAA at 1 and 3 hr. after inoculation with 10% (v/v) of a digest-broth culture shaken for 24 hr. Sample 1 was suitably diluted with CAA and the opacity of a range of dilutions measured. Duplicate samples 2 and 3 were centrifuged, and the cells washed twice with saline and total nitrogen measured by a standard micro-Kjeldahl technique. It was found that the relationship between opacity and bacterial nitrogen was the same for both sets of samples.

Protein. Soluble extracellular protein in culture fluid was measured, by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine albumin (Armour Ltd.) as a standard. In the purification process (Table 5), total protein was calculated from the absorption at 260 and 280 mμ as measured with a Unicam SP. 500 spectrophotometer, with the formula of Kalckar (1947).

Measurement of pH. pH was measured on a Pye Master pH meter.

RESULTS

Early experiments suggested that a main reason for low coagulase production was the appearance of non-coagulase-producing variants which rapidly outgrew coagulase-producing cells. This was tested as follows. A single coagulase-producing colony, recognized on a fibrinogen plate by the production of a large opaque halo around the colony, was inoculated into 20 ml. of 2% (w/v) Bacto-tryptone (Difco) and shaken on an inclined gramophone turntable for 24 hr. at 37°. A loopful (2 mm. diameter) of this culture was then inoculated into 20 ml. of 2% (w/v) Bacto-tryptone and shaken for a further 24 hr. Subculturing was continued in this manner for a total of five cultures. Each culture was plated on fibrinogen–agar to give discrete colonies and coagulase production assessed on the basis of halo diameter, when three distinct kinds were recognized: (*a*) large haloes about 7–9 mm. diameter; (*b*) reduced haloes about 2–4 mm. diameter; (*c*) halo absent. About 500 colonies were counted in each culture and the following was found. All of the colonies in the first and second cultures had large haloes. Of the cells in the third culture 90% gave large haloes and 10% had none. In the fourth culture the proportions were 25% large haloes, 74% without haloes and 1% reduced haloes. In the fifth culture no colonies produced large haloes, 95% were without haloes and 5% had reduced haloes. All three types had identical phage patterns. Rogers (1953) has noted a similar variation in the ability of *S. aureus* strain no. 524 to produce hyaluronidase.

In order to obtain culture supernatants containing the minimum amount of protein consistent with high concentrations of coagulase, it would have been desirable to grow both the inoculum and the inoculated culture in casein-hydrolysate medium. However, it was not possible to use casein-hydrolysate medium for growth of the inoculum, since this resulted in a culture containing a low concentration of coagulase of poor specific activity (Table 1). A similar result was obtained whether the inoculum consisted of whole culture or cells only.

When the inoculum was grown in digest broth and inoculated in casein-hydrolysate medium, coagulase in high concentration and of high specific activity was obtained whether the inoculum consisted of whole culture or cells only. Although the digest-broth culture contained approx. 500 μg. of soluble protein/ml. it was decided to use whole culture rather than cells only, for two reasons. If the inoculum consisted of cells only, then there was not only a somewhat lower yield of coagulase (Table 1) but, more important, a very substantial loss occurred when the cells were removed from the culture by filtration through Hyflo Super-Cel. Digest-broth cultures appeared to contain a substance which protected the coagulase from adsorption on the earth, and for this reason whole cultures in digest broth were used as inocula.

Shaking. All previous work on the production of staphylococcal toxins, commencing with that of Casman (1940), has shown that shaking is essential for obtaining rapid and maximal production. Since large volumes of culture medium were needed a comparison was made of some of the methods available. With a Vortex stirrer such as that used by Chain, Paladino, Callow, Ugolini & van de Sluis (1952), growth was adequate, but the coagulase yield was very low (about 0·1–0·2 unit/ml.). Most

Table 1. *Influence of the preparation of inoculum on production of coagulase by* Staphylococcus aureus *strain Newman in CAA medium*

A volume (50 ml.) of seed culture, shaken for 24 hr., grown in either digest broth or CAA (either whole culture or cells only) was added to 500 ml. of CAA and shaken in a penicillin flask at 37°. At the indicated times a 10 ml. sample was removed from each flask and immediately centrifuged. Coagulase (units) and soluble protein precipitable by trichloroacetic acid were measured in the supernatant.

Growth time (hr.)	Seed grown in digest broth and whole culture used (units/ml.)	(units/mg. of protein)	Seed grown in digest broth and cells only added (units/ml.)	(units/mg. of protein)	Seed grown in CAA and cells only added (units/ml.)	(units/mg. of protein)
1	1·2	19·0	0·70	21·4	0·12	6·0
1·5	1·35	13·5	1·0	20·8	0·2	5·7

successful was either of the following: (i) culture in penicillin flasks (cylindrical flasks 17 cm. diameter and 10 cm. deep) shaken on the horizontal table described above, or (ii) 20 l. bottles containing some 4 l. of culture and rolled in the horizontal position. Since the former were easier to handle and were available, they were used in all the experiments.

Initial culture density and time of growth. That the inoculation of 1 vol. of 24 hr. shaken digest-broth culture into 9 vol. of fresh casein-hydrolysate medium (i.e. 10%, v/v) gave the highest yield consistent with greatest specific activity is clear from Table 2, which is representative of several experiments giving similar results. A 20% (v/v) inoculation gave a slightly higher yield with lower specific activity, and 4% (v/v) gave a similar specific activity and lower yield. If, as in Fig. 2, the casein-hydrolysate medium was used with a 10% (v/v) inoculation then the highest specific activity was found between 80 and 90 min.

Bacterial growth, total extracellular protein precipitable by trichloroacetic acid and coagulase were measured at 20 min. intervals in five cultures grown under apparently identical conditions on 5 successive days. Maximum coagulase concentrations in the five cultures varied from 1·6 to 2·1 units/ml. and maximum specific activities varied

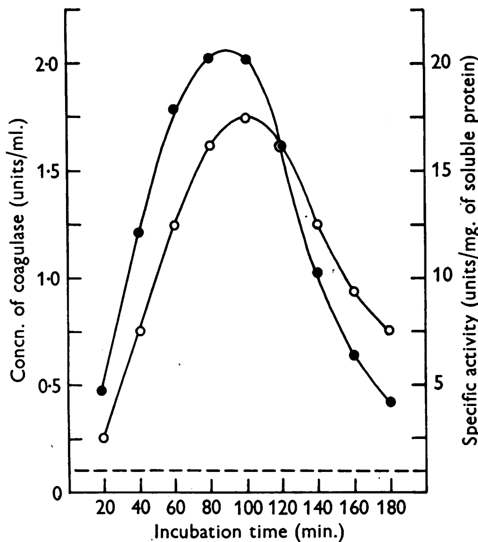

Fig. 2. Free coagulase (○) and specific activity (●) in casein-hydrolysate-culture supernatants as a function of incubation time. The broken horizontal line represents the inoculum level of coagulase.

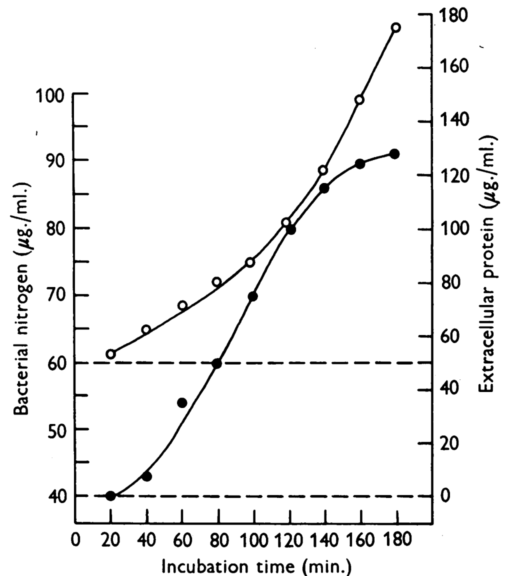

Fig. 3. Bacterial growth (●) and extracellular protein precipitable by trichloroacetic acid (○) in a casein-hydrolysate culture as a function of incubation time. The broken horizontal lines represent inoculum levels of bacterial nitrogen and extracellular protein.

Table 2. *Effect of initial culture density and length of incubation period on final yield and specific activity of coagulase liberated by* Staphylococcus aureus *strain Newman in CAA medium*

A digest-broth culture, shaken for 24 hr., containing 0·8 unit of coagulase/ml. and 500 µg. of soluble protein precipitable by trichloroacetic acid/ml. was diluted with CAA medium previously warmed to 37°. The volume of digest-broth culture indicated was diluted to give a final volume of 550 ml. and shaken at 37° in a penicillin flask. At the times stated a 10 ml. sample was removed from each flask and immediately centrifuged. Coagulase (units) and protein precipitable by trichloroacetic acid were measured in the supernatants.

	Coagulase concentration and specific activity in culture supernatants							
	Vol. (ml.) of digest-broth seed present/550 ml. of culture							
	275		110		55		22	
Growth time (hr.)	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)	(units/ml.)	(units/mg. of protein)
1	0·5	1·7	1·35	11	1·15	20	0·5	20
1·5	0·5	1·7	1·25	8	1·25	18	0·7	19
2	0·4	1·6	1·25	7	1·25	14	1·0	18
3	0·4	1·2	1·25	6·3	1·25	10	1·0	14

Table 3. *Effect of volume of culture grown in each penicillin flask on production of coagulase by* Staphylococcus aureus *strain Newman*

A volume (200 ml.) of a digest-broth culture shaken for 24 hr. was seeded into 2 l. of CAA medium previously warmed to 37°. The volume indicated was shaken in a penicillin flask at 37° for 80 min. Coagulase measurements were then made on culture supernatants.

Culture volume (ml.)	Concn. of coagulase (units/ml.)	Total units of coagulase produced
50	2·0	100
100	2·9	290
200	2·5	500
350	2·3	800
550	2·1	1160
750	1·5	1125

from 19 to 26 units/mg. of extracellular protein precipitable by trichloroacetic acid. Results for bacterial growth and extracellular protein precipitable by trichloroacetic acid were reproducible to within 10%. When each of these functions was plotted against time, curves of similar shape were obtained in each experiment. In all cases the highest concentration of coagulase was reached between 80 and 100 min. after inoculation and maximum specific activity between 70 and 90 min. after inoculation. The results obtained from one of these cultures are presented in Figs. 2 and 3. It should be noted that the liberation of coagulase occurred before the termination of rapid multiplication of the bacteria, and before the liberation of extracellular protein had reached its highest rate. The final conclusion drawn was that an inoculum of 10% (v/v) of a digest-broth culture, shaken for 24 hr. into casein-hydrolysate medium, giving a final concentration of 40 μg. of bacterial nitrogen/ml. when shaken for 80 min. in penicillin flasks, gave greatest yield of coagulase consistent with greatest specific activity.

Culture volume. Experiments were carried out to determine the greatest volume of culture, consistent with high yields of coagulase, which could be grown in a penicillin flask. The results of one such experiment are given in Table 3. In all cases about 500 ml. proved to be the maximum amount in which high concentrations of coagulase were obtained. When less than 500 ml. of culture was grown in each flask, higher concentrations of coagulase were obtained, but the total amount of coagulase was less.

Constituents and pH *of medium.* Each of the constituents of the casein-hydrolysate medium given under Methods was varied independently and the effect of this variation on coagulase production noted. The results, which are typical of several experiments, are given in Table 4. The minimum concentration of each constituent giving highest coagulase production was as follows: (i) casein hydrolysate, 2% (w/v); (ii) hydrated sodium β-glycerophosphate, 0·625% (w/v); (iii) glucose, 0·025% (w/v). These values did not vary in the different experiments.

It had previously been noticed that inoculation of the casein-hydrolysate medium with 0·1 vol. of a digest-broth culture shaken for 24 hr. reduced the pH from 7·6 to 7·4, and that after growth for

Table 4. *Effect of independent variation of concentration of casein hydrolysate, sodium β-glycerophosphate and glucose, and of initial* pH *of seeded culture on production of coagulase by* Staphylococcus aureus *strain Newman in CAA medium*

A volume (50 ml.) of digest-broth culture shaken for 24 hr. was seeded into 500 ml. of test medium previously warmed to 37°. The cultures were shaken in penicillin flasks for 80 min. at 37°. Samples were centrifuged immediately and all coagulase measurements were made on the cell-free supernatants. pH measurements were made on whole cultures.

(*a*) Varying concentration of casein hydrolysate

Concn. of casein hydrolysate (%, w/v)	Concn. of coagulase (units/ml.)
5	1·6
4	2·0
2	2·0
1	1·7
0·5	1·2

(*b*) Varying concentration of sodium β-glycerophosphate

Concn. of sodium β-glycerophosphate (%, w/v)	pH after growth for 80 min.	Concn. of coagulase (units/ml.)
3·75	7·0	1·6
1·25	6·8	2·2
0·625	6·8	2·2
0·25	6·8	1·7
Nil	6·75	1·8

(*c*) Varying concentration of glucose

Concn. of glucose (%, w/v)	Concn. of coagulase (units/ml.)
0·5	1·9
0·125	2·0
0·025	1·9
0·0125	1·5
Nil	1·2

(*d*) Varying initial pH

Initial pH of seeded culture	pH after growth for 80 min.	Concn. of coagulase (units/ml.)
6·50	6·40	1·0
6·85	6·65	1·4
7·10	6·90	1·8
7·40	7·15	1·9
7·55	7·35	2·0
7·70	7·40	1·8

80 min. the pH had fallen to between 6·8 and 7·2. It was therefore thought possible that the influence of sodium β-glycerophosphate might be due to buffering of the culture and that coagulase production might be influenced by pH alone. The point was investigated independently and it was found, provided that the initial pH of the inoculated culture was between 7·1 and 7·7, that there was no significant difference in the concentration of coagulase produced (Table 4). Furthermore it was found that the pH of the culture after growth for 80 min. was identical at concentrations of sodium β-glycerophosphate from 0·25 to 1·25 % (w/v).

In addition, the supplementary amino acids recommended by Rogers (1945) were tested and it was found that the addition of tyrosine (100 mg./l.) and tryptophan (20 mg./l.) to the basic medium either singly or together caused approx. 50 % increase in coagulase production. The addition of cystine (100 mg./l.) was without effect on the final coagulase content of the culture, but this point was not investigated until relatively late in the work and cystine was always included in the medium.

Removal of cells. Since delay in the removal of the cells from cultures grown for 80 min. under the conditions finally used resulted in the further liberation of soluble protein, it was important that the cells should be removed as quickly as possible. The most convenient and rapid method was by centrifuging for 7 min. at 3000 *g* followed by Büchner-funnel filtration with Hyflo Super-Cel.

In choosing Hyflo Super-Cel the following experiment was done. Filter pads were made from 2 g. of each of the following earths: Hyflo Super-Cel, Celite 535, Filter-Cel (all obtained from Johns Manville Co.), kieselguhr B.P. (acid-washed) and kaolin light B.P. (obtained from British Drug Houses Ltd., Poole, Dorset). When 200 ml. volumes of neutral, lightly spun cultures in casein-hydrolysate medium were filtered through the pads, some adsorbed the coagulase almost completely, whereas others failed to retain the bacteria. Good retention with low loss of coagulase was found in Hyflo Super-Cel pads, particularly if the earth was treated by boiling with 2N-NaOH for 1–2 min. It was very important that the same Hyflo Super-Cel pad should be used for as large a volume as possible in order to reduce the loss of coagulase due to adsorption.

When the majority of the bacteria had been removed from the culture by centrifuging it was found that one Hyflo Super-Cel filter pad, prepared as described in Methods, could be used to filter 20–25 l. of culture. If prior centrifuging was omitted, large numbers of bacteria passed through the pad after 2–3 l. of culture had been filtered. Small batches were cleared of bacteria by prolonged centrifuging without recourse to filtration.

Purification of coagulase

Stage I. Cadmium sulphate (670 g.) dissolved in about 2 l. of warm water was stirred into 246 l. of cell-free-culture supernatant containing coagulase at a concentration of 1·7 units/ml. The flocculent precipitate, which contained more than 90 % of the coagulase, was allowed to settle at 4° for 16 hr. Most of the supernatant fluid was decanted and the remainder removed by centrifuging. The precipitate was washed twice with cold water and resuspended in water as a thick slurry. 2N-HCl was added very slowly, with constant stirring at 4°, until a clear solution was obtained and the pH was 2·0. This solution was then dialysed for 48 hr. against a constant flow of KCl–HCl buffer, pH 2·0, *I* 0·1.

Stage II. Ammonium sulphate crystals were added to the 7825 ml. of solution at 4° to give a final concentration of 0·67 saturation. The precipitate was allowed to form for 1 hr., collected by centrifuging and dialysed at pH 2·0 for 24 hr. A certain amount of insoluble material was centrifuged down and discarded. The 594 ml. of supernatant contained 53 % of the coagulase at a concentration of 360 units/ml.

Stage III. The coagulase was reprecipitated by the addition of 1188 ml. of a saturated solution of $(NH_4)_2SO_4$ previously adjusted to pH 2·0 with 10N-HCl. After standing at 4° for 1 hr. the precipitate was collected by centrifuging, washed with 0·67 saturated $(NH_4)_2SO_4$ and dried *in vacuo* over P_2O_5. The yield was 27 g. of dried powder, containing 46 % of the original coagulase activity.

Stage IV. The powder was resuspended and dialysed against a constant flow of buffer, pH 2·0, for 48 hr., then adjusted to pH 2·7 and fractionally precipitated by the slow addition of a saturated solution of $(NH_4)_2SO_4$ previously adjusted to pH 2·7 with 10N-HCl. It was found that 65 % of the coagulase was precipitated between 0·10 and 0·20 saturation. Approximately a fourfold purification was achieved by this step. The precipitate was collected and dialysed against buffer, pH 2·0, for 24 hr. at 4°.

Stage V. This solution (49 ml.) was refractionated with $(NH_4)_2SO_4$ at pH 2·0. The precipitate which formed between 0·125 and 0·175 saturation, although containing only about 20 % of the activity, showed greatly enhanced specific activity.

Stage VI. On dialysis against Na_2HPO_4–citric acid buffer, pH 5·2 (McIlvaine, 1921), *I* 0·1, a precipitate formed, which was removed, leaving a solution of an electrophoretically homogeneous protein. It was not found possible to remove the remaining impurity in the fraction precipitated between 0 and 0·125 saturation, either by dialysis at pH 5·2 or by further fractionation with $(NH_4)_2SO_4$.

Table 5. *Purification of free coagulase produced by* Staphylococcus aureus *strain Newman*

For full details, see text.

	Stage of purification	Vol. (ml.)	10^{-3} × Total activity (units)	Yield (% of original activity)	Specific activity (units/mg. of protein)
	Culture supernatant fluid	$2\cdot46\times10^5$	410	100	25
I	Precipitated by cadmium sulphate (670 g.)	—	—	—	—
	Cadmium removed by dialysis for 48 hr. at pH 2·0	$7\cdot8\times10^3$	—	—	—
II	After pptn. in 0·67 saturated ammonium sulphate and dialysis at pH 2·0	594	215	53	—
III	Reprecipitated with 0·67 saturation of ammonium sulphate: dried over P_2O_5	27·0*	190	46	64·5
IV	Ammonium sulphate fractions, pH 2·7:				
	(i) 0·10 saturated ppt. (dissolved)	133	38	9·5	22
	(ii) 0·20 saturated ppt. (dissolved)	49	106	26	246
	(iii) 0·30 saturated ppt. (dissolved)	37	8·15	2	62
V	Ammonium sulphate fractionation of IV (ii), pH 2·0:				
	(i) 0·125 saturated ppt. (dissolved)	24	89	21·8	326
	(ii) 0·175 saturated ppt. (dissolved)	15·7	23·1	5·6	394
	(iii) 0·25 saturated ppt. (dissolved)	21	4·35	1·1	168
VI	Dialysed V (ii):				
	phosphate–citrate buffer, pH 5·2, *I* 0·1	18	19·8	4·8	430

* Weight (g.).

A summary of the purification procedure is given in Table 5.

Properties of purified coagulase

Stability. The dried powder obtained in stage III of the purification was stable for several months when stored over P_2O_5 *in vacuo* at 4°. After dialysis at pH 2·0 and freezing and storage at −18° at this pH for 1 week, there was a 30% loss of activity but there was no loss at 4° in 1 week. The corresponding losses at neutral pH were much greater, being 30 and 50% respectively.

The purified coagulase obtained in stage V was, however, much less stable. Approx. 50% was lost in 1 week at pH 2·0 or pH 7·0 at both −18° and 4°. No attempt was made to store the purified coagulase in $(NH_4)_2SO_4$ over P_2O_5.

Earlier attempts at freeze-drying, with an impure preparation, resulted in a high initial loss of coagulase activity, i.e. 20–40% loss on drying. The freeze-dried product stored at 4°, either *in vacuo* or in the presence of air, continued to lose activity, and after 1 month the loss was nearly 90%. Neither the initial loss nor the subsequent loss on storage could be prevented by the addition of any of the following: glucose, sucrose, glycine, cysteine, albumin, citrate or ethylenediaminetetra-acetic acid.

Biological properties. Purified coagulase is an extremely active clotting agent both *in vitro* and *in vivo*. As little as 75 $\mu\mu$g./ml. clotted human plasma in 24 hr. at 37°, and 22 μg./kg. body wt. killed rabbits by intravascular thrombosis when given intravenously. Smith & Johnstone (1956) first noted this fact. At least ten times this dose was given subcutaneously or intraperitoneally without damage. Mice possess little or no coagulase activator in their plasma and the intravenous toxic dose was some 30 times that for rabbits, being 750 μg./kg.

Purified specimens of free coagulase did not contain any of the following activities in detectable amounts: α-lysin (measured by the method of Duthie & Wylie, 1945), hyaluronidase (Rogers, 1954), penicillinase (measured by method 2 given by Duthie, 1947) or leucocidin (personal communication from Dr G. P. Gladstone).

Electrophoresis. The purified coagulase was investigated in the Perkins–Elmer Tiselius apparatus at pH 3·6 (Fig. 4), 5·2 and 6·8 in Na_2HPO_4–citric acid buffers (McIlvaine, 1921), *I* approx. 0·1. Boundary analysis showed a single symmetrical peak in each case. Another preparation with a specific activity of 400 units/mg. of protein, i.e. about 90% pure, was examined at pH 2·0 in KCl–HCl, *I* 0·1. This preparation contained two electrophoretic components. All measurements were made at 0°. The observed mobilities are presented in Fig. 5 as a function of pH and give a straight line corresponding to an approximate isoelectric point of pH 5·3. It must be stressed that this is only an approximate value since it was possible to make only one or at the most two measurements at each pH value. In order to obtain an accurate estimate

of the isoelectric point by this method it is necessary to measure mobility at several protein concentrations for each pH and extrapolate the results obtained to zero concentration, but this was not done.

Ultracentrifuging. Two samples, each having a specific activity of 400 units/mg. of protein, were examined in the Spinco model E ultracentrifuge: both preparations showed a simple asymmetrical peak. The first sample contained approx. 2% (w/v) of protein in Na_2HPO_4–citric acid buffer (McIlvaine, 1921), pH 6·8, $I \sim 0{\cdot}4$, and gave a sedimentation coefficient of 3·88. The second sample contained approx. 0·5% of protein in a buffer containing 0·05M-KCl, 0·025M-Na_2HPO_4 and 0·025M-KH_2PO_4, pH 6·8, $I \sim 0{\cdot}2$, and gave a sedimentation coefficient of 4·29. From $S = 4{\cdot}29$ a very approximate minimum mean molecular weight of 44 000 can be derived. This figure could conceivably be doubled by the unknown factors.

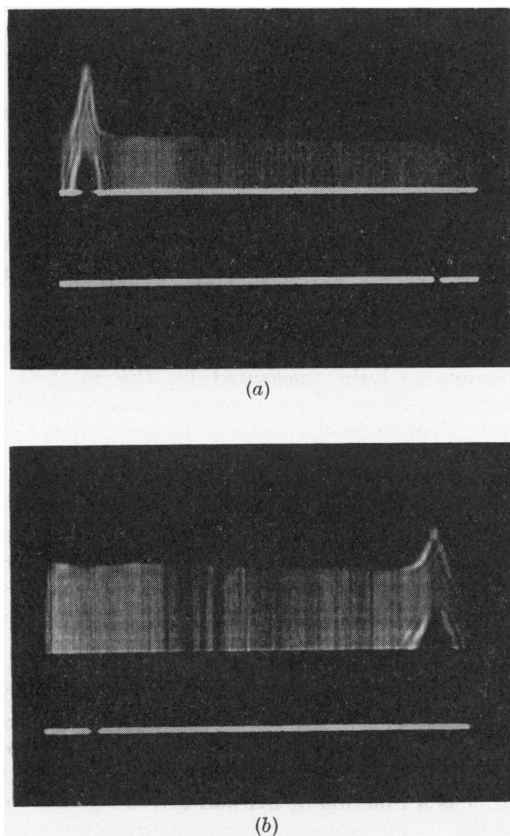

Fig. 4. Boundary patterns obtained on electrophoresis of purified free staphylococcal coagulase at pH 3·6 in Na_2HPO_4–citric acid buffer (McIlvaine, 1921), $I \sim 0{\cdot}1$; (*a*) ascending boundary, (*b*) descending boundary.

DISCUSSION

The purification was greatly simplified by the relative purity of the starting material, which had a specific activity of 25 units/mg. of protein and was therefore about 6% pure relative to the final electrophoretically homogeneous preparation. This relative purity depended not only on the peculiar character of the Newman strain (referred to by Duthie, 1954*b* as strain 2), but also on the nice adjustment of growth conditions, so that the bulk of the coagulase was formed within a short period (80 min.), during which time the coagulase content increased from about 0·1 unit/ml. to 1·75 units/ml. and the total extracellular protein increased only from 53 to 83 μg./ml. A further point was the rapid separation of the cells from the culture medium, thereby avoiding any further liberation of non-coagulase protein from the cells.

The relationship between bacterial growth, extracellular protein and the formation of both hyaluronidase and free coagulase has been extensively studied by Rogers (1954). It is doubtful if his results are directly applicable to the present studies since he used very much smaller inocula, but he noted the absence of any lag in the production of coagulase when fully grown cells were seeded into fresh warm medium. He also found the specific rate of coagulase formation to be slower than cell growth, but he did not find any decrease in the coagulase present after growth had ceased. This is contrary to the present studies, where there was rapid loss of coagulase after 2 hr. (Fig. 2). This

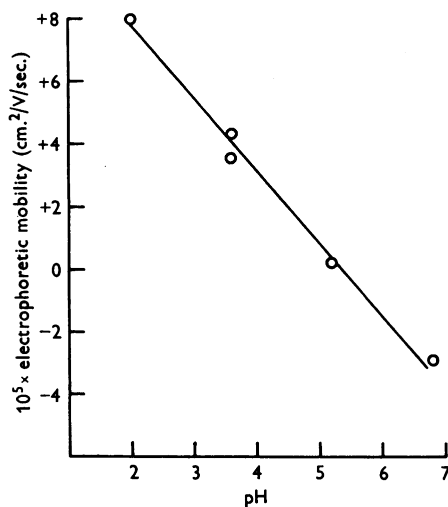

Fig. 5. Electrophoretic mobility of purified free coagulase at 0° plotted against pH. Protein strength in each ~1% (w/v). Buffers used were: KCl–HCl, pH 2·0, $I \sim 0{\cdot}1$; Na_2HPO_4–citric acid, pH 3·6, 5·2 and 6·8, $I \sim 0{\cdot}1$.

discrepancy may be due to differences in the medium used, since in our experiments growth in relatively rich media such as digest broth resulted in a decreased rate of loss of coagulase. After incubation for 16 hr. such cultures still contained about 1·0–1·5 units of coagulase/ml., compared with a maximum of about 2·0–2·5 units/ml. In the present study it was also found important to determine not only the essential constituents of the medium, but to use them in the correct concentration. Our studies on this point showed that, for the most part, Rogers's (1945) medium C was adequate for coagulase production, but not for the preparation of inoculum. In addition, casein hydrolysate was shown to contain inadequate amounts of aromatic amino acids, but the addition of cystine as in medium C was unnecessary. The addition of sodium β-glycerophosphate enhanced the production of coagulase. This was not due to its buffering capacity (Table 4*b*), but possibly because it was metabolized.

The instability of the enzyme at pH values above 2° excluded many of the adsorption techniques such as ion-exchange chromatography, since this involved working at above pH 2·0 for long periods. On the other hand, the stability of the enzyme at pH 2·0 made it possible to use such adsorbents as cadmium sulphate and aluminium phosphate, which could be dialysed away at this pH. Another important technique, that of alcohol precipitation, could not be used since coagulase together with its impurities was soluble in 95% ethanol at pH 2·0. Because of this, ammonium sulphate fractionation at low pH proved to be the only feasible method in the final stages of purification.

As can be seen from Table 5, the powder obtained after stage III contained nearly 50% of the original coagulase in a form which could be stored until needed. The subsequent low overall yield was due to the heavy loss in fraction V (i), where some 22% of the initial coagulase contained a protein impurity which could not be eliminated by subsequent fractionation. Fraction V (ii), 5·6% of the original coagulase, contained a single protein impurity which was easily removed by dialysis at pH 5·2.

The purified material obtained in the present study is the extracellular free coagulase which clots all fibrinogens, but only in the presence of a plasma activator. A certain specificity has been noted in this reaction, inasmuch as the free coagulase produced by a certain staphylococcal strain may react more readily with the plasma activator of one species than with another. Thus the free coagulase produced by strains of human origin clots bovine fibrinogen most readily in the presence of small amounts of human, rabbit and horse plasma, whereas clotting may not occur if sheep, guinea-pig, cow, dog or mouse plasma is used (Duthie, 1954*a*). In contrast with free coagulase, the majority of pathogenic staphylococci have on their surface a second factor which adsorbs the fibrinogen of certain species on to the cell surface, causing the cocci to adhere in gross floccules when shaken. This has been termed bound coagulase or clumping factor (Duthie, 1954*a*, 1955) and bears no relationship to free coagulase as far as can be discovered. It acts directly on the fibrinogens of certain species, and these are often not those whose plasma is clotted by the free coagulase produced by the same cells. It is antigenically distinct, and strains are easily found which produce one kind of coagulase and not the other. In the present studies variants of the Newman strain regularly appeared on subculture in 2% (w/v) Bacto-tryptone medium. These were coated with bound coagulase, but produced no free coagulase either on fibrinogen plates or in the medium.

The conception that there are two distinct coagulases has been recently challenged by Jacherts (1956) on somewhat inadequate grounds. He made one preparation of extracellular protein and two of protein extracted from bacterial cells, all of which had coagulase activity, which he found were identical in biological, immunological and physical properties. From this he concluded that free and bound coagulase as defined by Duthie (1955) are also identical. This conclusion is, however, based on a misinterpretation of the results. Washed staphylococcal cells possess a small amount of cell-associated hyaluronidase (Rogers, 1954), which can be obtained by crushing the cells with Ballotini beads or merely by storage in buffer overnight at 0–4°, when release occurs. Small amounts of cell-associated free coagulase were obtained by him in the same way. In the present study 0·1 unit of free coagulase was obtained on crushing the washed cells of 100 ml. of fully grown digest-broth culture and then extracting at pH 2·0. A very much larger amount (3·3 units) was obtained by extracting a similar volume of washed cells for a few minutes at pH 11·0 in the cold. Both extracts were identical with free coagulase and were quite distinct from bound coagulase. It is concluded that the material obtained by Jacherts was cell-associated free coagulase liberated by the treatment used, and that this bore no relationship to the bound coagulase described by Duthie (1955).

SUMMARY

1. The conditions under which free coagulase of high specific activity is liberated in the culture fluid have been investigated. Optimum conditions were obtained when a heavy inoculum of a fully grown digest-broth culture was seeded into a casein-hydrolysate medium and shaken for 1 hr. 20 min. at 37°.

2. The enzyme was isolated in an electrophoretically homogeneous form by adsorption on to cadmium sulphate, followed by fractional precipitation with ammonium sulphate.

3. On electrophoresis the purified enzyme had an isoelectric point of about pH 5·3. Ultracentrifuging a slightly impure preparation showed a minimum mean molecular weight of approximately 44 000.

4. The material was extremely toxic when injected intravenously into rabbits, and a concentration of 75 $\mu\mu$g./ml. clotted human plasma in 24 hr.

Our best thanks are due to Dr H. J. Rogers for many helpful discussions and for laboratory facilities, to Dr P. A. Charlwood for examination of material in the ultracentrifuge at the National Institute for Medical Research, Mill Hill, London, and to Glaxo Laboratories, Greenford, Middlesex for laboratory facilities. We also wish to thank Mr J. W. Ottewill and Mr W. Phillippe for their valuable help in constructing some of the equipment used. The work was made possible by a research grant from the South West Metropolitan Regional Hospital Board.

REFERENCES

Casman, E. P. (1940). *J. Bact.* **40**, 601.
Chain, E. B., Paladino, S., Callow, D. S., Ugolini, F. & van de Sluis, J. (1952). *Bull. World Hlth Org.* **6**, 73.
Clark, W. M. (1928). *Determination of Hydrogen Ions*, 3rd ed., p. 210. London: Baillière Tindall and Cox.
Duthie, E. S. (1947). *J. gen. Microbiol.* **1**, 370.
Duthie, E. S. (1954*a*). *J. gen. Microbiol.* **10**, 427.
Duthie, E. S. (1954*b*). *J. gen. Microbiol.* **10**, 437.
Duthie, E. S. (1955). *J. gen. Microbiol.* **13**, 383.
Duthie, E. S. & Lorenz, L. (1952). *J. gen. Microbiol.* **6**, 95.
Duthie, E. S. & Wylie, J. A. H. (1945). *Brit. J. exp. Path.* **26**, 130.
Jacherts, D. (1956). *Z. Hyg. InfektKr.* **142**, 502.
Kalckar, H. M. (1947). *J. biol. Chem.* **167**, 461.
Klemperer, R. & Haughton, G. (1957). *J. clin. Path.* **10**, 96.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Mackie, T. J. & McCartney, J. E. (1956). *Handbook of Practical Bacteriology*, 9th ed. Edinburgh: Livingstone.
McIlvaine, T. C. (1921). *J. biol. Chem.* **49**, 183.
Rogers, H. J. (1945). *Biochem. J.* **39**, 435.
Rogers, H. J. (1953). *J. Path. Bact.* **66**, 545.
Rogers, H. J. (1954). *J. gen. Microbiol.* **10**, 209.
Smith, D. D. & Johnstone, J. M. (1956). *Nature, Lond.*, **178**, 982.
Smith, W. & Hale, J. H. (1944). *Brit. J. exp. Path.* **25**, 101.
Stamp, Lord (1947). *J. gen. Microbiol.* **1**, 251.
Tager, M. (1956). *J. exp. Med.* **104**, 675.

The Phosphotransferase Activity of Phosphatases

1. SPECTROPHOTOMETRIC METHODS FOR THE ESTIMATION OF SOME PHOSPHATE ESTERS AND OTHER COMPOUNDS

By R. K. MORTON*
Department of Biochemistry, University of Cambridge

(*Received* 31 *July* 1957)

For the kinetic study of the 'phosphotransferase' (Dixon, 1949; Dixon & Webb, 1953) reaction catalysed by phosphatases (Morton, 1952, 1955*a*, 1958*a*, *b*) it became necessary to estimate phosphate esters without separation from the reaction mixture. It was required that the methods used would be accurate, sufficiently sensitive to measure between 0·05 and 0·2 μmole of a compound, and convenient enough to enable a large number of determinations to be handled.

Methods based on reactions catalysed by highly specific dehydrogenases appeared to meet all the above criteria; dehydrogenation of a substrate is coupled with the reduction of cytochrome *c* or diphosphopyridine nucleotide, which is estimated by changes in light-absorption at appropriate wavelengths. This paper describes the estimation of D-hexose phosphates, L-α-glycerophosphate, D-glyceraldehyde phosphate, dihydroxyacetone phosphate and phosphocreatine. Extension of these methods enables succinate, L-lactate and a variety of other compounds to be estimated.

* Present address: Department of Agricultural Chemistry, The Waite Institute, University of Adelaide, South Australia.

MATERIALS

Enzyme preparations

Yeast alcohol dehydrogenase. The crystalline enzyme was prepared from dried Fleischman's yeast by the method of Racker (1950).

Rabbit muscle L-*α-glycerophosphate dehydrogenase.* The preparation was based on the observations of Green (1936). Rabbit skeletal muscle (about 300 g.) was chilled in crushed ice immediately after removal from the animal, and finely minced and extracted twice (30 and 15 min.) with two