Technical methods

The use of papain-EDTA for Rh typing with 'incomplete' antisera

B. P. L. MOORE *From the National Reference Laboratory, Canadian Red Cross Blood Transfusion Service, Toronto*

Cysteine-activated papain (Löw, 1955) is an excellent reagent for Rh typing blood bank and ante-natal specimens by a one-stage technique using low-titre incomplete antisera, but it should only be used with incubation periods of 45 minutes or less; longer incubation tends to result in friable agglutinates.

Unactivated 1% papain (Stapleton and Moore, 1959) is better suited to incubation periods of 45 to 90 minutes, and this method has now been used successfully in this department for more than one million routine tests. Milk-clotting and serological tests have, however, shown a variation in the activity of batches prepared by different regional centres. It has now been found that a modified method of preparation and the addition of a small amount of di-potassium EDTA as a chelating agent (Kimmel and Smith, 1954) results in a more potent and stable extract which can safely be used with a papain: antiserum ratio of 2:1.

METHODS

PREPARATION OF PAPAIN-EDTA 1%. To 1 vol. 0.2 M phosphate buffer at pH 5.4, add 9 vol. of chilled, sterile 0.15 M saline. To 100 ml. of 0.02 M buffer-saline in a 100 ml. flask, add 1 g. papain (B.D.H.) and 0.05 g. (0.00125 M) K_2 EDTA. $2H_2O$.

Stopper the container tightly, and place on a Kahn shaker for 10 minutes. A small air-space is essential at this stage or too much powder will be left on the wall of the flask.

Leave at 4°C. overnight. Mix well, and filter through Whatman's No. 1 paper; this step should take place at 4°C. Dispense in 0.5-4 ml. aliquots. Label and cork the tubes and store at -20°C. for no more than two months. Once thawed, this product must not be refrozen.

METHOD FOR RH TYPING Suitable sera must react specifically in saline and albumin, by the indirect anti-globulin technique, and by the method to be described. In this study, the antisera were of our own manufacture, and had an albumin titre against heterozygous CDe/cde cells of 1/16 to 1/32.

Absorption controls are essential if the occasional re-appearance of trace amounts of anti-A, -B, -C, etc., is to be detected, e.g., A_1 dCe cells should be used to detect

the return of such unwanted antibodies to an anti-D serum.

Since cells coated with antibody are agglutinated by a mixture of papain and AB serologically inert serum (Löw, 1955), there is a chance that occasional 'false' reactions may occur. This danger is, in our experience, insignificant when testing blood donations or ante-natal specimens. However, since it is clinically important to avoid calling a D- negative ante-natal specimen D- positive, a negative control of papain and AB serum should be included when testing samples from such patients. Weiner and Nussey (1961) have emphasized this point, and have suggested that a one-stage papain test may be used for Rh typing patients before transfusion if such a control is included, but only when supplies of saline-active antisera are unobtainable.

Thaw a tube of papain-EDTA by holding it in the hand. Shake the tube well, and immediately add two volumes of papain to one volume of antiserum. Thoroughly mix the contents and use within 45 minutes.

In 7×55 mm. tubes place one drop of papain-serum and add one drop of a 4% saline suspension of the cells under test; the cells do not need to be washed in saline if the serum has been removed carefully. If 10×75 mm. tubes are used, the above volumes should be doubled.

Mix the contents and incubate for 30 to 90 minutes at 37°C. Longer incubation is not advised, but 10×75 mm. tubes may be centrifuged at $\times 120 g$ for one minute after 15 minutes' incubation. The results are read macroscopically, starting with the controls.

MILK-CLOTTING ASSAY The method used is essentially that of Balls and Hoover (1937) as modified by Hinkel and Alford (1951), Hinkel and Zippin (1951), and Löw (1955). Dried-milk powder, 20 g., is dissolved in 100 ml. of 0.1 M acetate buffer at pH 5.4, after which the solution is clarified through several thicknesses of gauze and stored at 4°C. Five ml. of milk is pipetted into a 16×150 mm. tube which is then placed in a 37°C. water-bath for 15 minutes and 0.5-3.0 ml. of enzyme extract warmed to room temperature added to the milk. The tube is tightly corked, and a stop-watch is started. The tube is then held horizontally below the surface of the water in the bath and intermittently rotated. The film of milk coating the tube thickens just before granularity appears. As soon as granules are seen, the time is noted. Results are conveniently expressed as 100V/Et units where V=volume of milk in ml., E=mg. enzyme added, and t=time in minutes. For maximum accuracy, E should be adjusted so that $t=1$ to 5 minutes (Hinkel and Alford, 1951).

RESULTS

It is not clear whether milk-clotting tests on papain extracts not activated by cysteine are a measure of proteolytic activity, but the results form an approximate and

rapid guide to the serological behaviour of a particular extract or method of preparation. Typical results are shown in Table I. The results on fresh extracts are reproducible with an error of $\pm 10\%$, but deterioration is rapid at 20°C ., and is still noticeable at 4°C .; extracts stored at

TABLE I
Milk Clotting 'Units'

Papain Preparation	Fresh Extract Alone	Extract + Serum 2:1
Stapleton and Moore	28	15
Papain-EDTA	53	30
Either, Seitz clarified	6	2
Papain-cysteine (Löw)	128	75
Seitz clarified	113	50

-20°C . lose activity more slowly. As might be expected papain-cysteine (Löw, 1955) is both the most active and the most stable preparation.

Serological tests confirmed the impression gained from the milk-clotting tests, and showed that papain-EDTA produced higher titration scores and more avid reactions than the 1% papain of Stapleton and Moore (1959). No difficulty was experienced with 'non-specific' reactions, nor did the presence of bovine albumin in concentrations of 7.5-15%, the range present in our antisera, result in any lack of specificity. Diluted 10 times in buffer-saline, papain-EDTA gave almost as high titration 'scores' as the undiluted product, whereas a prozone and a low titration 'score' occurred when papain 1% (Stapleton and Moore, 1959) was diluted three times. The stability of papain-EDTA at 20°C . was greater than that of the simpler extract, particularly in the presence of serum.

This new extract has now been in routine use in this service for several months with selected anti-D, -C+D, and -c sera. During this time no discrepancies have been noted between the latest results and those of former tests on the same persons. Preliminary studies have shown that papain-EDTA 1% also forms a satisfactory reagent for the detection of Rh antibodies by a modification of the two-stage technique of Lang and Lodge (1961).

REFERENCES

- Balls, A. K., and Hoover, S. R. (1937). *J. biol. Chem.*, **121**, 737.
 Hinkel, E. T., and Alford, C. E. (1951). *Ann. N. Y. Acad. Sci.*, **54**, 208.
 —, and Zippin, C. (1951). *Ibid.*, **54**, 228.
 Kimmel, J. R., and Smith, E. L. (1954). *J. biol. Chem.*, **207**, 515.
 Lang, B. G., and Lodge, T. W., (1961). *Vox Sang (Basel)*, n.s. **6**, 353.
 Löw, B. (1955). *Ibid.*, **5**, 94.
 Stapleton, R. R., and Moore, B. P. L. (1959). *J. Lab. clin. Med.*, **54**, 640.
 Weiner, W., and Nussey, A. M. (1961). *Lancet*, **1**, 257.

An improved plasma recalcified clotting test and its modification as a simple rapid heparin retarded clotting test

R. D. EASTHAM *Frenchay Hospital, Bristol*

Uncontrolled 'contact' activation and subsequent decay of activated plasma coagulation factors and variation in platelet substrate action during clotting are the two main factors which make the simple plasma calcium clotting time very variable (Margolis, 1957). Soulier (1959) found that as little as 1 mg. of bentonite resulted in rapid and complete activation of 1 ml. of plasma. Soya bean extract has been found to be a very satisfactory platelet substrate substitute both in the clotting of recalcified plasma and in thromboplastin generation tests (Connor and Carter, 1958; Hyun, Dawson, Butcher, and Custer, 1960).

It has been possible to develop a simple, accurate and reproducible plasma calcium clotting test, using optimum concentrations of soya bean extract and optimum amounts of bentonite. After exposure to bentonite fresh plasma is completely activated within three minutes, and by performing the test within the subsequent 10 minutes, activated factors do not decay. Since no tissue thromboplastin is added to this system, presumably mainly intrinsic thromboplastin is responsible for a normal plasma clotting time of between 34 and 43 seconds by this method. When heparin is added to the system in amounts sufficient to interfere with intrinsic thromboplastin generation, a simple rapid heparin retarded clotting test results, which takes less than three minutes to perform after the initial three minutes' activation of plasma.

PROCEDURE

REAGENTS The following are made up:—

Heparin solution Heparin solution (5 international units per ml.) is made up from a commercial preparation of initial strength of 5,000 units per ml. in 0.13 g. % parachlormeta-cresol in normal saline and kept at 4°C . (stable for at least one month).

Bentonite suspension Bentonite suspension (20 mg./ml. in normal saline) is stable at room temperature. Mix thoroughly before use. The working suspension is 2 mg./ml. in saline. Mix before use.

Soya bean extract Inosithin¹, 5 g. per 100 ml. in normal saline, is kept at -40°C . A working solution of 0.2 g. per 100 ml. saline is made up in a series of small tubes and kept frozen until required.

Heparin—bentonite working suspensions These are two, A and B.

- A 1 ml. stock bentonite suspension
- 1 ml. stock dilute heparin solution
- 8 ml. 0.85% saline.

¹The agents for Inosithin are V. A. Howe and Co. Ltd., 64 Pembridge Road, London, W.11.

Received for publication 13 October 1961.