and diffuse), the circle of reaction becoming smaller and more intense with decreasing antigen concentration. The control strip impregnated with the normal human serum is completely negative apart from one atypical spot at the site of application of the highest concentration of antigen.

DISCUSSION

As reported in an earlier paper (Feinberg, 1962), the time of antigen-antibody reaction, washing and staining can be considerably reduced. Taken together with the sensitivity of the test as now shown for precipitating auto-immune antibody, it becomes possible to carry out the entire test—from blood-taking to final result—within a day.

The technique could be considerably simplified by using a single 'microspot' of a 40 μg./ml. concentration of thyroglobulin, which approximates to dilution 6 on the strips in the illustration. This would readily detect auto-immune thyroiditis antibody in sera of patients having 1%, or even less, of the antibody contained in the serum of the patient used in this investigation.

Though the present investigation was restricted to auto-immune thyroiditis, the 'microspot' test should be equally applicable to other auto-immune conditions in which precipitating antibody circulates in the blood plasma, and may be useful in demonstrating such antibody in conditions where it has not yet been found. Such conditions could include rheumatoid arthritis, lupus erythematosus, atopic dermatitis, idiopathic adrenal atrophy, and Sjögren's syndrome. In the last, Jones (1958) found precipitating auto-antibodies to extracts of the lachrymal and salivary glands, though Anderson, Gray, Beck, and Kinnear (1961) subsequently showed these antibodies would react with antigens from a wide variety of tissues. They also demonstrated the presence of auto-antibody to thyroglobulin in some Sjögren sera. The 'microspot' test should also find a use in the demonstration of naturally, or artificially induced, heterophile antibody in man, as well as in the detection of low concentrations of antigens.

SUMMARY

A new specific antigen-antibody precipitation technique, the 'microspot' test, has been applied to the detection of the antibody arising spontaneously in auto-immune thyroiditis. It has been found that the technique can detect low levels of both the auto-immune antibody and the antigen against which it is directed. It is suggested that the technique could usefully be extended to the study of other proven or suspected auto-immune conditions.

I should like to acknowledge the photographic reproductions of Douglas F. Lawson.

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The estimation of β amino-isobutyric acid in human urine

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β Amino-isobutyric acid (B.A.I.B.) was identified independently by Crumpler, Dent, Harris, and Westall (1951) and Fink, Henderson, and Fink (1951), and was shown by Fink, Cline, Henderson, and Fink (1956) and Gartler (1959) to be a product of thymine metabolism. Examination of urine from victims of accidental whole body irradiation by Rubini, Cronkite, Bond, and Fliedner (1959) and by Gerber, Gerber, Kurohara, Altman, and Hempelmann (1961) showed significant increase of β amino-isobutyric acid. This suggested that the estimation of B.A.I.B. in urine would be helpful in indicating the extent of cellular damage after massive irradiation. Furthermore Awapara (1957) has shown significant increases of B.A.I.B. after nitrogen mustard therapy in cases of leukaemia, lymphosarcoma, and Hodgkin's disease, and measurement of this metabolite may be a useful index of D.N.A. catabolism in clinical medicine. For this purpose a relatively simple, quick, cheap and reliable method is required.

EXISTING METHODS OF DETERMINING B.A.I.B.

1 The method of Rubini *et al.* (1959) comprises decolorization with charcoal, desalting with resin, and two-way chromatography. After ninhydrin staining the unknown is compared visually with standards.

The advantages are ease, cheapness, and safety. Disadvantages are the impossibility of running large numbers of samples (including standards) at any one time and the use of visual methods of comparison. The problem of separation from urea, which is not stressed by Rubini, is discussed elsewhere.

2 In the high-voltage electrophoresis method of Gartler (1959), after separation and ninhydrin staining the paper is scanned. Advantages are speed and possibly accuracy, but the high cost and unfamiliarity of the

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apparatus combined with the hazard of a high-voltage system are serious drawbacks.

3 Using iron-exchange columns (*e.g.*, Jagenburg, 1959), the amino-acids are absorbed onto a column of ion-exchange resin and serially eluted with buffers in a fractionator. Final estimation is performed in a tube with ninhydrin and accuracy is probably high, although it is admitted that other amino-acids are sometimes included with B.A.I.B. Although reasonably quick for a single sample the method is not suited to multiple estimations.

4 The method of Gerber and Gerber (1960) has not been successful in our hands. By this method B.A.I.B. is converted to its dinitrophenyl derivative which is extracted and run on a one-dimensional chromatogram. The material is applied along a 4-in. line and the compound, after elution from the appropriate horizontal area, is measured spectrophotometrically with elaborate colour corrections. The method is reasonably rapid and several samples can be run at once. However, there are opportunities for loss of the compound, some of the reagents are hazardous (dinitrofluorobenzene is a vesicant and tetrahydrofuran is potentially explosive), and the final measurement appears to be subject to error.

EXPERIMENTAL

In evolving the method to be suggested the following procedures were examined.

Electrolytic desalting fails to remove urea, which is the chief cause of splitting and diffusion of the B.A.I.B. spot, thus causing difficulty in identification and sampling (Fig. 1).

Urea may be removed by urease but the impurities in urease preparations are themselves confusing.

If desalting is effected by ion-exchange resins certain critical considerations arise. The resin must be in the pure 'H' form initially, and after absorption many washes are necessary to remove the urea (Fig. 1).

Chromatography on 10-in. squares gives inadequate separation in a one-way run, while a two-way run produces a diffuse spot with some loss and consequent inaccuracy. Furthermore the number of samples and standards that can be run is, of course, drastically reduced.

The colorimetric reaction of Moore and Stein (1948) applied to the eluate obtained by washing out the unstained spot did not give reproducible results. This was due to the elution procedure and not to the reaction which gave accurate results with standard solutions. Sampling errors were also found to be a hazard with this method.

The use of a dilute staining solution followed by development of full colour in the test tube, as suggested by Landua and Awapara (1949), was not of value with the expected concentrations of β amino-isobutyric acid.

APPARATUS AND REAGENTS

PREPARATION OF RESIN COLUMN Zeo-Karb 225, 100-200 mesh, 8% cross linkage, purified according to Smith's (1960) technique and stored in the H form. A column of the resin, 5 × 0·9 cm., is prepared in a tube plugged at one end with glass wool. A 10 ml. pipette (bore = 0·9 cm.) does very well for the purpose.

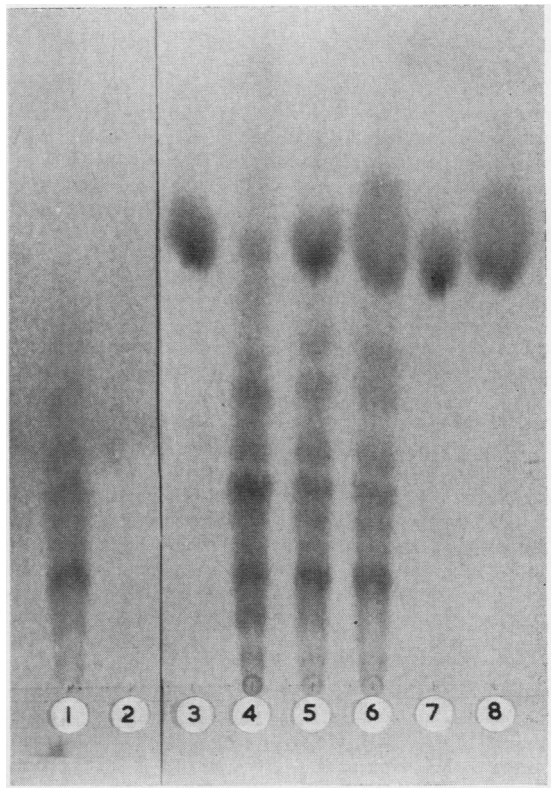

FIG. 1. *Lane 1 Urine from case of chronic myeloid leukaemia, 300 μg. nitrogen, unheated*
Lane 2 Standard B.A.I.B., 10 μg., unheated
Lane 3 Standard B.A.I.B., 10 μg., heated
Lane 4 Normal urine, 300 μg. nitrogen, heated
Lane 5 Urine from case of chronic myeloid leukaemia, 300 μg. nitrogen, heated
Lane 6 Urine from case of chronic myeloid leukaemia with 450 μg. urea added, heated
Lane 7 Standard B.A.I.B., 10 μg., heated
Lane 8 Standard B.A.I.B., 10 μg., with 450 μg. urea added, heated.

CHROMATOGRAPHY TANK A 12 in. universal strip Cromotank[1].

CHROMATOGRAPHY PAPER Whatman No. 1 23 × 57 cm. The amino-acid solutions are applied at 2·5 cm. intervals along a line marked across the sheet 10 cm. from one end. The paper is folded along a line 7 cm. from the same end.

CHROMATOGRAPHY SOLVENT This is n-butanol/acetic acid/water, 120/30/50.

[1]Shandon No. 2120

STANDARDS A set of 10 standards of pure B.A.I.B.[2] is prepared containing 1 to 10 μg./0·02 ml. of 10% isopropanol.

NINHYDRIN REAGENT This reagent was prepared according to the method of Clarkson and Kench (1948). It was found that an Analar grade of indanetrione hydrate was adequate for the purpose without further purification.

SPECTROPHOTOMETER A Hilger Uvispec was used.

TECHNIQUE

COLLECTION OF SPECIMENS Twenty-four-hour samples of urine are collected without preservative and stored at 4°C.

TOTAL NITROGEN This was estimated by the micro-Kjeldahl technique.

DESALTING Urine, 1 ml., is passed slowly through the 5 × 0·9 cm. resin column; the salts and urea are washed off with 35 ml. of water. The amino-acids are then eluted with 2N ammonia, and 15 ml. eluate is collected and dried down, taken up in alcohol, and dried down twice more. The residue is dissolved in 1 ml. water and stored at 4°C.

CHROMATOGRAPHY A volume of desalted urine equivalent to 300 μg. of total nitrogen is applied to the paper and 0·02 ml. of each of the standards applied at intervals between the urine spots, thus ensuring correct identification.

Chromatography is allowed to proceed for 16 hours at 23°C. (±2°C.). The papers are then removed and dried at bench temperature. After dipping in the ninhydrin reagent they are again dried at bench temperature and placed in an oven at 120°C. for one minute. The B.A.I.B. spots are then cut out, taking care not to touch them with the fingers, cut up, and eluted in 3 ml. of 50% ethanol for one hour. Blank sections of paper of the same size as the test and standard sections are cut out and eluted in the same way. The papers are removed and the tubes centrifuged to remove small fibres. The tests and standards are then read against their respective blanks in a 1 cm. cell at 570 mμ.

As a check to ensure that only B.A.I.B. is estimated a second run is made and, after staining, the papers are allowed to develop overnight at bench temperature. In these circumstances no colour develops with B.A.I.B. (see Fig. 1), and any colour in the same position is due to another amino-acid (Jagenburg, 1959). These have not so far been encountered.

RESULTS

STANDARD LINE As will be seen (Fig. 2) linearity is good up to 10 μg., and is reliably repeatable. In practice the 10 μg. standard is applied at intervals and a line drawn through the mean optical density of these standards. It has been found that linearity continues up to at least 50 μg. but above 10 μg. the spot becomes too diffuse for accurate sampling.

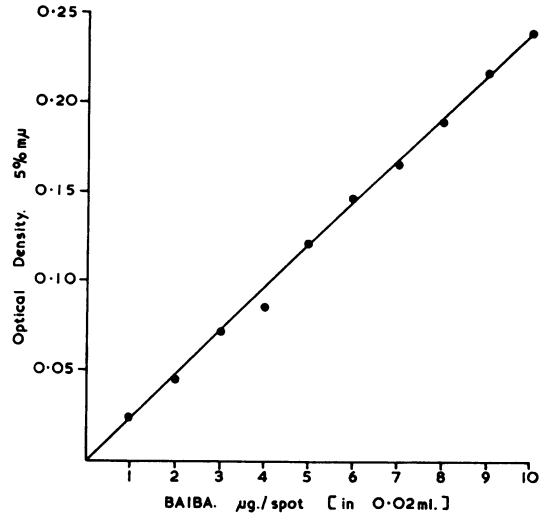

FIG. 2. *Standard line for β amino-isobutyric acid.*

RECOVERY EXPERIMENT Standard B.A.I.B. solutions were added to urine so that a range between 1·0 and 8·4 μg. per 0·02 ml. was obtained (Table I).

TABLE I

RECOVERY OF B.A.I.B. FROM URINE

Urine	*Calculated Concentration*	*Recovered Concentration*	*Percentage Recovery*
1	1·0	0·8	80
2	2·1	2·1	100
3	4·2	3·8	95
4	6·3	6·3	100
5	8·4	7·8	93

DUPLICATION EXPERIMENT Urines were put through the column in duplicate and then the eluates applied to the paper in duplicate (Table II).

TABLE II

DUPLICATION EXPERIMENTS

		Normal Urine (mg./day)	*Urine from Case of Chronic Myeloid Leukaemia (mg./day)*
Column 1	A	77	545
	B	67	504
Column 2	A	81	527
	B	77	486

[2]Messrs. Thomas Kerfoot & Co., Vale of Bardsley, Ashton-under-Lyme.

NORMAL VALUES Urines were collected from 12 normal males. Daily output was 33, 49, 51, 56, 67, 68, 70, 72, 92, 115, 153, and 205 mg. (mean 86 mg./day).

It has been suggested (Jagenburg, 1959; Gartler, 1959) that the population is divided into 'high' and 'low' excretors, the latter being in the majority, and that this distinction is genetically determined. Our preliminary results do not entirely bear this out; further work will be needed to demonstrate the importance of other factors, *e.g.*, total nitrogen excretion and the role of the spleen (Hillcoat, 1962), and thus whether two separate groups can indeed be distinguished.

SUMMARY

Methods available for the estimation of B.A.I.B. in urine are discussed, and a method that is cheap, reliable, and quick is described.

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A gel diffusion precipitin method for the estimation of C-reactive protein

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The precipitation of C-reactive protein with the non-specific somatic pneumococcal C-polysaccharide was first described by Tillett and Francis (1930). The inability to demonstrate the protein in health and its appearance during pregnancy and immediately after immunization has formed the basis of a useful but non-specific test with implications similar to, but not identical with, the erythrocyte sedimentation rate. C-reactive protein is an 'acute phase' protein associated with infections, rheumatoid arthritis, neoplasms, and other inflammatory and necrotic conditions. Estimates of the concentration of this protein have been used to measure the course of disease processes and their response to treatment.

Current methods for the detection and estimation of C-reactive protein utilize a specific antiserum generally raised in rabbits (MacLeod and Avery, 1941). The capillary precipitin method (Anderson and McCarty, 1950; Daguet, 1960) is the one in most common use; however, gel-diffusion precipitin (Fukuda, Heiskell, and Carpenter, 1959), complement-fixation (Muschel and Weatherwax, 1954; Rapport and Graf, 1956), and latex-fixation (Singer, Plotz, Pader, and Elster, 1957) techniques have also been developed. These latter methods have not found general application because of their technical difficulties and the length of time required for their performance.

The gel-diffusion precipitin method devised by Gell (1957) for γ globulin, siderophilin, and coeruloplasmin, and expanded by Soothill (1962), appeared to be adaptable for C-reactive protein and the possibility of this application has been investigated.

METHOD AND MATERIALS

ANTISERUM C-reactive protein antiserum Schieffelin (Scheiffelin and Co. New York, N.Y.) is a rabbit anti-human C-reactive protein antiserum, absorbed with whole human serum to ensure specificity.

STANDARD SERUM Serum from a case of rheumatoid arthritis with a high level of C-reactive protein (++++ capillary precipitin), hereafter treated as 100% C-reactive protein, was divided into 1 ml. portions and stored at −20°C.

BUFFERED AGAR One per cent Oxoid ion agar No. 2 in phosphate buffer *p*H 7·0 (50 ml. $NaH_2PO_4 \cdot 2H_2O$ 90 g./l., 200 ml. Na_2HPO_4 60 g./l., 250 ml. NaCl 8·5 g./l.).

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