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AUTOMATED DETERMINATIONS OF THYROID AND GASTRIC COMPLEMENT-FIXING ANTIBODY; COMPARISON WITH THE FLUORESCENT ANTIBODY AND MANUAL COMPLEMENT-FIXATION METHODS

W. J. IRVINE

M.R.C. Clinical Endocrinology Research Unit, 2 Forrest Road, and Department of Therapeutics, Royal Infirmary, Edinburgh

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SUMMARY

An automated technique has been established for the screening and titration of sera for complement-fixing antibody to gastric (parietal) cell and thyroid microsomal antigens. The automated method is of equal sensitivity to the manual complement-fixation technique but slightly less sensitive compared to the indirect fluorescent antibody method. The automated method has the advantage over the manual complement-fixation test of greater accuracy and reproducibility and the element of subjective interpretation is removed. It is less demanding on technician skill. The automated method requires only small amounts of sera but it consumes a considerable amount of antigen. Human antigen has been used in the present study.

The automated method for complement-fixation reactions has some advantages over the manual method for both routine clinical purposes and for the research laboratory.

A positive test for complement-fixing autoantibody to thyroid (Trotter, Belyavin & Waddans, 1957) has been shown to correlate in man with histological evidence of chronic thyroiditis (Roitt & Doniach, 1960; Buchanan *et al.*, 1962; Senhauser, 1964). Likewise, the presence of gastric complement-fixing antibody in the serum (Irvine *et al.*, 1962; Taylor *et al.*, 1962; Irvine, 1963) correlates with histological and functional evidence of chronic gastritis (Irvine *et al.*, 1965; Irvine, 1965). The detection of these autoantibodies is of value to the clinician when thyroid disease or atrophic gastritis is suspected. They are also of value in studying the familial aspects and the natural history of thyroid and gastric disorders. As both thyroid and gastric disorders are common, the number of sera which the clinical laboratory may be asked to examine may be large. An automated technique would therefore be valuable. The second reason for attempting to automate the complement-fixation method is to obtain better standardization in the technique itself. A preliminary report has been made (Irvine, 1966).

Correspondence: Dr W. J. Irvine, M.R.C. Clinical Endocrinology Research Unit, 2 Forrest Road, Edinburgh.

METHODS

Automated Complement-Fixation Method

Technicon Auto-Analyzer equipment was used (Technicon, Chertsey, Surrey, England; Chauncey, New York) and is illustrated in Fig. 1. It consists of an automatic sampler, proportioning pump (10 roller), double coil oil bath, colorimeter and recorder. The principle of the method is the same as the manual complement-fixation technique (*vide infra*) but the reagents are drawn into a system of transmission tubing where they are mixed and incubated. The rate of transmission through the system is determined by the internal diameter of the transmission tubing that is placed in the manifold of the proportioning pump. Mixing is achieved by putting the samples through a coil of glass tubing with the longitudinal axis

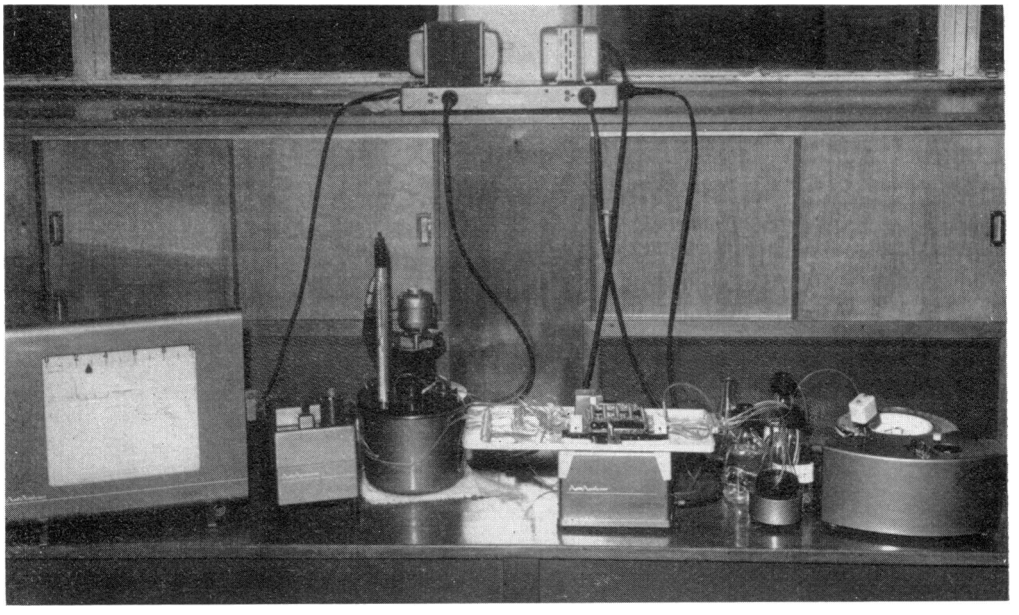

FIG. 1. The Auto-Analyzer consisting of (from right to left) the automatic sampler, proportioning pump, double coil oil incubating bath, colorimeter and recorder.

horizontal. Incubation without additional mixing is achieved using a glass coil with a vertical longitudinal axis. The duration of mixing and of incubation is determined by the length and the diameter of the tubing used. The internal diameter of the tygon plastic transmission tubing and of the glass mixing and incubating coils was 1·6 mm. Polyethylene tubing of 0·034 mm internal diameter was used to convey the individual reagents to the proportioning pump.

The samples to be tested were placed in the individual cups of the automatic sampler, while the other reagents were used continuously at a constant rate. The automatic sampler can be adjusted for the number of samples tested per hour and also for the period of sampling and for the period of washing between different samples. The fluid within the trans-

mission tubing is broken up into short segments by the introduction of bubbles of air at frequent and constant intervals; this minimizes diffusion of reagents within the system. The ratio of fluid to air in the system should be approximately 2:1, but the air must be removed before the samples pass through the colorimeter. 0·2 ml *Bryj* was added to each 500 ml of complement-fixation test diluent to act as a detergent and to adjust the surface tension of the fluid passing through the Auto-Analyzer so that a correct bubble pattern was obtained.

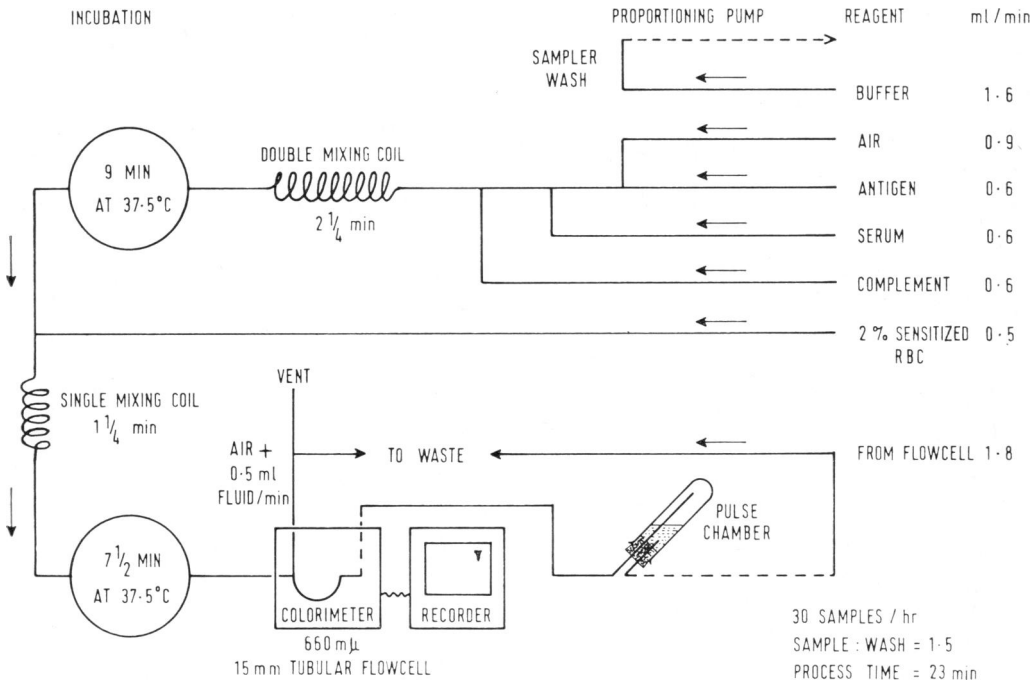

FIG. 2. The flow design for the automation of complement-fixation tests using saline tissue extracts and human sera. The approximate internal diameters of the tubes passing through the proportioning pumps in the order shown are: 0·073, 0·056, 0·045, 0·045, 0·073, 0·056, 0·040 and 0·090 in.

All reagents were added through capillary tubes using Technicon D_1 junction pieces. All joins in the system must be made perfectly. After each day's experiments the system was washed through with distilled water. The transmission tubing was replaced approximately every 2 weeks and all glass tubing and the flow cell was disassembled and washed with dichromate cleaning fluid and thoroughly rinsed. Attempts to wash the plastic tubing with various detergents resulted in erroneous complement titrations in spite of repeated washing with water and buffer. The flow diagram is shown in Fig. 2. The flow rates of the various reagents

was determined by direct measurement. The internal diameters (inches) of the tubing in the manifold of the proportioning pump were as follows: sampler wash, 0·073; air, 0·056; antigen, serum and complement, 0·045; re-sample, 0·073; air, 0·056; sensitized red cells, 0·040; and from flow cell, 0·090. It is to be noted that if a 5 roller proportioning pump is to be used the flow rates will be considerably greater. The success of this flow design depends on a perfect bubble pattern.

The following reagents were used in the present series of experiments: saline extracts of

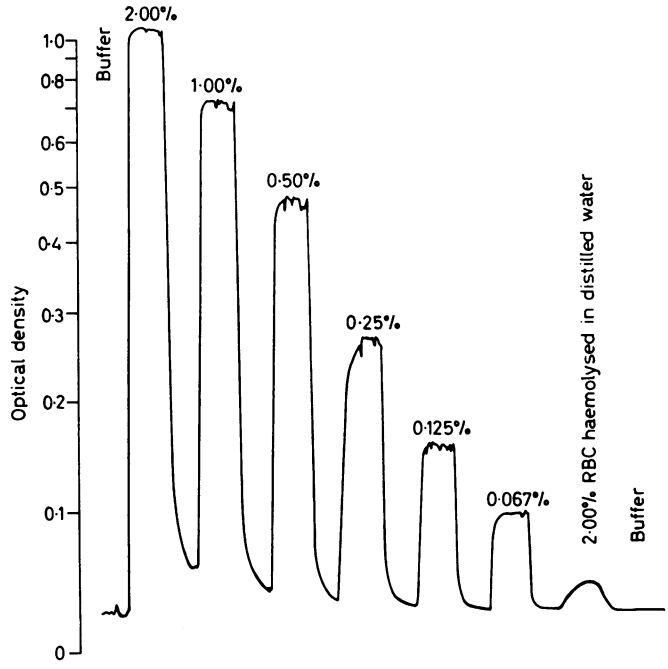

FIG. 3. Standardization of the Auto-Analyzer colorimeter with red cell suspensions of known concentrations. The colorimeter is sensitive to small changes in the concentrations of red blood cells in suspension but is insensitive to the amount of haemoglobin released as a result of lysis of the red cells.

human thyrotoxic tissue, of the mucosa of the body of human stomach obtained at partial gastrectomy for duodenal ulcer, and of rat liver; preserved guinea-pig complement (Stayne Laboratories Ltd, High Wycombe, Buckinghamshire, England); sera from patients with thyrotoxicosis, Hashimoto goitre, primary hypothyroidism, pernicious anaemia, simple atrophic gastritis and from random patients with unknown immunological status; fresh defibrinated sheep red cells obtained from the abattoir and used within 7 days; rabbit haemolytic serum (Stayne Laboratories Ltd, High Wycombe, Buckinghamshire, England); complement-fixation test diluent containing barbitone 0·575 g, sodium chloride 8·500 g, magnesium chloride 0·168 g, calcium chloride 0·028 g and barbitone soluble 0·185 g/l

(Oxoid, London, England). The saline tissue extracts were made by obtaining the fresh tissue at operation. The tissue was cut up finely and homogenized with an equal volume of complement-fixation test diluent. Three further volumes of the diluent were then added and mixed thoroughly with the homogenate which was then filtered through a layer of eight-ply gauze and centrifuged for 10 minutes at 1150 *g*. The supernatant, which contains the antigen, was pipetted off and stored at −20°C in 5 ml amounts. All sera were inactivated at 56°C for 30 min before use. The sheep red cells were washed in saline at least three times or until the supernatant after centrifugation was clear of haemoglobin and then centrifuged in buffer. A 2% suspension of the red cells was then sensitized with 0·16% rabbit haemolytic serum (haemolysin) at 37°C for 5 min. The cells were kept in suspension by a magnetic stirrer. The complement and the antigen in selected dilutions were kept in separate containers in an ice bath. All reagents, with the exception of the sera and diluent, were filtered through gauze before use. The diluent was filtered through Watman No. 1 paper.

As in the manual method the degree of complement fixation is assessed by the inhibition of haemolysis of the sensitized sheep red cells. In the automated method this was assessed colorimetrically using a turbidity filter (660 mμ). The results were recorded on optical density paper. The colorimeter and recorder were calibrated using complement-fixation test diluent (buffer) alone, suspensions of sheep red cells in known concentrations and haemolysed sheep red cells (Fig. 3).

Complement titration

A complement titration was done initially in the absence of antigen; the antigen and serum pick-up lines were placed in buffer and the complement pick-up line was attached to the automatic sampler which carried a series of cups containing the appropriate dilutions of complement. The complement titration was then repeated in the presence of the antigen dilution that had been selected (*vide infra*) for the screening of test sera for the corresponding antibody. Complement titrations in the presence and absence of thyroid antigen are shown in Fig. 4.

With the flow diagram as shown in Fig. 2, the haemolytic reaction for a given dilution of complement may not be maximal due to loss in concentration of the reagent by diffusion as it passes through the system when it is sampled for only a short period. This is illustrated in Fig. 4 by the observation that when a 1:25 dilution of complement is sampled for 20 sec only a proportion of the red cells are lysed (peak at 0·18 and 0·20 optical density scale in the absence and presence of complement, respectively). But if complement at the same dilution is fed into the machine continuously then 100% lysis is obtained over a period of 6 min. This is important in selecting the appropriate complement dilution that is to be used for the titration of antigen or for the detection of antibody; under these circumstances complement is supplied to the machine continuously. The sensitivity of the method for the detection of antigen or antibody is of course dependent on the amount of complement that is made available. The dilution of complement that is appropriate for the titration of antigen or detection of antibody has been found to be that dilution which when supplied at the rate of thirty samples per hour with a sample to wash ratio of 1:5 gives a recorded deflection of 0·7 optical density divisions (e.g. in Fig. 4 from a non-lytic base line of 0·90 to a peak height at 0·20 in the presence of the antigen dilution that is to be used in subsequent tests).

A complement titre is required at the beginning of each day's experiments and once the complement dilution has been constituted it must be kept in an ice-bath to minimize complement decay.

Titration of antigen

A titration of antigen was done in order to find the highest dilution that would still contain sufficient antigen to fix all the available complement in the presence of excess antibody. The antigen line was attached to the automatic sampler, the cups of which contained antigen in doubling dilutions. The complement pick-up line was placed in a

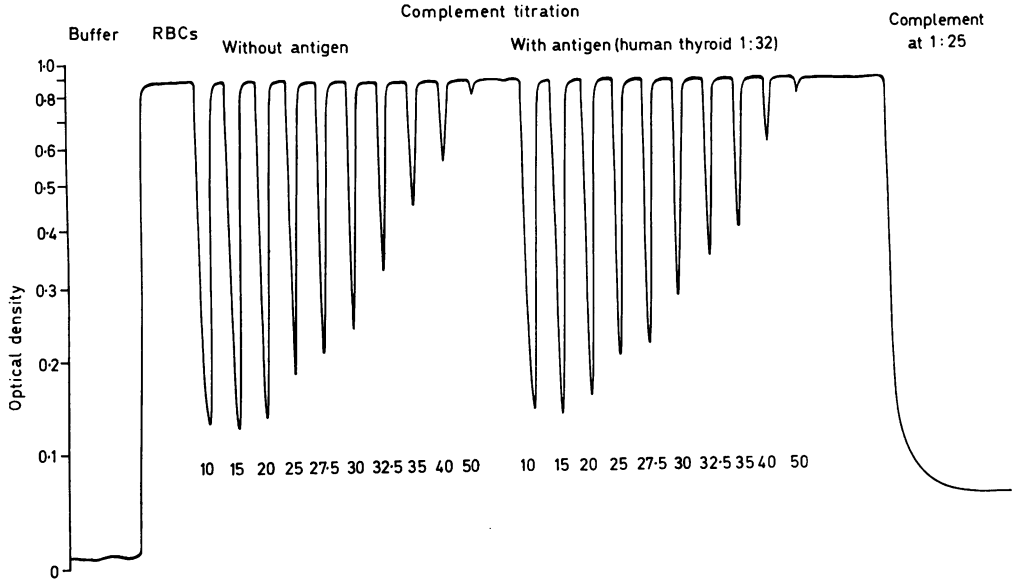

FIG. 4. Titration of preserved guinea-pig complement in the absence and presence of thyroid antigen (diluted 1:32). The dilutions of complement (1:10 to 1:50) are shown opposite each peak on the tracing. The complement titre was selected as 1:40. When the complement is sampled continuously in this dilution complete haemolysis is achieved in a period of 3 min. The difference between the colorimeter readings at the beginning and end of the tracing is due to the turbidity of the preparation of thyroid extract (see Fig. 5).

reservoir of the appropriate complement dilution and the antibody pick-up line was placed in a reservoir of a known positive serum that may be suitably diluted for the purposes of economy. Some few minutes were allowed for the system to come into equilibrium with regard to complement and to antibody before introducing the samples of antigen. This resulted in 100% haemolysis of the sensitized sheep red cells and established the base line, which was determined by the setting of the colorimeter and the turbidity of the serum. It is useful for localization purposes on the recorder paper to fill the first and last cups on the automatic sampler with 1% formalin in buffer. This results in a peak on the recorder chart at the beginning and at the end of the experiment as formalized cells are not susceptible to the lytic action of haemolysin and complement.

Fig. 5 illustrates the titration of a saline extract of human gastric body mucosa (G168). It is advisable to start with the most dilute samples because higher concentrations of proteinaceous material may well result in precipitation with resultant interference with the turbidity measurements of the colorimeter. The antigen titration should be repeated in the absence of antibody to check on the non-specific absorption of complement and the degree of turbidity of the different antigen dilutions. When each dilution of the antigen is sampled

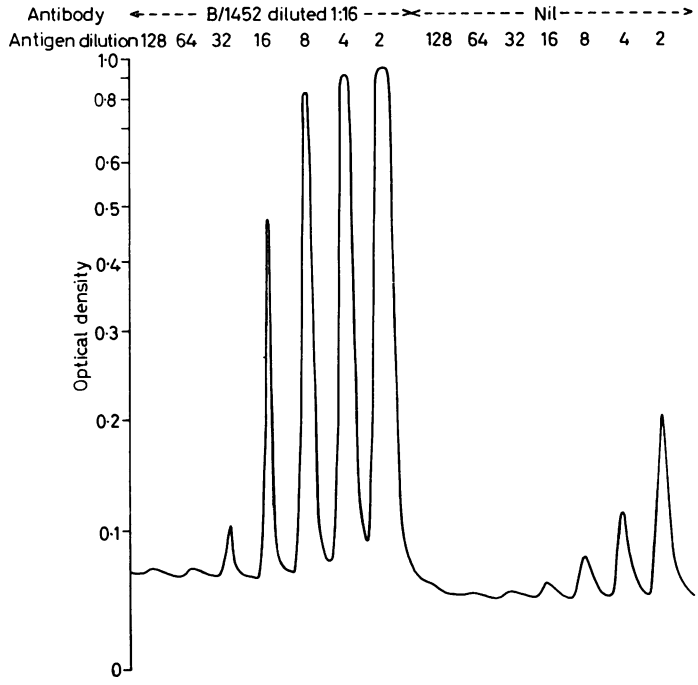

FIG. 5. Titration of a saline extract of human gastric mucosa (G.168) for antigenicity. A serum (B/4152) from a patient who was known to have high titres of gastric specific complement-fixing antibody was used in a dilution of 1:16. Complement was sampled continuously at the appropriate dilution (in this case 1:35). A control series of antigen dilutions is included without antibody to determine the non-specific binding of complement and the turbidity of the various dilutions of tissue extract. A titre of 1:16 was chosen as appropriate. When sampled continuously the antigen at this dilution would be sufficient so that the antigen–antibody reaction would be capable of fixing all the complement available.

for only 20 sec there is loss in concentration of the sample due to diffusion as it passes through the system. When sampled in this manner an antigen dilution that causes considerable but not total fixation of complement in the presence of excess antibody was found to be suitable for the screening of test sera for the corresponding antibody. In Fig. 5, for example, the dilution of antigen that was selected as suitable for the next stage in the procedure was 1:16. Gastric, thyroid and liver extracts were titrated in this manner using the corresponding organ-specific or non-organ-specific complement-fixing antibody.

Provided the saline extracts are stored in their concentrated form at −20°C and made up to the desired dilutions when required it was not necessary to repeat the antigen titrations except at infrequent intervals.

Screening and titration of sera for complement-fixing antibody

With complement and antigen being continually fed into the machine from reservoirs containing these reagents at the appropriate dilutions, the antibody pick-up line was attached to the automatic sampler, the cups of which contained a series of test sera in a

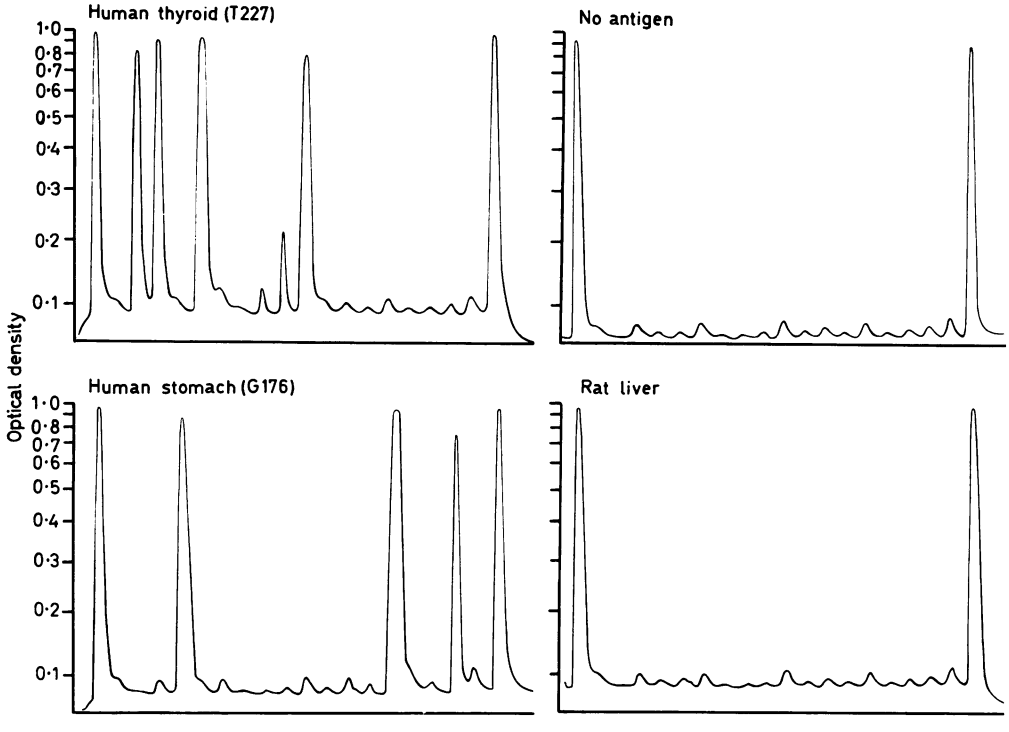

FIG. 6. The screening of eighteen sera for organ-specific complement-fixing thyroid and gastric autoantibodies using the Auto-Analyzer. The same sera were put through the machine four times with a different component on each occasion. A formalin peak is placed at the beginning and end of each run. For this initial screeening test all sera were used in a dilution of 1:4 after inactivation in a water bath at 56°C for 30 min.

dilution of 1:4. As before, the first and last cup of the automatic sampler should contain 1% formalin in buffer to help localization on the recorder chart. The same series of sera were retested using different antigens and in the absence of antigen to check for organ specificity and for anti-complementary effects. An example of such a series is shown in Fig. 6. For screening against thyroid, stomach, liver and for anti-complementary activity a total of 0·2 ml serum is required.

As in the titration of complement and of antigen using the automatic sampler, the reaction

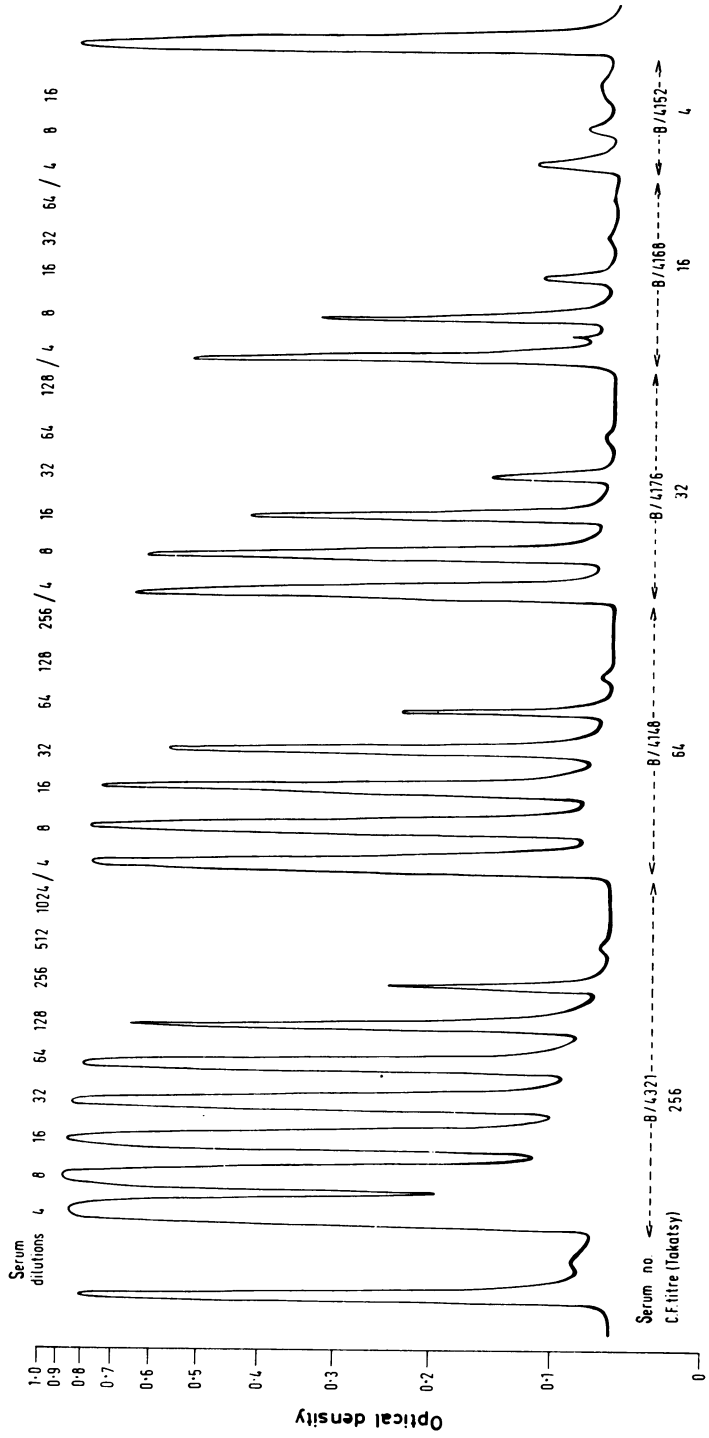

FIG. 7. The titration of sera for thyroid complement-fixing antibody. In this series human thyroid extract (T. 229) was used in a dilution of 1:32 and preserved guinea-pig complement in a dilution of 1:47·5. The test sera had previously been screened in a dilution of 1:4 (see Fig. 6) and had been shown not to absorb complement and not to be unduly turbid. The complement-fixation titres obtained by the Takatsy manual method are indicated.

is not maximal when the test serum is sampled for only 20 sec. Consequently, a peak on the tracing to be regarded as positive need only be a few optical density divisions (e.g. 0·03 divisions) provided the test sera does not give a similar peak with the liver extract or in the absence of antigen. Test sera giving an organ-specific reaction in a dilution of 1:4 can be titrated against the appropriate antigen as shown in Fig. 7.

Analysis of procedure

The various stages of the automated procedure were analysed. It is possible to reduce the amount of reagents used below those indicated in Fig. 2, but when this is done there is a tendency for adjacent peaks to overlap. It is likely that the internal diameter of the transmission tubing throughout the system would require to be narrower if smaller volumes of reagents are to be used.

A prolonged period of washing between each sample is required when the reagents have such a high protein content. A sample to wash ratio of 1:5 prevents any overlap between adjacent positive peaks except when the test sera or test antigen is present in high titre.

Various methods of joining the reagent lines after they have passed through the proportioning pump were studied. The most satisfactory results were obtained when capillary tubes were used for the addition of reagents to the main stream. The capillary tubes carrying reagents should lie so that bubbles of air are not trapped at their junctions with the main stream.

The most suitable incubation temperature and time for the reaction between antigen, antibody and complement was studied. It was found that the reaction was not closely dependent on temperature; it would occur at room temperature (20°C) about as efficiently as at 37·5°C. With prolongation of incubation more complement is fixed but there is a tendency for adjacent samples to contaminate each other and for complement to decay. It is therefore necessary to achieve a compromise between these three factors. Because the reservoirs of complement and of antigen require to be kept in an ice bath it was decided that a period of incubation at 37·5°C should be included.

Only a single mixing coil is necessary to achieve an even suspension of the sensitized red cells. The period of incubation for the lysis of the red cells was studied. At this stage the decay of complement is no longer important, but the contamination of adjacent samples with increased incubation is a complicating factor with prolonged incubation. Once again a compromise is achieved between allowing the reaction to go to completion and avoiding diffusion of reagents.

It is estimated that in the flow design as shown in Fig. 2 the fixation of complement and the lysis of the sheep red cells achieve approximately 80% completion.

Manual Methods for Detecting Thyroid and Gastric Complement-Fixing Antibody

In the indirect fluorescent antibody technique fresh unfixed air-dried sections of snap frozen human gastric mucosa from the body of the stomach were used (Irvine, 1963). The test serum (undiluted) was applied, the sections washed in buffer and then treated with horse anti-human globulin conjugated with fluorescein iso-thiocyanate (Progressive

Laboratories Ltd, Baltimore; Lot 3122) and then washed in phosphate buffer at pH 7·0. The duration of each stage was 20 min and the whole procedure was done at room temperature. The fluorescein–protein ratio of the conjugate was 8·8 μg/mg. The conjugate was absorbed with liver and dialysed free of fluorescein. A cover slip was mounted using 10% glycerine in buffer and the sections were examined under ultraviolet light using a Zeiss standard Universal microscope with 200 HBO mercury vapour lamp, dark ground condenser and filters BG 12 and 53. The intensity of staining of the parietal cells was graded as + +, or +, or negative, subjectively.

In the manual complement-fixation method the complement was titred in test tubes. A series of ten dilutions of complement was made in complement-fixation test diluent at 1:10, 1:20, 1:30, . . . 1:100. Two rows of test tubes were then set out. One volume (0·2 ml) of the corresponding dilution of complement was placed in each of the tubes according to the tube number from 1 to 10. Two volumes of complement-fixation test diluent were added using an automatic pipette to each of the tubes in row I and 1 volume of the diluent was added to each of the tubes in row II. One volume of the antigen in the dilution to be subsequently used in the screening of test sera was placed in each of the tubes in row II. One volume of 2% sheep red cells sensitized with haemolysin was then added to all tubes in rows I and II. The tubes were then mixed and placed in a 37°C water bath for 30 min. The complement titre was taken as the tube showing 100% lysis at this time.

The sera were screened and titred using the microtitre equipment supplied by Cooke Engineering Co., 735 North St Asaph Street, Alexandria, Virginia, U.S.A. All sera prior to testing were inactivated at 56°C for 30 min. In two rows of the microtitre trays duplicate doubling dilutions from 1:2 to 1:1024 were prepared for each serum using capillary loops (0·025 ml capacity). A control was added to the end of the first row and this contained saline in place of serum. An equal volume (0·025 ml) of antigen at the appropriate dilution (*vide infra*) was added to all the wells of the first row using the microtitre dropping pipette (1 drop). An equal volume of saline was added to all wells in the second row using a similar dropping pipette. Then to all wells in both rows was added 0·025 ml complement solution containing 2·0 minimum haemolytic doses per ml. After shaking, the plates were incubated at 37°C for 1¼ hr. Then to each well was added 0·025 ml 2% sensitized sheep red cells, the trays shaken and incubated for a further 45 min. The trays were then removed, gently shaken once more and replaced in the incubator for a further 30 min. The trays were then left at 4°C overnight and read next morning when they had reached room temperature. The end-point was taken as 50% haemolysis and was read by eye.

To determine the antigenicity of a tissue extract a chessboard titration was done in the microtitre plates using the same procedure as above but with dilutions of a known antiserum titrated against dilutions of the antigen.

ANALYSIS OF RESULTS WITH THE AUTO-ANALYZER

The findings obtained with the Auto-Analyzer using the flow design as shown in Fig. 2 were compared with the results obtained with the Takatsy microtitre complement-fixation method and the fluorescent antibody technique for gastric parietal cell antibody (Table 1) and for thyroid microsonal antibody (Table 2). The height of the peak of complement fixation shown by the Auto-Analyzer when the test sera were used in a dilution of 1:4

TABLE 1. A comparison of the methods for detecting parietal cell antibody

Indirect fluorescent antibody method (undiluted serum)	Complement fixation		No. of sera
	Manual (titre)	Auto-Analyzer (sera at 1:4 dilution) (height of peak)	
++	⩾32	+++	27
		++	10
		+	2
		Negative	—
++	⩾4⩽16	+++	—
		++	2
		+	9
		Negative	1
+	⩾4⩽16	+++	—
		++	1
		+	8
		Negative	6
+	<4	+++	—
		++	—
		+	3
		Negative	8
Negative	<4	+++	—
		++	—
		+	—
		Negative	155

correlated well with the titre of the gastric complement-fixing antibody indicated by the manual method and the intensity of staining in the indirect fluorescent antibody technique. The sensitivity of the Auto-Analyzer method is similar to that of the manual technique but slightly less than that of the indirect fluorescent antibody technique. It is to be noted that undiluted serum was used in the fluorescent antibody technique. No false positive results were obtained out of the 232 sera tested for gastric complement-fixing antibody. With regard to thyroid complement-fixing antibody, a similar correlation was found between the results of the manual, immunofluorescence and automated technique using the same sera (Table 2). The tires of complement-fixing thyroid antibody as determined in fourteen sera by the automated and by the manual methods are compared in Fig. 8. Provided the criteria for the selection of the complement titre in the automated method was strictly adhered to, the reproducibility of the titre for complement-fixing antibody as determined by the Auto-Analyzer was extremely precise on account of the graded height of the peaks as the antibody was titred out. The height of the individual peaks on repeated titration was also highly reproducible. By comparison the reproducibility in the manual method was poor at ± 1 doubling dilution.

TABLE 2. A comparison of the methods for detecting thyroid microsomal antibody

Indirect fluorescent antibody method (undiluted serum)	Complement fixation		No. of sera
	Manual (titre)	Auto-Analyzer (sera at 1:4 dilution) (height of peak)	
++	⩾32	+++	34
		++	7
		+	3
		Negative	—
++	⩾4⩽16	+++	2
		++	3
		+	5
		Negative	2
+	⩾4⩽16	+++	—
		++	2
		+	4
		Negative	5
+	<4	+++	—
		++	—
		+	6
		Negative	21
Negative	<4	+++	—
		++	—
		+	—
		Negative	138

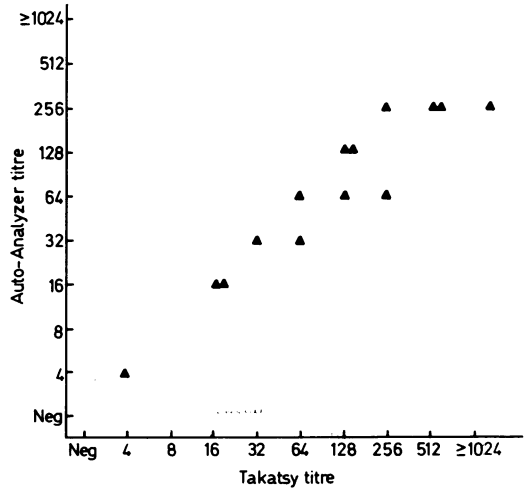

FIG. 8. Correlation between the titres of complement-fixing antibody as determined in fourteen sera by the Auto-Analyzer and the Takatsy manual method.

DISCUSSION

An automated technique for the detection of complement-fixing antibody to thyroid and to gastric mucosa has been established. The method has the advantage over the manual complement-fixation test of greater precision and reproducibility; a larger number of sera can be tested in a given period and the technique is not so demanding on technician skill. The subjective element in the interpretation of results has been removed. In an ordinary day's work, forty sera can be screened against thryoid, stomach, liver and in the absence of antigen. A disadvantage of the method as presently designed is the volume of antigen used. So far only human thyrotoxic tissue and human partial gastrectomy specimens have been studied. In large hospitals a sufficient number of thyroidectomies are likely to be done to ensure an adequate supply of thyroid tissue but this may not be the case with regard to gastrectomies. The species specificity of pareital cell antibody is wide and it is likely that the gastric mucosa from other species may prove to be suitable. Preliminary studies have suggested that pig gastric mucosa, but not dog, may be used as a source of parietal cell antigen. Further studies are required to determine the most suitable method of preparing the tissue antigens for use in the Auto-Analyzer in the hope that the period of washing between individual test sera may be reduced This would accelerate the rate of sampling and reduce the consumption of antigen per serum tested.

The Auto-Analyzer method should have general application to the clinical laboratory for the routine screening and titration of sera for gastric and thyroid complement-fixing antibody. It also has application to the research laboratory on account of the greater accuracy and reproducibility in the titration of sera and antigens.

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