Microdetermination of calcium and magnesium in biological materials

C. H. BOWDEN¹ AND VALERIE J. PATSTON

From the Department of Chemical Pathology, Westminster Medical School, London

SYNOPSIS The use of the dye calcon (1-(2 hydroxy-1-naphthylazo)-2-naphthol-4 sulphonic acid) for the estimation of calcium using E.D.T.A. and a commercial photoelectric titrimeter is described. The interfering effects of magnesium and phosphate have been overcome. The method has been extended to estimations on biological materials.

Results on 55 sera show that the E.D.T.A./calcon method gave slightly lower results ($-0.15 \text{ mg./100 ml.} \pm 0.029$) than the oxalate precipitation method.

Magnesium may also be estimated by incorporating the use of Eriochrome black T.

The dye calcon (1 (2 hydroxy-1-naphthylazo)-2-naphthol-4 sulphonic acid), which was investigated by Hildebrand and Reilley (1957) and adapted for use in serum calcium estimations by Golby, Hildebrand, and Reilley (1957), appears to be a real improvement on murexide, in both stability and colour change. Aqueous solutions of calcon produce a blue colour between pH values of 7.4 and 13.5. Below pH 7.4 and above pH 13.5 the indicator is red. At approximately pH 13, where magnesium is quantitatively precipitated as the hydroxide, only calcium ions react with calcon to form a red coloured complex. Thus under these conditions the calcium-calcon complex can be titrated with E.D.T.A. (ethylenediamine-tetra acetic acid) from a red to a pure blue end-point without interference from magnesium.

In our experience, however, the visual end-point described by Golby *et al.* (1957) is not sufficiently sharp to give highly accurate results. Also as sera vary greatly in tint the colour of the end-point will vary accordingly. We therefore made use of a commercial titrator and galvanometer, which gave a far more accurate determination of the end-point. A single calcium estimation using this apparatus takes less than three minutes. The method has been adapted satisfactorily for calcium estimations in serum, urine, food, and faeces.

EXPERIMENTAL

APPARATUS The EEL titrator (Evans Electro-selenium) used in conjunction with an EEL Unigalvo type 20

galvanometer was used throughout the work described. The instrument consists basically of a colorimeter with a built-in magnetic stirrer. Flanged beakers (4 ml. capacity) are supplied which are correctly located in the light beam which is focused at the centre of the solution in the beaker. Various Ilford filters can be selected for use as necessary.

As no error in the end-point is caused by moderate dilution of the sample it was unnecessary to use a micro-syringe when titrating with E.D.T.A. Therefore a 2 ml. Excello automatic micro burette² graduated in 0.01 ml. divisions was used. The burette was modified by fitting it with a diaphragm plastic tap³ which permitted accurate 0.01 ml. additions. The burette jet was either drawn out finely or the tap fitted with a hypodermic needle.

OPTICAL CONDITIONS FOR TITRATION The exact position of the absorption peaks, the molar extinction, and the stability of the colour of the free calcon and of the calcium complex are all dependent on pH. Above pH 13 the stability and the extinction of the free calcon is greatly reduced (Hildebrand and Reilley, 1957). However, the advantage of avoiding both the necessity of neutralizing acid extracts before titration and magnesium interference by using high pH levels outweighs the disadvantages of decreased dye stability and reduced optical density values. Under the working conditions described below the free dye had a loss of optical density of 1% at five minutes at wavelength 490 rising to 20% at 20 minutes. Under the same conditions the calcium-calcon complex showed no significant change.

DETERMINATION OF THE END-POINT FROM TITRATION CURVES Figure 1 shows the titration curve of a standard

¹Present address: Royal Air Force Institute of Pathology and Tropical Medicine, Halton, Aylesbury, Bucks.

Received for publication 29 September 1962.

²W. G. Flaig & Sons Ltd., 39, Waterloo Road, London, N.W.2.

³Townson and Mercer, Croydon, Surrey.

FIG. 1. Titration curve for dilute calcium standard with E.D.T.A. using calcon as indicator.

calcium solution using an Ilford 603 filter and galvanometer transmission scale.

The end-point can be determined without drawing a titration curve by noting the greatest difference in transmission scale deflection for each 0.01 ml. of E.D.T.A. added. This corresponds to the maximum rate of deflection and the end-point is taken as the second of the two readings showing a maximum difference.

REAGENTS The following were required:—

Stock calcium solution (0.01251M or 50 mg. Ca/100 ml.) 1.251 g., CaCO_3 anhydrous AR (dried at 120°C . and stored in a vacuum desiccator) is added to 150 ml. distilled water. After the addition of 25 ml. NH_4Cl the solution is made up to 1 litre.

Dilute calcium working solution (5 mg. Ca/100 ml.) Dilute the stock calcium solution 1:10 with distilled water.

Diamino-ethane-tetra acetic acid solution (E.D.T.A.) (0.0025 M) Diamino-ethane-tetra acetic acid disodium salt, 0.9306 g., is dissolved in 1 litre of distilled water.

Stock calcon indicator Calcon,⁴ 50 mg., is dissolved in 10 ml. methanol AR.

Dilute calcon indicator Dilute stock solution 1 : 25 with methanol.

2N Sodium hydroxide All aqueous reagents are prepared using ion exchange water and stored in polythene bottles.

STANDARDIZATION OF E.D.T.A. SOLUTION Into the 4 ml. beaker pipette 1 ml. dilute calcium standard, and add 0.5 ml. 2N NaOH and 0.2 ml. dilute calcon indicator. With medium to maximum sensitivity setting and using the Ilford 603 filter, titrate until the end-point begins to change colour, and re-set the galvanometer zero control to obtain a zero reading. Add the E.D.T.A. in 0.02 ml. or 0.01 ml. quantities. The end-point is determined as above. This can be interpolated to within 0.01 or 0.005 ml. if 0.02 ml. or 0.01 ml. additions are used.

The titration reading should be 0.5 ml. of E.D.T.A. for 1 ml. of the dilute calcium standard after allowance for the blank reading. The E.D.T.A. can be adjusted or a titration factor applied.

DETERMINATION OF REAGENT BLANK Using analytical reagents the blank values are extremely low (approximately 0.005 ml.) and to evaluate them it is necessary to follow Wilkinson's (1957) method of titrating a standard solution to past the end-point, adding more standard to the beaker and re-titrating.

Titration 2 — titration 1 = true reading of standard

Titration 1 — true standard reading = blank reading

TITRATION OF SERUM Into the 4 ml. beaker pipette 0.5 ml. serum and 0.5 ml. 2N NaOH and 0.2 ml. dilute calcon indicator and approximately 0.5 ml. of distilled water to adjust the level of the solution above the light path. Titrate as above using increased or maximum sensitivity for lipaemic or coloured sera.

When necessary satisfactory results can be obtained on 0.2 ml. of serum using titration additions of 0.01 ml. E.D.T.A.

CALCULATION Using 0.5 ml. serum calcium (mg./100 ml.) = titration reading $\times 10 \times 2$.

STORAGE OF CALCON SOLUTION Satisfactory results were obtained with stock calcon and dilute calcon solution stored at room temperature up to a period of eight weeks and three weeks respectively. Storage in a deep freeze cabinet or refrigerator considerably increased the period of stability.

OPTIMUM CALCON CONCENTRATION It was found that the optimum calcon concentration was that which gives a free dye extinction of about 0.2 to 0.3 at $490\text{ m}\mu$. Using the indicator specified above this corresponds to 0.2 ml. dilute indicator as in the method described. The indicators obtained from British Drug Houses and Hopkins and Williams had to be made up to double the concentration indicated. These indicators appear to give an inferior visual end-point but can be used with the titrimeter.

RESULTS

RECOVERY OF CALCIUM ADDED TO SERUM Varying amounts of calcium were added to three normal sera by adding stock calcium standard to the titration beaker after pipetting the serum. Table I indicates

⁴J. T. Baker Chemical Co., U.S.A., obtainable from Omni (G.B.) Ltd., 35 Dover Street, London, W.1.

TABLE I
RECOVERIES OF CALCIUM ADDED TO 11 SERA

	Calcium Added (mg./100 ml.)	Total Calcium Found (mg./100 ml.)	Calcium Recovered (mg./100 ml.)	% Calcium Recovery
Normal 1	—	8.6	—	—
	5.4	14.0	5.4	100
	10.8	19.4	10.8	100
	20.0	28.8	20.2	101
Normal 2	—	10.2	—	—
	0.6	10.8	0.6	100
	8.8	19.2	9.0	102
Normal 3	—	9.8	—	—
	0.4	10.2	0.4	100
	6.2	15.8	6.0	97
	13.0	22.6	12.8	98.5
Haemolysed serum	—	10.0	—	—
	2.0	12.0	2.0	100
	5.2	15.2	5.2	100
	7.8	17.8	7.8	100
Grossly haemolysed serum	—	9.0	—	—
	5.0	14.2	5.2	104
Lipaemic serum	—	11.2	—	—
	1.0	12.2	1.0	100
	5.2	16.4	5.2	100
	10.4	21.2	10.0	96
Grossly lipaemic serum	—	10.2	—	—
	5.0	15.4	5.2	104
	10.4	20.6	10.4	100
Jaundiced serum (total bilirubin = 3.4 mg./100 ml.)	—	8.4	—	—
	2.2	10.6	2.2	100
	7.2	15.8	7.4	102.8
Jaundiced serum (total bilirubin = 4.0 mg./100 ml.)	—	9.4	—	—
	0.4	9.8	0.4	100
	9.2	18.8	9.4	102
Jaundiced serum (total bilirubin = 6.0 mg./100 ml.) also lipaemic	—	8.8	—	—
	5.6	14.6	5.8	103.5
Jaundiced serum (total bilirubin = 18.4 mg./100 ml.)	—	9.6	—	—
	2.2	11.6	2.0	91
	8.4	18.0	8.4	100

the satisfactory recoveries obtained. Similar recovery experiments were carried out on lipaemic, haemolysed, and icteric sera. In such cases visual colour change varies according to the colour of the original serum, and, in most cases, the galvanometer sensitivity setting should be increased to give satisfactory deflection readings. In cases of gross lipaemia the maximum sensitivity setting should be used. Table I indicates satisfactory recoveries in all cases investigated.

EFFECT OF MAGNESIUM Magnesium is precipitated at the pH used in the method, and it was considered possible that calcium might be co-precipitated or that the precipitate formed might interfere with the end-point. Varying amounts of stock magnesium standard were therefore added to the titration beaker after pipetting the serum or calcium solution.

Table II shows satisfactory calcium recoveries even at levels of 100 mg.% magnesium. However, the heavy flocculant precipitates at high magnesium concentrations above 20 mg.% cause jumpy galvanometer readings due to interference with the light beam. Such values, however, are unlikely to be found in pathological sera.

EFFECT OF PHOSPHATE It was expected that high levels of phosphate would interfere with the end-point. Phosphate was therefore added to serum in the titration beaker using varying amounts of a solution of potassium dihydrogen phosphate equivalent to 50 mg. P/100 ml. Table III shows the results obtained. The interference with the end-point is characterized by a premature appearance of the blue end-point which then slowly reverts back to red. By slow titration this effect can be minimized up to a

TABLE II

EFFECT OF ADDED MAGNESIUM ON CALCIUM ESTIMATION

	Magnesium Added (mg./100 ml.)	Calcium Found (mg./100 ml.)
Standard calcium solution	—	10.0
	5.0	10.0
	10.0	10.0
	20.0	10.0
	50.0	9.8
	100.0	9.8
Serum I	—	9.4
	5.0	9.4
	10.0	9.4
	20.0	9.4
Serum II	—	10.0
	10.0	10.0
	100.0	10.2
Serum III	—	10.4
	5.0	10.4
	50.0	10.6
Serum IV	—	10.2
	5.0	10.2
	10.0	10.4
	20.0	10.2

phosphate level of 10 mg./100 ml. Bett and Fraser (1959) noted that the interference was directly related to the concentration of phosphate in the titration solution and not to the ratio of the calcium and phosphate ions. Thus by further dilution of the titration solution with 1 ml. or 2 ml. of water the level at which phosphate interferes can be raised to 15 to 20 mg./100 ml. If this dilution is carried out a corresponding increase in the amount of calcon dye should be made. Since 2N NaOH is used to give the high pH for the titration, the dilution can be made without going below pH 13. However, if many estimations

are to be carried out on materials with a high phosphate content it is desirable to use a morpholine nitrate-nitric acid precipitation technique discussed below.

EFFECT OF ANTICOAGULANTS Calcium concentrations were estimated on sera obtained from normally clotted blood and on plasma samples obtained by use of E.D.T.A. oxalate, heparin, and liquid citrate as anticoagulants.

The E.D.T.A. plasma gave zero values. Oxalated plasma appeared to retain a very small amount of calcium which approximated to 0.4 mg./100 ml. in all samples. Using liquid citrate as anticoagulant and allowing for dilution, calcium results were obtained which were very closely comparable with those on sera (mean difference = + 0.10 mg./100 ml. $\pm$ 0.009). Heparinized plasma also gave results which compared very closely with those found in sera from the same subjects (mean difference + 0.02 mg./100 ml. $\pm$ 0.003).

OXALATE PRECIPITATION AND E.D.T.A./CALCON ESTIMATION OF CALCIUM COMPARED

Calcium estimations by the Clark and Collip (1925) oxalate precipitation method (modified by Wheatley, 1944) and the calcon method described were made on 55 miscellaneous normal and pathological sera with the following results. The oxalate precipitation method gave a mean on 55 sera of 9.85 mg./100 ml. $\pm$ 1.26 (S.D.) and that of the calcon/E.D.T.A. method 9.70 mg./100 ml. $\pm$ 1.24 (S.D.). This gave a mean difference of + 0.15 mg./100 ml. $\pm$ 0.029

TABLE III

EFFECT OF ADDED PHOSPHATE ON CALCIUM ESTIMATION

	Total Phosphate Present (mg. P/100 ml.)	Calcium Found (mg./100 ml.)	Effect on End-point
Serum I	3.7	9.4	No interference
	5.7	9.4	No interference
	8.7	9.4	No interference
	13.7	9.2	Moderate interference
	18.7	9.0	Definite interference
	23.7	No accurate result	Gross interference
	53.7	No accurate result	Gross interference
Serum II	4.2	9.2	No interference
	6.2	9.2	No interference
	9.2	9.2	Slight interference
	12.2	9.2	Moderate interference
	14.2	9.2	Definite interference
	19.2	9.0	Gross interference
	24.2	No accurate result	Gross interference
Standard calcium solution	0	10.0	
	3.0	10.0	
	5.0	10.0	No interference
	11.0	No accurate result	Very slight interference
	20.0	No accurate result	Gross interference
	50.0	No accurate result	Gross interference
	100.0	No accurate result	Gross interference

(S.E.M.). The Student *t* test gave $p = < 0.001$. Thus there is a small but significant difference between the mean values of the two series, indicating that the E.D.T.A. results are slightly lower than those obtained with the oxalate precipitation method. However, the difference does not appear to be as great as that reported for other E.D.T.A. titrations by Wilkinson (1957) and Golby *et al.* (1957).

CALCIUM ESTIMATIONS ON BIOLOGICAL SPECIMENS USING MORPHOLINE NITRATE-NITRIC ACID EXTRACTS

Horner (1955) and later Baron and Bell (1959) described a quick method for preparing protein and phosphate-free extracts from biological samples without the preliminary ashing or precipitation of calcium. Phosphates and proteins are directly precipitated with a morpholine nitrate-nitric acid reagent and sodium tungstate leaving a clear acid supernatant which, by the addition of the normal quantity of NaOH, is ready for immediate titration with calcon and E.D.T.A.

REAGENTS FOR PRECIPITATION Two are required:—

1 *Morpholine nitrate-nitric acid reagent* (Horner, 1955; Baron and Bell, 1959) A 1:1 mixture of 25% v/v HNO₃ and 48% morpholine nitrate. 48% morpholine nitrate is made by adding 50% HNO₃ to 28 ml. morpholine until the solution is neutral followed by dilution with water to 100 ml. We found that it was essential to add 50% HNO₃ very slowly to morpholine cooled below 0°C. If the temperature is allowed to rise above 5°C. the resultant reagent may result in discoloration of the calcon giving poor end-points and high blank values.

2 *Sodium tungstate* 66% w/v Na₂ WO₄·2H₂O

MORPHOLINE NITRATE PRECIPITATION Two millilitres of the sample, prepared to contain approximately 20 mg. Ca/100 ml., 1 ml. sodium tungstate, and 2 ml. morpholine nitrate-nitric acid reagent are mixed thoroughly, allowed to stand for one hour at room temperature, and centrifuged for 10 minutes at 3,000 r.p.m.

TITRATION Pipette 1 ml. supernatant, 0.5 ml. 2N NaOH, and 0.2 ml. dilute calcon into a titration beaker and titrate as previously described.

mg.Ca/100 ml. prepared sample = test - blank $\times 10 \times 2.5$

RESULTS WITH URINE Direct titrations are possible on urine (0.5 ml. or 0.2 ml.) with low phosphate concentrations but may become rather difficult on urine with large concentrations of phosphate. The calcium content of 23 urines was therefore estimated by the morpholine nitrate method described and for comparison by preliminary oxalate precipitation of calcium followed by titrations with E.D.T.A. and calcon. With this method 1 ml. of 4% ammonium oxalate was added to 2 ml. of urine (pH 5) and 2 ml.

FIG. 2. Comparison of urine calcium estimations on 23 samples.

Ordinate By oxalate precipitation followed by E.D.T.A./calcon titration.

Abscissa By morpholine nitrate precipitation followed by E.D.T.A./calcon titration.

of water. After mixing and leaving for one hour the precipitate was centrifuged for 10 minutes at 3,000 r.p.m., the supernatant decanted off, and the precipitate dissolved in 0.5 ml. 2N HCl and made up to 4 ml. with water. One millilitre of solution (= 0.5 ml. urine) was titrated with E.D.T.A. and calcon. Figure 2 gives the results obtained and shows good agreement between the two methods. The morpholine nitrate method is a slightly quicker and probably more accurate method as it eliminates the need for the actual precipitation of calcium.

RESULTS WITH MILK AND FOOD The calcium content of milk and food was estimated by the rather laborious method of ashing followed by titration of the calcium present with E.D.T.A. and calcon and by direct morpholine nitrate precipitation followed by titration of the supernatant produced with E.D.T.A. and calcon. The good agreement between the two methods shown in Table IV indicates that the second rapid method is satisfactory.

RESULTS WITH SERUM The calcium content of 12 sera was estimated by direct titration with E.D.T.A. and calcon and by the morpholine nitrate method described. Figure 4 shows that the results are closely comparable but it has been found unnecessary to use this longer method for routine work. However, in

TABLE IV

ESTIMATION OF CALCIUM IN MILK AND FOOD BY TWO METHODS

Sample	Estimation after Morpholine Nitrate Precipitations (a)	Estimation after Ashing (b)	Difference between (a) and (b)
Milk (mg./100 ml.)			
I	120 120	127 125	- 6
II	128 133	112 112	+ 18
Food (mg. of total diet)			
I	660 660	635 652	+ 16
II	700 742	720 720	+ 1
III	375	395	-20

cases with grossly raised phosphate levels the end-points obtained are improved.

ESTIMATIONS OF TOTAL MAGNESIUM AND CALCIUM IN SERUM

Using the apparatus described the magnesium content of serum may also be estimated by first titrating the total calcium and magnesium present with E.D.T.A. at a pH of approximately 10.5 with Eriochrome black T indicator followed by titration of calcium only at a pH of approximately 13 with

E.D.T.A. and calcon as previously described. For each titration 0.5 ml. of serum is required.

The reagents used for total magnesium and calcium estimations are basically the same as described by Wilkinson (1957) but adjusted to suit the smaller volumes required for the apparatus by using 1 ml. of Eriochrome dye buffer solution which had the dye concentration reduced by 40%.

REAGENTS These are as follows:—

Stock magnesium standard (50 mg./100 ml.) $\text{Mg}\cdot\text{SO}_4\cdot 7\text{H}_2\text{O}$, 2.5328 g., is dissolved in 500 ml. ion exchange distilled water.

Dilute magnesium standard (5 mg./100 ml.) Dilute stock solution 1:10 with distilled water.

Ethanolamine (2 hydroxethanolamine, B.P. 173°C.) It may be necessary to re-distil this reagent.

Methyl alcohol absolute AR

Eriochrome black T stock dye solution Dissolve 400 mg. Eriochrome black T (G. T. Gurr Ltd.) in 100 ml. methyl alcohol, add 4 ml. ammonia AR 0.88 SG solution, and store in a polythene bottle. The dye is stable up to five or six months.

Dye buffer solution Eriochrome dye, 0.5 ml., and 0.5 ml. ethanolamine are made up to 50 ml. with distilled water. The dye buffer solution is made freshly each day.

METHOD The use of the apparatus is the same as described for the estimation of calcon. The Eriochrome black T colour change is sharper than that of calcon and the change of optical density is greater, so that the sensitivity settings of the galvanometer must be lowered.

CHECK ON E.D.T.A. STANDARDIZATION Into the titration beaker pipette 1 ml. or 1.2 ml. magnesium dilute standard and add 2 ml. of dye buffer solution. Titrate with E.D.T.A. until the full colour change has been observed and the E.D.T.A. titration figure giving the greatest galvanometer deflection noted.

1 ml. magnesium standard = 0.833 ml. E.D.T.A.

1.2 ml. magnesium standard = 1 ml. E.D.T.A.

It is useful to cross check the E.D.T.A. solution both with the magnesium standard and Eriochrome black T and with the calcium standard and calcon. After allowing for blank values there should be complete agreement of standardization.

TITRATION OF TOTAL CALCIUM AND MAGNESIUM IN SERUM Into the titration beaker pipette 0.5 ml. serum, add 1 ml. dye buffer solution, and titrate as before. Maximum accuracy is obtained using 0.01 additions of E.D.T.A. The pH of the dye buffer and serum is approximately 10.5.

Calcium only is estimated on a further 0.5 ml. of serum as described previously.

CALCULATION The titration for magnesium equals the total calcium and magnesium titre minus the titre for calcium only. $[\text{Mg (mg./100 ml.)}] = \text{titre Mg} \times 2 \times 5 / \text{standard reading (1 ml.)}$

RECOVERY OF MAGNESIUM ADDED TO SERUM Varying quantities of magnesium sulphate solution (as 50 mg.

TABLE V

RECOVERY OF MAGNESIUM ADDED TO A NORMAL SERUM

Magnesium Added (mg./100 ml.)	Magnesium Found (mg./100 ml.)	Magnesium Recovered (mg./100 ml.)	% Recovery
—	2.75	—	—
1.0	3.76	1.01	101.0
2.0	4.70	1.95	97.5
5.0	7.78	5.03	100.3
10.0	12.60	9.85	98.5
20.0	22.60	19.85	99.4

Mg/100 ml.) were added to a serum of known calcium content and after titration the recoveries were calculated.

Table V shows that the method gives satisfactory recoveries up to 20 mg. Mg/100 ml.

We wish to thank Professor N. F. MacLagan for his interest and help while these experiments were in progress.

REFERENCES

- Baron, D. N., and Bell, J. L. (1959). *J. clin. Path.*, **12**, 143.
 Bett, I. M., and Fraser, G. P. (1959). *Clin. chem. Acta*, **4**, 346.
 Clark, E. P., and Collip, J. B. (1925). *J. biol. Chem.*, **63**, 461.
 Golby, R. L., Hildebrand, G. P., and Reilley, C. N. (1957). *J. Lab. clin. Med.*, **50**, 498.
 Hildebrand, G. P., and Reilley, C. N. (1957). *Analyt. Chem.* **29**, 258.
 Horner, W. H. (1955). *J. Lab. clin. Med.*, **45**, 951.
 Wheatley, V. R. (1944). *Analyst*, **69**, 207.
 Wilkinson, R. H. (1957). *J. clin. Path.*, **10**, 126.

The November 1962 Issue

THE NOVEMBER 1962 ISSUE CONTAINS THE FOLLOWING PAPERS

Haemagglutination in acute hepatitis and other diseases
 PAUL TURNER, V. N. JHA, NUALA CROWLEY, and SHEILA SHERLOCK

An epidemiological study of haemagglutination in hepatitis
 JAMES R. MCARTHUR and NUALA CROWLEY

Blood clotting factors in cerebrospinal fluid
 STEFAN NIEWIAROWSKI, IRENA HAUSMANOWA-PETRUSEWICZ, and ZENON WEGRZYNOWICZ

Observations on the separate assessment of Prower-Stuart factor activities
 C. GARDIKAS, C. LYBERATOS, G. KALLINIKOS, and M. KALLINIKOU

An investigation into the use of activated and non-activated papain in routine rhesus blood grouping
 G. C. B. WINTER and B. E. WRATTEN

The contact phase of coagulation in the presence of heparin
 A. L. BLOOM

Muscle morphology in infantile protein malnutrition
 R. D. MONTGOMERY

The diagnosis of the scars of chronic pyelonephritis
 J. F. SMITH

Laryngeal dysfunction and the pulmonary syndrome of the newborn
 G. R. OSBORN and R. L. FLETT

Haematoxylin bodies in Hodgkin's disease
 N. CANDREVIOTIS

Resistance of *Salmonella typhi* to chloramphenicol
 B. RAMANARAYANA MURTI, K. RAJYALAKSHMI, and C. S. BHASKARAN

Part I *A preliminary report*

Part II *The mechanism of resistance*

The dispersal of staphylococci in hospital wards
 W. C. NOBLE

The dispersal of organisms from minor septic lesions
 B. T. THOM and R. G. WHITE

Serum tube identification of *Candida albicans*
 D. W. R. MACKENZIE

A simple colorimetric method for the determination of serum alpha-hydroxybutyric dehydrogenase activity
 S. B. ROSALKI

The incidence of cryoglobulinaemia as determined by a turbidimetric method
 L. BAGRATUNI

The determination of urinary 17-ketosteroids by an improved Zimmermann reaction
 R. N. BEALE, J. O. BOSTROM, and D. CROFT

Blood pyruvate concentration measured by a specific method in control subjects
 J. LANDON, J. K. FAWCETT, and VICTOR WYNN

The estimation of two alpha₁ glycoproteins (orosomucoid and another alpha₁ acid glycoprotein) in health and disease
 J. A. EASTON, J. HARDWICKE, and P. H. WHITEHEAD

Technical methods

An improved container for cells preserved by freezing
 J. H. L. PLAYFAIR and D. E. PEGG

Macroscopic demonstration of infarction in fresh brain slices
 D. N. RAINE

A routine immunofluorescence method for detecting autoantibodies to thyroid colloid
 R. C. NAIRN, T. GHOSE, I. B. PORTEOUS, and J. A. URQUHART

Book reviews

Index to volume 15

Copies are still available and may be obtained from the PUBLISHING MANAGER,

BRITISH MEDICAL ASSOCIATION, TAVISTOCK SQUARE, W.C.1, price 17s. 6d.