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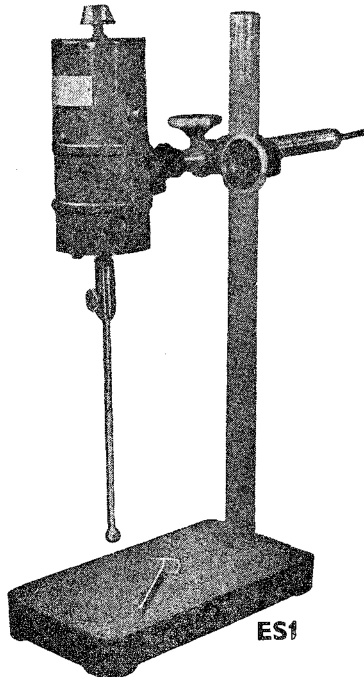

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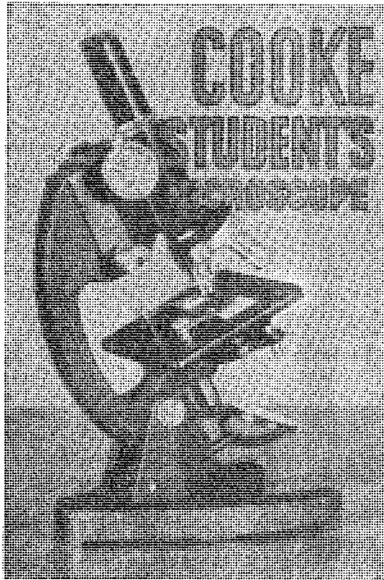

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A FURTHER NOTE ON THE SEROLOGICAL IDENTIFICATION OF SALMONELLA CULTURES

S. W. JOSLAND

Animal Research Station, Department of Agriculture, Wallaceville

In a previous paper (1948), the methods used at this station for the serological identification of salmonella were described. Since the publication of that paper, further experience in the use of these methods has been gained, and the purpose of this communication is to describe in greater detail the systematic technique now used here for the identification of salmonella cultures.

In the examination of a smooth culture conforming generally to the biochemical requirements of a salmonella, there are many antigenic possibilities which make a hit or miss approach costly in the use of sera and of time. A systematic routine is therefore necessary. Such a method is provided by the use of the polyvalent flagellar salmonella sera supplied by the Medical Research Council Standards Laboratory, Colindale, London. Notes on the constitution and use of these sera have been published by Bridges and Joan Taylor (1944), but for the benefit of those who do not have access to this article the composition of these sera are given below.

PSA serum contains factors c; i; k; lv; 1, 5; 1, 2; z_6; 1, 6.
PSB serum contains factors eh; r; y; z; z_{10}; enx; enz_{15}.
PSC serum contains the monophasic factors fg; mt; z_4; z_{23}; z_{25}; z_{14}; z_{27}; z_{29}.

It will be seen that the commonly met with factors, a, b and d are for some reason not included in these sera. For this reason we prepare a polyvalent serum containing these factors, which we designate PSD. Certain other factors are not incorporated in the MRC sera, but these occur in combination with factors which are included, so that for practical purposes the PSA, PSB, PSC and PSD sera give a full coverage of salmonella flagellar factors.

These sera are always used in tube tests, thus avoiding the confusing results often obtained by slide tests. Kauffman (1950) whose views must be treated with respect, contends that slide agglutination is not merely an orienting procedure, but is a method which, with proper technique, gives such definitive results, that in most cases tube agglutination is superfluous. Our experience conflicts with this contention, especially in relation to flagellar agglutination. It is our belief that most of the confusing reactions reported by bacteriologists who refer cultures to this station are due to reliance on the results of slide agglutination carried out without reference to the actual titre of the serum used.

For flagellar agglutination to occur efficiently the culture must be actively motile. To achieve this, all cultures are first passed through a semi-solid medium prepared from tryptose phosphate broth to which 0.4% agar and 8% gelatine are added. The medium is dispensed in petri dishes, and the inoculation made by stabbing the centre of the medium; after incubation overnight a motile organism will have spread through the body of the medium, and can be recovered from the edges, and recultured in broth.

Broth cultures are treated with formalin to give a 0.6% concentration. 2 c.c. of formalinised broth culture are then pipetted into each of four Wassermann tubes, and 1 drop of PSA, PSB, PSC and PSD serum is added to separate tubes which are placed in the water bath at 55°C. for 2 hours. If agglutination occurs it does so generally within a few minutes, but if no agglutination appears then the tubes are left in for 2 hours. The titre of M.R.C. salmonella sera is about 1/250, but we have found deterioration to occur rapidly in some of these diluted sera. Consequently sera other than polyvalent should be checked against homologous antigenic suspensions before being accepted as reliable. At Wallaceville we have found it desirable to prepare our own high titre polyvalent sera to the prescription of the MRC sera; while some lowering of titre does occur during storage of high titre sera, the margin of error is insignificant compared with that resulting from a loss of titre in diluted sera.

Should the organism be a salmonella other than S. pullorum or S. gallinarum, which are easily tested for by IX. XII. somatic

sera, agglutination will occur in one or more of the four tubes according to the presence of one or both of the flagellar phases. Consider the case of the flagellar antigens of S. bovis morbificans which are r; 1, 5. Should both phases be present, agglutination will occur with PSA and PSB sera. If phase 1 alone is present, agglutination will occur with PSB serum, while if phase II. alone is present agglutination will occur with PSA serum. On the other hand, if S. typhi-murium were the organism under examination, agglutination would occur with PSA serum only. This could be due to the presence of either phase singly, or both phases conjointly.

If agglutination occurs with PSC serum, then the organism will be a monophasic type. If agglutination occurs with PSA or PSB sera, the emulsion is tested with factor 1, 5 and factor enx sera, and then with sera for the more commonly occuring factors known to be present in the polyvalent serum or sera with which agglutination was obtained. Thus, if the organism is diphasic with both phases present, it should be possible to arrive at the flagellar components by these means.

Sometimes, however, only one phase of a diphasic salmonella can be identified; in this event the simplest procedure is to pass the organism through semi-solid agar to which have been added a few drops of high titre serum corresponding to the antigen already identified. A factor is identified when the suspension is agglutinated to titre by its homologous serum. It will be appreciated that any antigenic complex containing the factor 1 . . . will be agglutinated by any serum containing the 1 . . . agglutinin. Consequently where alternative 1 . . . combinations are possible, the use of single factor sera is necessary. Thus, to distinguish S. richmond VI. VII.; y; 1, 2 from S. bareilly VI. VII.; y; 1, 5; single factor sera for factors 2 and 5 must be prepared by absorption.

Cross agglutinations often present difficulties; these generally result from the presence of a common factor such as the somatic XII factor; sometimes they result from minor relationships not expressed in the Kauffman White Schema; Kauffman (1950) has drawn attention to the fact that the Schema makes no pretence of listing all the existing antigens. He has stressed the point that antigenic formulae are relative and reflect the existing state of our knowledge. Reference to Edwards (1943) will show the obscure minor cross agglutination possibilities existing in one batch of sera prepared at the Kentucky Salmonella Centre.

A routine such as that outlined above should identify the flagellar antigens. While this is being done the somatic antigens

can be identified by slide agglutination using group sera having a titre of approximately 1/50 to minimise cross agglutination due to common factors. In all cases, confirmation should be obtained by tube tests at 37°C. for 24 hours, using saline suspensions following alcoholisation. Cross agglutination is often of value in determining some of the factors, but in all doubtful cases recourse to single factor sera prepared by absorption is necessary. Again, knowledge of the flagella groupings is useful in limiting the somatic possibilities.

As a guide to the variety of sera which would be necessary for the identification of salmonella types found from human sources during the period 1948-51 in New Zealand, following is a list of these types identified at this station (1952).

S. paratyphi A	I II XII;	a;	—
S. paratyphi B	I IV V XII;	b;	1, 2.
S. typhi-murium.	I IV V XII;	i;	1, 2.
S. derby	IV XII;	fg;	—
S. bareilly	VI VII;	y;	1, 5.
S. bovis morbificans	VI VIII;	r;	1, 5.
S. typhi	IX XII; Vi;	d;	—
S. enteritidis	I IX XII;	gm;	—
S. anatum	III X XXVI;	eh;	1, 6.
S. orion	III X XXVI;	y;	1, 5.
S. newington	III XV;	eh;	1, 6.
S. cholerae-suis	VI VII;	c;	1, 5.

Sera with titres of 1/250 for the more commonly occurring factors may be purchased from the M.R.C. Colindale laboratories. It is recommended that in addition, stock cultures of those types necessary for the preparation of sera, for the preparation of suitable suspensions for checking, and where necessary for absorption, be maintained in larger hospital laboratories.

SUMMARY

A systematic procedure for the serological identification of salmonella cultures, in routine use at the Wallaceville Animal Research Station is described.

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ACUTE IDIOPATHIC PORPHYRIA

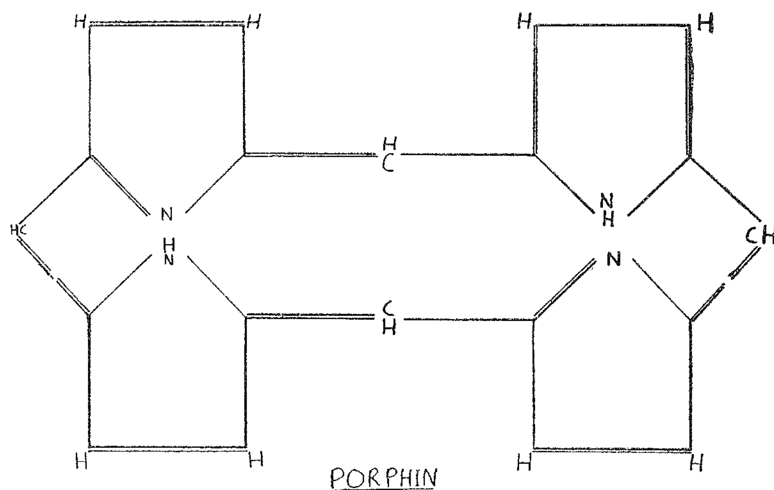
Including a case report and an account of diagnostic laboratory tests.

R. J. PATTERSON

(From the Department of Pathology, Auckland Hospital.)

Porphyrins are considered probable intermediate products in the building up of haemoglobin, haem being a ferrous complex of protoporphyrin. They are not proteins however, and contain no iron. Their place in human physiology is not completely understood, and Watson (1951) suggests that there is a likelihood that they have some part in cellular metabolism. It has been shown that glycine and acetic acid are connected with the synthesis of porphyrin in vivo and other amino acids such as proline, hydroxyproline and tryptophane have been suggested.

The parent substance of all porphyrins is porphin C_{20} H_{14} N_4 having the following structure:—



PORPHIN

Substitution of groups such as methyl, ethyl, vinyl, acetic or propionic, for the hydrogen atoms in the β and β^1 positions of its four pyrrole rings constitutes the chemical differences between them. For example, the coproporphyrin molecule (C_{36} H_{38} N_4 O_8) has four methyl (-CH_3) and four propionic acid (-CH_2 CH_2 COOH) groups, one of each being attached to each of its pyrrole rings. Similarly uroporphyrin (C_{40} H_{38} N_4 O_{16}) contains four

Case Report:

Mrs. E., a married woman aged 24, and the mother of two children, was admitted to Middlemore Hospital suffering from severe abdominal pain. Her admission note stated that she had had intermittent abdominal pain accompanied by frequent vomiting for 16 days. She had been constipated for five days and was given an enema, which seemed to aggravate her condition. When examined on admission, she was described as "a pale, distressed, young woman in some obvious pain." Her nervous condition suggested a trace of hysteria, so much so that the house surgeon noted the possibility of an exaggeration of her symptoms. The examination revealed tenderness over the whole left side, more particularly the L.I.F. A provisional diagnosis of strangulated left ovarian cyst was made and the patient admitted to a surgical ward.

At this stage her white count was 6,700, haemoglobin 14.9 gms./100 c.c. and sedimentation rate 7 mm. in 1 hour. Due to her difficulty in passing urine, no specimen was sent to the laboratory until the following morning. The specimen revealed no abnormality in colur when collected, but by the time it reached the laboratory, when it could safely be assumed to be at least two hours old, it had developed a deep brownish colour. On first examination this gave the appearance of a heavy concentration of urobilin. A closer examination, however, gave the impression that there was "something different" about it. Ehrlich's aldehyde test was strongly positive, but Schlesinger's test for urobilin was negative. There was a trace of albumin present and the deposit showed 10-15 leucocytes per H.P.F. The specimen was despatched, as a possible porphyria, to the central laboratory at the Auckland Public Hospital where this diagnosis was confirmed.

On the strength of the laboratory findings, no surgery was performed and the patient referred to a physician and transferred to the medical ward. Her pain persisted for 16 days, but gradually diminished in severity. She remained mentally disturbed and did not sleep at all well. During an attack she was given 100 mgms. of pethidine to which she responded very well, sometimes with "amazing rapidity." Urine specimens continued to darken on standing, and quantitative porphyrin tests performed one week after her admission gave the following results:—

Total Coproporphyrin	210	micrograms	per	100 cc.
Total Uroporphyrin	137	,,	,,	,, ,,

These figures are not typical of the usual case of acute porphyria, the coproporphyrin excretion being rather high and that of uroporphyrin lower than is normally experienced. It must

be borne in mind, however, that at this stage the major part of her crisis was probably over, so that her uroporphyrin excretion was diminishing.

After 19 days the patient seemed to have recovered. She was feeling well and made no complaints of pain. Her mental condition was normal, she had a good appetite and was sleeping well. She was subsequently discharged.

The clinical symptoms revealed by this woman, together with her urinary findings show that she was a case of acute idiopathic porphyria. Since her discharge she has been attending an outpatient clinic, but so far there has been no suggestion of any recurrence of a crisis, although her urine is still slightly discoloured. Quantitative porphyrin tests done recently on a casual specimen of her urine gave these results:—

Total Coproporphyrin	112	micrograms per 100 cc.
Total Uroporphyrin	64	„ „ „ „

She has complained of pain after lifting or walking, but the significance of these symptoms is doubtful. It is extremely likely, however, that a porphyria crisis will recur.

Tests for the examination of urines for porphyrins

I. General Inspection:

In cases of porphyria, particularly those of the congenital and acute varieties, there is a pigmentation of the urine varying in intensity from a light pink to a deep port wine or dark brown. This pigmentation is absent or only very slight in fresh specimens, but becomes darker on exposure to light, due to the transformation of porphobilinogen to porphobilin. This action may be hastened by boiling the specimen in a water bath for about 30 minutes or by the addition of an oxidising agent such as iodine. A fresh specimen, when examined through a spectroscope, usually gives the alkaline porphyrin spectrum in which at least two absorption bands are seen at approximately 575 and 535 mu. In specimens which have been standing, the presence of decomposition pigments may obscure this spectrum so that direct spectroscopic examination does not always yield satisfactory results.

II. Tests for Porphobilinogen:

In acute porphyria a chromogen, porphobilinogen, is excreted which gives a positive Ehrlich's test similar to that given by urobilinogen. The reactions differ, however, in that:

(a) Two absorption bands are seen in the spectroscopic examination of the porphobilinogen aldehyde complex (at 540-560 mu and at 480-510 mu) whereas with urobilinogen there is only the one (at about 560 mu).

(b) The pink colour produced by porphobilinogen is insoluble in amyl alcohol or chloroform, whereas that produced by urobilinogen may be extracted by either of these solvents.

The latter differentiation is the basis of a simple test for the demonstration of porphobilinogen, namely, that of Watson and Schwatz (Proc. Soc. Exp. Biol. & Med. 1941, *47,* 393).

Method: 2.5 ml. of urine is mixed with 2.5 ml. of Ehrlich's aldehyde reagent (0.7 gms. of p-dimethylaminobenzaldehyde in 150 ml. of concentrated HC1 + 100 ml. of distilled water) and 5 ml. of a saturated solution of sodium acetate, followed by a few mls. of chloroform. The mixture is then shaken thoroughly and allowed to settle. If the pink colour formed is due to porphobilinogen, it will remian completely in the supernatant aqueous layer, whereas if it is taken up in the chloroform layer, it is due to urobilinogen. Deviation from the quantities mentioned may give erroneous results. The test should be performed on a fresh urine, preferably one collected in a brown glass bottle.

A quantitative modification of the above method is as follows (Prunty, Biochem, J. 1945, *39,* 446).

Method: 5 ml. of fresh urine is acidified with 0.5 ml. of glacial acetic acid and any urobilinogen present removed by extraction with 10 mls. of ether. 5 mls. of Ehrlich's reagent (in this case 20 gms. of p-dimethyl-amino-benzaldehyde in 500 ml. of 36% W/W HC1 and 500 ml. of distilled water) is mixed with the urine and allowed to stand 80 seconds at room temperature, the intensity of the colour produced being read spectrophotometrically. A concentration of 1 unit/ml. is taken as giving an extinction at 550 mu of 0.545. Using the amounts mentioned, if x is the extinction at 550 mu of the solution, the number of units of porphobilinogen present per 100 ml. of urine, will be

$$\frac{X \times 200}{0.545}.$$

III. Quantitative Estimations of Porphyrins in Urine

Since porphyrins may be present as metallo-porphyrin complexes (usually with zinc), particularly in cases of acute porphyria, these must be broken up by preliminary acid treatment,

thus ensuring that the uroporphyrin-zinc complex is not extracted with the coproporphyrins by ether. 25 ml. of urine is made strongly acid by the addition of 2.5 ml. of concentrated HC1, the mixture being allowed to stand at least 2 hours (or overnight) at room temperature.

(a) Estimation of coproporphyrin (total coproporphyrin, including types I and III.)

The acidified urine is brought to approximately pH 4.0 by the addition of a saturated solution of sodium acetate until it is just alkaline to methyl orange. 50 ml. of ether is then added and the mixture shaken thoroughly in a separating funnel. The urine layer is run off and kept for further tests. The ether layer containing the coproporphyrins is washed twice with about a quarter of its volume of distilled water. The porphyrins are then extracted with 2 ml. lots of 0.1 N.HC1, three extractions usually being sufficient. It may be assumed, however, that extraction is complete when there is no detectable trace of fluorescence in the HC1 layer in ultra-violet light. An excess of coproporphyrin will give a pinkish tint to the extract and in ultra-violet light a brilliant orange-red fluorescence.

The quantitative estimation of coproporphyrin is made by measuring the optical density of the acid extract at the peak of the Soret absorption band at 401 mu. If there is an increased amount of coproporphyrin present it may be necessary to dilute the extract with 0.1 N.HC1 until the optical density is within the measurable range. Readings are taken at 400, 401 and 402 mu to ensure that the value for the peak of the absorption band is measured. Then since $E^{1\%}_{1\,cm.}$ at 401 mu for coproporphyrin is 8000 (Jope and O'Brien Biochem, J., 1945, *39, 239*), if x is the optical density of the coproporphyrin extract at 401 mu and y is the total volume, then $\frac{x\ y}{8 \times 25}$ mgs. of coproporphyrin are present in 100 ml. of the sample.

(b) Estimation of uroporphyrin.

(1) Wäldenstrom's porphyrin (sometimes called uroporphyrin III).

The pH of the urine remaining after the coproporphyrin extraction ((a) above) is adjusted accurately to pH 3.1. (For this a pH meter should be used). Two volumes of ethyl acetate are then

added, and the mixture shaken thoroughly for several minutes in a separating funnel. The urine layer is run off and kept for further examination. The ethyl acetate layer is washed twice with distilled water and the uroporphyrin extracted with successive lots of 0.5 N. HC1 until there is no fluorescence in ultra-violet light. The quantitative estimation is carried out as described by Sveisson, Rimington and Barnes (Scand. J. Clin. & Lab. Invest. 1949, *1*, 2). As before, the optical density of the extract is measured, this time at 405 mu, diluting if necessary with 0.5 N. HC1 until it is within the measurable range, and the total volume (y) noted. Then if x is the optical density at 405 mu of the extract, the number of mgm. of uroporphyrin present in 100 ml. of the specimen is given by the formula $\frac{x\ y}{5 \times 25}$ since $E^{1\%}_{1\text{ cm.}}$ at 405 mu for uroporphyrin is 5000.

Note: Wäldenstrom's porphyrin is not completely extracted by one lot of ethyl acetate, so that for greater accuracy two or more extractions should be made.

(2) Residual porphyrins (Uroporphyrin I).

To the urine remaining from the preceding extraction add 25 ml. of 3% (W/V) $Ca\ Cl_2$ then 50 ml. of N. NaOH. Centrifuge and decant the supernatant fluid. Wash the precipitate with 0.IN NaOH and again with distilled water. Dissolve in 25 ml. of 0.5 N. HC1 and filter. Examine the solution spectrophotometrically as under b (1) above, but applying Rimingtons' correction formula to eliminate the interference of co-existing pignents, viz.:—

$$D_{405} = \frac{2D'_{405} - (D'_{430} + D'_{380})}{1.844.}$$

Where D_{405} = true optical density at 405 mu.
D'_{405} = observed optical density at 405 mu.
D'_{380} = ,, ,, ,, ,, 380 mu.
D'_{430} = ,, ,, ,, ,, 430 mu.

Note.—If the uroporphyrin content of the specimen is very high and the phosphate content low, it is advisable to increase the quantity of the precipitate by the prior addition of a little Na_3PO_4. For each ml. of urine add 1 ml. of 0.2M. Na_3PO_4 and precipitate by the addition of 4 ml. of 3% $CaCl_2$ and 5 ml. of N. NaOH. It is essential that the $CaCl_2$ is present in excess to ensure precipitation of all the phosphate, otherwise adsorption will be incomplete.

The tests described are those in use at the Auckland Hospital central laboratory in the investigation of cases of porphyria. The amounts mentioned are the ones given in the original papers. It was found quite satisfactory, however, to use fractional quantities, provided, of course, that this was corrected for in the final calculation. These tests should be performed on 24-hour specimens so that the daily output may be ascertained. Such specimens should be collected in a dark Winchester containing about 5 grams of $NaHCO_3$. If there is likely to be any delay before examination a few mls. of toluene should be added.

Acknowledgments

I would like to thank Dr. F. H. Sims of the Pathology Department, Auckland Public Hospital, for his interest in this work and for his assistance in connection with it.

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"Where one man fails in an experiment, another succeeds. What is unknown in one age is clarified in the next. The arts and sciences are not cast from a mold; they are shaped and polished little by little, here a dab and there a pat as bears leisurely lick their cubs into shape. What my powers cannot solve, I still persist in sounding and trying out. By kneading and working over the new material, turning and warming it, I make it more supple and easier to handle for the man who will take it up after me. And he will do as much for a third. This is why I should not despair of difficulties or of my own incapacity—for it is only my own."

MICHEL DE MONTAIGNE

(As noted in J. Chem. Education, 1946, 23, 450, by Marvin Lowenthal, in "The Research Viewpoint.")

Some of the women candidates frankly admitted that they found the mechanical details of, say, a still, quite beyond them, nevertheless, one had actually dismantled an old still just to familiarise herself with the construction. Such questions must be expected, and if you find mechanical things difficult, dismantling, reassembling and drawing the various parts is the best way of fixing the details in your mind.

Use of Culture Media

Do not be reluctant to use enough culture media to ensure all the information you need by next day. E.g., if dealing with a mixed inoculum where you expect trouble in isolating one organism from among others, do not be afraid to use two plates inoculated heavily and lightly, instead of one. Indeed, you will save yourself valuable time on the second day, by being liberal with the culture media on the first day.

Use your skill here; don't be careless in applying the inoculum and then use several plates; but anticipate the difficulties on the basis of smears from the specimen.

Summary

1. Read the question carefully, and write an answer which covers the question.
2. Systematise or tabulate your answers as much as possible, both to indicate you have a grasp of the subject and also for your own and for the examiner's convenience.
3. Don't be afraid to ask for, and use sufficient media."

The Intermediate Examination was held in Christchurch on 29th and 30th of May last. The examiners were Dr. G. C. T. Burns, of Christchurch, and Mr. J. A. Samuel, of Dunedin. The latter has made some valuable remarks on the sitting of examinations which are published elsewhere.

We are pleased to congratulate the successful candidates who were:—Misses J. J. Crawshaw (Auckland), M. J. Grey (New Plymouth), S. Jenkins (Wellington) ,and B. Smith (Waipukurau), and Messrs. G. R. Rose and D. G. Till (Christchurch). It is a matter of congratulation that this examination in particular is having a very marked effect in stimulating the interest of the junior staff, thus forwarding the training, and experience, of these workers.

The successful candidates in the February Final examination which was reported in the April Journal, were Miss B. L. Broughton, Mr. J. W. Carroll, Mr. F. N. Corey, Miss P. Hildreth, Miss J. M. Mattingley, Miss J. M. Penrose, Mr. D. J. Philip, Miss G. G. Richards, Miss H. A. Simmonds and Miss L. E. B. Will.

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AN UNUSUAL TRANSFUSION HAZARD

Misses J. Grey and M. Brown, of the New Plymouth Laboratory, bring to our attention the short note in the correspondence column of the B.M.J. of 18/8/51, p. 417, pointing out that baby's cord blood should not be used for the typing of the mother, and gives an instance where a potential disaster was avoided.

The Significance of Fat in Sputum. The demonstration of fat in sputum is considered by many to be of importance in the diagnosis of pulmonary fat embolism and lipid pneumonia. In view of the disagreement on this subject a survey was made to determine the frequency of occurrence of fat in normal sputum. Two groups of patients were investigated, one with productive cough who provided bronchial sputum, the other group without cough who provided sputum from oral cavity. The unfixed sputum was placed on a slide, mixed with Sudan IV, covered with a coverslip and checked microscopically for fat. The results revealed fat in every instance. Bronchial sputum showed more fat than oral sputum. The concentration of fat in sputum was found to be unrelated to the type of pulmonary disease. It was concluded that fat is a normal constituent of sputum.—Neissle, W. (Amer. J. Clin. Path., Vol. 21), *430,* 1951.

A Micromethod for Prothrombin Time Determination. The usual methods require venipuncture, a procedure dreaded by patient, physician and technician alike when repeated frequently over an extended period. A method requiring 0.2 ml. of blood, obtained by deep finger puncture, is described. This micromethod is a modification of Shapiro's diluted plasma prothrombin time, and is comparable in accuracy to standard methods but is mainly designed for emergency use.—Isenberg, H. (J. Lab. & Clin. Med., Vol. 37), 807, 1951.

MEMBERSHIP OF THE ASSOCIATION

Although the question of membership of the Association is covered in the rules, the Secretary would like the following observations to be made:—

(1) Persons desiring to become members of the Association must make written application, themselves, to the Honorary Secretary.

(2) Their present position should be stated together with details of the work being done, the length of time employed, and the work done since being employed, together with the time spent in each type of work, if more than one type of work has been done. State "trainee," "laboratory assistant," etc.

(3) Give your educational qualifications, e.g., "School Certificate, "University Entrance," etc., and state whether you intend to proceed to the Intermediate and/or the final examination.

(4) Though not required by the rules, it is desirable that your Hospital Bacteriologist sponsor the application, by a letter confirming your application.

(5) Present applications should be made immediately, as it is desired to present the applications to the Council at the pre-Conference committee meeting. The list of members will be revised immediately after conference, and will be republished. Any corrections should be sent to the Secretary by Conference time.

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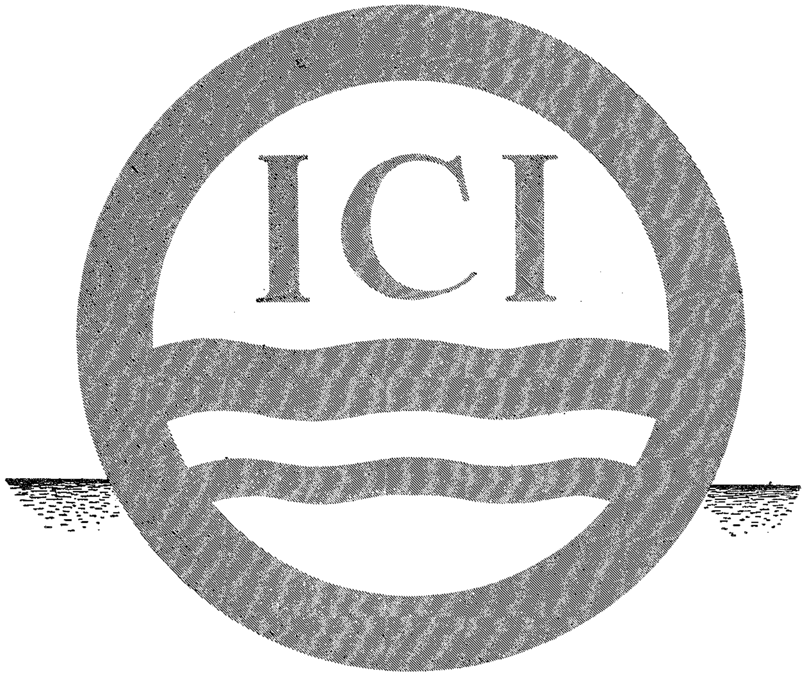
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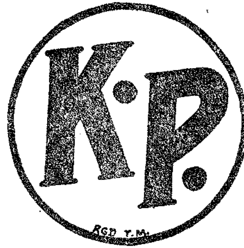

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