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NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS

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EDITORIAL

There is no more interesting side to modern pathological laboratory work than its evolution from that of a diagnostic aid to that of an agent for the control of specific treatments. While in some instances clinical observation is sufficient for control, in many others this must be supplemented by laboratory tests, and an increasing amount of such work is now part of the daily round.

While the brilliant conception of one man may lead to a major advance in human welfare, this is more often achieved by the integrated work of many, especially in these days when most major problems would be incapable of solution by an individual.

Such is the story of the substance known as dicoumarin, which is one of more than one hundred and fifty compounds either analogues or related in structure, of which more than forty have been shown to have prothrombin reducing properties. To Schofield and Roderick, two keen students of veterinary medicine and to an outstanding agricultural chemist Link must be given the credit for analysing the factors responsible for the haemorrhagic sweet clover disease in cattle which appeared in 1921 in North Dakota and in Ontario, and was proved by the brilliantly planned and executed researches of Link and his associates to be due to the change of coumarin, the aromatic substance in sweet clover into the haemorrhagic agent dicoumarin of the formula 3, 3 methylene-bis-(4 hydroxy-coumarin). It is of interest to note that this substance was first synthesised by Anschutz in 1903 and that his researches facilitated Link's work and that botanists are now endeavouring to develop sweet clover relatively free from coumarin.

Once identified, dicoumarin was rapidly made available in pure synthetic form, and in 1941 was first subjected to careful clinical studies in humans where its properties were found to be of value in certain diseases and particularly so in the prevention of post-operative thrombosis, especially when carefully controlled by daily blood prothrombin estimations. It can confidently be predicted that future work in our laboratories will include an ever-increasing amount of such control, the work of the laboratory being complementary to that of the clinician.

THE ESTIMATION OF PROTHROMBIN WITH REFERENCE TO VITAMIN K AND DICOUMARIN THERAPY

(W. C. CORSBIE)

(From the Pathological Laboratory, Public Hospital, Auckland.)

The advent of treatment with vitamin K and dicoumarin has focussed more attention upon prothrombin determination, and it is essential to have a reliable method of assessing its activity in routine laboratory use. Since the isolation of prothrombin is such a difficult matter, Quick (1934) devised an indirect measurement of the plasma prothrombin concentration. It is based on the theory of blood coagulation as expressed by the two equations—

(1) ProthrombinCa + thromboplastin = Thrombin.

(2) Fibrinogen + Thrombin = Fibrin.

The test measures the rate of thrombin formation, not the total thrombin formed, assuming that this rate is directly proportional to the prothrombin concentration. Decalcified plasma is furnished with an excess of thromboplastin and recalcified with a known amount of calcium, so that the test rests on the supposition that the concentration of prothrombin is the only variable. Since Aggeler et al (1946) state that with rare exceptions the variations in calcium and fibrinogen encountered in man do not affect the blood coagulation, this supposition is still not disproved. With slight changes the modifications suggested by Kelley and Bray (1939) have been used in this laboratory as a routine procedure and found satisfactory.

Glassware

Tubes approximately 70 X 10 mm. fitted with corks are used for the test.
They should be of clear, smooth-surfaced glass. The cleanliness of all glassware used in the test is important, but special care should be taken of these tubes. It is our custom to soak them in dichromate for 48 hours, making sure that they are well rinsed and dried before each use.

Tubes approximately 200 x 16 mm. fitted with corks are used for the collection of blood and for thromboplastin preparation.

Clean sheet glass approximately 16″ x 12″ is used for dehydrating the thromboplastin.

A glass pestle and mortar is of assistance in the preparation of the thromboplastin.

Reagents

Sodium oxalate M/10 (1.34 gms. per 100 ccs.).
Calcium chloride M/40 (0.38 gms. per 100 ccs.).

Both solutions are aqueous, but it is essential that the preparation of the latter be accurate. This can be done by assaying the amount of chloride present in a stock solution by the ordinary method for chlorides and diluting in accordance with this when a fresh solution is required.

Collection of Specimen

Several collection tubes containing exactly 0.5 cc. of the sodium oxalate solution may be prepared at the same time, but it is unwise to store these for more than three weeks. Avoiding prolonged venous congestion and using a dry syringe, withdraw 5 ccs. of blood, transfer 4.5 ccs. to the oxalate tube and invert the tube five or six times. The specimen should preferably be taken at least two hours after a meal.

Centrifuge the tube at 1500 r.p.m. and remove the plasma. If whole plasma is to be used for the test it may be stored in a refrigerator for 18 to 24 hours without appreciable lengthening of the prothrombin time, but if tests are to be made on dilutions of the plasma, these should be carried out within six hours of veni-puncture, as a lengthening of time has been noted in the greater dilutions of stored plasma.

Preparation of Thromboplastin

Cut a wedge from a lobe of fresh human brain which shows no visible trauma or infection. Remove the meninges and blood vessels, rinse in cold water and dab fairly dry with clean towel. Slice off the medulla and pound in a mortar until an even paste is made. Using a broad-bladed knife, spread the macerated brain evenly and thinly on to the glass plates, dry in the incubator until the material can be flaked off with a razor blade (24-28 hours), and store in screw-capped jars in the refrigerator. Brain so stored should remain potent for at least six weeks, and is used as the thromboplastin for the test.

Titre of Thromboplastin

This is given fully by Kelley and Bray. To 0.2 gm. of thromboplastin add 3.8 ccs. of normal saline, shake well and place in a waterbath at 56° C. for 10 minutes, shaking every three minutes. Cool quickly under the tap, centrifuge at 1000 r.p.m. for not more than three minutes to remove large particles only, and pipette off the turbid supernatant fluid.

From this 1 in 20 suspension prepare dilutions of 1 in 40, 1 in 80 and 1 in 160 in 0.5 cc. quantities. Also prepare a 1 in 30

dilution by adding 1 cc. of a 1 in 20 suspension to 0.5 cc. normal saline, and from this prepare dilutions of 1 in 60 and 1 in 120. Arrange in serial dilution and pipette 0.1 cc. of each dilution into each of four tubes. Make these into two duplicate sets and place in convenient racks. Obtain specimens of blood from two healthy, normal adults and proceed as for the test, using one plasma for each set of serial dilutions. The mean result for each dilution will give parallel low figures for three or four dilutions. Choose the central figure as the titre for the thromboplastin, making this the dilution for the test.

The results of a typical test should be:—

	1/20	1/30	1/40	1/60	1/80	1/120	1/160
Prothrombin time in seconds	19	19	20	21	22	25	31

The titre for this thromboplastin would be 1 in 30. If the titre for the 1 in 20 and 1 in 30 dilutions are not between 15 and 22 seconds, the titre must be regarded as unsatisfactory and the thromboplastin unsuitable. To date this has not occurred in this laboratory. The diluted thromboplastin will hold the correct titre for four to six days if stored in a refrigerator.

Test

Dilute the thromboplastin to the titre required and heat and centrifuge as above. Prepare plasma from the patient and a control plasma from a normal, healthy adult, using the method above. Both test and control are run in duplicate, but only one procedure will be described.

Place a tube containing approximately 0.8 ccs. of calcium chloride solution in the water bath at 37° C for about five minutes before the test is to be carried out. Add 0.1 cc. plasma and 0.1 cc. of diluted thromboplastin to a 90 x 10 mm. tube, shake gently, avoiding bubble formation, and place in a water bath at 37° C for two to three minutes. Do not leave the plasma-prothrombin mixture on the bench for longer than one hour or in the water bath longer than is required to warm the fluids. If a number of tests are to be carried out, it is better to split the calcium chloride solution required into several tubes containing not more than 1 cc.

Holding the tube in the bath, add quickly exactly 0.1 cc. of warmed calcium chloride solution, noting the exact time of its addition and examine the tube by running the fluid half-way down its length every two seconds. The end point is dramatically rapid, being the first sign of fibrinous clot formation. The clot may stay at the base of the tube or flow up the tube on inversion. With a result of under 32 seconds the difference between the first and second tubes should not be greater than two seconds. The two results are averaged and reported as:—Prothrombin time X secs. (Normal control, Y seconds.)

Treatment With Vitamin K

Many theories of blood coagulation have been advanced, but there seems a unanimity of opinion that thrombin has an inactive precursor here called "prothrombin." It is now thought that prothrombin is synthesised in the liver from vitamin K absorbed from the bowel, and that bile salts are essential for the absorption of the vitamin K. Prothrombin deficiency could then arise from a breakdown in the mechanism of the absorption of vitamin K and/or its utilisation by a healthy liver. The administration of vitamin K is an attempt to raise the prothrombin level by artificially feeding the liver with the necessary vitamin. It will be appreciated that if gross liver damage is present the treatment is of no avail.

Treatment With Dicoumarin

Dicoumarin, a haemorrhagic agent first obtained from spoiled sweet clover, has been successfully used in the prevention and extension of thrombosis. The drug has the effect of prolonging the prothrombin time by suppression of its action or inhibition of its formation. A change is also noted in the coagulation time and the sedimentation rate of erythrocytes, but these do not appear to run parallel to the prothrombin time. Unless the dosage can be rigidly controlled the danger from haemorrhage due to gross depression of prothrombin concentration is very real, and no treatment should be undertaken unless daily prothrombin estimations can be carried out. Whether dicoumarin is administered orally or by intravenous injection, regardless of large dosage, there is a latent period of 24 to 48 hours before its effects are demonstrable. Similarly, when the prothrombin concentration is once reduced by dicoumarin, it gradually returns to normal, which may take up to fourteen days. The disappointing results with vitamin K therapy reported by Quick (1942), Allen (1942) and Wright (1942), when trying to elevate the prothrombin concentration lowered by dicoumarin treatment, have been attributed to insufficient dosage by Barker et al (1945). All writers agree that transfusion with fresh citrated blood will temporarily raise the prothrombin level and haemorrhage will cease.

Clinical studies have shown that intravascular clotting is unlikely to occur if the prothrombin time is equivalent to a level of 30% or less, and that bleeding is unlikely to occur till the level falls below 10%. It must be remembered that as the prothrombin level decreases the prothrombin time increases. The administration of dicoumarin is designed so as to keep the daily level in the region of 20%.

Before treatment is commenced the prothrombin time upon whole and diluted plasma is ascertained. Dilutions corresponding to 10%, 20%, 30% and 40% of plasma in normal saline are made. Using correct titre thromboplastin, the prothrombin time is determined on the series in duplicate and the results averaged. Our average results over 40 cases has been:—Whole plasma, 21 secs.; 40% plasma in saline, 23 secs.; 30% plasma in saline, 28 secs.; 20% plasma in saline, 32 secs.; 10% plasma in saline, 62 secs. As these results are to be used as a guide for subsequent treatment it is essential that the collection and dilution of plasma be accurate. In the higher dilutions the dilution of fibrinogen does not allow the same semi-solid clot as is seen in whole plasma, but the white strands which form the end point are easily visible. Quick (1934) showed that plasma diluted with saline and with prothrombin free plasma gave identical results.

As a control for the daily test the plasma which has been obtained from the same patient the previous day should be used. The control plasma should give results within two seconds of the previous day's result.

The dicoumarin used in this hospital is prepared by Organon Laboratories, Ltd., each tablet containing 50 mgms. The usual oral dosage is 300 mgms. the first day, 200 mgms. the second day, and either 200 or 100 mgms. the third and succeeding days, depending on the prothrombin time. Barker et al (1935) in a survey of 1,000 patients found considerable variations in sensitivity to the drug, but maintain that if the prothrombin level is kept between 10% and 30% of normal, the risk from bleeding is small. If bleeding should occur it can be satisfactorily controlled by large intravenous doses (60 to 64 mgms.) of synthetic vitamin K, or transfusions of freshly-drawn, citrated blood.

Contraindications

Most writers agree on the following points given by Barker et al (1945). "On the basis of our experiences, we feel that the following conditions constitute definite contraindications to the use of dicoumarol (dicoumarin): (1) The presence of definite renal insufficiency; (2) the presence of definite hepatic insufficiency or hepatogenous jaundice, particularly if associated with prothrombin deficiency; (3) sub-acute bacterial endocarditis; (4) purpura of any type; (5) blood dyscrasia with tendency to bleed; and (6) recent operation on the brain or spinal cord. Dicoumarin should be given cautiously to patients who have (1) ulcerative lesions, open wounds, or potentially bleeding surfaces; (2) vomiting due to gastric or intestinal obstruction; (3) continuous or repeated gastric or intestinal drainage, or (4) dietary or nutritional deficiency."

Discussion

The repeated reference to "excess" thromboplastin used in the test is rather misleading, as the presence of an excess does not make up for any loss of potency in the thromboplastin employed as shown by Hurn et al (1945) and Quick (1942). In view of the repeated tests to be carried out, it is obvious that each batch of thromboplastin must be comparable. Hurn et al (1945), with an experience of more than 20,000 determinations of prothrombin time have found the preparation of thromboplastins which give consistent results with normal plasma is relatively simple, but that the preparation of thromboplastin giving consistent results with prothrombin-deficient plasma is difficult. Whether this is a feature peculiar to rabbit brain, I cannot say, but as many writers have reported variations in activity in thromboplastin prepared from rabbits from different localities, this may be so. Using human brain, we have not found this variation. A dilution curve of the same two normal adults has been plotted for each fresh batch of thromboplastin prepared, and yielded consistent results comparable to those of Hurn et al (1945) and running parallel to Quick's curve.

Thromboplastins prepared by the acetone extraction method of Aggeler et al (1946) and Quick (1942) have given a higher titre and a lower average normal prothrombin time. Our preparations are comparatively new, so comparisons are not available, but Aggeler reports no loss of thromboplastic activity over a period of two years. However, as Kark and Lozner (1939) and Hurn et al (1945) point out, the decrease in prothrombin concentration should be reflected by an appreciable prolongation of the time of the reaction. They consider that this active preparation does not show actual differences in prothrombin content that are apparent in less active preparations, and have reverted to the use of Quick's original method of preparation similar to the one described.

The writer has had no experience with Russel Viper Venom, or lung tissue as the origin for thromboplastin. Fullerton, using "Stypven" Viper Venom, obtained a dilution curve parallel to Quick's, the average normal whole plasma time being 22 seconds.

Aggeler et al (1946) maintain the dry oxalate mixture (6 mg. of ammonium and 4 mg. of potassium oxalate) routinely used in haemathology work has proved satisfactory. We have been unable to obtain correlation of results in diluted plasma, but have found only slight variations in whole plasma.

Magath (1939) has stated that the temperature of the bath

is important and should not vary over one degree. I feel unfit to comment, but have been able to duplicate results regularly using a covered water bath as used routinely for the Wasserman test.

The use of a 0.1 cc. pipette has been recommended by Kelley and Bray (1939) when adding the calcium chloride, but we have found that running 0.1 cc. out of a 1 cc. graduated pipette between marks is more reliable.

The question may then be raised as to what constitutes a normal prothrombin time. The answer depends almost solely on the thromboplastin in use. When this is standardised with whole and diluted plasma as suggested, the standard normal times will have been elucidated for each batch. Brinkhous (1940) reports: "The normal prothrombin level is remarkably constant, with but little variation from individual to individual. No sex variations in plasma prothrombin levels have been observed." Many articles are published pointing out the prothrombin concentration between the first and fifth day of life is dangerously low, and a relatively slight haemorrhage may produce a critical condition. Repeated references have been made to the fact that a great drop in prothrombin concentration must be encountered before an appreciable prolongation is noticed in the prothrombin time. A plasma may then be prothrombin deficient even to the extent of 60% of normal, and still give normal results with whole plasma, but this deficiency will show when prothrombin determinations are carried out on diluted plasma. Magath (1939), Fullerton (1940), Brinkhous (1940), and Aggeler et al (1946) have all suggested that the dilution of plasma will give a more accurate indication of the prothrombin concentration.

The dilution curve of Quick (1942) is printed with the curves of Fullerton, Aggeler, and our normal curve. For comparison a slightly deficient curve is plotted.

Summary

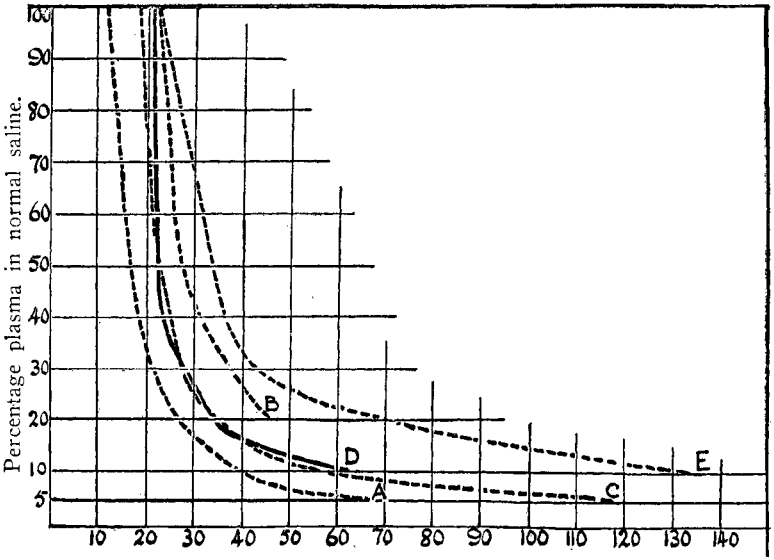
A routine method for the estimation of prothrombin time has been presented.

Numerous daily tests can be carried out and duplicated by any careful technician.

Human brain may be used as a source of thromboplastin. This can be prepared and standardised with comparative ease. Using thromboplastin prepared in this manner, the levels for dicoumarin treatment have been found to be:—Whole plasma, 18 to 22 seconds, 40%, 22 to 24 seconds; 30%, 25 to 30 seconds; 20%, 30 to 34 seconds; 10%, 60 to 64 seconds.

ACKNOWLEDGMENTS:

I wish to thank Dr. W. Gilmour, Director of this Department, for permission to publish this article, and Mr. J. T. Holland for his valuable technical assistance.



(A) Quick's curve using acetone extracted Rabbit brain.
(B) Fullerton's curve using "Stypven" Russel Viper Venom.
(C) Hurn's curve using thromboplastin "A" from Rabbit brain.
(D) Curve obtained in this laboratory using Human brain.
(E) Curve showing slight prothrombin deficiency noted only on dilution.

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HYDATID DISEASE

L. B. FASTIER

(From the Hydatid Research Dept., Otago Medical School, Dunedin.)

Human hydatidosis is caused by infestation with the cystic or larval stage of *T. echinococcus*. This parasite was first accurately described by Goerze in 1782, and since that date numerous papers have been published relating to the life history, diagnosis and treatment. Since this article deals with the laboratory aspect of the subject, it will be assumed that the zoological and pathological facts are known.

Research on the experimental production of the disease in laboratory animals has been hampered by the fact that no completely susceptible animal exists. From the work carried out by Miss E. Batham, formerly of this Department, it is possible to reproduce cysts in mice by the intraperitoneal inoculation of scolices or ova provided that the reticulo-endothelium of the host is partially destroyed by splenectomy. On reviewing the results of such experiments it would seem that unless these were statistically analysed the relative number of animals which would have to be employed to obtain a significant figure for the percentage of "takes" would be enormous. Other common animals have been used, among these rats, guinea pigs and rabbits. It can be said that the second animal is entirely refractive to any surgical method for scolex implantation. The situation is complicated somewhat by the occurrence of cysts of *T. crassicollis* in the livers of normal laboratory rats, but once their appearance is recognised the danger of mistaking them for *T. echinococcus* is rare. It is of interest to note that the Casoni test in experimental animals is always negative, even though gross cysts may be found post-mortem.

From the clinical laboratory viewpoint the question of diagnosis is of more interest than the semi-academic research work briefly outlined above. The diagnosis of hydatid disease lies in both Radiological and Pathological Departments. Neglecting the former, we find the examination of a suspected case consists of: (a) Complete blood count and differential for eosinophilia (indicating generally a helminth infestation); (b) Casoni test; (c) Precipitin test; (d) Complement fixation test; (e) Examination of sputum for evidence of pulmonary hydatidosis. Included in this group may be the examination of aspiration material from pleural cavities, etc., but I refrain from allocating this a further classification letter on account of the very real danger of obtaining material in this manner owing to

hydatid fluid leakage from the puncture giving rise to an active sensitization of the patient usually resulting in acute anaphylactic shock.

The most unfortunate aspect of the disease is the usually symptomless condition until the growing cyst reaches a size as to cause discomfort by mechanical pressure on the surrounding organs. This usually manifests itself on non-specific clinical signs. Usually notice is first given to the patient by the appearance of some "queer tissue" in the sputum or by an inexplicable urticaria.

Of the examinations outlined above, the Casoni and complement fixation tests are by far the most specific, and, provided that adequate controls are employed, the latter is the test of preference. The successful performance of this test lies in the use of a satisfactory antigen, and as such several workers have advocated the replacement of pooled hydatid fluid by chemically purified "Hydatid Antigen." Thus Fairley (1922) recommends an alcoholic scolex extract; while Dennis (1937) has prepared a trichloracetic acid precipitable substance from cyst fluid. Anyone familiar with the variable scolex content of hydatid fluid will appreciate the technical difficulties attached to the preparation of the former antigen. It is in this connection that an interesting observation on hydatid antigen production may be made. Hydatid fluid rich in scolices makes an excellent antigen in the Casoni and complement fixation tests, those devoid of scolices are without such properties. Such a situation offers several hypotheses as to the origin of the reactive substance. It may be derived from the metabolic properties of the scolex or from the degeneration of the scolex itself. At the same time the germinative endothelium of the cyst may be involved. Embryogically speaking, no difference should be found between the chemical composition of either of these tissues, as the scolex is derived directly from the cystic endothelium by some unknown stimulus. Under these circumstances it would seem that the scolex is the major site of antigen production, and work has shown that in all probability a metabolic process is involved.

Briefly, the line which research work has been following in this Department has been the investigation of the chemical fractionation of the scolex, membrane and fluid with a view to the isolation of one or more active hydatid principles which could be used for standardised antigens in the serological reactions. To do this both complement fixation and sensitisation of guinea pigs with ultimate kymographic recording of the uterine contractility have been employed. The results derived from these examinations have shown that at least one active substance is polysaccharide in nature and can be isolated from all three components of the hydatid cyst.

The above review makes no pretext of covering even a fraction of the wide field of hydatid research; it is to give laboratory workers some idea of the technical difficulties attached to the study of a disease with which many are familiar only by reference to text books.

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MORPHOLOGICAL VARIATION IN SALMONELLA AERTRYCKE DUE TO THE EFFECT OF PENICILLIN

L. G. ECCERSALL.

(From the Pathological Laboratory, Public Hospital, Auckland.)

During a minor outbreak of meningitis in which *H. influenzæ* was found to be the causative organism in two cases, a cerebrospinal fluid was received from a clinical case of meningitis, the direct film of which showed pus and many Gram-negative bacilli. This was cultured on blood agar and cooked blood agar and incubated overnight. Large colonies were present on both plates, and subcultures were made into peptone water sugars. A further specimen of the fluid again showed pus and Gram-negative bacilli in direct film, but in contrast to the first specimen in which the organisms were regular in size and shape, large numbers of filaments were present, together with swollen and irregularly staining

forms. This pleomorphism was certainly suggestive of *H. influenzæ,* but the large colony formation and heavy growth of the organism on solid medium were not consistent with *H. influenzæ*. Subcultures showed acid and gas in glucose, mannite, dulcite and maltose; lactose and saccharose were not fermented, the organism was motile and agglutinated polyvalent *Salmonella* antiserum to full titre, and was finally identified as *S. aertrycke.*

The marked pleomorphism of the organism had no apparent explanation, until these findings were compared with those described by Fennel of the bizarre morphology of *B. ærogenes* due to the effect of penicillin, isolated from the urine of a leprous patient. The case under discussion had been treated with penicillin and it appeared that herein lay the explanation of the morphological variation of the infecting organism.

Accordingly glucose peptone water media was prepared in 10cc. amounts, with 1% of Andrade's indicator and penicillin added to give concentrations of 1, 2, 3, 4 and 5 units per cc. An emulsion of a single colony of the organism was made in broth, and one drop inoculated into each tube of glucose peptone water as well as into a control tube which contained no penicillin.

After eighteen hours' incubation all tubes showed growth and stained films were prepared. The control tube showed small, regularly staining Gram-negative bacilli, typical of any organism of the *Salmonella group*. The tube containing one unit of penicillin per cc. showed good growth and Gram-negative bacilli which appeared to be slightly thicker than normal, but no filaments were found. The tubes containing two and three units of penicillin per cc. showed moderate growths and large numbers of Gram-negative filaments. The tube containing four units of penicillin per cc. showed only light growth and large numbers of Gram-negative filaments. In the tube containing five units of penicillin per cc. a very light growth resulted, films showing many Gram-negative filaments and swollen forms varying in size, shape and staining. These were obviously degenerate forms.

Extending the results of the in vitro experiment, it is considered that the marked pleomorphism of the *S. aertrycke* in vivo was due to the effect of penicillin.

Fennel's article suggests that penicillin prevents the normal process of binary fission from taking place, and "it is easy to visualise a bacillus growing and growing, unable to shake off its mate, unable to divide, and simply becoming longer and longer and, at times, from over-nutrition, becoming very obese in the middle."

Summary

Salmonella aertrycke, the causative organism from a fatal case of meningitis, showing extreme morphological variation in

vitro and in vivo, due to the anti-bacterial effect of penicillin, has been described.

ACKNOWLEDGMENTS:

I wish to thank Dr. W. Gilmour, Director of Pathology Auckland Public Hospital, for permission to publish this article, and Mr. R. W. Litherland for the photomicrographs.

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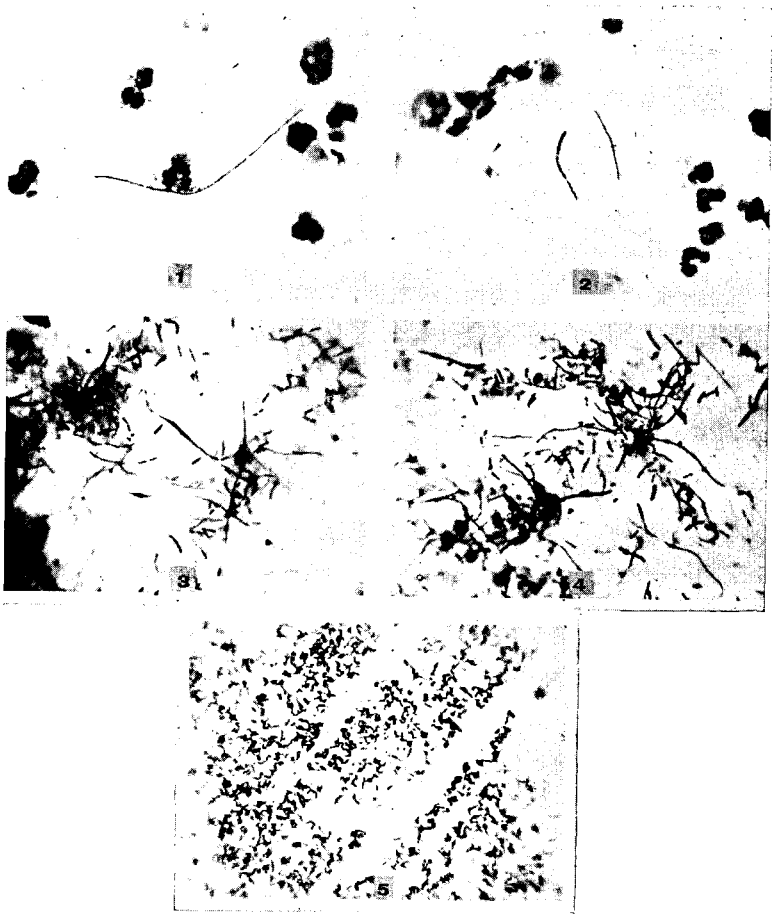

PHOTOMICROGRAPHS: X 570.

(1) Filaments in direct film
(2) Swollen filaments in direct film
(3) and (4) Filaments and irregularly staining forms from the culture in glucose peptone water containing 5 units of penicillin per cc.
(5) Film from the control culture in glucose peptone water containing no penicillin

THE ESTIMATION OF PROTEIN IN CEREBROSPINAL FLUID

(J. B. BROWN and I. M. COLE)

(From the Pathological Laboratory, Public Hospital, Auckland.)

The estimation of cerebrospinal fluid protein has become of increasing importance in this laboratory in the last few years, as shown by the number of estimations performed. Whereas in 1940 there were 91 estimations of protein in these fluids, in 1945 the number had risen to 1046, and already in the first quarter of this year 373 estimations have been performed, and it is now a routine to estimate the protein on all cerebrospinal fluids sent to the laboratory.

The basic method of estimation has been that of Ayer, Dailey and Fremont-Smith (1931). Till the beginning of 1945 this was used without modification, but while the estimation is a simple one, the accurate preparation of the standard is tedious and, even though stored on ice and preserved by the addition of thymol, has been found to be unstable.

On acquiring a Coleman No. 11 spectrophotometer, the method was adapted and a standard curve constructed from a series of accurate standards prepared in accordance with the original instructions. Two separate normal sera were each diluted to approximately 100 and 50 mgm. of protein per 100 ccs. The nitrogen content of these diluted sera was estimated in duplicate by the micro Kjeldahl method, using a titration technique and the protein calculated by subtracting the non-protein nitrogen estimated by the same method and multiplying by the factor 6.25. These known concentrations were further diluted so that two series of protein concentrations ranging from 10 to 100 mgms per 100 ccs. were obtained. These standards were then precipitated in the ordinary way and the density-concentration curve constructed, using the spectrophotometer at 700mu. where it was found that changes in particle size such as occur on standing produced no effect on the density curve. This curve obeyed the Beer Law of proportionality of density with concentration, and since then has been used in all estimations of protein in spinal fluid.

As this method was not suitable for small laboratories, a search was made for a satisfactory, stable standard capable of being used with an ordinary colorimeter. King and Haslewood (1936) described a formazine suspension in gelatin as a solid standard for the estimation of protein. This method was fully investigated, but failed to give the desired accuracy. However, when formazine was investigated as an aqueous suspension excel-

lent correlation was obtained between estimations of protein with the spectrophotometer, and with the colorimeter using the formazine standard.

Preparation of Standard

25 ccs. of 10% aqueous hexamine (B.P.) were mixed with 25 ccs. of 1% aqueous hydrazine sulphate (A.R.) and stood at room temperature for 15 hours. The precipitate of formazine obtained was mixed carefully by gentle shaking until the precipitate was evenly dispersed throughout. This was the stock solution. To make the working standard, 13 ccs. were diluted with 200 ccs. of distilled water, the resulting fluid being an artificial standard equivalent to 50 mgms. of protein per 100 ccs. This standard was well dispersed, formed a turbidity rather than a precipitate, settled slowly and could be immediately prepared for use by a gentle shake.

Method of Use

To 1 cc. of C.S.F. was added 1 cc. of distilled water and 2 cc. of an aqueous 5% sulphosalicyclic acid solution, and the mixture allowed to stand for five minutes. It was then mixed gently and compared in the colorimeter, the comparison being that of density. The concentration of protein in the unknown was then calculated by the following formula.

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{50}{1} \quad \text{mgms.}$$

In cases of C.S.F. with high protein content, smaller amounts of fluid were used, made up to 2 ccs. with distilled water and 2 ccs. of 5% sulphosalicyclic acid added. The results were then multiplied by the appropriate dilution factor.

Discussion

An excellent density match was obtained in all cases, there being no difference in the spectral transmission curves as shown by an analysis of both the artificial standard turbidities and that of a true protein by the spectrophotometer.

Approximately 50 protein estimations have been carried out as a routine procedure firstly by the spectrophotometric method and then by the method described with no special precautions and completing the readings before calculating results. The results of 10 consecutive and typical readings are given here, the first figure being the spectrophotometer reading and the second the colorimeter reading: 41/40, 105/110, 160/150, 34/30, 74/70, 22/20, 134/135, 34/38, 26/30, 31/30.

This artificial standard was found to be stable and has antiseptic properties. After a trial period of three months the stock

standard was unchanged in all respects. The diluted standard was checked over a period of two months at frequent intervals by means of the spectrophotometer and no changes were observed.

Summary

A satisfactory artificial standard is described for the estimation of the protein content of C.S.F. and allied fluids, which is easy to make and appears to be stable and remain free from contamination even when kept at room temperature.

ACKNOWLEDGMENTS:

We wish to thank Dr. W. Gilmour, Director of this Department, for permission to publish this article.

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Ayer, J. B., Dailey, M. E., and Fremont-Smith, F. (1931). Arch. Neurol. and Psychiat. 25, 1033.

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THE USE OF FLUORESCENCE MICROSCOPY IN THE EXAMINATION OF SPUTUM FOR M. TUBERCULOSIS—A PRELIMINARY REPORT

(I. W. SAUNDERS)

(From the Bacteriological Laboratory, New Plymouth Hospital.)

Since the beginning of April, 1946, I have examined 220 films of sputum by the method of fluorescent microscopy using the method of H. Lempert (Lancet, 1944,2,818) in parallel with duplicate films examined by Mr. G. Meads and stained by the normal Ziehl-Neelsen method. The search times were respectively three and five minutes.

Of these films, each method obtained 21 positives out of a total of 22 by both methods, the two negative films being still negative after long search, but the patient in each case was suffering from pulmonary tuberculosis.

Objects other than tubercle bacilli were found to fluoresce, but these did not cause any difficulty owing to their shape differing from that of the bacilli.

Advantages of the method appear to be in the greater speed of examination and the ability to cover a large area in a short time, while the disadvantages are inability exactly to check morphology as with the classical method, the cost of the apparatus (about £10) and the tedium of looking at a practically black field, there being but a few shapeless fluorescent particles in negative specimens.

CONFERENCE OF THE NEW ZEALAND ASSOCIATION OF BACTERIOLOGISTS, 1946

The Second Annual Conference of the New Zealand Association of Bacteriologists was held in Palmerston North on August 3 and 4, 1946.

The following delegates were present:—Mr. H. L. Haden (*Whangarei*), Miss W. Corsbie and Messrs. D. Whillans, A. M. Murphy and I. M. Cole (*Auckland*), Misses J. H. MacDiarmid, K. Riley and Mr. M. N. Keenan (*Hamilton*), Miss M. Corrin (*Rotorua*), Mr. W. Carruthers (*Gisborne*), Messrs. I. W. Saunders and M. O. Ekdahl (*New Plymouth*), Miss M. R. Harrow and Mr. E. L. F. Buxton (*Wanganui*), Miss I. Munro and Mr. G. W. McKinley (*Waipukurau*), Misses S. Kirkland and C. Anderson and Messrs. S. O. Jarratt and G. R. George (*Palmerston North*), Mrs. L. S. Moroney (*Hastings*), Messrs. J. Pierard, N. J. Ellison and W. N. Nuttall (*Wellington*), Mr. J. J. G. Peddie (*Wallaceville*), Mr. V. J. Hawke (Nelson), Messrs. D. H. Adamson, H. E. Foster and W. B. Gibson (*Christchurch*), and Mr. J. H. A. Ward (*Timaru*).

Mr. Nash, chairman of the Palmerston North Hospital Board, opened the Conference in the new Nurses' Tutorial Block and spoke warmly commending the work of the Association to date. He mentioned especially the Journal of the Association and wished the Association every success.

Mr. E. L. F. Buxton, President of the Association, thanked Mr. Nash on behalf of the Association for the hospitality shown by the Palmerston North Hospital Board, and spoke of the activities of the Association since the first annual meeting. He mentioned the incorporation of the Association and the recognition of the Association by the Pathologists, the Hospital Boards' Association and the Department of Health as some of the more important events of the year. He was also pleased to note that the membership of the Association now stood at 84.

The election of the Council for the year resulted as follows:—*President,* Mr. E. L. F. Buxton; *Vice-Presidents,* Messrs. J. J. G. Peddie and N. J. Ellison; *Secretary-Treasurer,* Mr. S. O. Jarratt; *Members of Council,* Messrs. D. H. Adamson, G. W. McKinley and D. Whillans; *Editor,* Mr. D. Whillans.

The Conference then elected the following honorary members of the Association:—The Director-General of Health, Professors C. E. Hercus and E. F. D'Ath, Drs. W. Gilmour and E. F. Fowler (*Auckland*), T. H. Pullar (*Palmerston North*),

P. P. Lynch and J. O. Mercer (*Wellington*), A. B. Pearson (*Christchurch*), M. Fitchett (*Hamilton*), D. J. A. Doyle (*Napier*), K. Uttley (*Timaru*) and D. Allen (*Invercargill*).

After lunch provided by the Hospital Board at the Cafeteria, the general business of the Association was brought forward.

(1) Salary scales for members not covered by the present scale. After considerable discussion the following committee was elected to receive letters on the subject and report to the next Council meeting:—Messrs. D. H. Adamson (convener) and Messrs. J. H. A. Ward and V. J. Hawke.

(2) Some exception was taken to the wording of the Schedule for the Department of Health's Certificate, and the following committee was set up to receive letters on the subject:—Mr. N. J. Ellison (convenor) and Messrs. J. Pierard, J. J. G. Peddie, with Mr. D. Whillans as corresponding member.

(3) The Editor spoke of the policy of the JOURNAL and the steps leading up to the purchase of the printing press and accessories. He also spoke on the Constitution as printed and the Association's Certificate of Membership.

(4) Mr. G. W. McKinley spoke on behalf of Mr. E. L. F. Buxton on the necessity of a preliminary examination to be held at the end of three years and to be run by the Health Department. In view of the wide divergence of opinion of what was desirable and necessary before approaching the Health Department, a further committee to receive letters was set up. This was Mr. I. W. Saunders (convenor), with Messrs. M. O. Ekdahl, G. R. George, V. J. Hawke, G. W. McKinley, H. E. Foster and J. H. A. Ward as corresponding members.

After a number of matters of a routine nature were covered, the Conference adjourned till 7.30 p.m., when Mr. Hurran, of Glaxo, Ltd., gave a most informative talk on the production of penicillin in England during the war. This was followed by a paper by Mr. G. W. McKinley on laboratory methods in the investigation of tuberculosis and a short talk on the practical points in the isolation and identification of tubercle bacilli by Mr. D. Whillans. The meeting closed at 10 p.m. after a delightful supper.

On Sunday a tour conducted by Mr. Barrett, Publicity Officer, was made of Massey Agricultural College. Dr. Whitehead, of the Dairy Research Institute, spoke to members on the work of cheese starters and 'phage troubles, and the subject proved to be of considerable interest to members as shown by the number of questions asked.

The afternoon was spent by Council members in a series of Committee meetings, and it was decided to accept the invitation of Christchurch to hold the next Conference there in August, 1947.

A SIMPLE NON-RETURN VALVE

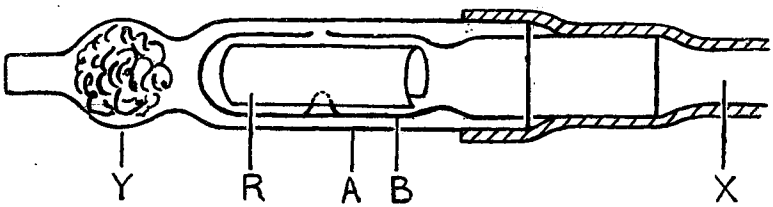
(P. H. CURTIS)

(From the Pathological Laboratory, Public Hospital, Auckland.)

Used extensively in the Middle East as an airway and forced pressure valve on all transfusion sets, this piece of apparatus has proved efficient either for exerting pressure or creating suction. The model described below is a modification which makes the rubber flap self-positioning.

Construction

Take a piece of standard glass tubing of 7mm. bore, 7cms. in length, close one end and blow a hole of diameter 1mm. through the tube 1cm. from the closed end and diametrically opposite this hole indent a small pip. Now form a shallow waist around the tube 2cm. from the closed end. (See diagram.)



Next take a rectangular piece of thin rubber sheeting (R) 1.75cm. long and 1.5cm. wide (a piece of surgical glove is suitable for this purpose), cover with French chalk and insert into the tube lightly rolled, sliding it past the waist to the bottom of the tube so that the blown hole is completely covered and there is an edge of the rubber sheet on each side of the pip.

Now take a piece of glass tubing (A) 6cm. long, open at both ends, and of sufficient bore to leave a clearance of 1mm. between its inner wall and the outside of the smaller tube (B). Near one end form a bulb into which a plug of cotton wool is inserted to act as a filter.

The open end of B is then inserted into the end of a firmly-fitting piece of rubber tubing to a distance of 3.5cm. and the tubing reflected on the glass for about 2cm. The open end of A is then slid over the closed end of B till it touches the reflected rubber, which is then rolled back over the open end of A to make an airtight joint. Reference to the diagram will make this clear. The advantage of this type of joint is that it is easily dismantled for cleaning and sterilising.

For pressure attach a Higginson's syringe at Y and connect the lead at X ot the pressure container, while **for suction** the Higginson's syringe is attached to the rubber tube from X and Y is attached to the container in which a suction is to be produced.

Principle

When at rest, air may pass through the valve both ways without restriction, but when the pressure of the air in B is greater than that in A, the resultant thrust on the rubber flap closes the valve. (Note that if the valve is being used to produce pressure, all bungs, etc., on which pressure will be exerted must be fixed firmly into their containers.)

Summary

In cases of forced blood transfusion, this valve has proved most effective, and if operated in the reverse way will maintain a steady suction for the bleeding of donors. It will also provide the necessary continuous suction when placed between the suction bottle and the filtrate container of a Seitz apparatus.

ACKNOWLEDGMENTS:

I wish to thank Dr. W. Gilmour, Director of this Department, for permission to publish this article, and Messrs. D. Whillans and J. B. Brown for their valuable suggestions and assistance.

HERE AND THERE

Small additions consisting of a laboratory for the Bacteriologist, an office, and a ladies' cloak room have just been completed at the Bacteriological Laboratory, Wanganui Hospital.

The Editor would appreciate comments on the JOURNAL as well as pars, suggestions, etc. He reminds contributors that owing to the holiday season the deadline for contributions to the JANUARY issue will be November 16th, 1946. Material sent before this will be very welcome.

Mr. J. B. Brown, M.Sc., Biochemist at the Pathological Department, Auckland Hospital, recently delivered a paper on "An Application of Spectrophotometry to Complex Ions" at the combined Annual Conference of the N.Z. Institute of Chemistry and the Royal Institute of Chemistry, at Wellington.

Mr. N. J. Ellison is anxious to gather interesting material for practical demonstration at the next Annual Conference of the Association. Material should be sent to him C/o Dr. P. F. Lynch, Kelvin Chambers, The Terrace, Wellington.

This quarter's great thought: While Link was responsible for elucidating the chemistry of Dicoumarin, it was Chain who worked out the extraction and purification of Penicillin.

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★ Printed 12,000 pages,
★ and all Association stationery.

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