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THE

TECHNIQUE OF ABDERHALDEN'S SERUM REACTION

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THE TECHNIQUE OF ABDERHALDEN'S SERUM REACTION.

In November of last year a series of experiments were commenced with the object of determining the value of Abderhalden's serum reaction—dialysis method—in the diagnosis of cancer. The results obtained by following exactly Abderhalden's technique were inconclusive, as the examples given later show. The principles of the reaction have been stated and the chief details of the dialysis method described so often that it is unnecessary here to do more tham emphasise certain points in so far as they bear upon the modifications in the method to be suggested in this paper.

Ninhydrin, the reagent used to test for the decomposition products of proteins, reacts with numerous other substances, as was shown by Neuberg.[1] Dextrose is the most important of these substances owing to its presence in blood serum. When 0·2 c.c. of a 1 per cent. solution of ninhydrin is boiled for a minute with a dilute solution of glucose in distilled water a transitory red colour is obtained. If normal saline be substituted for the distilled water the colour obtained is more or less permanent. The same reaction takes place with other sugars—e.g., galactose, maltose, &c. This formation of colour with sugars probably explains the various reddish shades of purple often obtained in testing dialysates in experiments with blood serum. Abderhalden's explanation[2] of these colours is that either the experiment has been carelessly carried out or the blood contained acids or alkalies.

[1] Biochemische Zeitschrift, 56, 500, 1913.
[2] Abwehrfermente, fourth edition, p. 275.

In carrying out a test 0·2 c.c. of ninhydrin solution is added to 10 c.c. of a solution of albumin, peptone, or amino-acids, and the mixture boiled for one minute. In order to secure regular boiling and to prevent "bumping" Abderhalden recommends "boiling sticks" made of wood and specially prepared; the small pieces of unglazed porcelain or broken glass ordinarily used by chemists are equally efficient. An extraordinary stress has been laid on "boiling for exactly one minute." Whilst it is obviously necessary to boil the tubes an equal length of time—and experience has shown that one minute's boiling is sufficient—the importance attached to this rule naturally suggests that a method by which the tubes are heated together would be preferable to that of boiling each tube separately in the Bunsen flame. Such a method has been adopted in the apparatus devised for the purpose by Abderhalden,[3] who nevertheless uses it only for testing tubes in preliminary experiments with dialysing sacs and not for actual serum experiments. A simple and satisfactory method for solutions of leucin is to place the test-tubes containing the dialysates to be tested in boiling water and to remove them again in nine minutes. In order to reduce the amount of evaporation the tubes may be lightly corked after they have been two minutes in the bath.

In comparing the results of the ninhydrin tests it is sometimes difficult to judge small differences of tint when the colours are compared in ordinary test-tubes. This difficulty has been overcome by the use of tubes 23 cm. long by 9 mm. bore, made of colourless glass. The boiling with ninhydrin is carried out in a test-tube, and the liquid is then poured through a small funnel into one of the long narrow tubes. A comparison of colour is made by holding the tubes on a sheet of white paper in a good light and looking directly on the column of liquid. Small differences are easily detected in this way. These tubes are also well adapted for a comparatively rough but very useful method of obtaining an idea of the quantitative differences in colour. 1 c.c.

[3] Münchener Medizinische Wochenschrift, No. 5, p. 233, 1914; and Abwehrfermente, fourth edition, p. 232.

of the liquid with the weakest reaction to ninhydrin is placed in one of the long tubes and distilled water added until only the faintest violet colour is visible when the column of fluid is looked at through the open end of the tube. As a rule—at least when 0·5 c.c. of blood serum is used as the minimum dose in an experiment—it is not necessary to add any water at all. This tube is now the standard colour and volume. A cubic centimetre of liquid is taken from each of the ninhydrin tests and diluted until a standard volume is of a standard colour. The amount of water added is a measure of the depth of colour.

Examples of Results obtained by using Abderhalden's Technique.

1. Sera from rats bearing a tumour (rat tumour 9) and from normal rats were obtained after starving the animals for 18 hours. The sera were clear and free of hæmoglobin and were used immediately.

Serum.	Substrate.	Ninhydrin test.	Biuret test.
1·5 c.c. normal.	1 g. (approximately) rat tumour 9.	+	–
1·5 c.c. tumour.	,, ,,	+	–

2. The experiment was repeated.

Serum.	Substrate.	Ninhydrin test.	Biuret test.
1·5 c.c. tumour.	Rat tumour 9.	++	–
1·5 c.c. ,,	,,	+++	–
1.5 c.c. normal.	,,	++	–
1.5 c.c. tumour.	Nil.	–	–
1·5 c.c. normal.	,,	+	–

3. Rabbit placenta was prepared as substrate; a pregnant rabbit and a buck were starved for 18 hours and then bled

from the ear vein. The sera obtained were clear and free of hæmoglobin.

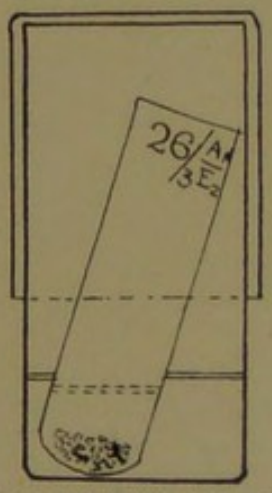
Serum.	Substrate.	Ninhydrin test.	Sulphosalicylic acid test.
0·5 c.c. from doe.	Rabbit placenta.	–	–
1·0 c.c. ,,	,,	–	–
1·5 c.c. ,,	,,	++	–
2·0 c.c. ,,	,,	(+)	–
0·5 c.c. ,,	Nil.	–	–
1·0 c.c. ,,	,,	(+)	–
1·5 c.c. ,,	,,	+	–
2·0 c.c. ,,	,,	+	–
0·5 c.c. from buck.	Rabbit placenta.	–	–
1·0 c.c. ,,	,,	–	–
1·5 c.c. ,,	,,	++	–
2·0 c.c. ,,	,,	+++	–

There was not sufficient serum from the buck to set up the control of serum alone.

It is obviously impossible to draw any conclusion from such results. The most striking point is the irregularity of the depth of the ninhydrin test of the dialysates. In the last experiment quoted, for example, the dialysate from the test, 2 c.c. of serum from pregnant rabbit plus rabbit placenta, contains less ninhydrin-reacting substances than the dialysate of the preceding test with 1·5 c.c. of serum. Abderhalden's explanation of such results[4] is that the digestion of the substrate has proceeded further (more amino-acids produced) in the test in which 1·5 c.c. of serum was employed than in the other case. The explanation is not satisfactory, however, since the conditions under which the tests were made were similar. The following experiment shows that the dialysing tubes were the source of error. Forty of Schleicher and Schüll's dialysis tubes, No. 579 A, 16 mm. by 50 mm., were well

[4] Abwehrfermente, English translation, pp. 200-201.

soaked in water and then tested with 2·5 c.c. of a 5 per cent. solution of egg-white. The tubes which permitted egg-white to pass were discarded; the remaining impermeable tubes were well washed with water, sterilised in the autoclave, and then tested with 2·5 c.c. of a 5 per cent. solution of Witte's peptone. The sacs were separated into groups according to the depth of the ninhydrin reaction. In my experience with about 100 of these sacs the largest group of equal permeability out of 40 consists of 5 or 6. The largest group of sacs thus obtained were well washed and sterilised in the autoclave and again tested with peptone; the ninhydrin reactions were strikingly unequal.



Elongated Petri dish with dialysing sac *in situ*.

This alteration in the permeability with sterilisation renders the sacs practically useless, and it was therefore decided to attempt to prepare dialysing tubes of collodion. At the same time the Erlenmeyer flasks recommended by Abderhalden were replaced by elongated Petri dishes of suitable size. (See Figure.) For the dialysing sacs described below the inner cylinder measures 4 cm. by 8 cm., the outer 4·5 cm. by 5 cm. These cylinders are washed separately, put together, and wrapped in paper, and then sterilised in hot air.

Preparation of dialysing sacs.—By numerous experiments with solutions of varying concentrations of pyroxylinum in alcohol and ether of varying proportions it was found that sacs of almost any degree of permeability could be prepared. For the purposes of the Abderhalden reaction the following concentration of collodion is required: gun cotton, 2·6; absolute alcohol, 33 c.c.; and ether, 67 c.c. The gun cotton is dried to constant weight by exposure to air; the alcohol is poured over it, contained in a stoppered bottle. After 24 hours the ether is added, and the clear solution obtained is ready for use.

Schleicher and Schüll's extraction thimbles, No. 603, 22 mm. by 80 mm., are taken and shortened by a centimetre. A number and the number of times the thimble is to be immersed in collodion and the relative proportions of ether and alcohol are written with India ink on each tube, thus, for sac No. 26:— $26/_3\frac{A_1.}{E_2}$ By this means a record of the behaviour of each sac is easily kept.

The sacs are now boiled in a mixture of equal parts of alcohol and ether in order to displace and disperse the air from the interstices of the paper. They are then filled and covered with collodion of the composition given above. After the lapse of an hour the tubes are taken out of the collodion, allowed to become almost dry, and then replaced for ten minutes. This is repeated, and the tubes when almost dry are placed in distilled water. By these processes the paper of each thimble becomes impregnated with collodion, and six layers (three inside and three outside) of the same material are formed.

It is important to remember that the concentration of the collodion is rapidly altered by evaporation of ether, and to a less extent of alcohol. The collodion should therefore be freshly prepared and a fair number of sacs (say 16) made at one and the same time. This is easily done if sufficient collodion is prepared to make 25. The thimbles are boiled in the alcohol-ether mixture contained in a saucepan; the fluid is poured off, and the thimbles are then covered with the collodion. The lid of the saucepan is fixed tightly to reduce evaporation.

The freshly made sacs are quite impermeable to solutions of egg-white, and are easily permeable to a 2 per cent. solution of Witte's peptone or to silk-peptone of a much lower concentration. After being used several times—and consequently sterilised several times—the collodion tubes become more permeable. It is now possible to distinguish between minute amounts of Witte's peptone, as the following experiment illustrates, and at the same time the sacs are impermeable to albumin.

The delicacy of ninhydrin as a reagent for Witte's peptone is rather better than 1 in 1000—i.e., 0·01 grm. in 10 c.c. To 20 c.c. of water

0·22 grm. of Witte's peptone was added and 2 c.c. of the solution placed in each of half a dozen collodion sacs and the sacs dialysed for 18 hours against 20 c.c. of distilled water; the dialysates give a faint and equal reaction with ninhydrin. 0·26 grm. of Witte's peptone was dissolved in 20 c.c. of water, and in each of six collodion sacs 2 c.c. of the solution was placed and dialysed for 18 hours against 20 c.c. of water. The dialysates were tested with ninhydrin and gave an equal result of a distinctly deeper tint than the dialysates from the first set of tubes. It is thus possible to distinguish between 0·02 grm. and 0·024 grm. (approximately) each contained in 2 c.c. of water. In other words, the degree of permeability of the collodion sacs to peptone corresponds at least to the delicacy of the ninhydrin reaction with the same substance. And since the substances to be detected in an actual experiment with blood serum are in my experience invariably amino-acids, the delicacy of these sacs is even greater than the experiments above indicate.

When the sacs have been in use for some time (six to eight weeks) and have been sterilised in the autoclave about 20 times they become permeable to albumin, and are then useless for the purposes of serum tests. There is thus a gradual increase in permeability, and it is only during the middle part of the "life" of a sac that the requirements of the Abderhalden reaction are complied with ideally—viz., (*a*) impermeability to serum albumin and globulin; (*b*) easy permeability to Witte's peptone; and (*c*) unalterability with sterilisation. Since, however, in an actual experiment amino-acids are always tested for—peptones in my experience being never found in the dialysate—the early life of a sac is perfectly satisfactory. But it is obvious that in any given experiment the sacs employed should have been made together at the same time, and should have been autoclaved an equal number of times.

The results obtained with such tubes will be published in a subsequent paper, but it may be pointed out here that by the use of graduated doses of serum and by invariably testing the dialysate with sulpho-salicylic acid a constant record of the

permeability of the sacs is kept. Since sulphosalicylic acid detects 1 part of albumin in 40,000 of water, it is clear that any alteration in the behaviour of the sac towards albumin is indicated at the earliest possible moment and before quantities recognisable with ninhydrin can pass. And, further, by the use of graduated doses of serum any alteration in the permeability of the sacs to amino-acids would be indicated by discordancy in the results of the ninhydrin tests.

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