THE THREE-PHASE MODIFIED BENDIEN REACTION

BY

C. H. G. PRICE, M.D.

(From the Department of Pathology, Bristol General Hospital)

For many years clinicians have felt the urgent need for some simple but reliable laboratory test for the presence of active malignant disease. In other fields of medicine there has been very noticeable improvement in the results of treatment, this in many cases being due to the diagnosis of the morbid condition at an early stage which had not previously been possible, but which, with the introduction and recognition of some specific test, was placed upon a rational and scientific basis. This unfortunately has not been the case with malignant disease, and it must be admitted that a really satisfactory diagnostic laboratory method has yet to be elaborated to aid and supplement the experience of the skilled clinical observer. It is true that many have attempted to demonstrate changes in the blood of cancerous patients, and, from minor non-specific alterations from the normal, numerous tests have been evolved, accompanied by extravagant claims as to their value in diagnosis. There has, however, been a deplorable absence of confirmation of the results obtained with the several tests in the hands of all save the originators, whose enthusiasm may at times have gained the upper hand of their judgment.

One aid to diagnosis which has perhaps enjoyed a longer period of popularity than others has been the Bendien vanadic acid serum test. There is little doubt that in its original form it was merely a method of demonstrating the increase of serum protein which may occur with active progressive malignant disease. Boyland (1932a) concluded that it was based upon certain non-specific changes in serum that could also be demonstrated in other morbid states besides active cancer. The spectrographic examination which was performed in the original Bendien test has also been shown to be valueless (Smith *et al.*, 1931; Groeneveldt and Kromme, 1931), and it has long since ceased to be used.

Cronin Lowe (1933a, 1933b, 1934) introduced a modified form of the Bendien test for which he claimed great accuracy in diagnosis. This was his so-called "three-phase test"; in principle it consists of the quantitative comparison of the amounts of flocculation produced in a given specimen of serum which had been treated with a standard solution of vanadic acid, and the amount of flocculation obtained with the same standard after the serum had been subjected to ether extraction (Phase C) and also to heat inactivation at 56° C. for half an hour (Phase B). The originator of this test maintained that if the increase of flocculation produced in Phase C (as compared with the flocculation in the original untreated serum) was greater than the decrease of flocculation found in Phase B, then active malignancy was present in the individual from whom the specimen of blood was obtained. In other words, the quantitative estimation of the flocculated material gave, therefore, a fraction

$\frac{C-A}{A-B}$, which when greater than unity was said to be pathognomonic of active cancer. It was also claimed that a specimen of blood from a person in normal health or suffering from some benign disease would, with the three-phase test, yield a fraction less than unity. In the series of cases reported here the interpretation of the fraction was recorded as indefinite in a number of specimens where

the difference between the denominator and numerator of the fraction was less than three times the maximum error of the process used.

The claims made for this three-phase test were so great that if this work could be confirmed a most valuable weapon had been forged for the fight against cancer, and in view of the results of Jones and Woodhouse (1936), who obtained agreement of 75 per cent. between serological and clinical findings, it was decided to investigate a series of cases with this particular test. Practically all the 427 specimens examined in my series were obtained without any knowledge of the clinical condition of the patient at the time of performing the test, and it was only some days later, after the result of the test had been obtained, that details were available as to the diagnosis and treatment of the case. In this series, therefore, the test was performed with true impartiality, and the interpretation of the clinical state of the patient at the time of collecting the specimen of blood was entirely the work of another observer, whose notes were made at that time. The technique described by Cronin Lowe (1933a) was closely followed as regards the performance of the test itself; the only modification was in the method of estimating the amount of flocculation produced by the action of the vanadic acid standards upon the various specimens of serum. This modification, together with the results of a number of experiments on the mechanism of the test, is described below.

A small preliminary series of forty-seven specimens of serum were examined with the three-phase test, a nephelometer being used to estimate the amount of flocculation. This is Series A (Table I).

TABLE I.—Results in Series A (47 Specimens)

Disease	No. Examined	Positive	Negative	Indefinite
Active malignant . .	21	8 (38%)	12 (57%)	1 (5%)
Non-malignant . .	26	7 (27%)	19 (73%)	—

It will be seen that there was agreement between the results of the three-phase test and the clinical findings in twenty-seven specimens examined (58 per cent.), and in nineteen cases (40 per cent.) the result of the serum test was incorrect.

Following upon this a series of 398 specimens of serum were examined with the three-phase test, a photo-electric absorptometer being used for all estimations. This is Series B (Table II).

TABLE II.—Results in Series B (398 Specimens)

Disease	No. Examined	Positive	Negative	Indefinite
Group 1 : Active malignant . .	143	55%	37%	8%
Group 2 : Inactive malignant (clinical cures)	138	37%	57%	6%
Group 3 : Non-malignant . .	99	42%	53%	5%
Group 4 : Hodgkin's disease and leukaemia	18	50%	50%	—

Distribution of Positive and Negative Results

In the two series, therefore, 445 specimens of serum were examined. Owing to the controversial nature of the morbid process in the cases in Group 4, these were excluded when calculating the figures given below. The total number of specimens analysed is therefore reduced

to 427. A positive result was obtained with 185 specimens of serum. Of these, an active malignant process was present in ninety-four cases (51 per cent.). A negative result was obtained in 215 specimens: a non-malignant lesion was present in 148 of these (69 per cent.). An indefinite result was obtained with the other twenty-seven specimens of serum examined.

From the examination of this series of 427 specimens of serum tested the fact therefore emerges that there was agreement between serological results and the clinical picture in only 55.5 per cent. of them. For purposes of comparison the results obtained by other workers using this three-phase test are given in Table III.

TABLE III

	No. of Specimens	Percentage of Agreement
Cronin Lowe (1933a)	600 (about)	95
Allott (1933)	13	69
Patterson and Adler (1933)	59	60
Jones and Woodhouse (1936)	450	75
Nielsen (1936)	550	81.5
Oliver (1937)	276	56
Price (present series)	427	55.5

The majority of the specimens examined in Series B fall into four large groups—namely, rodent ulcer, epithelioma (of various sites), carcinoma of the breast, and non-malignant diseases. These are now considered as separate groups (Table IV).

TABLE IV.—Principal Groups occurring in Series B

	No. Examined	Positive	Negative	Indefinite
Group I (Rodent ulcer, 75) :				
Active	15	7 (47%)	8 (53%)	—
Inactive*	60	20 (33%)	37 (62%)	3 (5%)
Group II (Epithelioma, various sites, 98) :				
Active	54	29 (54%)	22 (41%)	3 (5%)
Inactive*	44	15 (34%)	26 (59%)	3 (7%)
Group III (Carcinoma of breast, 66) :				
Active	43	26 (60%)	14 (32%)	3 (8%)
Inactive*	23	12 (52%)	10 (43%)	1 (5%)
Group IV (Non-malignant diseases)	99	42 (42%)	52 (53%)	5 (5%)

* Inactive = clinical cures.

On consideration of the figures given above the following points emerge.

(a) The most favourable results were obtained in Group III (carcinoma of the breast), where in active cases 60 per cent. of the specimens tested gave a positive result with the three-phase test. When the total number of cases of active malignant disease is considered, amounting to 143 specimens of blood examined, this figure drops to 55 per cent.

(b) On examination of Group IV (non-malignant diseases), 42 per cent. of the specimens still yielded a positive result with the test. This is only 18 per cent. less than was found in Group III, where the most favourable results were produced.

(c) In the group of 138 specimens from cases of malignant disease that had been treated (Table II) and where no clinical activity of the neoplastic process could be demonstrated, the positive results obtained amounted to 37 per cent. It must be admitted that among the cases in this group there must be quite a number in which there would be some recurrence of activity of the growth within a short period, and in which

there was probably symptomless activity even at the time when the blood specimen was obtained. Therefore it would be expected that in this particular group a higher percentage of positive results would be obtained than in the non-malignant category. This, however, was not the case.

(d) On examination of the whole series from the point of view of sex it was found that of the positive group of results, 52 per cent. of the samples of blood were from female patients and 48 per cent. from males. In the group of specimens which yielded a negative result with the three-phase test, 49 per cent. of the sera were derived from females and 51 per cent. from males. In comparing these figures with those showing the association of active malignancy with a positive result of the three-phase test, it will be seen that the female sex is associated in slightly greater proportion with a positive result than is active malignant disease (52 per cent. to 51 per cent.). Since the sex of the patient was entirely a random factor and quite unselected, these figures suggest that the association of an active malignant process with a positive result to the three-phase test was also a matter of chance.

(e) It is true that over the total series of 427 specimens negativity of the three-phase test is to a higher degree associated with non-malignant conditions. (This was, in fact, nearly 70 per cent.) It must, however, be noted also that a greater proportion of negative results was obtained over the whole series examined (50 per cent. negative to 43 per cent. positive), and also that out of the total number of 427 specimens of serum tested, 263 (61 per cent.) were from cases of non-malignant disease (or clinically cured malignant conditions). The result of this would be a somewhat greater possibility for a negative result of the test to be associated with some innocent lesion.

The cases were also analysed from the point of view of the age of the patients from whom the blood specimens were taken. Here it was found that over the fourth to seventh decades, into which categories the majority of the cases fall, the proportion of negative results of the tests tends to equal that of positive results. In other words, there is a one in two chance that a specimen of serum can give a positive result with the test (or a negative result, excluding the small number of indefinite results). The figures in the following table suggest that the larger the number of specimens examined the nearer to equality will be the number of positive and negative results obtained.

TABLE V

Age	Positive	Indefinite	Negative	Total
0—10	1 (33%)	—	2 (67%)	3
11—20	8 (73%)	—	3 (27%)	11
21—30	5 (25%)	4	11 (55%)	20
31—40	15 (56%)	1	11 (41%)	27
41—50	32 (47%)	4	33 (48%)	69
51—60	41 (44%)	10	43 (46%)	94
61—70	43 (50%)	4	39 (45%)	86
71—80	19 (32%)	5	35 (59%)	59
81—90	1 (20%)	—	4 (80%)	5

The Test in Prognosis

In fifty-eight cases it was possible to obtain specimens of blood for examination on two or more occasions. In only eighteen (31 per cent.) of these was the test of any prognostic value. In a number of the patients examined it was found that several results might agree with the clinical condition and progress of treatment, and then be followed by a result that was obviously and entirely incorrect. In a number of the cases examined it was noticed that the tube in which "initial flocculation"

occurred would move to the left (that is, "initial flocculation" would take place with a more dilute vanadic acid standard) as the patient's state deteriorated. This is due to the increase of serum proteins which is usually but not invariably associated with active progressive malignancy, and is by no means specific for that condition.

A number of cases were also followed up for a period of six months after the last blood test had been performed. Information was available concerning twenty-six cases classed as "clinical cures." These patients had all been tested with the three-phase reaction, and had yielded uniformly positive results. Of these twenty-six cases, twenty-five (96 per cent.) had shown no recurrence of any activity, and in one there had actually been great improvement in the general clinical condition. During the time these cases were under observation not one had shown evidence of renewed activity. During this same period twenty-eight cases of active malignant disease were also investigated; these people had all given negative results with the three-phase test. Of the twenty-eight cases twenty had shown definite recurrence of malignant activity (71 per cent.); of these, nine patients had died directly as a result of malignant disease. Seven cases had actually shown improvement in the clinical condition (25 per cent.). One had remained stationary, with no obvious clinical change. In addition, thirty-three cases from the non-malignant group were also examined over the same period. These patients had all given positive results with the three-phase test. Not one of them had shown the development of any malignant process so far as could be determined by clinical examination. With regard to the group of "clinical cures" it was also noted that those cases giving a negative or non-malignant result with the three-phase test showed a higher incidence of recurrence of activity than did those cases from the same group which yielded a positive result.

Mechanism of the Three-phase Test

This initial flocculation is defined by the originator of the test as the first tube in which there appears, after standing for twenty-four hours, a deposit of 1 to 2 mm. of precipitated material produced by the interaction of the serum and vanadic acid standards. It can easily be shown that this phenomenon is closely connected with the serum colloids. In a protein-free filtrate prepared from serum no such flocculation can be demonstrated even on the addition of the most concentrated of the vanadic acid standards (No. 30). Again, in the case of cerebrospinal fluid, which contains the serum crystalloids in roughly the same concentration as occurs in serum (except of course the chlorides), but in which there is only a very small amount of colloidal material, no flocculation can be produced.

Boyland (1932b) has shown that the position and amount of flocculation produced with a given serum could be quantitatively modified by the addition of small amounts of globulin. He has also shown that the albumin/globulin ratio influences the amount of flocculation that may occur. These points were confirmed by several simple experiments. Boyland concludes that the amount of flocculation produced is governed by three factors: (1) the albumin/globulin ratio, (2) the total amount of protein present, and (3) the alkali reserve of the serum. In connexion with this third point it was found by experiment that the process of ether extraction (Phase C of the test) of a serum was accompanied by an alteration of the pH of that serum, which became more acid.

Adjustment of the reaction of a specimen of serum *in vitro* by the addition of dilute mineral acid was also found to be associated with an increase in the amount of flocculation produced in that serum, even though it was not subjected to ether extraction. Examination of specimens of serum which had been subjected to heat inactivation revealed that the reaction had become more alkaline, this being accompanied by a decrease in the amount of flocculation produced by the addition of any given vanadic acid standard (Phase B). This decrease of flocculation could also be produced by the alteration of the pH of the serum by the addition of weak alkali.

It was thought that the serum cholesterol might play some part in determining the actual amount of flocculation that was produced with any specimen of serum. In 398 samples of serum, therefore, in addition to performing the three-phase test, the level of the serum cholesterol was also estimated. It was not found possible to demonstrate any quantitative relation between the value obtained for the serum cholesterol and (a) the amount and position of the "initial flocculation," (b) the amount of flocculation in Phase B, and (c) the amount of flocculation in Phase C. One small point was, however, noted with regard to the serum lipoids. With a specimen of serum it was found possible to produce a curve (amount of flocculation plotted against time) showing the inactivation process which occurs during the period of heating at 56° C. A similar phenomenon could be demonstrated with a solution of serum proteins in normal saline. When, however, the proteins were rendered lipoid-free by washing thoroughly with ether, after separation and drying of the protein, the process of inactivation (as shown by decreased flocculation) could not be reproduced in a solution made from this material. It appears, therefore, that the serum lipoids are necessary for the production of inactivation by heat of a serum, as shown by this method, but further investigation did not disclose evidence of any quantitative relation between the amount of cholesterol present and the degree of flocculation of any serum with a given vanadic acid standard.

These results suggest that the alterations that occur in Phases B and C of the three-phase test are partly due to changes produced in the reaction of the serum when it is subjected to heat inactivation (Phase B) or to ether extraction (Phase C). Mere alteration of the pH of the serum, however, would not reproduce the range of alteration of the amount of flocculation produced by the two processes described—that is, Phases B and C—and it seems possible that the serum lipoids may be partly responsible, or that some qualitative change occurs in the serum colloids themselves. It should, however, be pointed out that the basic processes of the three-phase test do not serve as an index of any particular property of blood serum or serve to measure any specific constituent. The test as such is therefore entirely empirical. It can also be shown with many specimens of serum that if the result of the test is based upon the A, B, and C phases corresponding to the "tube of initial flocculation," a totally different result may be obtained by estimating the result on either the set of three tubes to the left or right of the point where "initial flocculation" occurs.

Technique

The method of performing the three-phase test is well described by Cronin Lowe (1933a). His technique was carefully followed, except in the actual method of quantitative estimation of the amount of the flocculated material produced in the test. The apparatus used for this purpose

was originally a Zeiss interferometer, an instrument of great precision, but very costly and of little value in routine pathological work. For reasons of economy, therefore, a Leitz nephelometer was chosen for estimating the flocculation produced in the test. Forty-seven specimens (Series A) were examined in this way, but control experiments with this instrument showed a large visual error, especially when estimating a depth of fluid greater than 12 mm. The nephelometric method of estimation was in consequence discarded, and a Hilger "Spekker" photo-electric absorptiometer was used for all the other estimations (Series B). This instrument was chosen for the following reasons: (1) its initial cost is relatively low, and it is capable of performing any type of colorimetric analysis; (2) there is no visual matching of colour fields, so that personal error is minimized; (3) its intrinsic error is low (± 1.25 per cent.); (4) over the range of turbidities examined the absorption curves of serum albumin and serum globulin are practically linear, as are also the curves of mixtures of these substances; and (5) the absorption curve of cholesterol was also found to be linear from zero to 500 mg. per 100 c.cm., and this instrument was successfully used for some 500 cholesterol estimations performed upon the present series of specimens: for this analysis the method described by Kamlet (1934) was employed, the error for this process being found to be ± 5 per cent.

The method that was used for the preparation of turbid fluids from the flocculated material obtained in the three-phase test is now given in detail. The technique described by Cronin Lowe (1933a) was religiously followed as far as the stage of estimation. The set of three tubes (one from each phase) showing an initial flocculation in Phase A (untreated serum) were centrifuged at 2,000 r.p.m. for five minutes. The supernatant fluid was aspirated with a fine suction pipette, and N/80 caustic soda was added up to the 2 c.cm. graduation. The packed deposit was then dissolved by gentle agitation. These tubes were allowed to stand for twenty-four hours, when it was found that the yellow colour due to traces of vanadic acid was almost completely discharged. This was of considerable importance when estimating visually in the Leitz nephelometer. To each tube was added 8 c.cm. of a standard solution containing 20 per cent. of salicyl-sulphonic acid and 20 per cent. of glucose. This mixture was slowly inverted three times to mix and allowed to stand for ten minutes; in the case of the Series A specimens these opaque fluids were estimated visually in the nephelometer against a standard glass turbidity wedge set at a depth of 10 mm.

In the case of the specimens in Series B the opaque fluids were estimated in the absorptiometer against a water standard set at 1.000. In each case the results were obtained in the scale units of the instrument used for the estimation, but since only relative units were necessary to compare Phases A, B, and C, it was not deemed of importance to convert these scale units into some absolute units such as grammes or milligrammes. The maximum error found in control experiments with the absorptiometer was ± 2.55 per cent., and there was the added advantage that distilled water could be used as the standard for comparison, being a colourless fluid.

Summary

Over a series of 427 specimens of blood examined with the three-phase modified Bendien test there was agreement of serological results with clinical findings in 55.5 per cent.

Evidence suggests that the larger the number of cases or specimens which are tested, the nearer to equality becomes the proportion of positive and negative results. In other words, the association of active malignancy with a positive result is a matter of chance.

Consequent upon this, and supported by subsequent history, the test is of no value whatever either in the diagnosis of malignant disease or in the control of treatment or prognosis in that condition. The test, in my hands, has shown itself to be entirely unreliable.

There is no quantitative relation between a positive result of the three-phase test on a sample of serum and the level of the cholesterol in that particular specimen. Neither was it possible to demonstrate any connexion between the serum cholesterol figure and the amount of flocculation in Phases B and C of the three-phase test.

A brief description is also included of some experiments performed regarding the mechanism of the three-phase test. Two separate methods of estimation of the amount of flocculation occurring in the test are also briefly discussed, together with the main reasons for their adoption.

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In view of the large and rapidly increasing amount of work on endocrinology it has been decided to establish a journal devoted to this subject called the *Journal of Endocrinology*. This periodical will be issued quarterly, and will publish communications "which advance knowledge concerning the internally secreting glands, the mode of their actions, the nature of their secretions, and the disorders of their functions." A committee formed for the promotion of the new journal has appointed Professor E. C. Dodds as editor, and Dr. R. L. Noble as assistant editor. An editorial board has been formed consisting of Dr. P. M. F. Bishop, Professor C. R. Harington, Dr. A. S. Parkes, Dr. F. G. Young, and Dr. S. Zuckerman. Papers intended for publication should be submitted to the Editors of the *Journal of Endocrinology*, Courtauld Institute of Biochemistry, Middlesex Hospital, Mortimer Street, London, W.1. The subscription rate for each volume of four issues will be 30s., and should be paid direct to the publishers of the new journal, the Oxford University Press, Amen House, Warwick Square, E.C.4.