COMPARISON OF THE ERYTHROCYTE SEDIMENTATION RATE, C-REACTIVE PROTEIN, SERUM DIPHENYLAMINE, AND TETRAMMONIUM TESTS IN RHEUMATIC FEVER AND RHEUMATIC HEART DISEASE

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The difficulty in assessing the activity of the disease process in rheumatic fever and rheumatic heart disease is apparent from the large number of laboratory tests which have been used from time to time. In a previous study (Eastham, Szekely, and Davison, 1958) we have attempted to assess the value and limitations of the C-reactive protein test as a measure of rheumatic activity in a group of unselected cases of rheumatic fever and rheumatic heart disease. In the present study, using the same clinical material, we have correlated four laboratory tests in the hope that it might be possible to demonstrate a distinct advantage of one test over the others.

In acute rheumatic fever, serum electrophoresis shows that the albumin content falls, while alpha-1, alpha-2 and gamma globulin concentrations rise (Dole, Watson, and Rothbard, 1945). Ernstone (1930) showed that plasma fibrinogen concentration rose and the haematocrit level fell. According to Jackson, Kelly, Smith, Wang, and Routh (1953), these changes are reflected in the rise in the erythrocyte sedimentation rate.

Roantree and Rantz (1955) showed that C-reactive protein occurred in the serum early in the course of acute inflammation. Coburn, Moore, and Haninger (1953) demonstrated that the serum diphenylamine reaction paralleled the erythrocyte sedimentation rate, and Jacox (1951) showed a similar correlation between the serum tetrammonium turbidity and the erythrocyte sedimentation rate in cases of rheumatic fever.

The last two tests were, therefore, selected for comparison with the erythrocyte sedimentation rate and the serum C-reactive protein in our series.

Methods and Material

I. The erythrocyte sedimentation rate (E.S.R.) was read at one hour in 200-mm. tubes of 2.5 mm. internal diameter (Westergren, 1921) using citrate solution as already described. Any reading of more than 10 mm. in one hour was regarded as abnormal.

II. The serum tetrammonium turbidity reaction (TET) was estimated by the method of Jacox (1951). The turbidity was read in 1 cm. cells in a twin-cell Spekker absorptiometer, using an Ilford spectrum violet filter No. 601. Cetyl-dimethyl-benzyl-ammonium chloride was used in place of octyl-dimethyl-benzyl-ammonium chloride, since it has an identical effect on serum protein (Jacox, 1953). The barium sulphate standard suspension (Jacox, 1951) gave an extinction value of 0.585. The upper limit for normal serum was taken as 0.295.

III. The serum diphenylamine reaction (DIP) was estimated by the method of Coburn and others (1953), using the sensitive diphenylamine reagent of Ayala, Moore, and Hess (1951). Correction for the serum blank was not made in view of the findings of Coburn, Bates, Hahn, and Murphy (1956). The reaction was read at 530 mμ in 1 cm. cells in a Unicam S.P. 600 spectrophotometer. The instrument readings were brought into line with those of Coburn and his co-workers. This was done with the help of Dr. E. L. Hess, Ph.D., who kindly supplied a suitable calibration graph; from this reference sucrose standards were prepared. The normal range of coefficient of extinction for our instrument was 0.246-0.379, with an average of 0.330 (this value corresponds to the colour given by a 0.106 g. per cent. solution of pure sucrose).

IV. A capillary qualitative test for the presence of serum C-reactive protein was made (Anderson and McCarty, 1950), using C-reactive protein antiserum—Schieffelin.

The four tests were performed on 362 blood samples taken from 191 patients. 182 cases, out of the 191 cases, on whom all four laboratory tests were performed, were divided clinically into the following groups:

- (1) *Active rheumatic carditis* (11 cases)
- (2) *Chronic valvular heart disease*. No certain evidence of rheumatic activity, but past or present cardiac failure (50 cases)
- (3) *Chronic valvular heart disease*. No certain evidence of rheumatic activity and no past or present cardiac failure (114 cases)
- (4) *Chorea* (7 cases)
- (5) *Non-rheumatic* (9 cases):

Congestive cardiac failure	..	7
Rheumatoid arthritis	..	1
Erythema nodosum	..	1
- (6) *Normal pregnant women*.—Sera from 48 cases were examined using three of the tests, but on these no erythrocyte sedimentation rate estimations were made.

In 23 cases, four or more serial blood samples were tested over a period of weeks.

Results

Fig. 1 shows the results obtained from one patient over a period of 360 days, and demonstrates similar trends in the tests used. When the serum diphenyl-

amine and tetrammonium reactions are compared as in Fig. 2 (opposite), only a very rough direct correlation is apparent.

None of the sera from the 48 pregnant women contained C-reactive protein; the range for the diphenylamine reaction with the sera was from 0.189 to 0.357 (mean 0.252); the corresponding range for the tetrammonium reaction was from 0.038 to 0.290 (mean 0.170).

When the serum diphenylamine reaction and erythrocyte sedimentation rate were compared no simple correlation was seen. Very similar results were obtained when the serum tetrammonium reaction and erythrocyte sedimentation rate were compared. Although the majority of sera gave negative results with the diphenylamine reaction and the tetrammonium reaction, this was certainly not the case with either the erythrocyte sedimentation rate or the C-reactive protein (Table I, opposite).

Fig. 3 (overleaf) shows the haemoglobin concentration, corresponding erythrocyte sedimentation rate, and presence or absence of C-reactive protein in each of the 362 blood samples tested.

The numbers of positive and negative C-reactive proteins at various erythrocyte sedimentation levels are shown in Table II (overleaf).

Fig. 1.—Serial readings of erythrocyte sedimentation rate, C-reactive protein, tetrammonium, and diphenylamine reactions in a case of active rheumatic carditis over a period of 360 days.

TABLE I

Group	Erythrocyte Sedimentation Rate		C-reactive Protein		Diphenylamine Reaction		Tetrammonium Reaction		Total	Pregnancy
	Negative	Positive	Negative	Positive	Negative	Positive	Negative	Positive		
(1) Active Carditis ..	0	11	0	11	8	3	7	4	11	Non-pregnant
(2) Chronic Valvular Disease ..	15	28	13	30	39	4	41	2	43	Non-pregnant
Cardiac Failure at Some Time in Past or Present	0	7	3	4	7	0	7	0	7	Pregnant
(3) Chronic Valvular Disease ..	52	42	87	7	94	0	94	0	94	Non-pregnant
No Cardiac Failure at Any Time	3	17	17	3	20	0	20	0	20	Pregnant
(4) Chorea	3	4	7	0	6	1	7	0	7	Non-pregnant
(5) Non-Rheumatic Cases	4	5	3	6	8	1	8	1	9	Non-pregnant

Discussion

The diphenylamine reaction apparently depends on sialic acid, which is derived from serum mucoprotein (Ayala and others, 1951; Coburn and others, 1956; Hess, Hahn, and Ayala, 1956; Werner and

Odin, 1952), and mucoprotein is mainly associated with the serum alpha globulin fraction (Linko and Waris, 1955). The diphenylamine reaction should, therefore, reflect mainly changes in alpha globulin, and more particularly in the alpha-1 fraction,

Fig. 3.—Erythrocyte sedimentation rate readings plotted against corresponding haemoglobin concentrations in 362 blood samples.
x = Positive C-reactive Protein. O = Negative C-reactive Protein

TABLE II

C-Reactive Protein	Erythrocyte Sedimentation Rate (mm./hr)					Total Sera
	0-15	16-30	31-45	46-60	61 Upwards	
Positive	38	30	16	17	37	138
Negative	128	58	21	15	2	224

although the protein-bound polysaccharide present in the gamma globulin fraction must have an effect.

Coburn and others (1953) found that the diphenylamine reaction correlated well with the erythrocyte sedimentation rate in cases of rheumatic fever, and subsequently claimed that it was a more useful test than either the erythrocyte sedimentation rate or the C-reactive protein (Coburn and others, 1956).

The tetrammonium reaction of Jacox (1951) depends almost entirely on the alpha globulin concentration in the serum (Saifer and Zymaris, 1956). It was found by Jacox and Gale (1951) that it could be used as a non-specific test of "acute phase serum", correlating well with the erythrocyte sedimentation rate and the serum bacteriocidal activity on *B. subtilis* in cases of acute rheumatic fever.

Serial readings made in 23 cases showed good correlation between the four tests. In Fig. 1 the results obtained for one patient over nearly one year are shown. The patterns followed by the tests are similar, but, while the serum C-reactive protein is frequently positive and the erythrocyte sedimentation rate is abnormal, the diphenylamine and tetrammonium reactions are only abnormal during one period. Since the diphenylamine and tetrammonium reactions appear to follow similar courses in serial readings on one case, it seemed possible that the two tests would correlate directly in a series of random readings. This should apply particularly in the early stages of rheumatic activity since the alpha globulin increases before the

gamma globulin fraction and the tetrammonium reaction is known to depend almost entirely on the alpha globulin, whilst the diphenylamine reaction is influenced by changes in both alpha and gamma globulin.

Fig. 2 shows that direct correlation may be considered to be present, but that the degree of relationship is poor. Only three sera with negative C-reactive protein tests were outside the normal limits for the other two tests.

Although Yocum and Doerner (1957) claim that serum C-reactive protein migrates with the beta globulin fraction, and Hedlund and Brattsten (1955) are of the opinion that it migrates with gamma globulin, it is more probable that C-reactive protein, on electrophoresis, runs with the alpha-1 globulin fraction (Hedlund, 1947; Perlman, Bullowa, and Goodkind, 1943; Shackman, Heffer, and Kroop, 1954). Wood and McCarty (1951) found that the serum test was positive when 1 mg. C-reactive protein per 100 ml. serum was present. In acute cases of rheumatic fever, the level could rise to more than 33 mg. per 100 ml. This amount is still only a fraction of the total alpha globulin.

Fearnley, Pirkis, de Coek, Lackner, and Meanock

(1955) used the diphenylamine reaction in the assessment of activity in rheumatoid arthritis, and they found a wide overlap of diphenylamine readings when normal controls were compared with active cases. They also found that the diphenylamine reaction was not abnormal in any of the active cases in which the erythrocyte sedimentation rate was normal. While the diphenylamine reaction follows the pattern of the erythrocyte sedimentation rate in any one case of acute rheumatism (Fig. 1), one reading alone cannot be used with any degree of certainty to detect activity, unless it is grossly in excess of the upper limits of normal. This also applies to the tetrammonium reaction. The erythrocyte sedimentation rate and diphenylamine reaction in 362 blood samples demonstrated that, within the normal range of the diphenylamine reaction, grossly abnormal erythrocyte sedimentation rate readings occurred which could not be explained by the presence of anaemia alone. No simple correlation between the diphenylamine reaction and the erythrocyte sedimentation rate was apparent. Similar results were obtained when the tetrammonium reaction and the erythrocyte sedimentation rate were compared in the same blood samples.

The erythrocyte sedimentation rate is affected by the plasma fibrinogen and globulin concentrations (particularly alpha and gamma globulin fractions) and by the packed cell volume. With the Westergren method, the influence of the latter factor is reduced, since whole blood is diluted with sodium citrate solution and the use of 200-mm. columns delays the onset of packing. In Fig. 3 the haemoglobin concentration is plotted against the erythrocyte sedimentation rate of the blood specimens examined; with a haemoglobin concentration of 15 g. per 100 ml., a reading of 110 mm./1 hr is seen to be possible (a corresponding Wintrobe reading could not exceed 45 mm./hr).

The findings in Table I strongly suggest that erythrocyte sedimentation rate and C-reactive protein readings are much more sensitive than the diphenylamine and tetrammonium reactions. Thus, in the "active carditis" group of eleven cases, the erythrocyte sedimentation rate was raised and the C-reactive protein was positive in all cases, whereas the diphenylamine and tetrammonium reactions were abnormal on only three and four cases respectively. In the second group of fifty cases of "chronic valvular disease with a history of cardiac failure at some time", the erythrocyte sedimentation rate and C-reactive protein were more frequently positive than either of the other two tests. The erythrocyte sedimentation rate was more frequently positive

than the other tests in the third group of 114 cases of "chronic valvular disease with no history of cardiac failure". In particular, the erythrocyte sedimentation rate was raised in seventeen out of twenty of the pregnant women in this group (nine of the pregnant women had haemoglobin concentrations of less than 12 g./100 ml.). The erythrocyte sedimentation rate was also raised in 42 out of the 94 non-pregnant women in this group, and again, nine of the cases with a raised erythrocyte sedimentation rate had haemoglobin concentrations of less than 12 g./100 ml. Thus anaemia is not the complete cause of a raised erythrocyte sedimentation rate, even though evidence of acute inflammation, as shown by positive C-reactive protein tests in ten cases in this group, is lacking in the remaining 49 cases. In the absence of anaemia, the presence of a raised erythrocyte sedimentation rate must be regarded as pathological. It is known that, on recovery from an acute inflammatory process, the C-reactive protein becomes negative before the erythrocyte sedimentation rate returns to normal, and therefore it must be assumed that these cases were recovering from an acute inflammatory condition or had reached a chronic inflammatory phase. In this third group of cases the diphenylamine and tetrammonium reactions were all negative.

In the fourth group the total of seven cases of chorea is too small for any accurate conclusion to be drawn; no case had a positive C-reactive protein, whereas four had a raised erythrocyte sedimentation rate. Table II shows that the majority of blood samples contain C-reactive protein only when high erythrocyte sedimentation rate readings are obtained.

Other workers have compared a number of the tests used to detect serum changes in acute inflammation. Harris, Friedman, and Tang (1957) compared four such tests, and found that the erythrocyte sedimentation rate fell with the onset of cardiac failure. With hormone therapy, the erythrocyte sedimentation rate fell, the C-reactive protein became negative, and the antistreptolysin-"O" titre fell; on cessation of treatment, both the erythrocyte sedimentation rate and C-reactive protein reaction showed a "rebound" phenomenon. These authors also observed that, after the C-reactive protein test became negative, the serum mucoprotein already being normal, the erythrocyte sedimentation rate remained abnormal, particularly in adolescent girls.

In a series of observations, Adams (1956) compared the serum mucoprotein, serum C-reactive protein, serum non-glucosamine polysaccharide, and antistreptolysin-"O" titre, and concluded that the C-reactive protein was only remotely related to the concentration of the mucoprotein and non-glucosa-

mine polysaccharide. He also observed that there existed a direct relationship between these two latter entities, but that the relationship was not consistent.

Comparison of a series of ten tests by Müller and Kähler (1956) showed that the C-reactive protein, erythrocyte sedimentation rate, and serum copper levels were reliable indices of activity. The serum iron, Takata Ara reaction, Weltmann reaction, thymol turbidity, total white cell count, polymorphonuclear lobe count, and body temperature measurement, were much less reliable. The body temperature was the most useful and the Takata Ara reaction the least useful of these latter tests. The tests listed by Fischel (1957) attempt to estimate the numerous complicated changes which occur in the blood at different rates during the course of an inflammatory process. Since these are essentially non-specific in nature, and the exact stage of the disease process is not known in any given case, it is hardly surprising that simple direct correlation has not been found.

Critical appraisal of the results obtained by other workers and of our own experience suggests that the two most useful tests are those which are most simply performed, namely, the erythrocyte sedimentation rate and the C-reactive protein reaction.

Summary

The Westergren erythrocyte sedimentation rate, serum C-reactive protein test, serum diphenylamine reaction, and serum tetrammonium turbidity reaction, were compared in 362 samples of serum from 217 patients, with the following results:

- (1) The serum diphenylamine reaction and tetrammonium turbidity reaction are not sensitive enough to determine the presence or absence of rheumatic activity.
- (2) The erythrocyte sedimentation rate and C-reactive protein test appear to be much more sensitive, and are more easily and rapidly performed.
- (3) The erythrocyte sedimentation rate is more frequently abnormal in negative cases than the C-reactive protein test, and is influenced by both anaemia and polycythaemia.
- (4) Since the C-reactive protein reaction detects 1 mg. per 100 ml. serum, it may well be too sensitive, although the knowledge that a patient's serum contains no C-reactive protein is very useful.

Similar comparisons of tests used in the assessment of activity of the rheumatic process are discussed.

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REFERENCES

- Adams, F. H. (1956). *J. Pediat.*, **49**, 16.
 Anderson, H. C., and McCarty, M. (1950). *Amer. J. Med.*, **8**, 445.
 Ayala, W., Moore, L. V., and Hess, E. L. (1951). *J. clin. Invest.*, **30**, 781.
 Coburn, A. F., Bates, R. C., Hahn, J. W., and Murphy, P. (1956) *J. chron. Dis.*, **3**, 140.
 —, Moore, L. V., and Haninger, J. (1953). *A.M.A. Arch. intern. Med.*, **92**, 185.
 Dole, V. P., Watson, R. F., and Rothbard, S. (1945). *J. clin. Invest.*, **24**, 648.
 Eastham, R. D., Szekely, P., and Davison, K. (1958). *Ann. rheum. Dis.*, **17**, 314.
 Ernstone, A. C. (1930). *Amer. J. med. Sci.*, **180**, 12.
 Fearnley, G. R., Pirkis, J., de Coek, N., Lackner, R., and Meanock, R. I. (1955). *Ann. rheum. Dis.*, **14**, 226.
 Fischel, E. E. (1957). *Amer. J. Med.*, **22**, 429.
 Harris, T. N., Friedman, S., and Tang, J. (1957). *Amer. J. med Sci.*, **234**, 259.
 Hedlund, P. (1947). *Acta med. scand.*, Suppl. 196, 579.
 — and Brattsten, I. (1955). *Scand. J. clin. Lab. Invest.*, **7**, 99.
 Hess, E. L., Hahn, J. W., and Ayala, W. (1956). *Proc. Soc. exp. Biol. (N.Y.)*, **91**, 528.
 Jacox, R. F. (1951). *J. Lab. clin. Med.*, **37**, 721.
 — (1953). *J. clin. Invest.*, **32**, 661.
 — and Gale, R. J. (1951). *J. Lab. clin. Med.*, **37**, 728.
 Jackson, R. L., Kelly, H. G., Smith, E. K., Wang, P., and Routh, J. I. (1953). *Amer. J. dis. Child.*, **86**, 403.
 Linko, E., and Waris, E. (1955). *Scand. J. clin. Lab. Invest.*, **7**, 141.
 Müller, W., and Kähler, H. J. (1956). *Dtsch. med. Wschr.*, **81**, 1410.
 Perlman, E., Bullowa, J. G. M., and Goodkind, R. (1943). *J. exp. Med.*, **77**, 97.
 Roantree, R. J., and Rantz, L. A. (1955). *A.M.A. intern. Med.*, **96**, 674.
 Saifer, A., and Zymaris, M. C. (1956). *Clin. Chem.*, **2**, 195.
 Shackman, N. H., Heffer, E. T., and Kroop, I. G. (1954). *Amer. Heart J.*, **48**, 599.
 Werner, I., and Odin, L. (1952). *Acta Soc. Med. upsalien*, **57**, 230.
 Westergren, A. (1921). *Acta med. scand.*, **54**, 247.
 Wood, H. F., and McCarty, M. (1951). *J. clin. Invest.*, **30**, 616.
 Yocum, R. S., and Doerner, A. A. (1957). *A.M.A. Arch. intern. Med.*, **99**, 74.

Comparaison de la vitesse de sédimentation érythrocytaire et des réactions à la protéine C-réactive, à la diphenylamine et au tetrammonium dans la maladie de Bouillaud

RÉSUMÉ

La vitesse de sédimentation érythrocytaire (Westergren), la réaction à la protéine C-réactive, la réaction à la diphenylamine et la réaction de turbidité au tetrammonium furent comparées en 362 prélèvements de sang de 217 malades, avec des résultats suivants:

- (1) La réaction à la diphenylamine et la réaction de turbidité au tetrammonium ne sont pas assez sensibles pour déterminer la présence ou l'absence d'une activité rhumatismale.
- (2) La vitesse de sédimentation érythrocytaire et la réaction à la protéine C-réactive semblent être beaucoup plus sensibles, et plus faciles et rapides.
- (3) La vitesse de sédimentation érythrocytaire est plus souvent anormale dans des cas négatifs que la réaction à la protéine C-réactive et est influencée aussi bien par une anémie que par une polycytémie.
- (4) La réaction à la protéine C-réactive décèle 1 mg. par 100 cc. de sérum et il se peut bien qu'elle soit trop sensible, mais il est utile de savoir que le sérum du malade ne contient pas de protéine C-réactive.

On discute des comparaisons similaires des réactions employées dans l'évaluation de l'activité du processus rhumatismal.

Comparación de la velocidad de sedimentación eritrocitaria y de las reacciones a la proteína C-reactiva, a la difenilamina y al tetramonio en la enfermedad de Bouillaud

SUMARIO

La velocidad de sedimentación globular (Westergren), la reacción a la proteína C-reactiva, la reacción a la difenilamina y la reacción de turbiedad al tetramonio fueron comparadas en 362 muestras de sangre de 217 enfermos, con los resultados siguientes:

(1) La reacción a la difenilamina y la reacción de turbiedad al tetramonio no son bastante sensibles para determinar la presencia o la ausencia de la actividad reumática.

(2) La velocidad de sedimentación eritrocitaria y la reacción a la proteína C-reactiva parecen ser mucho más sensibles y de realización más fácil y rápida.

(3) La velocidad de sedimentación eritrocitaria es más a menudo anormal en casos negativos que la reacción a la proteína C-reactiva y se ve afectada tanto por la anemia como por la policitemia.

(4) La reacción a la proteína C-reactiva revela 1 mg. por 100 cc. de suero y es, quizás, demasiado sensible, pero el conocimiento de que el suero de un enfermo no contiene proteína C-reactiva es útil.

Se discuten similares comparaciones de reacciones empleadas en la valoración de la actividad del proceso reumático.