ROUTINE LABORATORY PROCEDURES IN ARTHRITIS AND RELATED DISORDERS*

BY

EDWARD F. HARTUNG AND MARY E. MAHOOD

From the Arthritis Clinic, Bellevue Hospital, Fourth Division, New York University Post-Graduate Medical School

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The differential diagnosis of the arthritides and related disorders is mainly on clinical grounds, since there are few specific tests. The only exceptions are the infectious arthritides, where a causative organism can sometimes be demonstrated. However, a number of laboratory procedures are of confirmatory importance in diagnosis, and these may be listed as follows:

- (1) Erythrocyte sedimentation rate
- (2) Serum uric acid determination
- (3) C-reactive protein detection
- (4) Sensitized sheep-cell agglutination titre
- (5) Anti-streptolysin O titre
- (6) Lupus erythematosus cell test.

Superficially these appear simple to perform and interpret. The pitfalls are due to:

- (i) the multiplicity of methods recommended, each method resulting in appreciably varying results,
- (ii) the lack of unanimity of workers as to the best method,
- (iii) the fact that some tests require considerable experience unless detailed instructions are laid down for the technician,
- (iv) disagreement as to the normal values.

The diagnosis and treatment of arthritis and rheumatism are, of course, phases of internal medicine, and as our knowledge of these conditions increases it is increasingly evident that rheumatology, if it is a specialty, which we doubt, can never be divorced even for a moment from internal medicine in general. Therefore, it is important to stress that the particular laboratory procedures mentioned here are purely supplementary to the general laboratory procedures required in the diagnosis of internal medical conditions. In some hospitals and clinics, however, it may be found expedient, when the amount of work warrants it, to set up a specialized laboratory to perform tests of particular significance in arthritis and related disorders.

Since we have recently set up such a laboratory, it occurred to us that the experience gained in selecting the most practical and accurate procedures, and in standardizing the details of technique, would be of value to others who contemplate the establishment of such a laboratory.

There are many methods for each test, and we offer the following, with their normal values, as our selection of the most practical and accurate techniques.

(1) Erythrocyte Sedimentation Rate

There is not one step of any proposed technique for the performance of the sedimentation rate that is not subject to variation in the hands of clinicians, technicians, and investigators. It appeared to us, after many years of experience with the various methods, that the modified Westergren is the most accurate as well as the one most commonly used. Simple as it is, however, the pitfalls are numerous. Probably the most common source of error is in differently cleaned glassware.

Materials:

- (a) A chemically clean tube containing 0.5 ml. 3.8 per cent. sodium citrate.
- (b) Westergren pipettes (thoroughly cleaned and dried) at least 300 mm. long and calibrated from 0 to 200 mm. The internal diameter should be at least 2.5 mm.
- (c) Westergren supports to hold the tubes *exactly* vertical.

Technique.—Use a dry syringe to obtain the venous blood, and add 4.5 ml. to the 0.5 ml. of 3.8 per cent. sodium citrate. Mix thoroughly to prevent clotting. Draw a column of blood 200 mm. up into the Westergren pipette. Place the pipette into the support. Care must be taken to see that the pipette is exactly vertical. The fall of the column of red cells is read at the end of one hour. The result is reported as the number of mm. of

fall per hour. The test must be completed within 2 hours after the blood is drawn from the patient.

The pipettes are cleaned by immediate rinsing, and by soaking in detergent overnight. The next day they are rinsed twenty times in tap water and twice in distilled water, and then thoroughly dried.

Normal Values.—From zero to 15 mm. for men, and up to 20 mm. for woman (Coggeshall, 1953). While it is recognized that the number of red blood cells affects the rate, correction is not usually considered of enough clinical value to make the calculation worthwhile (Poole and Summers, 1952). Fasting and post-prandial specimens do not show significant variations (Hartung, unpublished data).

(2) Serum Uric Acid

Analysis of the serum has been shown to give the most accurate results (Bensley and others, 1947). If other specimens of blood are used, as whole blood or plasma, care must be taken to make note of the entirely different normal values. The method we prefer is a modification of that of Buchanan, Block, and Christman (1945).

Reagents:

- (a) *Urea Cyanide Carbonate* (Christman and Ravitch, 1932).—25 g. pure sodium cyanide and 50 g. anhydrous sodium carbonate are dissolved in 400 ml. distilled water. If heat has been applied to hasten solution, cool and add 75 g. urea. After solution is complete, make up to a volume of 500 ml. and filter. A slight precipitate will settle from this solution on standing, but the value of the solution for the determination remains unimpaired for several months.
- (b) *Arsenophosphotungstic Acid* (Benedict, 1922).—In a litre pyrex flask dissolve 100 g. pure sodium tungstate (Baker or Merck) in 600 ml. water. Add 50 g. pure arsenic acid (As_2O_5), 25 ml. phosphoric acid 85 per cent., and 20 ml. concentrated hydrochloric acid. Boil the mixture for about 20 min., cool, and dilute to 1 litre. The reagent keeps indefinitely in a dark bottle.
- (c) *Standard Uric Acid Solution*.—The stock solution should contain 1 mg. uric acid per 1 ml. It may be purchased at this concentration, or it may be made according to the published formula (Folin, 1933).

For the determination, 1 ml. stock solution is diluted to 100 ml. Therefore, 1 ml. dilution is equal to 0.01 mg. uric acid. The dilute standard is not stable and should be made up fresh for each determination.

- (d) *Sulphuric Acid, Sodium Tungstate Mixture*.—One part, by volume, of 10 per cent. sodium tungstate plus eight parts, by volume, of M/12 sulphuric acid. The pH of the mixture should be 2 ± 0.1 .

The mixture should be prepared fresh on the day of the determination.

- (e) *Serum*.—1 ml. patient's serum. It should be clear and not haemolized.

Technique.—In a 50-ml. Erlenmeyer flask, mix 1 ml. patient's serum and 9 ml. sulphuric acid, sodium tungstate mixture. Let the mixture stand for 10 min. to assure complete precipitation of the protein. Filter through a No. 42 Whatman filter.

In a 50-ml. volumetric flask, put 5 ml. of the clear filtrate; to it add 2.5 ml. urea cyanide carbonate, 1 ml. arsenophosphotungstic acid, and dilute to 50 ml. with distilled water. At the same time prepare a blank of distilled water plus the reagents, and a standard containing 5 ml. the dilute standard in place of the patient's serum.

Let the colour develop for 1 hour, then read on a photo-electric colorimeter using 660 wave length. Set the machine at zero with the blank.

Calculations:

$$\frac{\text{Unknown reading}}{\text{Standard reading}} \times 0.05 \times \frac{100}{0.5} = \text{mg. per 100 ml.}$$

Alternative Method.—Uric acid may also be determined by the uricase method of Buchanan and others (1945), but for serum the difference is not significant (personal communication from Dr. T. F. Yü, 1955).

All the tubes are cleaned by soaking them overnight in detergent, rinsing them twenty times in tap water and twice in distilled water, and thoroughly drying them.

Normal Values.—These normal values are those below 6.0 mg. per cent. for men and below 5.5 mg. per cent. for women (Jacobson, 1938; Mull, 1943).

(3) C-Reactive Protein

C-reactive protein is a specific protein which appears in the body in response to a variety of inflammatory processes. It has been found in patients with pneumococcal pneumonia, acute rheumatic fever, staphylococcal osteomyelitis, and subacute bacterial endocarditis (Tillett and Francis, 1930). It has also been found in the serum of patients with rheumatoid arthritis (Hedlund, 1947) and a variety of other diseases, as myocardial infarction and cancer (Löfström, 1943). It is not, therefore, of much diagnostic significance, but indicates an acute inflammatory condition, and can be used as a measure of the response to therapy.

Materials (Anderson and McCarty, 1953):

- (a) Capillary tubes with an internal diameter of from 0.5 to 1 mm. and 6 mm. long
- (b) Clear serum (plasma from oxalated or citrated blood will not react)
- (c) C-reactive protein antiserum (should be centrifuged before each using)
- (d) A plasticine block.

Technique.—Draw the antiserum up into the capillary tube to about 1 to 1.5 cm. Then add an equal part of the patient's serum. This can easily be done by slanting the tube of serum and dipping the tip of the capillary tube into the serum. There must not be an air bubble between the two sera. The end of the capillary tube is sealed with plasticine and placed upright in a plasticine block. The tubes are then incubated at 37° C. for 2 hours and refrigerated at 4° C. for 24 hours. They are read immediately upon removal from the refrigerator. They are reported as 1† to 4†. A 1† is a narrow band of white precipitate at the point of interphase, while in a 4† the precipitate fills the tube. A 2† and 3† are the stages of precipitation in between.

Normal Values (Anderson and McCarty, 1953).—Normal serum contains no C-reactive protein. The presence of a precipitate shows that an active inflammatory process is present. If a series of tests are done over in a few weeks, the progress of the disorder may be studied.

(4) Sensitized Sheep-cell Agglutination

Heller Modification of Rose Test (Heller, Jacobson, and Kolodny, 1949)

As found by Rose, Ragan, Pearce, and Lipman (1948), the sera of patients with rheumatoid arthritis in the active stage contains a substance which agglutinates sheep erythrocytes sensitized with specific haemolysin to much higher titres than normal sheep cells. Heller and others (1949) found that the factor in the serum responsible for the agglutination of normal sheep cells was present in both normal and rheumatoid individuals, and that this normal agglutinin is variable and independent of the capacity of the sera of patients with rheumatoid arthritis to agglutinate sensitized cells. It can, therefore, be selectively absorbed without significantly influencing the titre against sensitized sheep cells. The titre thus obtained reflects the concentration of the factor in the serum associated with rheumatoid arthritis.

Materials:

- (a) *Normal Sheep Cells.*—One volume of sheep cells is mixed with one volume of Alsevers solution as a preservative. The Alsevers solution is prepared as follows:

2.05 g. dextrose, 0.8 g. sodium citrate, and 40.2 g. sodium chloride are dissolved in 100 ml. distilled water. The pH should be from 6.1 to 6.2 and can be adjusted with 10 per cent. citric acid. It is sterilized in the autoclave at 15 lb. for 20 min.

The cells must be thoroughly washed. This is done by shaking them with normal saline, centrifuging at 2,000 r.p.m. for 10 min., removing the supernatant, and repeating the procedure three

times. The supernatant of the last washing should be water clear. The packed cells are used for absorption (explained later). A 2 per cent. suspension in saline is also made from the packed cells. A part of the latter is diluted again with saline to make a 1 per cent. suspension for use in the test.

- (b) *Determination of Basic Agglutination Titre.*—The agglutination titre of rabbit anti-sheep haemolysin, obtained commercially, is determined as follows:

The haemolysin is prepared in dilutions of 1 : 100, 1 : 200, 1 : 300, and so on up to 1 : 1,000. Eleven 10 × 75 mm. tubes are put in a rack. To the first ten tubes add 0.50 ml. saline, and to the last 0.75 ml. saline. Add 0.25 ml. of the various dilutions of haemolysin to the first ten tubes. Add 0.25 ml. of the 2 per cent. suspension of the sheep cells, previously prepared as above, to each of the eleven tubes. Incubate in a water bath at 37° C. for one hour. Refrigerate for 24 hours and read upon removal from the refrigerator. In the determination use a dilution double the last one which shows agglutination. For example, if the last tube to show agglutination is a 1 : 100 dilution, prepare the haemolysin for the determination in a 1 : 200 dilution.

- (c) *Preparation of Sensitized Sheep Cells.*—To a 2 per cent. suspension of normal cells as prepared above, add an equal volume of haemolysin as determined in Section (b). The mixture of the two should be allowed to stand for one hour before using.

- (d) *Patient's Serum.*—Clear serum is obtained from a clotted specimen of blood and inactivated at 56° C. for 30 min. For absorption of non-specific agglutinins, four volumes of serum are added to one volume of packed sheep cells and allowed to stand at room temperature for 40 min. with gentle shaking at intervals. The mixture is centrifuged and the clear serum is again added to a volume of packed cells and allowed to stand for another 40 min. The serum is again centrifuged, and the clear serum is ready for the test.

Technique.—Two series of two-fold dilutions of absorbed serum (prepared as above) are made in 0.5 ml. normal saline.

The first series consists of three tubes with the serum dilutions ranging from 1 : 3.5 to 1 : 14. To this series is added 0.5 ml. of 1 per cent. suspension of normal sheep cells, making a final dilution of 1 : 7 to 1 : 28.

The second series consists of ten tubes with the serum dilutions ranging from 1 : 3.5 to 1 : 1,792. To this series is added 0.5 ml. sensitized sheep cells making a final dilution of 1 : 7 to 1 : 3,584.

The tubes are shaken gently, incubated in a 37° C. water bath for 1 hour, and then refrigerated over night. Readings are made after gentle shaking, and recorded as complete agglutination, partial agglutination, or no agglutination. The test is valid only if there is no

agglutination in the tubes containing normal cells, thus indicating complete absorption of the normal sheep cell agglutinins. The result reported is the titre of the last tube showing partial agglutination.

All of the glassware is soaked overnight in detergent, rinsed twenty times in tap water and twice in distilled water, and then oven-dried.

Normal Values (Heller and others, 1949).—Cases with active peripheral rheumatoid arthritis give titres of 1 : 28 or higher with complete agglutination in the first two tubes. For this reason, this value is considered to be the lower limits of a positive reaction.

(5) Antistreptolysin-O Titre (Todd, 1938)

The presence of a significant amount of the antibody, antistreptolysin-O, in human sera is an indication of a recent infection by a Group A streptococcus. The antistreptolysin-O titre is used as an aid in the diagnosis of rheumatic fever, in which the titre is increased, and in the differential diagnosis of diseases simulating rheumatic fever, but which rarely show an increased titre.

Materials:

- 0.1 ml. patient's serum
- 0.9 per cent. saline
- Freshly drawn rabbit blood
- 0.1 ml. standard serum (known titre)
- Streptolysin-O reagent (Difco).

Technique:

(a) *Preparation of Rabbit Blood*.—Use a sterile 18 or 19 gauge needle, a 10-ml. syringe, and 100 ml. 0.9 per cent. saline. Draw 10 ml. blood directly from the rabbit's heart and add it to the 100 ml. saline. Centrifuge at 2,000 to 2,500 r.p.m. for 7 min. Remove supernatant. Add another 100 ml. saline, mix, and centrifuge again for 7 min. Remove supernatant. Make a 5 per cent. suspension of the packed cells with 0.9 per cent. saline.

(b) *Dilution of Unknown Serum*.—Add 0.1 ml. of the patient's serum to 4.9 ml. 0.9 per cent. saline, thus making a 1 : 50 dilution. Place ten tubes, size 100 by 13 mm., in a rack. Using the 1 : 50 dilution, proceed as follows:

Tube No.	1 : 50 Serum (ml.)	Saline (ml.)	Serum Dilution
1	1.0	0.0	50
2	0.8	0.2	62½
3	0.6	0.4	83
4	0.4	0.6	125
5	0.32	0.68	156
6	0.24	0.76	208
7	0.20	0.80	250
8	0.16	0.84	312
9	0.12	0.88	416
10	0.10	0.90	500

(c) *Standard Serum*.—With each group of tests a serum with a known titre is run. A dilution must be

made that will fall in the range of the serum dilution. As an example, if a titre of 8,900 is used, the serum must be diluted 1 : 5,000 to fit within the given ranges. Place thirteen tubes, size 100 × 13 mm., in a rack and proceed as follows:*

Tube No.	1 : 5,000 Dilution (ml.)	Saline (ml.)	Serum Dilution
1	0.80	0.2	6,240
2	0.70	0.3	7,140
3	0.65	0.35	7,700
4	0.60	0.40	8,300
5	0.55	0.45	9,100
6	0.50	0.50	10,000
7	0.45	0.55	11,100
8	0.40	0.60	12,500
9	0.35	0.65	14,300
10	0.30	0.70	16,700
11	0.25	0.75	20,000
12	0.20	0.80	25,000
13	0.0	1.0	

(d) *Test*.—All the above serum dilutions must be added to the tubes before the streptolysin reagent is rehydrated. The reagent is only stable for a short time. Rehydrate the reagent by adding 25 ml. distilled water as directed by the manufacturers. Add 0.5 ml. streptolysin reagent to each tube. Shake gently. Place racks in a 37° C. water bath for 15 min. Take out and add 0.5 ml. of 5 per cent. suspension of rabbit red blood cells. Shake gently. Put back into water bath for 45 min. Remove from bath and refrigerate for 24 hrs. Read immediately upon removal from refrigerator. The end-point is read as the last tube showing no haemolysis. The standard serum should read 8,300 plus or minus one tube. If it does not, the test is invalid.

Calculate:

$$\frac{8,900 \text{ (or known titre)}}{\text{Standard reading}} \times \text{Reading of serum}$$

The pipettes are soaked in chromic acid cleaning solution overnight, rinsed twenty times in tap water and twice in distilled water, and oven-dried. The tubes are immediately rinsed, boiled in detergent for 5 min., rinsed twenty times in tap water and twice in distilled water, and oven-dried.

Normal Values (McEwen and Ziff, 1955).—Anything above 200 units is considered significant of an infection with haemolytic streptococcus. However, more important is a progressive rise of titre.

(6) Lupus Erythematosus Cells

A substance in the gamma globulin fraction of plasma from patients with systemic lupus erythematosus causes the "L.E." phenomenon to occur *in vitro* in the presence of normal blood. The L.E. phenomenon consists of the phagocytosis by a polymorphonuclear leucocyte of a mass of desoxyribonuclear leucocyte.

Technique.—Using a china-marking pencil, make three

* Personal communication from A. W. Beauheimer, Department of Microbiology, New York University.

circles on a slide. Put a drop of whole fresh blood in each circle.

Place the slide in a Petri dish kept moist by means of a damp piece of filter paper, and incubate for 20 min. Remove from incubator and wash off clots with normal saline. Flood slide with patient's plasma. Put cover slips over circles. Again place in moist Petri dish and re-incubate for one hour. Remove cover slips and air-dry slide. (It is important that the slides be air-dried.) Stain with MacNeal tetrachrome stain. Examine under the microscope, allowing at least 10 min. to a slide (Schultz, Baum, and Ziff, 1955).

In examining the slides look for the following:

- "Haematoxylin bodies",
- "Rosettes",
- "L.E." cells.

"Haematoxylin Bodies."—These are dark-purplish staining material varying in size and shape. The material is most often homogenous in consistency, similar to the L.E. inclusion, but more darkly stained. They have been shown to be depolymerized deoxyribonucleic acid, the same material which when phagocytized forms the L.E. cells.

"Rosettes."—These are clusters of polymorphonuclear leucocytes surrounding a "haematoxylin body". The appearance of a typical rosette with a free cloudy mass in the centre is highly suggestive but not diagnostic of lupus. Often these rings surround clumps of platelets. The latter are pseudo-rosettes and are not significant.

"L.E." Cells.—A typical L.E. cell must be found before the preparation can be called positive. This is

a neutrophilic polymorphonuclear leucocyte which has engulfed a purplish-staining homogenous mass which is so large that the nucleus is pushed to one side of the cell. The mass is chemically the same as the "haematoxylin body", but after it is engulfed by the cell it may vary in staining quality from pale blue to a dense purple. It is always homogenous in appearance and lacks any chromatin structure; this differentiates it from a "tart cell" with which it is often confused. The inclusion may vary in size from that of a single lobe of a polymorphonuclear leucocyte to the size of two leucocytes. Occasionally more than one inclusion is found in a cell (Dubois, 1953).

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