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SERUM PROTEIN CHANGES IN RATS WITH ARTHRITIS INDUCED BY MYCOBACTERIAL ADJUVANT

J. S. LOWE

Imperial Chemical Industries Limited, Pharmaceuticals Division,
Alderley Park, Macclesfield, Cheshire.

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Abstract—Arthritis has been induced in rats by injecting a mixture of dead tubercle bacilli in liquid paraffin into the right hind-foot pad and resulting changes in the serum proteins have been studied. Throughout the arthritic condition no changes occurred in the level of total serum protein. However, immediately following the injection of adjuvant, the serum albumin fell and a pre-albumin band appeared in the starch gel electrophoretogram. The albumin level reached a minimum two days after the injection and then started to rise again; simultaneously the pre-albumin band disappeared. Ten days after the injection secondary arthritic lesions appeared in the ears, tail and fore and hind paws. These were accompanied by a striking fall in the level of serum albumin which remained low until the lesions began to regress naturally. Simultaneously profound changes occurred in the electrophoretogram. These included the appearance of pre-albumin, post-albumin and slow α_2-globulin bands and a fall in the fast α_1-globulin. By day fourteen these changes were even more pronounced and by day sixteen two further bands appeared—a second pre-albumin and a protein migrating between the origin and the slow α_2-globulin. These last bands had only a transient existence: they were not present in sera taken at day twenty. The changes in the electrophoresis pattern reverted to normal as the condition regressed. The adjuvant arthritis was accompanied by an elevated glycoprotein level in the α_1- and β-globulins. It has been shown that the majority of the changes in the serum proteins are associated with inflammation. The relationship between these serum changes and those seen in human arthritis is discussed.

THE production of adjuvant-induced arthritis in rats was first described by Stoerck, Bielinski and Budzilovich[1] and by Pearson.[2, 3] The development of this arthritic syndrome in rats may be conveniently followed by measuring the changes in thickness of both hind feet.[4] Following the injection of a mixture of dead tubercle bacilli in liquid paraffin into the right hind foot pad, the foot rapidly increases in size during the first three days. Thereafter the swelling diminishes slightly until about 7 or 8 days after the injection when there is a further swelling. At approximately day 10, inflamed lesions, called secondary lesions, appear in the ears and tail and in joints in the fore paws and hind feet. Fourteen days after the injection the lesions have usually proliferated so extensively that both hind feet up to and including the ankle joint are red and swollen. Subsequently the inflammation subsides leaving pale granulomatous swellings around the joints. This condition seems nearer to human rheumatoid arthritis than any other laboratory model so far investigated.

The present paper describes the changes occurring in the serum proteins of rats with developing adjuvant-induced arthritis.

METHODS

Induction of arthritis

The arthritis was produced by injecting 0·05 ml of a fine suspension of dead tubercle bacilli (human strains, PN, DT and C grown for 8 weeks, killed by steam and dried in a vacuum oven; from Ministry of Agriculture Veterinary Laboratories, Weybridge, Surrey) in liquid paraffin B.P. (5 mg/ml, w/v) into the right hind foot pad of specific-pathogen-free male albino rats (Alderley Park Strain 1) weighing approximately 200 g. Blood samples were removed either from the tail, when approximately 60 μl were drawn into a glass capillary tube which was then sealed and centrifuged to separate the serum, or collected by cardiac puncture under ether anaesthesia.

Electrophoretic techniques

(a) Starch-gel electrophoresis was carried out by the method of Smithies[5] using a bridge-buffer containing boric acid (8·552 g/l) and sodium hydroxide (60 ml N/1) pH 8·9. Starch gels were prepared in a buffer containing boric acid (1·3605 g/l) and sodium hydroxide (8·8 ml N/l) pH 8·9. Starch—hydrolysed (Connaught Medical Research Laboratories, Toronto) was used for the preparation of all the starch gels.

(b) Paper and cellulose acetate membrane electrophoresis was conducted in a Shandon Universal Electrophoresis tank using barbitone buffer pH 8·6, $\mu = 0{\cdot}03$ for paper and the buffer recommended by Owen[6] for cellulose acetate.

(c) Zone electrophoresis on ethanolysed cellulose was carried out in an apparatus based on that of Porath.[7] The only difference lay in the construction of the tube in which the supporting material was held. This was made from a Pyrex tube with an even inner surface, length 121 cm, ext. dia. 4·2 cm, int. dia. 3·9 cm. A B45 socket was sealed on to the top of the tube and 13 cm from the bottom three identations were made. These acted as a support for a perforated disc of Darvic (I.C.I. Ltd., Plastics Division) which in turn was covered with No. 1 Whatman filter paper cut to size. The perforations of the disc occupied 40% of its area and thus allowed satisfactory conduction of the current. The column was packed with Munktell ethanolysed cellulose (Gallenkamp & Co. Ltd.) suspended in barbiturate buffer pH 8·6, $\mu = 0{\cdot}03$, by Porath's method except that the suspension of cellulose in buffer was not stirred during the packing procedure. Porath's conditions for electrophoresis were followed using 1,200 V at 65 mA for 36–40 hr.

Staining methods

(i) Cellulose acetate membranes were stained for 15 min in naphthalene black (0·2% w/v) dissolved in methanol (9 parts) and glacial acetic acid (1 part). Excess stain was removed by washing in 10% (w/v) glacial acetic acid in methanol.

(ii) Glycoproteins on cellulose acetate membranes were stained by the method of Bodman.[8]

(iii) Starch gels were stained in naphthalene black solution as described by Smithies.[5]

Serum protein analysis

(i) Total serum proteins were determined by the biuret procedure.[9]

(ii) For determination of albumin, serum was subjected to electrophoresis on a cellulose acetate strip. The strip was stained with naphthalene black, dried and cut to

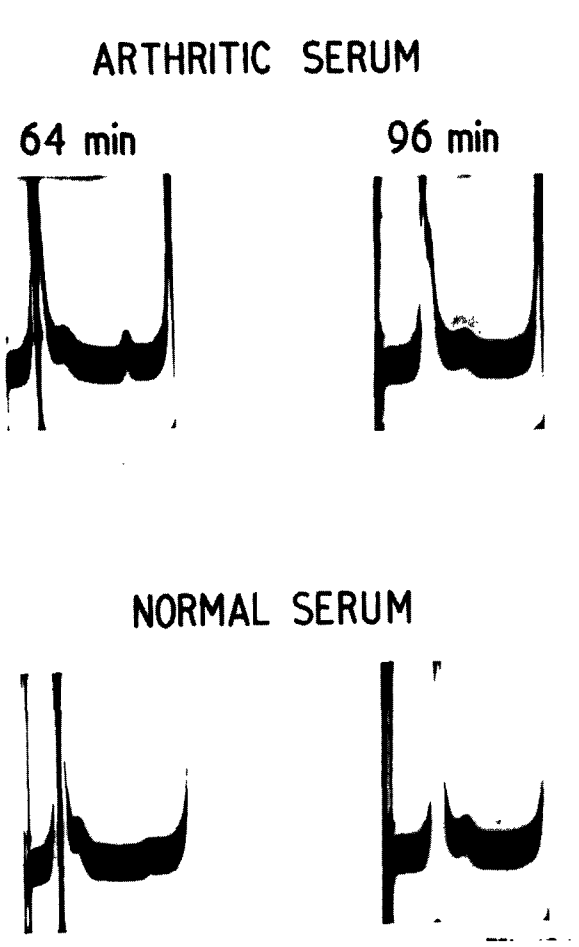

FIG. 1. Ultracentrifuge studies on normal and arthritic rat serum. Top: arthritic serum. Bottom: normal serum. Photographs on the left are of runs of 64 min duration, those on the right of 96 min duration. Both runs were at 59,780 rev/min and sedimentation is from left to right.

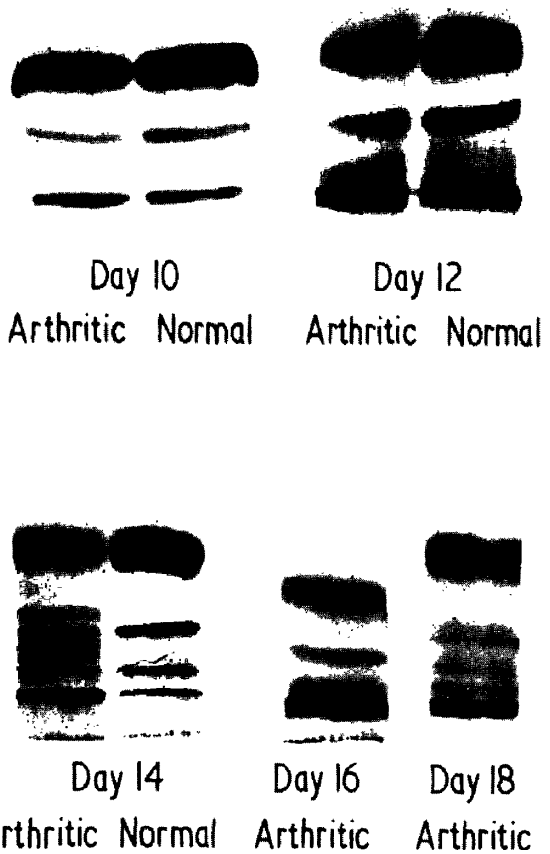

FIG. 5. Starch gel electrophoretic patterns of normal and arthritic rat sera.
Top. L. to R. Day 10. arthritic, normal.
Day 12. arthritic, normal.
Bottom. L. to R. Day 14. arthritic, normal.
Day 16. arthritic.
Day 18. arthritic.

separate the albumin from the other serum proteins. The two pieces were dissolved separately in mixtures of chloroform (9 parts) and ethanol (1 part) and the extinction of the solutions measured at 600 mμ. In calculating the albumin as a percentage of total protein no allowance was made for the different amounts of dye taken up by the albumins and globulins.

Sedimentation velocity measurements

Sedimentation velocity runs were carried out by Dr. W. E. F. Naismith of Fibres Division, Imperial Chemical Industries Limited, Harrogate, using a Spinco Model E analytical ultracentrifuge. Serum was centrifuged at 59,780 rev/min (250,000 *g*).

RESULTS

Serum protein changes in rats, fourteen days after injecting adjuvant into the footpad

Paper electrophoresis showed that serum taken from rats with secondary lesions fourteen days after the injection of adjuvant into the foot pad had a lower level of albumin than serum from controls. Analytical ultracentrifuge studies showed that serum from arthritic rats had a higher proportion of fast sedimenting component than that from controls and the main peak in the sample of arthritic serum was probably beginning to split into two components after centrifuging for 96 min or longer (Fig. 1). Electrophoresis on strips of cellulose acetate and starch gel confirmed that there were marked differences between normal and arthritic sera.

Identification of bands on starch gel electrophoretograms of normal and arthritic rat serum

At the time this work was performed there were no details published regarding the classification of the various protein fractions revealed on starch gel electrophoresis of normal rat sera. It was therefore necessary to correlate the bands seen on paper electrophoresis with their location on starch gel. Normal rat serum (35 ml) and 14 day arthritic rat serum (43 ml) were separated by zone electrophoresis on a Porath column (Fig. 2). Samples from several tubes of the eluate were submitted to either paper electrophoresis, cellulose acetate electrophoresis or both. In this manner the contents of the tubes were bulked to give six fractions, viz, A: containing albumin, B: albumin + α_1-globulin, C: α_2-globulin + trace of β-globulin, D: α_2-globulins + β-globulins, E: β-globulins and F: γ-globulins. The distribution of each of these fractions on starch gel electrophoretograms was then studied and a pattern of the bands produced by normal and arthritic rat sera on starch gel electrophoresis was built up. These results were extended and confirmed by two dimensional paper starch gel electrophoresis following the method described by Poulik.[10] The findings are summarized in Fig. 3.

Changes in total serum protein, albumin and starch gel electrophoresis pattern during the development of the arthritic syndrome

Since preliminary studies of normal and 14-day arthritic rat sera had revealed differences in the serum protein pattern, it was decided to examine the serum proteins at intervals to determine when these changes occurred and how quickly they reverted to normal. Twenty-six rats received the usual i.d. injection. Two were killed on day 0 and two on each succeeding alternate day until day 22 and the final two were killed on day 30. Blood was withdrawn by cardiac puncture. The serum was assayed for

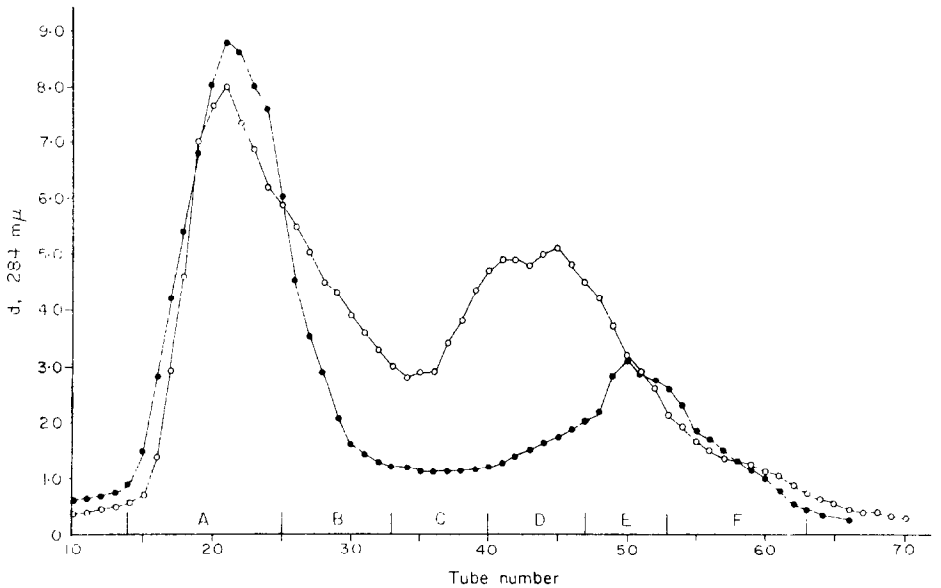

FIG. 2. Zone electrophoresis of normal and arthritic serum on a Porath column using ethanolysed cellulose as the supporting medium. (—○—○—) distribution of proteins from arthritic serum, (—●—●—) distribution of proteins from normal serum. Fractions A to F were cut as indicated.

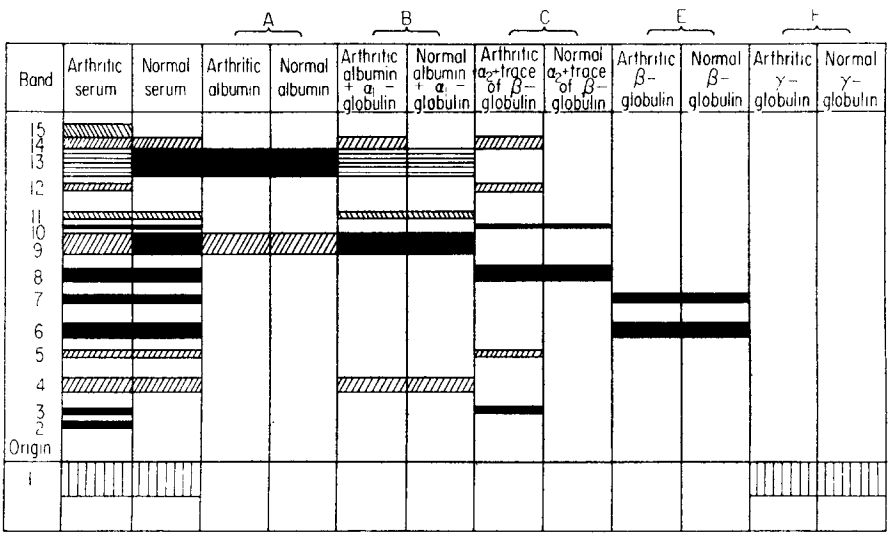

FIG. 3. Identification of protein bands seen on starch gel electrophoretograms of normal and arthritic rat sera. The identification of the various fractions A–F given on the top row of the figure, are those determined by paper electrophoresis. Fraction D is not shown since its pattern was similar to that of Fraction C.

total protein and albumin and the starch gel electrophoresis pattern determined. The thickness of two hind feet were measured at frequent intervals.

There was no change in the total serum protein concentration throughout the duration of the experiment (Fig. 4) but following the injection into the foot pad the albumin level immediately began to fall reaching a minimum value after 2 days (Fig. 4). Between day 2 and day 10 the albumin concentration increased slightly but never attained its original value. Ten days after injection the albumin level fell strikingly and remained low until the experiment was terminated 30 days after the injection had been given. The onset of secondary lesions, as indicated by the thickness of the hind foot which did not receive an injection of adjuvant, coincided with the second fall in albumin at day 10. (Fig. 4). There was almost an inverse relationship between the changes in serum albumin levels and the thickness of the injected foot.

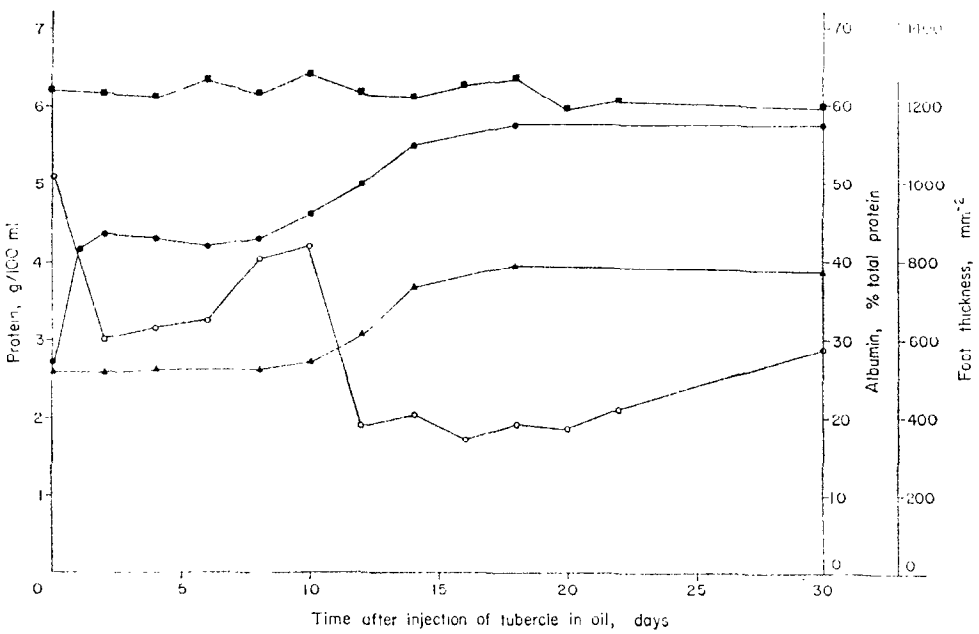

FIG. 4. Changes occurring in total serum proteins, (—■—■—), serum albumin (—○—○—) and foot thickness (—●—●— injected foot; —▲—▲— other hind foot) in a group of rats given an injection of adjuvant into the right hind foot pad.

In the following description, the band numbers refer to those of Fig. 3. Examination of the starch gel electrophoretograms revealed that two days after the injection of the adjuvant, the serum albumin band was less intense and a pre-albumin band (band 14) was present. From day 2 to day 10 the intensity of the albumin band increased and the pre-albumin band disappeared making the pattern indistinguishable from that of normal rat serum. (Fig. 5). On day 12 a pre-albumin and a slow α_2-globulin band

(band 3) appeared and there was a decrease in the intensity of the albumin and fast α_1-globulin bands (band 9). By day 14 the differences between normal and arthritic rat serum were striking. The intensity of the albumin and fast α_1-globulins were further diminished, the pre-albumin band was still present and the post-albumin bands (bands 10–12) which were scarcely detectable in normal serum were well resolved. Furthermore, the proteins migrating between the fast α_1-globulins and the slow α_1-globulins (bands 5–8) were more pronounced than in the normal rat serum. Between 16 and 18 days after injection the pattern was similar to that observed at day 14, except for the appearance of a new band (band 2) between the origin and the slow α_2-globulin. This protein has not yet been classified, but it has a transient existence since serum taken 20 days after the injection was like 14-day serum. By day 30, the electrophoretic pattern was about normal except for a less intense serum albumin band.

These experiments have been repeated by taking a series of blood samples from individual rats at different times after the injection of adjuvant into the foot pad. The results confirmed the findings described above but an occasional rat has been found which did not show the rise in albumin between day 2 and day 10.

Changes in serum glycoproteins during development of arthritis

Sera from 16-day arthritic rats were subjected to electrophoresis on cellulose acetate and the membranes were stained for glycoprotein by the periodic-acid–Schiff method. Comparison of these results with those obtained from normal rat serum indicated that sera from arthritic rats had elevated glycoprotein levels in the α_1- and β-globulins.

The relationship between serum protein changes and the appearance of secondary lesions

When secondary lesions appear in rats injected with adjuvant, the total amount of inflamed tissue in the animal is considerable. It therefore appeared likely that the changes seen in the serum proteins at this time might result from the massive and widespread inflammation. In an attempt to reproduce this degree of inflammation in an acute fashion a group of rats was injected with adjuvant at multiple sites (both ears and both hind feet). Sera taken during the first three days after these injections had serum protein patterns similar to those of rats 14 days after a single injection except for the absence of the slow α_2-globulin and the unidentified protein. Apart from these two, the other changes thus appear to result from the widespread inflammation.

As a further check on this point two rats were given an injection (i.p.) of tubercle bacilli in saline (1·0 ml of 5 mg tubercle bacilli/ml) (Group 1), two rats an injection (i.d.) of adjuvant into the foot pad (0·05 ml of 5 mg tubercle bacilli/ml) (Group 2) and a further two rats (Group 3) were given both the i.p. and i.d. injections. The serum albumin levels were examined in each animal at intervals up to 11 days after the injection. From Fig. 6 it is clear that in all three groups the serum albumin fell, but, the fall was most marked in the animals receiving the tubercle bacilli in saline i.p. In only one group however (Group 2) did the animals develop arthritic lesions. Starch gel electrophoresis confirmed this fall in serum albumin and showed that only rats given adjuvant into the foot pad (Group 2) developed the full arthritic serum pattern.

It is clear from this experiment that both the vehicle and route of injection are of importance in the development of the arthritic syndrome. Neither rats given tubercle bacilli in saline i.p. nor rats given both tubercle bacilli in saline i.p. and tubercle bacilli in oil i.d. developed arthritis. The mechanism of this reaction is not understood but work is being done to investigate this point and will form the subject of a further publication from these laboratories.

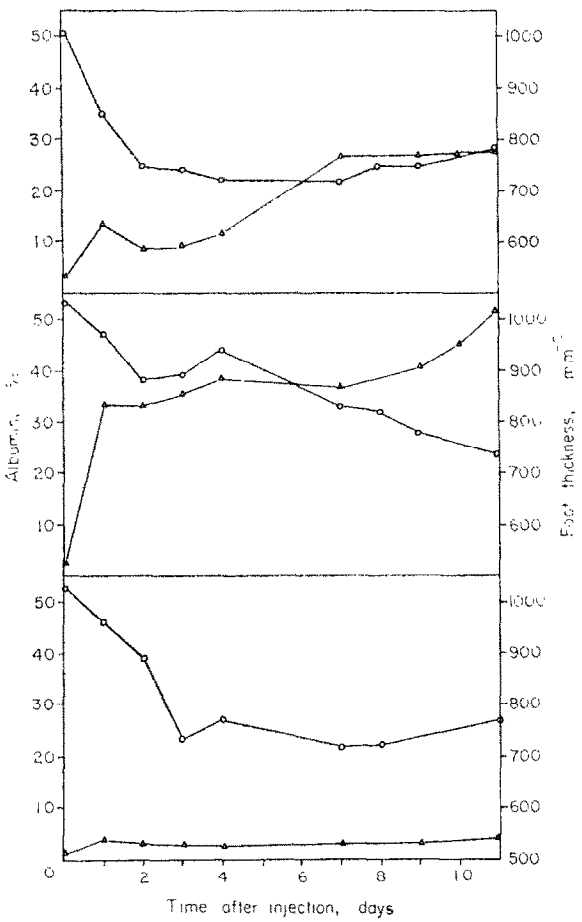

FIG. 6. Serum albumin levels (—○—○—) and thickness of injected foot (—△—△—) of rats given tubercle bacilli in various media by different routes. Bottom. Group I given tubercle bacilli (1·0 ml of 5 mg/ml) in saline i.p. Middle. Group II given tubercle bacilli in liquid paraffin (0·05 ml of 5 mg/ml) into the foot pad. Top. Group III given tubercle bacilli in saline (1·0 ml of 5 mg/ml) i.p. and tubercle bacilli in liquid paraffin (0·05 ml of 5 mg/ml) into the foot pad.

DISCUSSION

During the course of this work two papers have appeared on the starch gel electrophoresis of normal rat serum.[11, 12] The present work has revealed only minor differences from these two published accounts and these concern the post albumin components. In normal rat serum we found only two post-albumin bands regularly but

arthritic serum always contained three and on rare occasions four zones. Two of these are classified as α_2-globulins (bands 10 and 12) and one as α_1-globulin (band 11). Both Espinosa[11] and Beatson *et al.*[12] described four post-albumin zones; Espinosa classified these as α_1-globulins and Beaton *et al.*[12] designated two as α_2-and two α_1-globulins. It is not surprising that confusion arises in this area since conventional methods of zone electrophoresis produce poor resolution, moreover, a small difference in experimental method may result in a different distribution of the individual proteins. It may be preferable to term the zones 'α_1–α_2' globulins. Minor differences between the work from the three laboratories may also be due to strain differences between the animals used.

The slow α_2-globulin (band 3) seen in the starch gel electrophoretograms of sera from rats with secondary lesions has been observed in sera from pregnant, young and tumour-bearing rats,[13] from foetal and lactating rats, partially hepatectomized rats and rats bearing Walker 256 carcinomas[14] and also from rats injected with heavy metals such as cadmium, mercury, beryllium, copper and manganese.[15] What appears to be the same protein has recently been identified by Kelleher and Villee[16] in foetal but not in maternal rat serum thus confirming some earlier work by Darcy.[17, 18]

The electrophoretic patterns of sera from rats with fully developed adjuvant-induced arthritis are similar to those described by Paoletti, Riou and Truhaut[19] for serum from rats given intraperitoneal injections of trypan blue. They found a fall in serum albumin and fast α_1-globulin, increase in other α- and γ-globulins and post-albumins together with the appearance of the slow α_2-globulin. They commented that "some pre- or post-albumin fractions which are otherwise drowned in the albumins, may be individualized because of the disappearance of some of the albumin substances". Because of the known hepatic toxicity and carcinogenic properties of trypan blue, Paoletti *et al.*[19] argued that the abnormal serum protein pattern might be due to liver damage. However, in rats with adjuvant induced arthritis liver damage is negligible so that their argument is not tenable in this sutuation. A more plausible explanation for the appearance of the slow α_2-globulin is that advanced by Heim[14] who calls it "Reproduction Associated Protein" (R.A.P.) and points out that it appears in serum whenever large-scale synthesis of tissue or milk occurs. In adjuvant arthritis there is a synthesis of tissue, e.g. in the granuloma formation in the arthritic lesions.[4] This reasoning is supported by the fact that the slow α_2-globulin was never observed in rats with massive inflammation unless they exhibited arthritis.

The characteristic changes occurring in the adjuvant arthritic rat serum are the fall in serum albumin and appearance of aberrant globulins. It is interesting to compare these findings with those reported in human rheumatoid arthritis. Once again a decrease in serum albumin and an increase in the globulin fractions has been observed,[20] some of the globulins being associated with carbohydrate.[21] These changes are more marked as the severity of the disease increases and revert to normal during periods of remission. Shetlar, Shetlar, Payne, Neher and Swenson[22] have reported that an elevation of serum globulins and a lowering of serum albumin occurs during active phases of experimental arthritis in the pig. Once again, remission of the condition was accompanied by a reversal of the serum protein changes.

It is clear from these remarks that the serum changes occurring in the arthritic rat closely parallel those seen in arthritic humans and pigs. The significance of the fall in serum albumin is not understood but since it always accompanies acute phases of

inflammation it could be merely a reflection of the increase in capillary permeability. Further work is in progress to clarify this point. Moreover, as shown in the present work, decrease in albumin can occur without an arthritic condition, and in arthritis it probably only reflects the inflammation which accompanies the condition.

There have been no reports of a slow α_2-globulin appearing in the serum of human arthritics but the method usually employed (paper electrophoresis) could not detect it. On the other hand the serum of most patients with rheumatoid arthritis contain an unusual type of macroglobulin called rheumatoid factor. No similar protein was found in arthritic rat serum so perhaps the slow α_2-globulin and the protein of band two could be analogous proteins. However, slow α_2-globulin is probably associated with the granuloma formation which leaves only the protein of band two, which has a transient existence, as a possible relative of rheumatoid factor. There is at present no evidence for or against this idea.

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