

CHANGES PRODUCED IN MOUSE PLASMA PROTEINS BY ACUTE BACTERIAL INFECTIONS*

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An enormous literature has built up in recent years concerning the changes in plasma proteins of man and laboratory animals which correlate with a variety of disease states. The reviews of Leutscher (1) and more recently of Petermann (2) provide general discussions of the subject but leave one with the impression that while a great deal is known, little is understood. Perhaps one reason for this is that in themselves, groups of proteins such as α -, β -, and γ -globulins, euglobulins, macroglobulins, and even albumin have few intrinsic connotations other than their methods of preparation or detection. Many individual proteins, however, do have significance based on their known chemical or biological capacities. More recently individual constituents have been investigated and their variation correlated with pathological conditions.

Immunoelectrophoresis has proven very useful in detecting and following such variation. Since first applied by Williams and Grabar to abnormal sera from myeloma patients (3), its effectiveness has been expanded to the study of a large selection of anomalies and diseases (4).

Infectious diseases have not received such attention, however. Where attempts with human material have been made, reports have been inconclusive or, according to some, unimportant (5). Nevertheless, in view of published findings concerning the conventional electrophoretic profiles of plasma proteins in infectious disease, it seemed likely that immunoelectrophoresis would demonstrate some of the individual protein modifications. It also seemed that a productive approach would be the examination of carefully controlled experimental infections in laboratory animals before a study of human specimens, however carefully selected, from hospital wards. This report will deal with variations of immunoelectrophoretic patterns of mouse plasma induced by laboratory manipulation of normal samples and by treatment of mice with toxic and infectious agents.

Materials and Methods

Immunoelectrophoresis was performed on all samples in veronal buffer pH 8.2, 0.05 N, at 4 volts per cm as described elsewhere (3, 6). Because of the large number of samples to be

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analyzed and small size of individual samples, the micromethod of Scheidegger (7) was employed. Washed, dried slides were stained with bromphenol blue (8).

Antisera were prepared in rabbits by hyperimmunization over a period of 6 months. After an initial intramuscular injection of normal mouse plasma in Freund's adjuvant, each animal received at least 35 intravenous injections of 5 times diluted, pooled plasma. These were administered on 3 successive days in alternate weeks, until trial bleedings showed satisfactory complexity or until any improvement ceased to be significant. Sera from eight of ten original rabbits were collected. Terminal and late trial bleedings were pooled in each case but samples from different animals were not mixed. They were filtered and stored in ampules at 4°C with 0.01 per cent merthiolate. Some antisera were more suitable for general application in immunoelectrophoresis; some were deficient in antibody to constituents of particular interest. One might be especially valuable for the examination of one phenomenon, while another was better suited for the demonstration of another plasma modification. The illustrative material presented in this paper was prepared from results obtained with serum R-15-131-1. This antiserum contained antibody to most constituents studied but not to several α - and β -globulins with which they might be confused.

Mouse serum and plasma samples were obtained from the infraorbital sinus after rupture of the ophthalmic venous plexus. A description of this useful technique has been recently published by Riley (9). To obtain plasma, the mice were injected intravenously with 0.2 mg of sodium heparinate *v.s.p.* (Connaught) in 0.2 ml of physiological saline.

Mice employed for these experiments were Swiss albino, designated Nelson-Collins Swiss (NCS), from the colony at The Rockefeller Institute. Their origin, treatment and care, and some of their characteristics have been described by Dubos and Schaedler (10). Although considered completely normal with respect to state of health, it should be pointed out that their intestinal flora is profoundly different from commercially available laboratory animals, in that they are free of *Escherichia coli*, *Salmonella sp.*, and *Proteus vulgaris*.

Hemolytic titre of mouse complement was determined by serial dilution of samples in NaCl-veronal buffer supplemented with 0.005 M Mg^{++} and 0.0015 M Ca^{++} (11). Fresh sheep cells in Alsever's solution from Cappel Laboratories, Westchester, Pennsylvania, were sensitized in 500 times diluted rabbit hemolysin. 0.5 ml of an 8 per cent suspension of sensitized cells was added to tubes containing 0.5 ml of mouse plasma dilution. All tests were prepared in an ice bath, incubated 1 hour at 37°C, then chilled in the ice bath before rating. Ratings were recorded as $\pm(1/2)$ to 4 by increments of 1/2, 1 signifying a minimum frankly positive reaction.

Microbiologicals employed were as follows:—

Lipopolysaccharide (endotoxin) of *Serratia marcescens* was kindly supplied by Dr. Maurice Landy.

Whole, heat-killed *Serratia* organisms were washed and lyophilized after 15 hours growth in tryptone broth.

Zymoan, Lot 513171, insoluble yeast polysaccharide was procured from Fleishman Laboratories.

Diphtheria toxin-antitoxin specific precipitate was prepared with 1350 Lf units of crystalline toxin MS-770 from Dr. C. G. Pope and 1350 Lf units of horse antitoxic serum No. 15 from Dr. I. H. Lepow. Precipitate was washed 3 times with cold buffered saline.

Mycobacterium tuberculosis var. *bovis* (Vallee) was grown for 7 days in Dubos 1A medium with Tween. For infection of mice 0.2 ml of 5 times diluted culture were administered intravenously.

Micrococcus pyogenes var. *aureus* (Giorgio) was grown overnight in Penassay broth and diluted 3 times with saline for intravenous injection.

RESULTS

A reference pattern for normal mouse plasma (NMP) is shown in Fig. 1 *a*. From several antisera at our disposal, each of which gave somewhat different patterns because of differing antibody constitution, antiserum R-15-131-1 was chosen for most of the later studies. All of the earlier experiments were repeated using this antiserum. The conditions for obtaining this pattern were carefully controlled particularly with respect to quantity of reactants employed, development time, and staining procedure.

Certain precipitation lines were selected by the experimental conditions. After suitable identification they were labeled only if:

1. They were modified in some way by the conditions of the experiment, or the treatment of the experimental animals.
2. They were reactions of components about which something is known other than their mere presence in an immunoelectrophoretic pattern, and which, from reports in the literature, might be expected to be altered by the treatment of the materials.
3. They would serve as orientation points for the identification and detection of alterations in those reactions fulfilling requirements 1 or 2.

Plasma constituents in the first category will be dealt with specifically in the presentation of the results. These include albumin (*A*), α_1 -*vc*, α_2 -*vm*, fibrinogen (ϕ), and β_1 -*C* and β_1 -*D* on the normal pattern; haptoglobin (α_2 -*H*) and γ -*X* on pathological patterns. The other constituents labeled on the reference pattern, α_2 -macroglobulin (α_2 -*M*), transferrin (*T*), β_2 -macroglobulin (β_2 -*M*) and γ -globulin fulfill requirements for categories 2 and 3.

β_1 -*C* shows remarkable variation depending on whether the sample is taken as serum or plasma, and how long and at what temperature the sample is allowed to age before immunoelectrophoretic analysis. Quite frequently in serum, particularly if it is allowed to stand in the presence of a poorly formed or fragmented clot before separation, β_1 -*C* becomes bimodal in electrophoretic distribution with a second concentration maximum in the β_2 region of the pattern. This component has been named β_1 -*D*. For this reason plasma was used for most experiments. This conversion occurs also in plasma standing at room temperature for 4 hours or overnight at 4°C producing the pattern seen in Fig. 1 *b*. Normal β_1 -*C* precipitation arcs are obtained in nearly all cases, however, in which the experimental conditions permit the analysis of the sample shortly after being drawn from the mouse. Müller-Eberhard has described the conversion of β_1 -*C* to β_1 -*A*, in human serum, β_1 -*A* having a higher mobility than β_1 -*C* (12), and Clausen and Heremans (13) have reported that the mouse homologue, which was consistently bimodal in their experiments, cross-reacted with human β_1 -*C*. Müller-Eberhard has reported purified human β_1 -*C* to be associated with the activity of a subcomponent of *C*'₃ in hemolytic systems

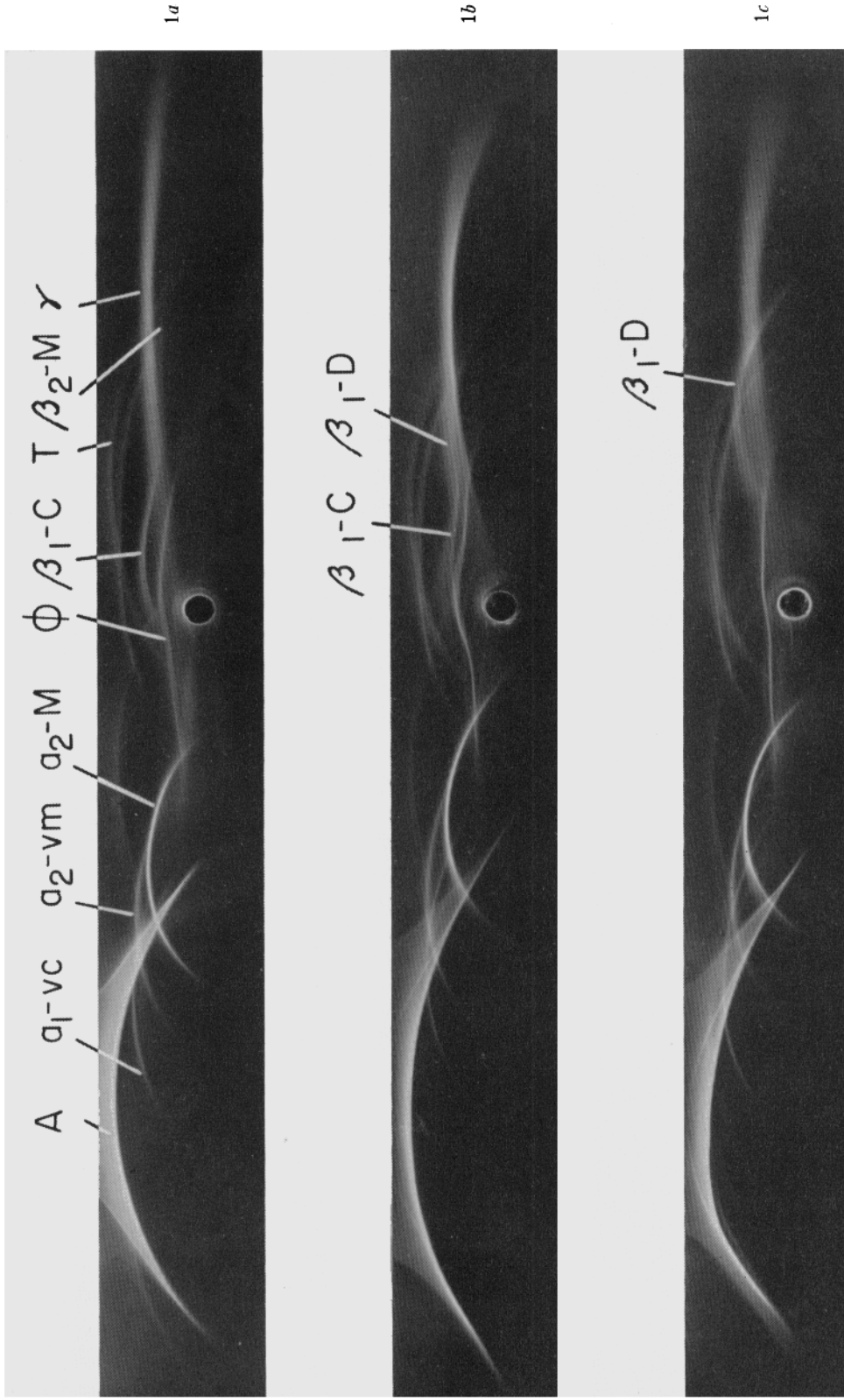


FIG. 1. Immunoelectrophoretic patterns of normal mouse plasma. FIG. 1 a, reference pattern for freshly drawn plasma; β_1 -C appears as single component; FIG. 1 b, various treatments effect a partial dissociation of β_1 -C into two components, β_1 -D migrating with a lower mobility; FIG. 1 c, aging or more severe treatment effects total conversion to β_1 -D.

(14). We have performed various experiments to demonstrate the lability of β_1-C in mouse plasma. These provide evidence that mouse β_1-C is affected by physical or chemical treatments in the same way as its human serum counterparts and, circumstantially, as C' activity.

Effect of Addition of Gram-Negative Lipopolysaccharide to Serum.—One ml of pooled normal mouse serum, which showed only slight conversion of β_1-C , was treated with 0.5 mg of *Serratia* lipopolysaccharide. After standing at room temperature 20 minutes the sample was centrifuged and subjected to electrophoretic analysis on the same slide with the untreated control sample. Identical experimental samples were prepared using 0.5, 1.0, and 2.0 mg of whole, heat-killed, lyophilized *Serratia* organisms. The *Serratia* lipopolysaccharide and the 0.5 mg of *Serratia* organisms produced a partial conversion of β_1-C , while 1.0 and 2.0 mg of whole killed *Serratia* produced patterns showing only β_1-D similar to Fig. 1 c.

Effect of Gram-Negative Lipopolysaccharide in Vivo.—Eight mice were bled for normal serum samples, after which six received by the intravenous route 1.0 mg *Serratia* lipopolysaccharide in 0.2 ml saline, two mice receiving only saline. After 150 minutes all mice were bled a second time. First and second samples were analyzed on the same slide by immunoelectrophoresis. Five additional mice received 1.0 mg of *Serratia* organisms following the control bleeding. The sera of the two mice which received saline were unchanged by the experimental procedure, and all control samples contained clear β_1-C with a small conversion component. Of the six samples from mice which had received lipopolysaccharide, four showed no trace of β_1-C or β_1-D , two a suggestion of β_1-D . Three of the five samples from mice which had received whole organisms were not significantly altered, two appeared devoid of β_1-C or β_1-D .

Absorption by Specific Immune Precipitate.—Diphtheria toxin-antitoxin precipitate was added to 0.5 ml samples of fresh pooled mouse plasma. A control sample and three experimental samples treated with 0.03, 0.1, and 0.3 ml packed precipitate respectively were incubated 1 hour at 37°C. Immunoelectrophoresis demonstrated slight conversion of β_1-C in the control but complete dissociation to β_1-D by 0.03 ml of specific precipitate. In the sample absorbed with 0.3 ml only a faint trace of β_1-D remained.

Absorption with Zymozan.—0.4, 2.0, and 4.0 mg of zymozan were used to absorb 1 ml samples of the normal plasma pool used for the specific precipitate absorption. Immunoelectrophoretic analysis revealed that the 4 mg/ml concentration effected complete conversion.

Fig. 2 illustrates and summarizes some of the results from experiments on the conversion of β_1-C to β_1-D . Table I summarizes the results of experiments attempting to relate hemolytic complement titres of samples subjected to these treatments to the assumption that β_1-C is involved with C' activity. It is important to note that the hemolytic titre of mouse plasma is typically quite

low in the sheep erythrocyte-rabbit hemolysin system. β_1 -C and/or β_1 -D is present in substantial quantity in mouse plasma, as indicated by the relatively strong and rapidly developing line of precipitate in gels. If β_1 -C is associated with the complement system it would not seem that the hemolytic titer should

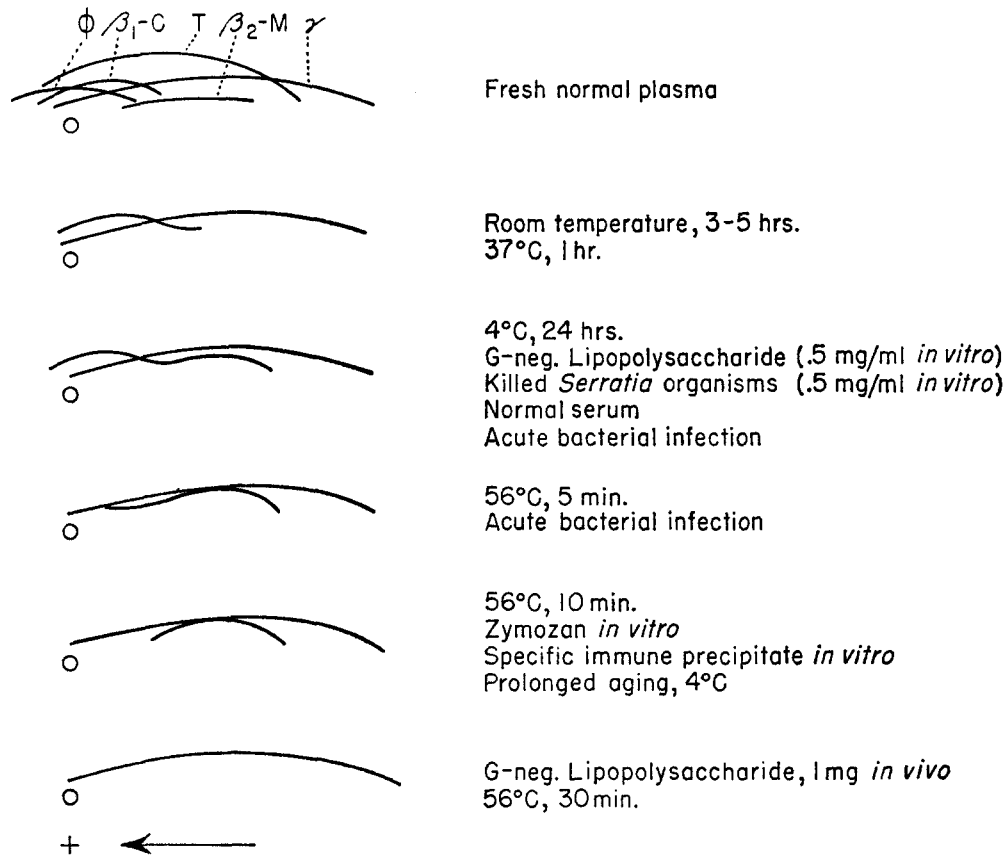


FIG. 2. Progressive alteration of β_1 -C. Diagrams illustrate typical patterns observed for treatments imposed on samples or on mice before samples are taken.

be quantitatively limited by this component. The hemolytic titer of mouse plasma is reduced slightly merely by incubation for 1 hour at 37°C, and virtually eliminated by standing overnight even at 4°C, neither treatment producing complete conversion of β_1 -C to β_1 -D. The table indicates that only the larger amount of specific precipitate reduces hemolytic activity below a titer of 10, at which time only traces of β_1 -D are detectable by immunoelectrophoresis. Absorption with lesser quantities of specific precipitate leads to nearly complete conversion of β_1 -C to β_1 -D but does not eliminate hemolytic activity. If mouse

β_1 -C is associated with C' activity, as its human homologue appears to be, then it is active in amounts undetectable by immunoelectrophoresis, or β_1 -D, its conversion product, is active as well. Short of the isolation of the protein it is unlikely that this question can be resolved. Considering its extreme lability this also could prove difficult.

Experimental tuberculous infections in mice might be expected to induce changes in plasma proteins which immunoelectrophoresis would detect. Twenty 6-week-old male mice were bled for normal plasma samples 2 days before infection with bovine *M. tuberculosis*. Subsequent samples were taken at weekly intervals up to 4 weeks. Fig. 3 presents the results typical for this experiment. Twelve mice survived for the 3-week sample, six for the final sample. One week after challenge an α_2 globulin is present in appreciable quantity which is not

TABLE I
Hemolytic Activity of Mouse Plasma

NMP dilution		10	20	40	80	160
NMP	—	2½	1½	1	±	
	1 hr. 37°	2½	1½	1-		
NMP + specific precipitate	0.06 ml/ml	2	1½	1		
	0.2 ml/ml	1½	1			
	0.6 ml/ml	±				
NMP + zymozan	0.4 mg/ml	2	1½	1		
	2.0 mg/ml	2	1			
	4.0 mg/ml	1½				

detectable in normal plasma. By staining with benzidine-peroxide reagent (15) we were able to show that this component was haptoglobin (α_2 -H). In all mice β_1 -C was partially dissociated to β_1 -D and both components seemed elevated. At 3 weeks all infected animals showed a marked increase in γ -globulin levels and the presence of a low mobility γ -globulin. This latter component is tentatively designated γ -X. Schultze, who has described the component in human serum, has identified it as C-reactive protein (CRP) (16).

Staphylococcal infections induce the same changes outlined for plasma from tubercular mice. Acute staphylococcal infections have been more thoroughly examined since the effects are produced more rapidly, and since fewer special precautions are necessary during the manipulations. A series of patterns could be presented here which would appear identical to Fig. 3. Such an experiment was performed concurrently with the tuberculosis experiment. The difference seemed to be only one of time, the α_2 -H being detectable the 1st and 2nd day following infection and the increased γ -globulin along with the appearance of γ -X on the 5th or 6th day.

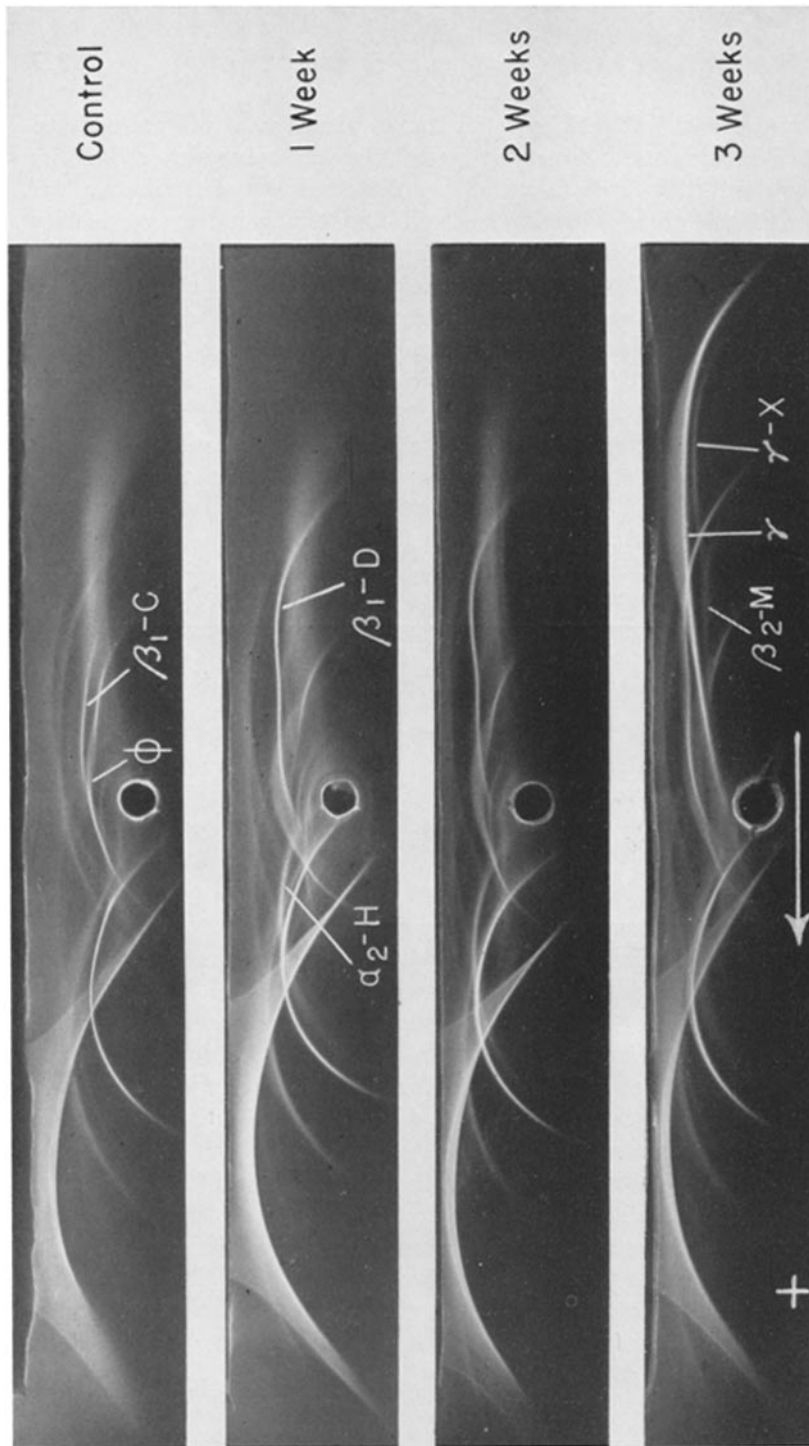


FIG. 3. Immunoelectrophoretic patterns of plasma samples from a single mouse before (control) and during (1, 2, and 3 weeks) infection with *M. tuberculosis*.

We have tried several experiments to demonstrate CRP activity of γ - X and other aberrant proteins, all without success. No constituent is depleted by absorption with whole pneumococci, even in massive amounts. Nor is a precipitin line developed by anti-human CRP sera from sheep, cat or rabbit or by sheep anti-rabbit CRP. There is no evidence therefore that mouse γ - X is C-reactive or that it is even the homologue of human γ - X described by Schultze.

Fig. 4 *a* is a comparison of a normal plasma pool with a pool from infected mice. Twelve 6-week-old females were infected with 3×10^8 staphylococci by the intravenous route. Samples were taken 4 days later and examined individually as well as pooled. Although there was individual variation in response to the infection, most samples gave patterns similar to the pool illustrated in Fig. 4 *a*. Albumin (*A*) has a distinctly higher mobility. An α_1 -globulin, which in plasma from infected mice becomes faint or undetectable, is labeled α_1 -*vc* (variable concentration) in the pattern of the normal pool. This component is one in which there was considerable individual variation even among normal mice. An α_2 -globulin also acquires a higher mobility and is labeled α_2 -*vm* (variable mobility).¹ Fibrinogen (ϕ) is seen with a β_2 component, and β_1 -*C* is largely converted to β_1 -*D*. Haptoglobin (α_2 -*H*) is moderately strong.

In the series for the mouse infected with *M. tuberculosis* illustrated in Fig. 3 α_2 -*H* appears less important in the 3 week sample. It was later discovered, however, that the antibody which developed α_2 -*H* was antihemoglobin and that the density, indeed the detection of the haptoglobin depended upon the amount of bound hemoglobin. Frequently, even with acute phase samples, no haptoglobin line developed. If such a sample were supplemented with 0.05 volume of a 1:4 erythrocyte lysate, haptoglobin could always be detected if present. Fig. 4 *b* shows normal and infected samples thus handled. In the normal pattern the hemoglobin reaction is developed but not the haptoglobin line. The pathological pattern, on the other hand, shows both components in a reaction of immunochemical identity indicating that the antibody responsible is indeed specific for a lysate protein bound to the α_2 -*H* component. This slide was stained with benzidine-peroxide reagent for the peroxidase activity characteristic of hemoglobin.

The distribution of protein concentrations observed by direct staining with bromphenol blue or azocarmine immediately after electrophoresis indicated a considerable increase in all globulin fractions but γ , and a significant decrease in albumin (Fig. 4 *c*). The distribution of lipids stained with oil-red (15) was not significantly altered although the concentration in the α_1 region was somewhat depleted (Fig. 4 *d*). Some individuals did not show such marked increase in globulin or decrease in albumin. Some appeared perfectly normal with respect

¹ α_1 -*vc* has two distinct mobility expressions in normal mice, consequently a mobility change in this protein unless controlled with a normal sample from the same mouse would be meaningless. α_2 -*vm* has, so far as we can ascertain, quite uniform mobility in the normal.

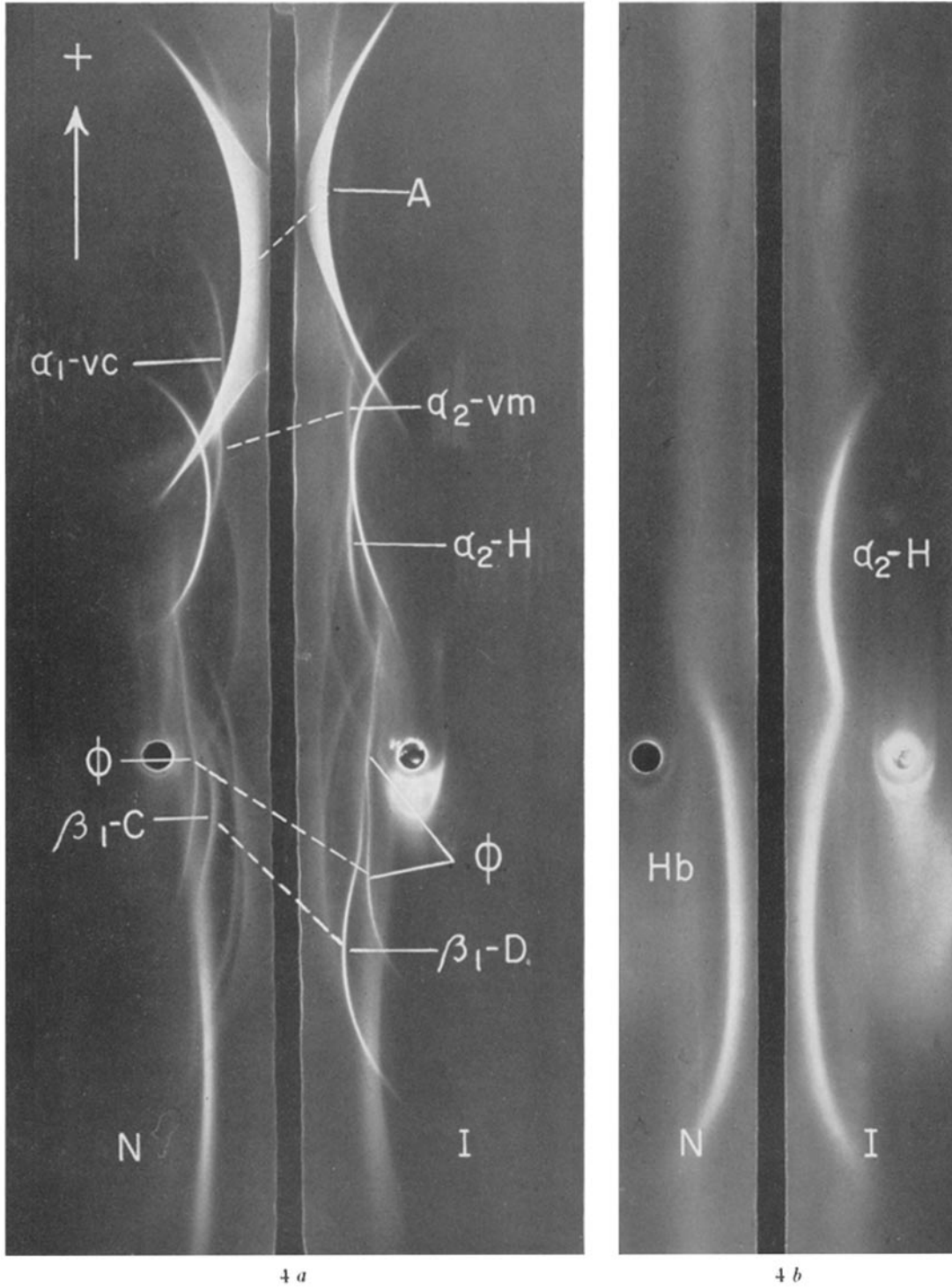


FIG. 4. Comparative study of plasma protein modifications resulting from acute staphylococcus infection. FIG. 4 a, immunoelectrophoretic patterns of normal (N) and infected (I) plasma pools; FIG. 4 b, immunoelectrophoretic pattern obtained by the addition of hemoglobin to plasma samples. The slide was stained with benzidine-peroxide reagent to detect hemoglobin and haptoglobin-hemoglobin reaction lines.

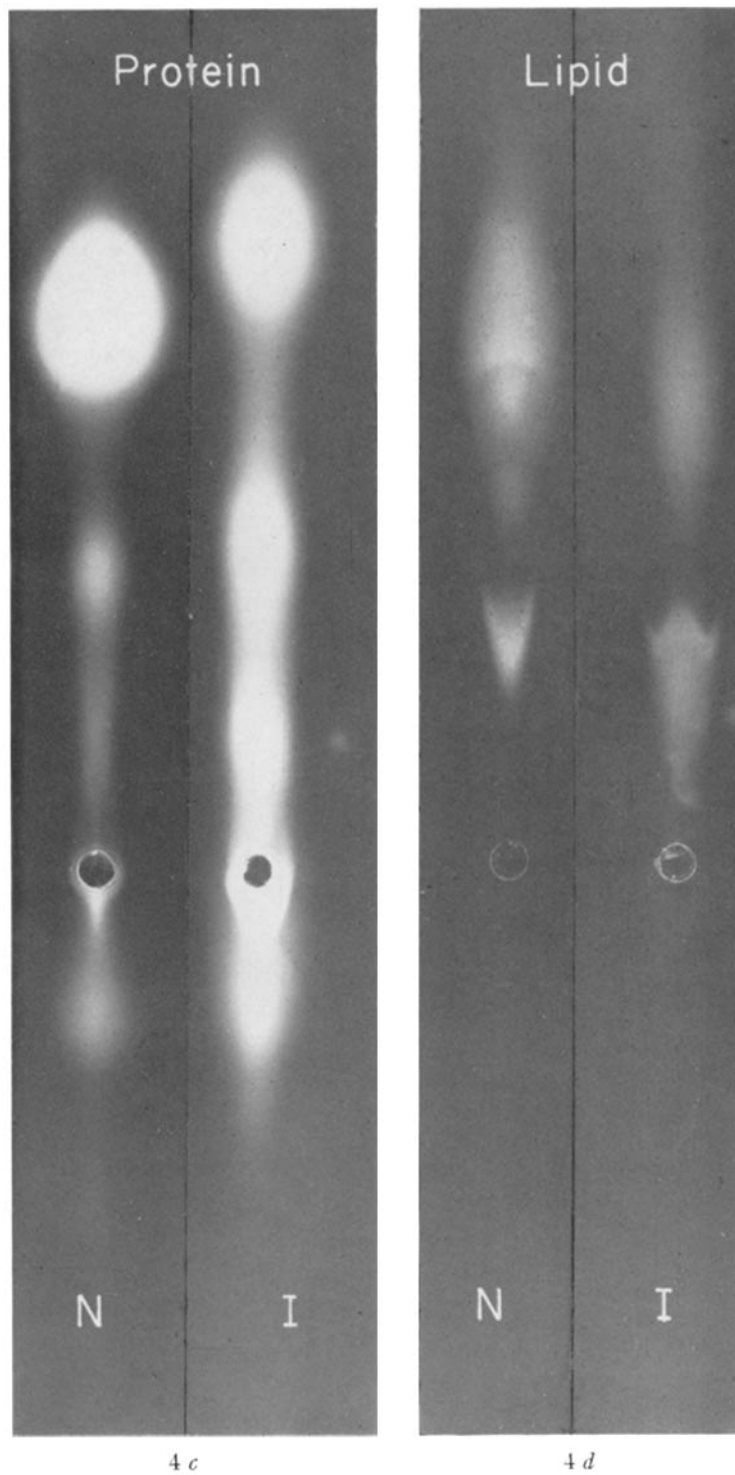


FIG. 4 *c*, slide stained for protein distribution in plasma from normal and infected mice. FIG. 4 *d*, slide stained with oil-red-O for distribution of lipids. See text.

to protein distribution. In some individual samples there was a marked depletion of lipid staining material in the α_2 and α_1 regions and an increase in the ρ zone.² Since we did not have pre-infection samples from these individuals, however, it was not possible to determine whether this effect was characteristic of the infectious state.

DISCUSSION

Changes Related to Experimental Infections.—Numerous investigators have studied human pathological specimens by moving boundary and zone electrophoresis and have reported many variations from normal serum patterns. It is impossible to relate their findings to those presented here because of differences in species and variations of technique. Seibert *et al.* (18) have examined scores of tuberculosis sera. Other findings have been reviewed recently by Petermann (2). One fact upon which there is general agreement is that in acute infections globulin concentrations go up while albumin concentrations go down. One of the more sensitive globulin regions is the α zone where both α_1 and α_2 components have been reported to vary in absolute concentration with different disease states. These changes may be due to lipoproteins, glycoproteins, ceruloplasmin, haptoglobins, and in many cases otherwise unidentified proteins.

We can offer no explanation for our observation of a higher mobility of albumin in acute staphylococcal infection and the similar shift of α_2 -*vm* (an otherwise unidentified α -globulin). Such shifts do not occur simply upon dilution of plasma even to 1/16. Mobility changes do introduce complexity into the interpretation of simple increase of protein concentration in the α_1 zone. Albumin moving out could leave the impression of increased α_1 , and α_2 moving in could increase such an impression, unless some means other than protein estimation were available for the positive identification of α_1 globulins. The same questions could be raised in the case of the β_1 -C \rightarrow β_1 -D conversion in the mouse and of the β_1 -C \rightarrow β_1 -A conversion in human. Müller Eberhard (12) estimates β_1 -C in human serum to constitute 0.5 per cent of total proteins so that in this system, the conversion might not loom very significant in terms of total protein profile. While it appears that β_1 -C and/or β_1 -D is more concentrated in mice in acute phase plasma, no estimate has been attempted.

The elevation of haptoglobin in plasma from mice with acute infections could be significant in this regard. Although its absolute concentration could not be determined from immunoelectrophoretic studies, and although there are considerable mobility shifts observed in the constituents of neighboring mobili-

² The greek letter ρ is used to designate the electrophoretic zone occupied by components migrating more rapidly than albumin. It was introduced by Williams (17) and Williams and Grabar (3) to conform with the Greek letter system for electrophoretic classifications of plasma proteins. We feel it is greatly to be preferred over the loose and ambiguous term "pre-albumin."

ties, it is clear that the appearance of α_2-H could change the α profile in almost any quantitative electrophoretic study. This is particularly true when it is considered that α_2-H is undetectable in the plasma from normal 6- to 8-week-old mice.

The appearance of α_2-H and the disappearance of $\alpha_1-\nu$ are of considerable theoretical interest. It is well known that haptoglobin fluctuates with toxic and infectious stress (19) and it is tempting to propose mechanisms which would explain the fluctuations in terms of a disease process. Study of the phenomenon in laboratory animals, however, would seem to be a necessary starting point. If α_2-H represents a protein synthesized by a tissue or cell type suddenly stimulated by infectious or toxic agents, the location of the site of its formation might provide some valuable clues to the host-parasite relationship. It may be pertinent to mention that mice treated with Gram-negative endotoxins also showed marked elevation of haptoglobin one day following intravenous injection.

In the case of $\alpha_1-\nu$, several possibilities might be investigated to elucidate its depletion or disappearance from pathological samples:

1. It is a protein with a high turnover rate and the tissues or cells responsible for its elaboration are gravely affected by the infection.
 2. It is removed from the circulation by affected tissues or some serological reaction.
 3. It is converted into some form which does not precipitate the antibody.
- Experiments are in progress to determine the tissue of origin for α_2-H , $\alpha_1-\nu$ and $\gamma-X$.

NCS mice have very low γ -globulin levels, perhaps due to the very limited intestinal flora and to the strict methods employed to control infection in the colony. Although we have not as yet investigated the rise in γ -globulin during infections in other mice it may well be that the increase would be less spectacular. In NCS mice we estimate an increase of nearly fourfold. In mice raised under less protective conditions, showing normally higher γ -globulin levels, the same absolute increase would represent considerably less proportional rise.

Preliminary studies on samples from human patients covering a variety of bacterial and metabolic diseases were begun in order to determine which of the alterations observed in the mouse would be most interesting to pursue further. To date, the number of samples analyzed from each type of infection or disease is insufficient to provide a basis for correlation. However, most of the changes described for the mouse have been detected in human patients.

Haptoglobin, though usually present in detectable quantities in the normal adult human, is demonstrably elevated in certain bacterial infections. β_1-C is frequently seen partially converted or dissociated to β_1-A in freshly drawn serum from patients, but was found normal in several lung involvements including Friedlander and pneumococcal pneumonia.

Certain α -globulins in human serum other than haptoglobulin are known to be genetically variable (20) and also variable in susceptibility to concentration and mobility changes in disease states (2). Because of this, and since normal samples from individual patients were not available, no attempt has been made to find a human analogue for α_1 -*vc* in the mouse.

SUMMARY

The immunoelectrophoretic patterns of plasma proteins from mice are altered significantly by acute infections. Some proteins are dissociated into two or more components, some showed striking increase in plasma concentration, others are depleted, and certain ones appear which are undetectable in normal samples.

β_1 -C dissociated into two electrophoretic components under a variety of conditions in addition to infections. Endotoxins and killed organisms *in vivo*, and specific precipitate absorption, heat and aging *in vitro* produced this change. Endotoxins injected into mice also induced a rise in haptoglobin though not as sharply or predictably as acute infection.

Preliminary results with samples from hospital patients with acute diseases are discussed. It was concluded that study of experimental diseases in laboratory animals by these techniques could provide a fruitful basis for the investigation of the plasma protein changes in similar human diseases.

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