PLASMA PROTEIN FORMATION IN VITRO BY TISSUES FROM MICE INFECTED WITH STAPHYLOCOCCI*

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Numerous changes have been observed in human serum proteins in the course of infections. The most consistent of these appear to be decreased albumin levels and elevation of α_2- and γ-globulins (1–3). Increased levels of serum glycoproteins have been reported in chronic infectious diseases (4). One of the α_2-glycoproteins which is most markedly increased in pneumonia and other acute inflammatory disorders is haptoglobin (5). In many of these conditions, the increase in glycoproteins is accompanied by the appearance of the acute phase protein, C-reactive protein (6).

Experimentally induced infections in animals afford a more controlled means of studying such plasma protein changes. Immunoelectrophoretic analysis of plasma samples taken from mice in the course of experimental bacterial infection have revealed several qualitative and quantitative changes in plasma proteins, among which was the appearance of 3 constituents not detected in the plasma prior to infection (7).

A method developed recently, employing autoradiography of immunoelectrophoretic patterns, has permitted the study of sites of synthesis of various plasma proteins (8, 9). Use is made of the fact that some tissues will produce isotope-labeled plasma proteins when cultured *in vitro* in a medium containing C^{14}-labeled amino acids. In the present investigation this method has been used to study plasma protein synthesis by tissues from mice with staphylococcal infections, in an attempt to determine the source of those proteins in which changes were observed.

Materials and Methods

Mice.—Rockefeller NCS female mice were obtained from the colony of The Rockefeller Institute at 6 to 8 weeks of age. The mice are maintained "specific-pathogen-free," and it has

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been reported that their intestinal flora is comprised largely of lactobacilli (10). Such mice have low serum levels of γ-globulin and most have no detectable haptoglobin. All mice receiving staphylococci were infected within two days after removal from the colony.

Micrococcus pyogenes var. *aureus* (Giorgio) was grown 18 to 20 hours at 37°C in penassay broth. Such cultures contained between 2 to 2.5 × 10^8 viable units per ml. Mice were challenged by the intravenous route with 0.2 ml of an appropriate saline dilution. The dosage in viable units (v.u.) per mouse is given in the description of the specific experiments.

Mouse Plasma.—Mice were heparinized (0.2 mg iv) 15 minutes prior to sampling. Plasma samples were separated from blood drawn from the retro-orbital plexus. Serum samples were obtained at the time the animals were killed. All mice were sampled the day before infection. Mice selected for the experiments were required to have had normal IEA patterns prior to infection and, subsequently, patterns typical for their stage of infection 1 day before their tissues were cultured.

Tissue Cultures.—Organs to be cultured were removed under sterile conditions. Spleens from 2 to 3 animals were pooled; livers were cultured individually, except in the first of the three reported experiments, for which livers of 3 animals were pooled. Spleens were minced and weighed amounts (approximately 50 mg) were cultured in roller tubes with 2 ml of medium as described previously (8). Liver slices were prepared with a Stadie slicer, and about 500 mg were cultured for 5 to 6 hours in 3 ml of medium in Ehrlenmeyer flasks under an atmosphere of 95 per cent O_2 and 5 per cent CO_2 (11). The medium consisted of Hanks' balanced salt solution, to which were added 0.5 per cent ovalbumin, glucose (to 22 mM), mixtures of vitamins (12), penicillin (200 u/ml) and amino acids (13), from which lysine and isoleucine had been omitted. Uniformly labeled C^{14}-L-lysine (605 μc/mg) and C^{14}-L-isoleucine (675 μc/mg), obtained from the Institut Pasteur, were added to a concentration of 1 μc/ml each. All cultures were preincubated for ½ hour in the medium lacking these amino acids, in order to dilute non-labeled lysine and isoleucine in the tissue. In one of the 3 experiments (Experiment II), streptomycin was added to the medium to a concentration of 10 μg/ml. After the culture period, media were frozen with the tissues, thawed once, and centrifuged at 10,000 *g* for 20 minutes. Supernatants were dialyzed against 2 changes of 0.015 M NaCl for 48 hours, and concentrated 20-fold by lyophilization. In one experiment, the liver tissue was homogenized in 0.15 M NaCl with a glass homogenizer, and the supernatant fluid concentrated 20-fold as above.

Antisera.—Antisera were prepared in rabbits as described in a previous publication (7, 8). Three of the most potent antisera were selected for this study, R-1, R-3, and JJI.

Immunoelectrophoresis.—Microelectrophoresis was carried out in 1.5 per cent agar in 0.038 M veronal buffer, pH 8.2, at 5 volts/cm for a period of 90 minutes. Antibody diffusion was allowed to continue for 24 hours at 5°C. Slides were washed free of unreacted protein, dried, and stained with bromphenol blue. In the acid range, this dye was considered to provide better images for photoenlargement than amido black.

The culture medium concentrates were applied 3 times (2.5 μl) to the antigen wells, allowing adequate time between fillings for all fluid to be imbibed by the agar gel. When serum carrier was used, one filling of serum from either normal or infected animals was employed prior to addition of culture fluids to the antigen well. For better visualization of certain precipitation lines, carrier serum was used both undiluted and 5 times diluted. Patterns for all samples were developed with each of the three antisera described, and in many cases supplementary analyses were made with 3 times diluted antisera. Antisera R-1 and R-3 contained no detectable antihaptoglobin, but both contained antihemoglobin. If both the medium concentrate and the carrier serum were low in hemoglobin, one filling with a dilute mouse erythrocyte lysate (approximately 0.2 per cent hemoglobin) allowed the visualization of haptoglobin in the IEA patterns when present.

Hemoglobin, haptoglobin with bound hemoglobin, and other components with peroxidase activity were identified by staining with a benzidine-peroxide reagent (14).

Autoradiography.—Kodak royal pan film was exposed for 2 weeks in contact with the dried, unstained IEA slides. Images were developed for 4 minutes at room temperature in Kodak DK60a developer.

Histology.—Representative pieces of cultured tissues were fixed for 5 hours in a mixture of Zenker's solution 90 per cent, and neutral formalin 10 per cent. Sections were stained with methyl green–pyronin (15).

RESULTS

Table I shows the cumulative mortality data for groups of ten mice infected intravenously with four different doses of staphylococci. It is seen that 4.5×10^7 v.u. per mouse resulted in the early death of more than half the animals. Early mortality dropped very rapidly within a very narrow dose range. At doses below 2.5×10^7 v.u. death was occasional but erratic with respect to time; at 1×10^7 v.u. mortality was rare.

TABLE I

Cumulative Deaths in Groups of 10 Mice Challenged with Staphylococci (iv)

Group	Dose	4 days	10 days
A	4.5×10^7	6	6
B	3.6×10^7	3	*
C	3.0×10^7	1	3
D	2.5×10^7	0	2

* Survivors in this group were used for experimental purposes at 4 days (*cf.* Experiment III).

Experiment I.—A group of ten mice were challenged with 2×10^7 v.u. Control animals received 0.2 ml of sterile saline. At 4 and 10 days post-challenge, livers and spleens of three infected animals were pooled for tissue culture. Mesenteric lymph nodes from four animals were used. For culture of control tissues the organs of four non-infected animals were pooled.

The results of the examination of patterns of all tissues in all combinations of antisera and carriers are recorded in Table II. Analyses of homogenates prepared from liver slices after culture showed a distribution of protein labeling similar to the corresponding culture fluids. The amount of labeled serum protein in the homogenates, however, was considerably less than in the medium concentrates. The results recorded, therefore, are those obtained with medium concentrates.

Experiment II.—Three groups of five mice were challenged with 2×10^7 v.u., 1×10^7 v.u., and sterile saline, respectively. Tissues from mice receiving the higher dose were cultured on the 4th day, and tissues from the other groups on the 11th day after injection. Spleen, lymph node, and heart tissues from

TABLE II

*Summarized Observations of Plasma Proteins Labeled by Mouse Tissues in Vitro**
Experiment I

Serum protein‡	Pooled spleen			Pooled liver			Pooled lymph node		
	C	4 days	10 days	C	4 days	10 days	C	4 days	10 days
ρ(nid)	+	+	+						
ρ-α_1				+	++	++			
A				+	+	++			
α_2(nid)						+			
α_2-vm§	+		+			+			
α_2-H	++	+	++	tr	+	++			
α_2-M	++	++	++	tr	+	++	+	tr	+
β_1-I					+	++			
β_1-C-D	+	+	++	tr	+	++	+		+
T				tr	+	++			
β_2-M	+	+	+						
γ_1			+				+	tr	
γ_2	+	+	+++			+	+	+	+
γ_3	tr	+	++						
SLC					+	+			

* tr = trace or questionable labeling. +, ++, +++ = relative degree of C^{14} labeling of IEA precipitates.

‡ Key to nomenclature.

ρ(nid) = unidentified ρ component.
ρ-α_1 = α_1 with a rapid (ρ) component produced during infection.
A = albumin.
α_2(nid) = unidentified α_2.
α_2-vm = α_2 whose mobility appears to increase during infections (7).
α_2-H = haptoglobin with bound hemoglobin.
α_2-M = α_2-macroglobulin.
β_1-I = β_1 shown to have peroxidase activity.
β_1-C-D = β_1-C thought to be subcomponent of C′-3 system (7). Considered mouse homologue of human β_1-A-C.
T = transferrin.
β_2-M = β_2 macroglobulin (γ_1-M).
γ_1 = rapidly migrating γ-globulin showing partial-identity spur with γ_2.
γ_2 = type γ-globulin with intermediate to broad range mobility.
γ_3 = slow migrating γ-globulin which appears during infections (originally designated γ-X (7)).
SLC = slow liver component labeled by liver *in vitro* and produced in quantity during infection.

§ Identification of labeled α_2-vm is tentative.

two mice in each group were pooled. Liver slices from two mice in the respective groups were cultured separately. The significant results of this experiment are recorded in Table III.

Experiment III.—Ten mice were challenged with 3.6×10^7 v.u. Spleens and

livers were cultured from two animals at 3 and 11 days following infection. As in Experiment II, spleen tissues were pooled and liver slices were cultured separately. No tissues from non-infected mice were cultured. The results are recorded in Table IV.

Of the proteins labeled by the spleen, those which showed increased labeling following intravenous staphylococcal infection were β_1-C-D and the immune globulins. γ_2 and β_2-M were formed by normal spleen, as demonstrated in a previous report (8). 5 to 10 days after infection, γ_2 and γ_3 concentrations were increased in the circulation (7) and it is illustrated in Fig. 1 that their formation

TABLE III

*Summarized Observation of Plasma Proteins Labeled by Mouse Tissues in Vitro**

Experiment II

Serum protein‡	Pooled spleen			Liver					
	C	4 days	11 days	C		4 days		11 days	
				Mouse 1	Mouse 2	Mouse 1	Mouse 2	Mouse 1	Mouse 2
ρ(nid)	+	+	+						
ρ-α_1						+	+		
A				+	+	++	++	++	++
α_2-H	+	++	+			+	+	tr	tr
α_2-M	+	+	+				tr		
β_1-I						+	+		
β_1-C-D		+	+				+		
T				+		+	++	+	+
β_2-M	+	++	+						
γ_1		+	+						
γ_2	+	+++	++					+	
γ_3									
SLC						+	++		

* See footnote*, Table II.

‡ See footnote‡, Table II.

by the spleens of infected animals taken during this stage was indeed strikingly increased. γ_1, a globulin which appears to cross-react with γ_2, also became evident on the autoradiographs. Other proteins formed by the spleen (α_2-M, haptoglobin-hemoglobin complex, and an unidentified ρ), appeared similar in control and infected mice. Transferrin, which was found labeled by spleen in earlier experiments using different strains of mice (8, 16), was not labeled by the spleens of NCS mice in the present experiments.

Differences in plasma protein production between mesenteric lymph nodes from infected and from normal mice were not observed. The intravenous route of infection with staphylococci apparently affected immune globulin formation in the spleen much more than in the lymph nodes.

Perhaps the most interesting observations in these experiments concern the striking increase in labeling of many plasma proteins in cultures of liver tissue from infected as compared to control animals.

Livers from control animals showed weak but consistent labeling of albumin and transferrin, and variable labeling of ρ-α_1, α_2-M, α_2-H, β_1-C and β_1-I. Proteins which showed the most marked increase in labeling in culture fluids

TABLE IV

*Summarized Observations of Plasma Proteins Labeled by Mouse Tissues in Vitro**

Experiment III

Serum protein‡	Pooled spleen		Liver			
	3 days	11 days	3 days		11 days	
			Mouse 1	Mouse 2	Mouse 1	Mouse 2
ρ(nid)	+	+				
ρ-α_1			++	++		+
A			++	++	+	++
α_2-H	+	++	++	++	tr	+
α_2-M	+	+	++	++	tr	+
α_2(nid)			+	+		
β_1-I			++	++		+
β_1-C-D	+	+	++	++	+	+
T			++	++	tr	+
β_2-M	+++	++				
γ_1		+				
γ_2	++	+++			+	+
γ_3		+				
SLC			++	++	+	++

* See footnote*, Table II.

‡ See footnote‡, Table II.

from infected mouse livers were ρ-α_1, α_2-H, α_2-M and β_1-C. A less pronounced increase was seen in albumin, transferrin, and β_1-I. A slow-migrating protein, designated SLC, which cross-reacts with β_1-I, was detected only in cultures of livers from infected animals. It was shown that the β_1-I-SLC complex has marked peroxidase activity. γ-globulin formation was present only in cultures of livers taken late after infection, and was correlated with infiltration of portal spaces by lymphocytes and plasma cells observed in histological section. IEA patterns of liver culture medium concentrates are illustrated in Fig. 2.

Heart tissue, cultured in Experiment II, produced no detectable labeling of any proteins in the immunoelectrophoretic patterns.

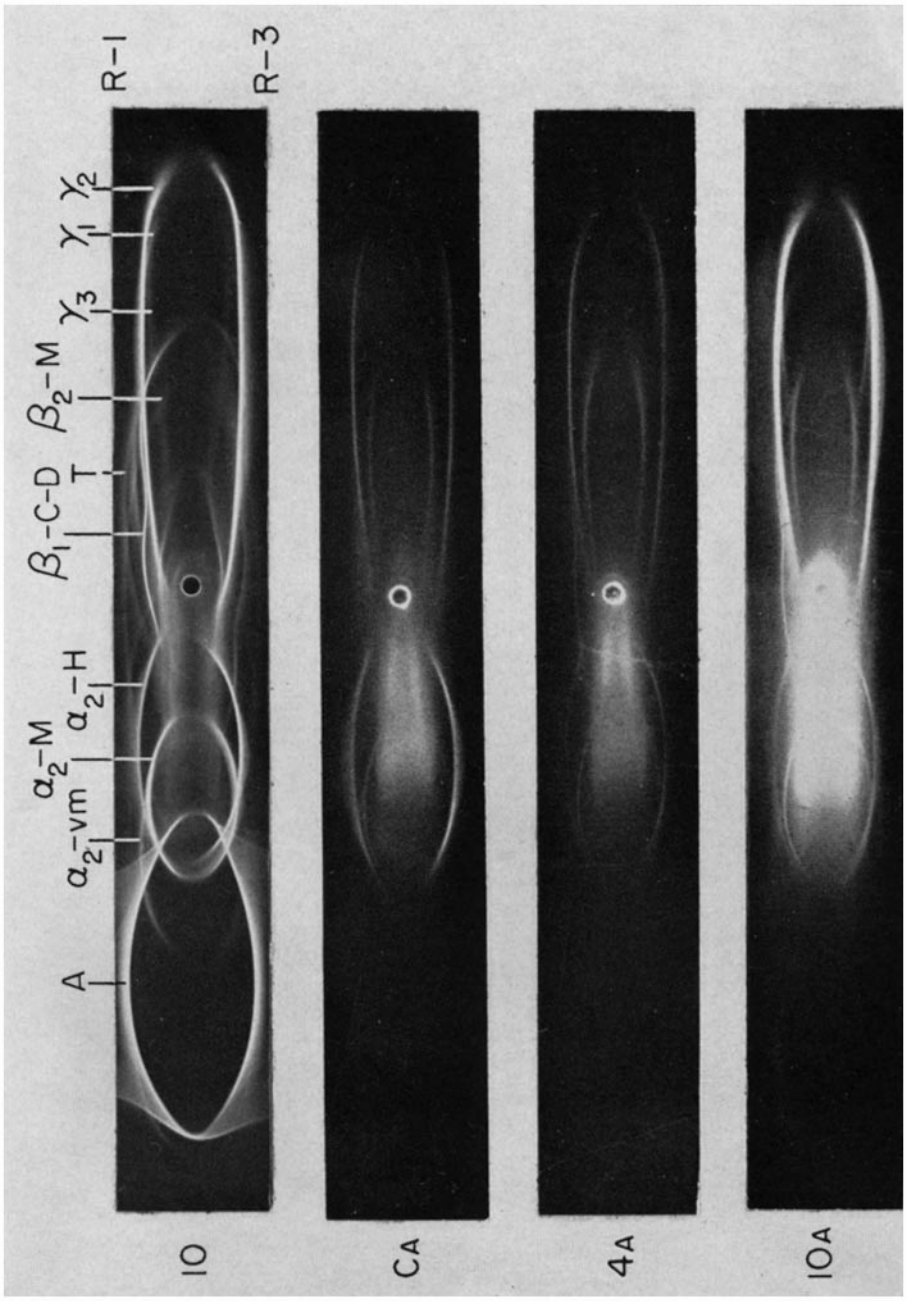

FIG. 1. Representative IEA patterns from Experiment I. *10*, IEA pattern of plasma from a mouse infected with Giorgio strain 10 days previously. Upper patterns in each plate were developed with antiserum R-1, lower patterns with antiserum R-3. This plasma was used as carrier for culture medium concentrates. *CA*, Autoradiogram of IEA slide on which medium concentrate of normal spleen culture was added to plasma. *4A*, autoradiogram of a slide with added medium concentrate of a culture of spleen tissue from 4-day infected mice. *10A*, autoradiogram of a slide with added medium concentrate of a culture of spleen tissue from 10-day infected mice. For protein nomenclature see footnote‡, Table II. Note trace of ρ′nid) in the autoradiogram.

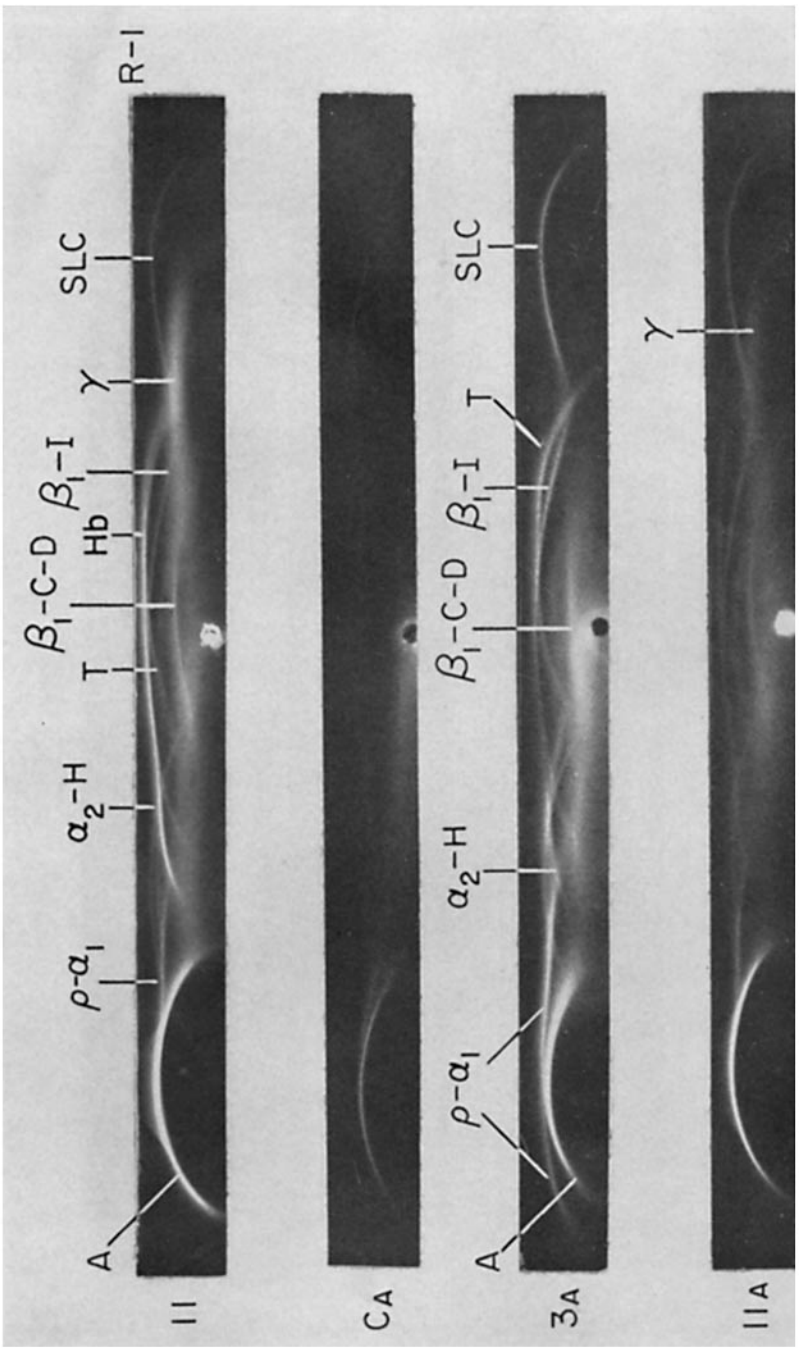

FIG. 2. Representative IEA patterns from Experiment III. *11*, IEA pattern of culture medium concentrate of liver from an 11 day infected mouse. Patterns illustrated were developed with antiserum R-1. *CA*, *3A*, and *11A* are autoradiograms of IEA slides of medium concentrates of cultures of liver slices from a normal control mouse (*CA*), a 3 day infected mouse (*3A*), and an 11 day infected mouse (*11A*). Note that the SLC and β_1-I arcs join in autoradiograms *3A* and *11A*. No serum carrier was used.

DISCUSSION

Increased activity in immune globulin formation in NCS mice after intravenous infection with staphylococci is relatively easy to demonstrate since production of these proteins by tissues from control animals was relatively low. This is also apparent from the low concentration of circulating γ-globulin *in vivo*, and is probably due to the specific-pathogen–free state of these mice and the non-typical intestinal flora consisting almost exclusively of lactobacilli and enterococci (10). Increased synthesis, and the consequent increase in circulating γ-globulin following infection, are not surprising since the animals would be expected to show an active immune response in such circumstances. The fact that two proteins cross-reacting with γ-globulin are also synthesized by the spleens of the infected animals suggests that these proteins belong to the group of immune globulins (17).

Enhanced labeling of serum proteins by liver tissues is more difficult to interpret. Many of the factors controlling protein homeostasis are not known. Furthermore, several proteins labeled by liver cultures in these experiments were also labeled by cultures of other tissues.

Both α_2-M and haptoglobin have been shown to stain for hemoglobin in immunelectrophoretic patterns (8). Certain enzymes bind to α_2-M (18). Labeling of either of these proteins in culture fluids, therefore, does not necessarily represent synthesis of the protein *in vitro*. Indeed, α_2-M becomes labeled in almost any culture of living tissue. In view of this, it cannot be determined whether increased labeling of α_2-M by cultures from infected animals represents increased synthesis.

The problem with haptoglobin is somewhat less difficult, since it is labeled only by hematopoietic tissues and by the liver. Addition of cold hemoglobin does not dilute labeling of haptoglobin in liver cultures, but does in the spleen cultures. It is, therefore, more likely that haptoglobin is formed in the livers of infected animals.

β_1-A-C has been identified as a component of C'3 in human serum (19). Labeling of this protein by various human tissues including lymph node and lung has been observed (9). In the present study, labeling of β_1-C-D, presumed to be the mouse homologue of β_1-A-C (7), occurs in both liver and lymphoid tissue of infected animals, although in normal animals it occurs mainly in the spleen and lymph node. Further studies are needed to show which cell type common to these tissues may be responsible for formation of this protein.

The cellular source of many of the serum proteins within the liver remains undecided. Since most of the serum protein production appears specific for the liver, the hypothesis that their formation can be ascribed to liver parenchymal cells is appealing. It has to be stressed, however, that the role of the Kupffer cells in the formation of serum proteins has not been evaluated in these studies, and needs further investigation.

The increased labeling of proteins such as ρ-α_1 and SLC appeared unequivocally to be the result of increased production of these substances stimulated by the infection. Concentrated fluids from cultures of liver tissue from infected mice, but not control mice, contained quantities of these proteins sufficient to contribute their characteristic precipitate lines to the IEA patterns of a carrier serum. That these lines were also intensely labeled indicated increased synthesis. Enhanced synthesis of many of the other serum proteins was more difficult to establish. Quantitative differences between the amino acid pools in livers from control and from infected mice could conceivably have a differential effect on the labeling *in vitro*. Although all liver tissues were preincubated before culture in order to dilute unlabeled lysine and isoleucine, no determinations of the free amino acids were made to rule out such effects. If it can be assumed from the reported data, however, that the livers from infected mice produced more serum protein than the control livers, the changes described in the previous report (7) can be accounted for.

Unfortunately, the acute phase protein known as C-reactive protein in the human, has not yet been identified in mouse serum, and its synthesis could not, therefore, be studied here. Recent observations with monkey tissues (20) indicate that this protein is also formed by the liver, and only by the livers of infected animals.

An increased rate of albumin synthesis in liver tissue from infected animals appears incompatible with an apparent decrease in circulation as reported in the earlier study (7). One possible explanation for this discrepancy might be an even higher rate of albumin leakage from the circulation during acute infection. In the later stages of intravenous staphylococcal infections, most of the viable organisms recoverable from the tissues of mice are found in the kidney and numerous abscesses occur in this organ (21). It has also been observed that urinary protein increased significantly during staphylococcal infections in mice (22).

While increased protein production is observed early in infection, as in the case of those proteins formed by the liver, or late in the case of most of the immune globulins, no firm conclusions can be drawn about the relationship between changes in serum protein production and the course of infection. Such a study would require larger groups of mice, more frequent sampling of tissues, and more quantitative detection methods.

The changes observed in these experiments are not restricted to staphylococcal infection alone. It has been reported that infection of NCS mice with *Mycobacterium tuberculosis* induces the conversion of β_1-C to β_1-D and an increase in haptoglobin to detectable levels, both within a week. Injection of Gram-negative endotoxin had a similar, but more variable effect (7). In preliminary studies it has been found that either loss of blood or intraperitoneal administration of 100 to 200 μg of endotoxin induces, within 24 to 48 hours,

increased labeling of plasma proteins by liver tissue *in vitro* similar to that observed in staphylococcal infections. Such an effect could not be shown after repeated injections of stilbestrol, in spite of pronounced increase in RES activity of such livers with respect to carbon clearance (23).

SUMMARY

Isotopic labeling of plasma proteins *in vitro* by tissues from staphylococcal-infected and control mice was examined, using autoradiography of immunoelectrophoretic patterns. The results indicate that in infected animals there is hyperactivity of liver tissue in producing albumin, α- and β-globulins, and of spleen tissue in producing immune globulins. The observed increase in labeling of some proteins by tissues from infected mice is to a large extent correlated with an increase of these proteins in the serum.

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