SITES OF FORMATION OF IMMUNE GLOBULINS AND OF A COMPONENT OF C'_3*

I. A New Technique for the Demonstration of the Synthesis of Individual Serum Proteins by Tissues in Vitro

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(Received for publication, May 9, 1961)

Various methods have been used to isolate tissues or cell types from animals in order to determine the sites of plasma protein formation. Studies on the effect of extirpation of (1, 2) and of damage to (3–6) liver and spleen have indicated the role of these organs, but more specific evidence has been obtained with the use of perfused isolated organs (7–9) and of tissue culture (10–19). The results of such studies have shown that the liver is the main, if not the only site of albumin formation; and moreover, that most of the α- and some of the β-globulins are formed in the liver. The formation of clotting factors (16) and fibrinogen (7, 13) by the liver was also demonstrated; and the studies of Rice *et al.* (20, 21) indicate that this organ may perhaps be involved in the production of complement components. Espinosa (9) found some α_2-globulin formation in the spleen, but did not determine the nature of this protein. Experiments with hemopoietic and lymphoid organs have demonstrated that lymphoid tissue in spleen, lung, bone marrow, lymph nodes, and intestinal mucosal lining are responsible for most if not all of the gamma globulin formation (17–19).

The demonstration of serum protein synthesis *in vitro* has been accomplished in either of two ways. One either has to show a net increase of the specific protein within the *in vitro* system (12), or the incorporation of a radioactive precursor, which in most instances is an amino acid (11). Further difficulties are inherent in characterizing or isolating the protein under study. In order to do this, methods have been used which rely on the measurement of physical and chemical properties (11, 13, 14, 17, 18) or biological activities (16) or on precipitation with a specific antiserum (12, 15, 19).

In systems where mixtures of tissue and serum proteins may be present, physicochemical separations and isolation techniques normally used with

* These studies were supported by grant E-3076 from the United States Public Health Service.

‡ United States Public Health Service Senior Research Fellow SF 522.

serum proteins are not readily applicable since the properties of most tissue proteins under such conditions is not known. A method which would simultaneously characterize physicochemical and immunological properties of serum proteins would, therefore, have distinct advantages if it could be applied to the demonstration of serum protein synthesis.

With the development of immunoelectrophoresis (22–24) it has become possible to demonstrate immunologically many individual components in the serum protein groups of different electrophoretic mobilities.

The present study describes the use of a method based on a combination of incorporation of labeled amino acids into serum proteins by tissues *in vitro*, and subsequent autoradiographic demonstration of the labeled proteins in immunoelectrophoretic patterns. This method has provided information on the site of synthesis of a few serum constituents such as the immune globulins and a component of C'_3.

Materials and Methods

Preparation of Tissue Cultures.—Organs to be cultured were removed under sterile conditions; they were minced and about 50 mg of each organ (wet weight) was cultured in each of 2 or 4 roller tubes for 24 to 48 hours. Each tube received 2 ml of a medium consisting of Hanks' balanced salt solution to which were added glucose (up to 22 mM), inositol (2 mg/liter), and mixtures of vitamins (25), antibiotics, and amino acids (26) from which either isoleucine or lysine 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, Paris, were added to a concentration of 2 μc/ml. In a few experiments, however, the sole source of amino acids consisted of a hydrolysate of C^{14}-labeled algal proteins (Volk Radiochemical Co., Chicago) in a concentration of 5 μc (100 μg) per ml of medium. In a few experiments, the medium contained 5 per cent mouse serum, but in later experiments 0.5 per cent ovalbumin was used.

Immunoelectrophoresis.—The culture media were frozen with the tissues, thawed once, centrifuged at 10,000 *g* for 20 minutes, and the supernatants dialyzed against 0.15 M NaCl for 24 to 48 hours. The culture fluids were subsequently concentrated 10 to 20-fold using either carbowax (27), or vacuum dialysis (Membranfiltergesellschaft, Göttingen), and were then subjected to immunoelectrophoretic analysis.

The microimmunoelectrophoresis was carried out according to the method of Scheidegger (28) using a barbital buffer, pH 8.6, u 0.075. In those experiments where mouse serum was not added to the medium, it was found preferable to add, separately, various dilutions of carrier mouse serum to the same well as the culture fluids. In these cases, the antigen reservoir was filled once with undiluted or diluted mouse serum. After the carrier serum had been absorbed into the agar (3 to 4 minutes) culture fluid was added twice to the same reservoir. During the development of the immunoelectrophoresis precipitation lines (20 hours at room temperature), the labeled proteins from the culture fluids were precipitated by the antiserum together with the carrier serum proteins. The purpose of diluting the carrier mouse serum was to concentrate the radioactivity into a sharp line for those components of the carrier serum and culture fluids that were in antigen excess when undiluted serum was used. The antiserum used to develop the immunoelectrophoretic patterns was a rabbit antiserum prepared against whole mouse serum.[1]

[1] A few slides were developed with rabbit antimouse sera which were kindly given by Dr. C. A. Williams, The Rockefeller Institute, New York. This antiserum was used in particular because it always showed a sharp β_2-macroglobulin line.

The immunoelectrophoretic diagrams were stained with amido black 10B according to the method of Uriel and Scheidegger (29), or with azocarmine (30), usually subsequent to autoradiography. Special staining techniques were employed to identify the precipitation lines of haptoglobin (30) and ceruloplasmin (30). These special stains were employed prior to drying of the slides and autoradiography. Peroxidase activity after immunoelectrophoresis was determined by washing the slides for 24 hours in 0.15 M NaCl, and then incubating them for 30 minutes in a saturated solution of benzidine hydrochloride, in acetate buffer, pH 4.7, containing hydrogen peroxide. Ceruloplasmin oxidase activity was demonstrated after washing, by incubation for 24 hours in a solution containing 5×10^{-3} M *p*-phenylenediamine in acetate buffer, pH 5.7, u 0.1.

Autoradiography.—Sheet film (Kodak ortho contrast, Kodak ortho royal, and Kodak royal pan) was cut into strips the size of microscope slides. A strip of film was placed between the dried immunoelectrophoresis slide and a blank slide, and these were clamped together by a tight rubber band. The film was placed so that its emulsion surface and the very thin layer of dried gel on the immunoelectrophoresis slide were in direct contact. Each slide with film was individually wrapped in aluminum foil, and placed in a slide box. During exposure, the box was kept at 4°C. Ortho contrast film was exposed for 7 weeks and developed in Kodak D11 developer, at 4°C for 5 minutes. Ortho royal and royal pan films were exposed for 4 to 7 days, and developed in Kodak DK-60a developer for 4 minutes. After the films had been developed, the corresponding immunoelectrophoresis slides were stained with amido black, and the immunoelectrophoresis compared with its autoradiograph.

Antisera.—Rabbit antimouse serum (JJI) was prepared by immunizing rabbits with a mixture of 2 ml of mouse serum and 2 ml of complete Freund's adjuvant injected into the footpads and subcutaneously. After an interval of 4 weeks, the animals were repeatedly challenged with 2 ml of a 1:10 dilution of serum, injected intraperitoneally, and were bled 7 days after the last injection. The best antiserum selected for these studies showed at least 23 lines upon immunoelectrophoresis with normal mouse serum. The distribution of these lines was as follows: 1 ρ, 1 albumin, 5 α_1-, 7 α_2-, 8 β-, and 1 γ-globulin (Fig. 1). The presence of 2 of these lines (ρ and β_2-macroglobulin) was variable when this antiserum was used. After addition of excess hemoglobin to the serum used as an antigen, still another β-globulin line appeared. Other antisera against mouse serum described in the literature are those of Heremans *et al.* (31), and of Wemyss and Williams (32). The distribution of the lines developed by antiserum JJI throughout the serum groups of different electrophoretic mobility resembled those of Clausen and Heremans (33) to a great extent. However, it is known that variations in the amounts of antibody present in various antisera against different constituents of serum can result in different positions of the precipitation lines, particularly in the α_2-region. The authors found it, therefore, difficult to compare their patterns with those described by Heremans *et al.* (31). Clausen *et al.* (34) have stated that their $\alpha_{2\text{-}II}$-globulin shows the staining characteristics of haptoglobin. In the present studies, the major protein showing the specific staining properties of haptoglobin in the α_2-region was somewhat closer to the antibody trough and had a different shape than the $\alpha_{2\text{-}II}$-globulin of Clausen *et al.* (34). After addition of hemoglobin, not only the haptoglobin line but portions of a few lines closer to the antigen reservoir usually showed some peroxidase activity, *i.e.*, a part of the albumin and of 1 or 2 α_2-globulins. Under the conditions of these experiments hemoglobin apparently combined not only with haptoglobin, but was also associated with these other precipitation lines. In the β-globulin region, 1 line of similar mobility and shape as transferrin showed slight peroxidase activity before addition of hemoglobin; whereas, another line of similar position was revealed after addition of excess hemoglobin. This latter line was the only line formed by isolated mouse hemoglobin (Fig. 2).

The slides stained for ceruloplasmin oxidase activity demonstrated 1 line which was parallel to the haptoglobin and somewhat closer to the antibody trough. The ceruloplasmin and hapto-

globin lines represented two of the three slow α_2-globulins of similar mobilities. The lines corresponding to albumin, α_2-macroglobulin[2], transferrin, a component of C'_3 ($\beta_{1c\text{-}1d}$, 32, or $\beta_{3\text{-}I}$, 35), β_2-macroglobulin (36), and γ-globulin could be readily recognized. The transferrin line was verified by immunoelectrophoresis and autoradiography of serum incubated with Fe^{59} (34).[3]

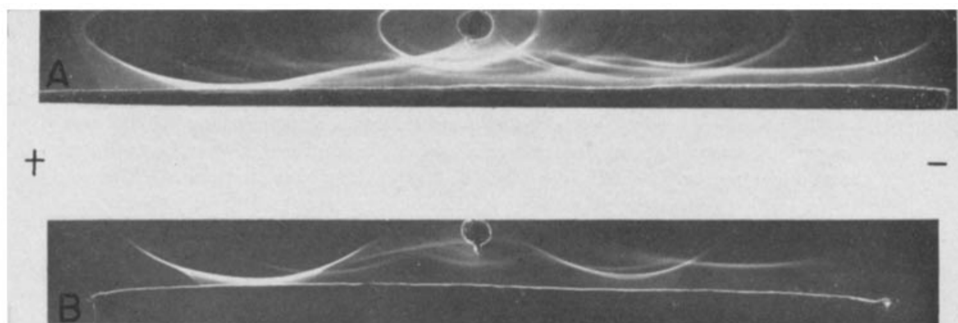

FIG. 1. Immunoelectrophoretic patterns of undiluted (*A*) and of 1:5 diluted (*B*) mouse serum developed with undiluted rabbit antimouse serum (JJI). Stained with amido black.

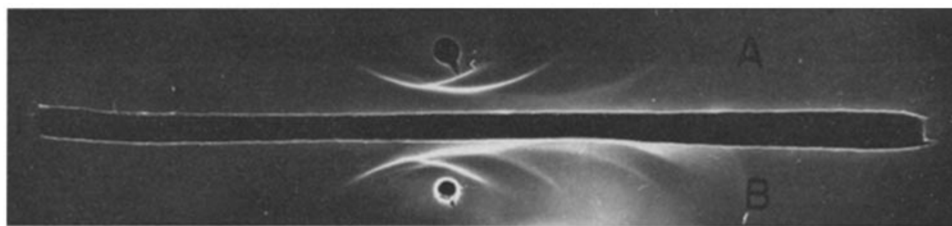

FIG. 2. Immunoelectrophoresis of undiluted mouse serum after addition of small (*A*) and larger (*B*) amounts of hemoglobin, developed with antimouse serum (JJI) and stained for peroxidase activity. Note portion of albumin line, α_2-macroglobulin, haptoglobin, and β-globulin lines with peroxidase activity (*A* and *B*), and hemoglobin line only after addition of excess hemoglobin (*B*).

EXPERIMENTAL PROCEDURES AND RESULTS

Preliminary Experiments.—

Experiment I: Spleen tissue of a normal mouse was cultured in a medium containing a mixture of C^{14}-labeled amino acids and 0.5 per cent ovalbumin. The proteins of the culture fluids were precipitated in 24 per cent Na_2SO_4 and redissolved in 0.2 ml saline.

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[2] Dr. E. C. Franklin, Department of Medicine, New York University School of Medicine, was so kind as to perform ultracentrifugation on some normal mouse serum. After centrifugation for 6 hours at 100,000 *g*, examination of the supernatant and pellet fractions with immunoelectrophoresis showed that the protein responsible for the formation of this α_2-line had a greater sedimentation constant than any of the other α_2-globulins.

[3] The Fe^{59} citrate was supplied by E. R. Squibb and Son, Long Island City, New York.

Experiment II: Spleen tissue of two mice was cultured in a medium containing C^{14}-labeled lysine and 5 per cent mouse serum. The culture fluids were concentrated about 10-fold by dialysis against carbowax.

The autoradiographs of immunoelectrophoretic diagrams prepared with the concentrated culture fluids from both these experiments showed radioactivity in the γ-globulin lines. There was also a vague zone of radioactivity which appeared parallel to and on the concave side of the very sharp γ-globulin arc in the autoradiographs prepared from the spleen cultures. It was difficult to determine to which β-globulin line of the immunoelectrophoretic pattern this corresponded. No radioactivity was observed in any other lines.

Experiment III: Spleen tissue of a normal mouse was cultured in a medium containing C^{14}-labeled lysine and 0.5 per cent ovalbumin. Control culture tubes containing normal mouse serum instead of tissue were included in this experiment. A similar amount of mouse serum (0.2 ml per 2 ml of medium) was added to the spleen tissue culture fluids after culturing and removing the excess labeled amino acids by dialysis. Both culture fluids and controls were concentrated (about 20-fold) by vacuum dialysis.

Four different sets of immunoelectrophoretic patterns with autoradiographs were prepared with each fluid. The autoradiographs obtained with control fluids were negative, whereas, the spleen culture fluids showed radioactivity in at least 4 different precipitation lines. Of these lines, 1 was the gamma globulin line, 1 was a β-globulin probably corresponding to the double curvature line of $\beta_{1c\text{-}1d}$, and 2 corresponded to lines in the α_2-globulin region.

Demonstration of Formation of γ-Globulin, β_2-Macroglobulin, and $\beta_{1c\text{-}1d}$-Globulin by the Spleen, and of Albumin and α-Globulins by the Liver.—

Improvement of the technique: In the preliminary experiments there was evidence of antigen excess in several of the precipitation arcs. The sensitivity of the autoradiography depended greatly upon concentrating all the radioactivity into a sharp line. Therefore, in subsequent experiments, the culture fluids were concentrated without any addition of mouse serum, and the immunoelectrophoretic diagrams developed by adding separately from the culture fluids, different dilutions of mouse serums to the antigen well in order to show more sharply the precipitation arcs that would otherwise be in antigen excess. Two different mouse sera were selected as carrier sera, one showing a clear β_2-macroglobulin line, and the other showing a sharper $\beta_{1c\text{-}1d}$-line. Moreover, some patterns were developed with diluted antiserum JJI (1:3) and with two different antisera.

Furthermore, lengthening of the culture period from 24 to 48 hours resulted in greater incorporation of labeled amino acids by liver cultures.

Experiment IV: In this final experiment, liver, spleen, and brain (with meninges) of two normal mice were cultured in media containing 0.5 per cent ovalbumin. 2 tubes with 50 mg of each organ were prepared with a medium containing C^{14}-lysine, 2 similar tubes with a C^{14}-isoleucine–containing medium. The culture fluids were

concentrated (15- to 40-fold) by vacuum dialysis. Various dilutions of carrier serum and of antimouse serum were used to develop approximately 10 sets of immunoelectrophetic patterns with autoradiographs from each concentrated culture fluid. A few patterns were stained for haptoglobin prior to autoradiography.

The results are listed in Table I. It can be seen from the table that the culture fluids from *brain tissue* showed no radioactivity in any of the immunoelectrophoretic arcs

TABLE I

*Radioactivity of Immunoelectrophoretically Separated Mouse Serum Proteins from Tissue Culture Fluids**

Tissue	C^{14}-amino acid	Carrier mouse serum	Albumin	α_1-globulins		α_2-globulins			β-globulins				γ-globulin
				I	II	Haptoglobin	Ceruloplasmin?	Macroglobin	Peroxidase activity	Transferrin	β_{1c-1d}	β_2-macroglobulin	
Liver	Lysine	Undiluted	+	—	—	—	—	—	—	—	—	—	—
		1:5 diluted	++	++	—	—	—	—	—	—	—	—	—
	Isoleucine	Undiluted	++	++	++	+	+	—	—	—	—	—	—
		1:5 diluted	+++	+++	—	—	—	—	+	+	—	—	—
Spleen	Lysine	Undiluted	—§	—	—	++	—	—§	—	++	++	+++	+++
		1:5 diluted	—	—	—	+	—	—	—	++	++	—	+++
	Isoleucine	Undiluted	—§	—	—	++	—	—	—	++	++	+++	+++
		1:5 diluted	—	—	—	+	—	—	—	++	++	—	+++
Brain	Lysine	Undiluted	—	—	—	—	—	—	—	—	—	—	—
		1:5 diluted	—	—	—	—	—	—	—	—	—	—	—
	Isoleucine	Undiluted	—	—	—	—	—	—	—	—	—	—	—
		1:5 diluted	—	—	—	—	—	—	—	—	—	—	—
Spleen	Lysine	Undiluted‡	—§	—	—	++	—	—	—	+	++	++	+++
	Isoleucine	Undiluted‡	—§	—	—	++	—	—	—	+	++	++	+++
Liver	Isoleucine	Undiluted‡	—	++	++	—	—	++	—	—	—	—	—

* The intensity of the radioactive lines on the autoradiographs was graded from + (just visible) to +++ (very dark).

‡ These patterns were developed with 1:3 diluted antiserum; all other patterns with undiluted antiserum.

§ Portions of these lines were radioactive owing to hemoglobin binding.

in spite of the fact that brain tissue metabolized well *in vitro* under the conditions of these experiments. On the other hand, both spleen and liver tissue culture fluids showed multiple radioactively labeled proteins.

The *spleen tissue* incorporated radioactive amino acids into proteins precipitated in the γ-globulin line, the β_2-macroglobulin line, the double curvature β-globulin line (β_{1c-1d}, 32), and in the transferrin line (Fig. 3). In addition, there are 1 or 2 lines in the α_2-region and a small piece of the albumin line that were radioactive and also stained for peroxidase activity. 1 of the α_2-lines could be recognized as haptoglobin; the other protein and the slowest moving fraction of the albumin apparently also

complexed with hemoglobin from the culture fluid. (These were the same lines as the ones onto which hemoglobin, when added to normal mouse serum, conferred peroxidase activity).

The only line with peroxidase activity that was not radioactive in spleen cultures was the β-globulin. This line had such a weak peroxidase activity that, if it contained

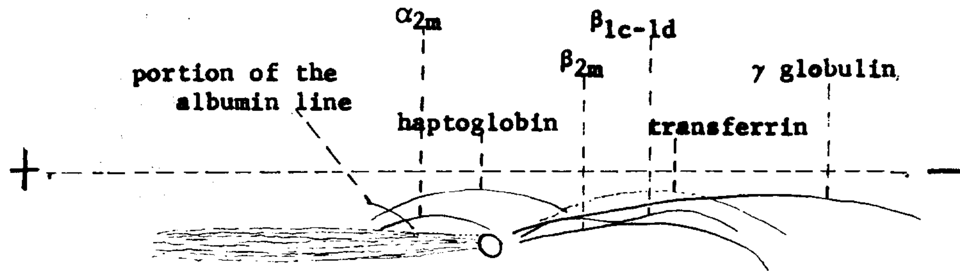

FIG. 3. Tracing of autoradiograph made from an immunoelectrophoretic pattern of spleen tissue culture fluid with undiluted mouse carrier serum, developed with a 1:3 dilution of antimouse serum (JJI). The trailing effect of radioactive material was only observed with spleen culture fluids.

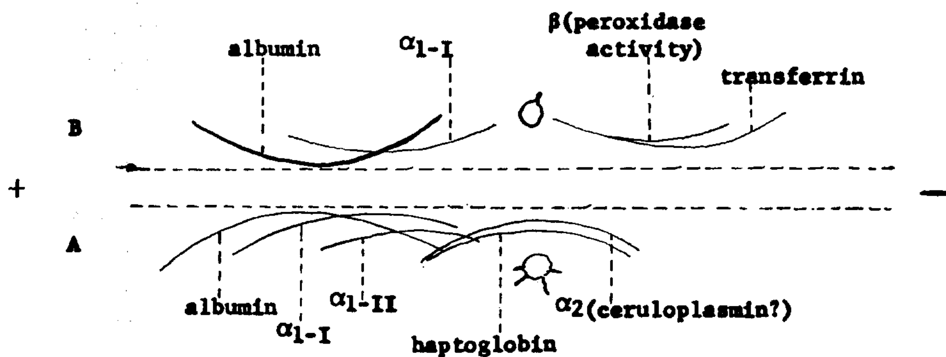

FIG. 4. Tracing of autoradiograph made from an immunoelectrophoretic pattern of liver tissue culture fluid, with undiluted (*A*) and with 1:5 diluted (*B*) mouse carrier serum, developed with undiluted antimouse serum (JJI).

hemoglobin at all, it could not have bound nearly so much as the other peroxidase-positive lines. There was never enough free hemoglobin in the fluids and carrier sera to form a separate hemoglobin line in the immunoelectrophoresis diagrams. The use of diluted carrier serum to develop the immunoelectrophoresis diagrams with the spleen culture fluids did not enhance the sensitivity of the technique (Table I), but dilution of the antiserum and use of different antisera (see footnote 1) resulted in a better separation of the β_{1c-1d} from the β_2-macroglobulin line (Figs. 3 *A* and 3 *B*).

The *liver tissue* incorporated C^{14}-isoleucine into albumin, into $2\alpha_1$-globulins, perhaps corresponding to α_{1-I}-and α_{1-II}-globulin of Heremans *et al.* (31) (Fig. 4), into α_2-

globulins, and into 2 β-globulins. 1 of these β-globulins was the arc with weak peroxidase activity described above, while the other was transferrin, as identified by reactivity with Fe^{59}. Of the 3 radioactive α_2-globulins 1 corresponded to haptoglobin, 1 to α_2-macroglobulin, and 1 either to ceruloplasmin itself or to the line immediately next and parallel to ceruloplasmin.

The liver culture fluids in which C^{14}-lysine had been used were not concentrated to the same extent (0.3 ml) as the one with C^{14}-isoleucine. This probably accounts for the apparent difference in sensitivity between the results obtained from the liver cultures with the two different amino acids. No such difference was found with spleen cultures if either culture fluid was concentrated to 0.1 ml (40-fold). Dilution of the carrier serum (1:5) in the immunoelectrophoresis resulted in sharper lines and increased sensitivity in detecting radioactive proteins such as albumin, α_1, and transferrin. Dilution of the antiserum (1:3) resulted in an increased sensitivity for 1 of the α_2-globulins (Table I).

DISCUSSION

The results of these experiments indicate that the combination of tissue culture with immunoelectrophoresis-autoradiography can be used to demonstrate the sites of synthesis of various serum proteins *in vitro*.

The most interesting observation in this study seems to be that the $\beta_{1c\text{-}1d}$-line is radioactive when spleen, and not when liver or brain tissue, is cultured in the presence of labeled amino acids. This line may represent the homologue in mouse serum (32, 35) of a human protein ($\beta_{1a\text{-}1c}$), isolated by Müller-Eberhard and shown to be one of the components of human C'_3 (37). Results obtained with human tissues show that the human $\beta_{1a\text{-}1c}$ is also formed by lymphoid tissue (38).

Other proteins labeled by spleen and not by liver or brain tissues are the gamma globulin and β_2-macroglobulin, confirming earlier results obtained with rabbit and human tissues *in vitro* (20, 29). On the other hand, liver and not spleen or brain tissue appear to form albumin and two α_1-proteins. This is also consistent with the available data from the literature (7, 11, 12).

Interpretation of the results with some of the α- and β-globulins proves difficult. The function of many of the proteins seen in immunoelectrophoresis is unknown. Some of the serum proteins carry other proteins such as enzymes, hormones, etc., and can become labeled in systems employing incorporation of labeled amino acids *in vitro* because the substance they carry is formed by the cultured tissues. In the present experiments, this may very well explain why haptoglobin becomes labeled upon culturing mouse spleen, a hemopoietic organ. The labeling of the haptoglobin in the culture fluids is either due to the formation of haptoglobin, itself, or to the binding of synthesized, labeled hemoglobin. The latter possibility seems most likely since, under the conditions of these experiments, hemoglobin also appeared to complex with part of another α_2-globulin and of albumin as revealed by specific staining of the corresponding portions

of the lines in the immunoelectrophoretic diagram. These protein complexes also appeared labeled in the autoradiographs.

The weak labeling seen in a few of the α_2- and β_2-globulins cannot be readily interpreted in these experiments because of the lack of knowledge concerning the individual properties of these proteins. The α_2-globulins labeled by the liver tissue appear to be haptoglobin and two other proteins, one of which resembles α_2-macroglobulin, and the other possibly ceruloplasmin. Weak labeling was observed in the transferrin line both with liver and spleen tissue cultures. This finding is similar to the results obtained with monkey tissues (38).

The present study shows that specific results can be obtained with this method since control culture tubes with a tissue such as brain, and incubation of serum in culture medium, were consistently negative. Moreover, results obtained with C^{14}-lysine and with C^{14}-isoleucine incorporation are similar. In experiments not reported here, however, where a medium was employed containing mouse serum and a mixture of labeled amino acids, some of which are known to attach themselves non-specifically to proteins through S-S bonds, weak, non-specific labeling of serum proteins was obtained in the control tubes. Such a random mixture of labeled amino acids was therefore avoided in subsequent experiments.

The major advantage of the technique is that it enables one to study the synthesis (turnover) of individual proteins without the need of specific antisera against single serum components. The results obtained with β_2-macroglobulin have demonstrated that some lines can be more readily detected with autoradiography than from the corresponding immunoelectrophoretic pattern on the slide. However, a quantitation of amounts of serum proteins formed cannot readily be accomplished with the use of the present method, whereas an approximation can be obtained when specific antisera against single components are available. It may prove helpful to compare the results obtained with both methods in order to estimate the sensitivity of the autoradiographic technique and to determine the optimal conditions of the immunoelectrophoretic patterns. Such studies are now in progress.

Improvements in the sensitivity of the technique without inducing nonspecific results may result from the use of more than one selected labeled amino acid in the culture media, and of specific, non-denatured, carrier proteins and different antisera to develop the immunoelectrophoretic patterns. This method can be applied most readily to the demonstration of *in vitro* synthesis of proteins with a comparatively high turnover rate (40). Prolonging the culture period from 24 to 48 hours improved the results, but no attempts have been made to culture longer. A disadvantage is that some tissues, liver in particular, are difficult to culture *in vitro*. The method of autoradiography of immunoelectrophoretic diagrams could be adapted to perfusion experiments, where higher rates of protein formation *in vitro* are usually obtained (41). The use of other labeled precursors

(42) with this method may also provide valuable information regarding the sites of serum protein formation. In future experiments, this method will be applied to the study of certain abnormal serum proteins present in the serum of diseased mice.

SUMMARY

The development of a new method for the determination of the sites of serum protein formation has been described. The method involves the incorporation of C^{14}-labeled amino acids by tissues cultured *in vitro*, and subsequent autoradiography of immunoelectrophoretic patterns prepared from a mixture of culture fluids and carrier serum with an antiserum against the carrier serum.

This technique has been used to demonstrate formation of γ-globulin, of β_2-macroglobulin, and of a component of C'_3 by mouse spleen tissue, and of various other serum proteins by liver tissue. The specificity and sensitivity of this method have been discussed, and some of its advantages and pitfalls were mentioned.

In addition, a rabbit antimouse serum was prepared, and the immunoelectrophoretic patterns obtained with mouse serum were compared with those described in the literature.

The technical assistance of Mr. Norman Goldberger and Miss Ethel Jacobson is gratefully acknowledged.

BIBLIOGRAPHY

1. Tarver, H., and Reinhardt, W. O., Methionine labeled with radioactive sulfur as an indicator of protein formation in the hepatectomized dog, *J. Biol. Chem.*, 1947, **167,** 395.
2. Rowley, D. A., The formation of circulating antibody in the splenectomized human being following intravenous injection of heterologous erythrocytes, *J. Immunol.*, 1950, **65,** 515.
3. Rappaport, A. M., Clarke, D. W., and Stewart, M., Effect of liver ischemia on plasma in the dog as measured by electrophoretic analysis, *Proc. Soc. Exp. Biol. and Med.*, 1952, **80,** 585.
4. Van Dommelen, C. K. V., Schulte, M. J., Brandt, K., van Leeuwen, L., and Wadman, S. K., Abnormally low α_2- and β-globulin levels in serious hepatic insufficiency, *Acta. Med. Scand.*, 1959, **165,** 211.
5. Brown, R. K., Read, J. T., Wiseman, B. K., and France, W. G., The electrophoretic analysis of serum proteins of the blood dyscrasias, *J. Lab. Clin. and Med.*, 1948, **33,** 1523.
6. Jacobson, L. O., Robson, M. J., and Marks, E. K., The effect of X-radiation on antibody formation, *Proc. Soc. Exp. Biol. and Med.*, 1950, **75,** 145.
7. Miller, L. L., and Bale, W. F., Synthesis of all plasma protein fractions except gamma globulin by the liver. The use of zone electrophoresis and lysine-ϵ-C^{14} to define the plasma proteins synthesized by the isolated perfused liver, *J. Exp. Med.*, 1954, **99,** 125.

8. Miller, L. L., Bly, C. G., and Bale, W. F., Plasma and tissue proteins produced by non-hepatic rat organs as studied with lysine-ϵ-C^{14}. Gamma globulins the chief plasma protein fraction produced by non-hepatic tissues, *J. Exp. Med.*, 1954, **99**, 133.
9. Espinosa, E., Production of α- and γ-globulin by the isolated and perfused rat spleen, *Nature*, 1959, **184**, 1801.
10. Roberts, S., and White, A., Studies on the origin of the serum proteins, *J. Biol. Chem.*, 1949, **180**, 505.
11. Peters, T., and Anfinsen, C. B., Production of radioactive serum albumin by liver slices, *J. Biol. Chem.*, 1950, **182**, 171.
12. Peters, T., and Anfinsen, C. B., Net Production of serum albumin by liver slices, *J. Biol. Chem.*, 1950, **186**, 805.
13. Dancis, J., Braverman, N., and Lind, J., Plasma protein synthesis in the human fetus and placenta, *J. Clin. Inv.* 1957, **36**, 398.
14. Radding, C. M., Bragdon, J. H., and Steinberg, D., The synthesis of low and high density lipoproteins by rat liver *in vitro*, *Biochim. et Biophysica Acta*, 1958, **30**, 443.
15. Marsh, J. B., and Whereat, A. F., Synthesis of plasma lipoprotein by rat liver, *J. Biol. Chem.*, 1959, **234**, 3196.
16. Pool, J. G., and Robinson, J., *In vitro* synthesis of coagulation factors by rat liver slices, *Am. J. Physiol.*, 1959, **196**, 423.
17. Askonas, B. A., and White, R. G., Sites of antibody production in the guinea pig. The relation between *in vitro* synthesis of antiovalbumin and γ-globulin and distribution of antibody-containing plasma cells, *Brit. J. Exp. Path.*, 1956, **37**, 61.
18. Askonas, B. A., Humphrey, J. H., and Porter, R. R., On the origin of the multiple forms of rabbit γ-globulin, *Biochem. J.*, London, 1956, **63**, 412.
19. Thorbecke, G. J., Gamma globulin and antibody formation *in vitro*. I. Gamma globulin formation in tissues from immature and normal adult rabbits, *J. Exp. Med.*, 1960, **112**, 279.
20. Rice, C. E., Boulanger, P., and Plummer, P. J. G., Parallel studies of complement and coagulation. V. The effect of chloroform, *Canad. J. Comp. Med.*, 1952, **16**, 216.
21. Rice, C. E., Boulanger, P., Plummer, P. J. G., and Annau, E., Parallel studies of complement and blood coagulation. VII. The effect of ethionine, *J. Immunol.* 1952, **68**, 673.
22. Poulik, M. D., Filter paper electrophoresis of purified diphtheria toxoid, *Can. Med. Sc.*, 1952, **30**, 417.
23. Graber, P., and Williams, C. A., Jr., Méthode permettant l'étude conjugée des propriétés électrophorétiques et immunochimiques d'un mélange de protéines. Application au serum sanguin, *Biochim. et Biophysica Acta.*, 1953, **10**, 193.
24. Williams, C. A., Jr., and Graber, P., Immunoelectrophoretic studies on serum proteins. I. The antigens of human serum, *J. Immunol.*, 1955, **74**, 158.
25. Eagle, H., Oyama, V. I., Levy, M., Horton, C. L., and Fleischman, R., The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid, *J. Biol. Chem.*, 1956, **218**, 607.

26. Neuman, R. E., and McCoy, T. A., Growth-promoting properties of pyruvate oxalate and α-ketoglutarate for isolated Walker carcinoma 256 cells, *Proc. Soc. Exp. Biol. and Med.*, 1958, **98,** 303.
27. Kohn, J., A simple method for the concentration of fluids containing protein, *Nature*, 1959, **183,** 1055.
28. Scheidegger, J. J., Une microméthode de l'immunoélectrophorèse, *Internat. Arch. Allergy*, 1955, **7,** 103.
29. Uriel, J., and Scheidegger, J. J., Électrophorèse en gélose et coloration des constituants, *Bull. Soc. chim. biol.*, 1955, **37,** 165.
30. Uriel, J., Les réactions de caractérisation des constituants protéiques après électrophorèse ou immunoélectrophorèse en gélose, *in* Analyse Immunoélectrophorétique, (P. Grabar and P. Burtin, editors), Paris, Masson et Cie., 1960, 33.
31. Heremans, J., Clausen, J., Heremans, M. Th., and Rask-Nielsen, R., Immunoelectrophoretic characteristics of normal mouse serum as a basis for studying pathological changes in serum of mice carrying transplantable malignant growths, *J. Nat. Cancer Inst.*, 1959, **22,** 45.
32. Williams, C. A., Jr., and Wemyss, C. T., Jr., Changes produced in mouse plasma proteins by acute bacterial infection, in press.
33. Clausen, J., and Heremans, J., Immunoelectrophoretical investigations of mouse serum protein fractions obtained by ammonium sulphate fractionation, *Acta Physiol. Scand.*, 1960, **48,** 471.
34. Clausen, J., Rask-Nielsen, R., Christensen, H. E., and Munkner, T., Two transplantable mouse hepatomas associated with an increase of metal combining β-globulin (transferrin) in serum, *Cancer Research*, 1960, **20,** 178.
35. Clausen, J., and Heremans, J., An immunologic and chemical study of the similarities between mouse and human serum protein, *J. Immunol.*, 1960, **84,** 128.
36. Rask-Nielsen, R., Lontie, R., Clausen, J., Christensen, N. E., Heremans, J. F., and Brauns, G., l'Ultracentrifugation des protéines sériques dans trois sortes de leucémies transplantables de la souris, *Rev. franç. études. clin. et biol.*, 1960, **5,** 1000.
37. Müller-Eberhard, H. J., Nilsson, U., and Aronsson, T., Isolation and characterization of two β-glycoproteins of human serum, *J. Exp. Med.*, 1960, **3,** 201.
38. Asofsky, R., and Thorbecke, G. J., Sites of formation of immune globulins and of a component of C'_3. II Production of immunoelectrophoretically identified serum proteins by human and monkey tissues *in vitro*, *J. Exp. Med.*, 114, 471.
39. Levene, H., Franklin, E. C., and Thorbecke, G. J., 7 S and 19 S gamma globulin formation by human lymph nodes *in vitro*, *J. Immunol.*, in press.
40. Niklas, A., and Maurer, W., Über die Neubildung einzelner, getrennter Serumeiweiss Fraktionen nach oraler gabe von S^{35}-L-methionin an Ratten, *Biochem. Z.*, Berlin, 1952, **323,** 89.
41. Miller, L. L., Physiological factors and liver cell function, *J. Histochem. and Cytochem.*, 1959, **7,** 235.
42. Kunkel, H. G., and Bearn, A. G., Phospholipid studies of different serum lipoproteins employing P^{32}, *Proc. Soc. Exp. Biol. and Med.*, 1954, **86,** 887.