SOME EFFECTS OF IRRADIATION ON THE IMMUNOCHEMICAL AND PHYSICOCHEMICAL IDENTITY OF SERUM PROTEINS

by

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Submitted to the Department of Zoology and the Faculty of the Graduate School of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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June, 1955

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CHAPTER I

INTRODUCTION

In protein systems molecular excitations and ionizations which occur during absorption of radiant energy produce structural and chemical alterations in the nature of the proteins. These alterations are manifested in many of the measurable properties of protein solutions such as viscosity, solubility, optical density, coagulability, enzymatic activity, and others. Since the early studies by Hardy (1903), extensive consideration has been given to the biochemical effects of irradiation on proteins. Discussions by Dale (1952), Barron and Finkelstein (1952), and the reviews of Arnow (1936), Fricke (1938), and Ord and Stocken (1953) give a comprehensive and exhaustive presentation of previous work.

The mechanisms by which radiant energy produces its effects on proteins are not understood, but the fundamentality and importance of these effects in producing the syndrome of radiation sickness is recognized. Consequently, this research was undertaken to further the understanding of some of the biochemical factors involved when proteins are irradiated.

Ionization is considered the primary effect produced by radiation, but this may be followed by secondary effects, which

seem to be associated with the existence in the body of toxic substances following irradiation. H_2O_2 formed on irradiation of water, ammonia liberated in irradiated amino acid solutions, and histamine-like substances produced in the irradiated body have been demonstrated and are among the substances which have been postulated as being radiation-induced poisons. Body proteins may be altered by irradiation to such an extent as to become "foreign" elements and perhaps antagonists in their own physiological environment. The plausibility of this last speculation was examined.

I wish to express my gratitude to Dr. Charles A. Leone, who, as my graduate advisor, inspired the undertaking of this project and whose suggestions and criticisms throughout the progress of the investigation and preparation of this manuscript were generously offered. My gratitude is also expressed to Dr. A. Byron Leonard for his interest in this work and for his critical review of this manuscript. To Dr. Frank E. Hoecker, Director of the Radioisotope Research Laboratory; Dr. J. D. Stranathan, Chairman of the Department of Physics; and to Dr. David Paretsky and Dr. E. L. Treece of the Department of Bacteriology at the University of Kansas a debt of gratitude is owed for making available to me certain facilities for carrying on this work.

CHAPTER II

THE EFFECT OF *IN VITRO* X-RAY IRRADIATION OF PROTEINS AS REVEALED BY THE QUANTITATIVE, TURBIDIMETRIC, PRECIPITIN REACTION

Since it is recognized that irradiation damages proteins, it seemed appropriate to investigate the effects of irradiation on proteins *in vitro*, and study any alterations by serological methods. In order to elucidate some of the biochemical factors involved, an investigation was made on the effects of X-ray irradiation of hemocyanin on its serological activity using turbidimetric analyses and two dimensional agar-diffusion tests.

Materials and Methods

Hemocyanins* in the sera of the American lobster, *Homarus americanus*, were the proteins chosen to be irradiated. The hemocyanin used was native material, which had been sterilized in a Seitz filter, bottled in serum vials and stored in the cold. Rabbits were used to produce antisera against hemocyanin according to the procedures of Leone (1949).

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*Obtained from a collection of sera of invertebrates maintained in the Department of Zoology in accordance with research contract NR163-012, Nonr 147(00) with the Office of Naval Research, Department of the Navy.

With slight modification the procedures described by Boyden and Defalco (1943) and Leone (1949) for the turbidimetric analysis of precipitin reactions have been used. The changes include: (1) the use of 2.0 ml. as the volume of antigen rather than 1.7 ml. and (2) the use of a new method for making the antigen dilution series. The Libby photronreflectometer (1938) was utilized to titrate antigen-antibody reactions. In quantitative titrations a doubling dilution series of twelve tubes of antigen is prepared; each tube receives the same amount of antiserum, 0.3 ml., and the antigen-antibody reactions are allowed to proceed for a measured period of time. A plot of the values of turbidities resulting from the reaction (ordinate) against the antigen dilution (abscissa) produces a peaked curve characteristic for the reacting system.

Utmost care was taken in preparing antigen dilutions. Only National Bureau of Standard calibrated pipettes were used. A method was devised for preparing the dilutions by making only whole-volume transfers (table 1). Antigens to be used in comparative turbidimetric tests were adjusted to have equal protein concentrations, (tube A in table 1), and the various dilutions were prepared from the contents of this tube. Future references to the protein concentrations of antigens used in tests will be made with respect to tube No. 1. Since, in all tests, the doubling dilution series is used, the concentration of protein in any tube can be calculated when that of tube No. 1 is known. In the doubling dilution

Table 1.

Method for the preparation
of antigen dilutions for quantitative turbidimetric tests.

	TUBES								
contents	A	B	C	D	E	F	G	H	I
ml. diluent	--	3	7	15	3	7	15	3	7
ml. antigen solution	--	1(A)	1(A)	1(A)	1(D)	1(D)	1(D)	1(G)	1(G)

The contents in each of the above tubes were mixed well and a final dilution series was prepared for each antigen:

	TUBES											
contents	1	2	3	4	5	6	7	8	9	10	11	12
ml. diluent	--	1	--	--	--	1	--	--	--	1	--	--
ml. antigen solution	2(A)	1(A)	2(B)	2(C)	2(D)	1(D)	2(E)	2(F)	2(G)	1(G)	2(H)	2(I)

series the protein concentration in any tube is half that of the tube preceding it in the series. For tests with rabbit-antisera a diluent of 0.9 per cent saline solution buffered with phosphates was employed. The buffered saline was made 0.0040 M with respect to Na_2HPO_4 and 0.0027 M with respect to KH_2PO_4.

All turbidimetric tests were performed in duplicate and the mean turbidity values were calculated from the results of duplicate tests. For each test two control tubes were prepared; an antigen control containing 2.0 ml. of antigen in the same concentration as in tube No. 1 plus 0.3 ml. of saline diluent, and an antiserum control containing 0.3 ml. of antiserum plus 2.0 ml. of saline diluent. Turbidities resulting from reactions were calculated by subtracting from the mean turbidity values of duplicate tests the turbidity values of controls.

The effect of the irradiation of antigen on the precipitin reaction was also studied using an agar-plate precipitin test. The modified Ouchterlony test (1948) as described by Leone, Leonard and Pryor (1955) was utilized. In this test the antigen-antibody reaction takes place in agar contained in a Petri plate. The agar in the plates has been poured so that five reservoirs, or wells, are formed in the solid media. Four of these wells are arranged in a square, the adjacent edges of each well being 20 mm. apart. The fifth well is situated in a central position. Each well is 10 mm. x 10 mm. and approximately 3 mm. in depth. In the tests,

antiserum and antigen are put in separate wells such that the incident planes of diffusion are at right angles to each other. Reactions occur in the agar, and zones of precipitation appear between the wells. The agar diffusion test combines two activities, the diffusion of the solutions in the agar and the serological reaction between the agents, both of which are subject to analysis in a single test.

Total serum protein determinations were made colorimetrically using phenol reagent of Folin and Ciocalteu (1927) according to the method of Greenberg (1929) by means of a Leitz Rouy-photometer.

Irradiations were achieved with a Westinghouse, 150 KV maximum, industrial type unit and with a General Electric, Maxitron Two-Fifty unit. Tube voltages of 140 KV and current of 5 m.a. with no accessory filters were used. With the use of the Westinghouse unit, samples were exposed at a distance of 50 cm. from the tube target and with the Maxitron Two-Fifty a distance of 21.5 cm. was employed. The dosage rates measured at these distances with a Victoreen r-meter in air were 43 and 1,075 r./minute respectively. Samples to be irradiated were placed in dialyzing tubing (visking sausage, diameter 19 mm.), which was sealed at both ends with rubber stoppers. The tubing containing the protein solutions was suspended in air perpendicular to the X-ray beam and irradiated.

Experimental Results

Two samples of five ml. aliquots taken from the same stock solution of Homarus hemocyanin contained 2.90 gms. of protein per 100 ml. One of the portions was exposed to a total level of 1,000 r.; the other was given an exposure of 3,000 r. The X-irradiations were administered in single doses at 43 r./minute. A third five ml. aliquot, handled in the same manner as were the two others but without being subjected to irradiation, served as a control. All samples remained in the dialysis bags for approximately 75 minutes. This uniformity of timing was intended to equalize the opportunity for pervaporation of water from each sample, although loss of water was negligible. A slight turbidity was noted in the sample which had received 3,000 r.

The three samples of hemocyanin were quantitatively compared by turbidimetric serological tests performed six hours and 96 hours after irradiation. In the tests performed at six hours, tube No. 1 of the antigen dilution series contained a protein concentration of one part to 380. The antiserum was used undiluted. These tests were performed at room temperature. The reaction time was 60 minutes. In these tests the whole turbidity curves were not obtained. Only a postzone of the titration was represented in the curves and there was an apparent progressive shift of the curves to the right on the horizontal axis with increasing levels of irradiation administered to the hemocyanin. In order to obtain the whole turbidity curves for

the tests performed at 96 hours, the antigen dilution series were prepared from first tubes containing a protein concentration of one part to 92 and the same antiserum was used but was diluted with three parts of physiological saline (Bolton, 1947). The reaction time and temperature were the same. These results are presented in figure 1,A.

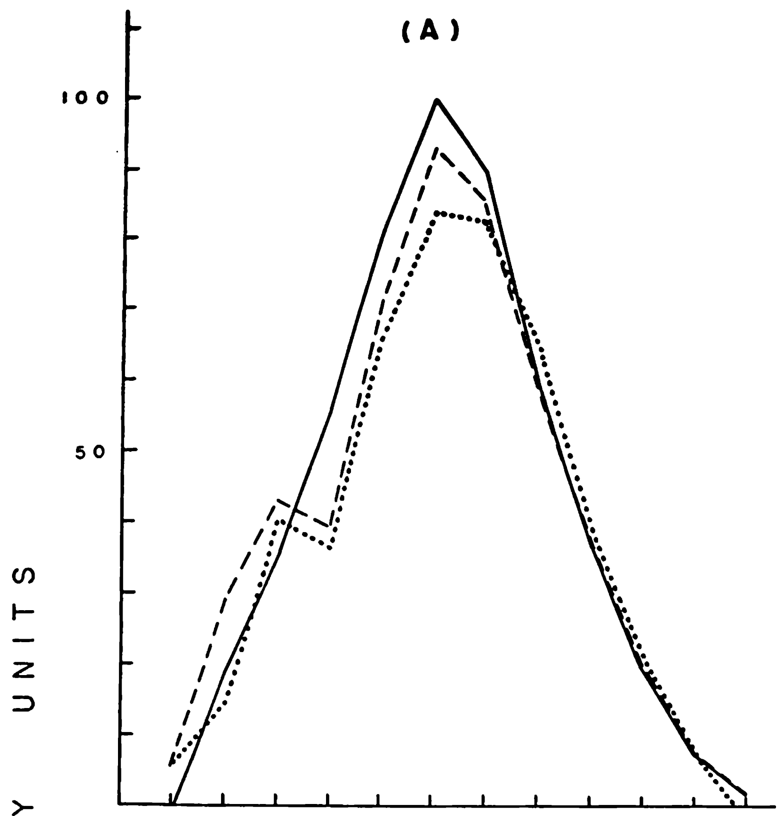
For the agar-diffusion precipitin tests, diluted antiserum was put in the center well of the plate; some of the 1,000 roentgen-irradiated sample was placed in one outer well; a 3,000 roentgen-irradiated sample in another, and the remaining two wells were filled with non-irradiated hemocyanin. An antigen dilution of 1:1,000 (parts protein per parts solution) was used. Photogram recordings were made of the tests at 104 hours and at 208 hours. The zone of precipitation of the 3,000 roentgen-irradiated sample was slightly wider than those of the other two samples.

From a second stock solution of hemocyanin, which contained 1.42 gms. of protein per 100 ml., three five-milliliter portions were taken for irradiation. One aliquot was given an exposure of 4,000 r., another 5,000 r. and the third 12,000 r. Single doses of X-irradiation were administered at 1,075 r./minute. Determinations of protein concentrations in the samples following irradiation revealed no change in the sample receiving 4,000 r. In the other two samples an increase in protein concentration was found. The sample which had received 5,000 r. had a protein concentration of

Figure 1. Comparative serological turbidity curves obtained from tests involving the reaction of irradiated and non-irradiated samples of hemocyanin with anti-hemocyanin sera.

(A) legend: —— non-irradiated hemocyanin; - - - 1,000 r., and ····· 3,000 r. irradiated samples.

(B) legend: —— non-irradiated hemocyanin; - - - 4,000 r., ····· 5,000 r., and —·— 12,000 r. irradiated samples.



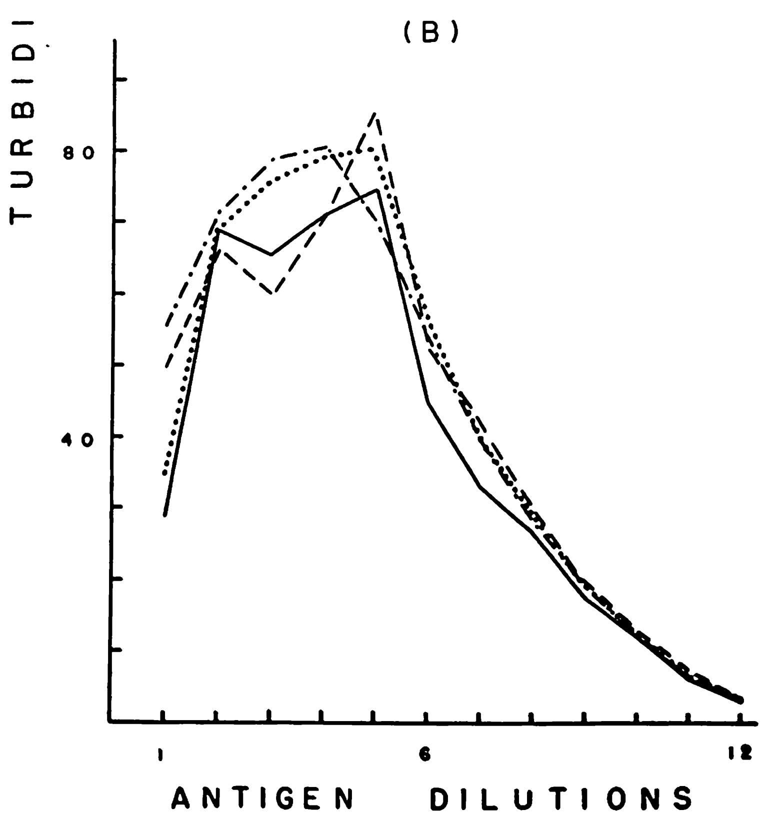


Figure 1.

1.58 gm./100 ml., and the 12,000 roentgen-irradiated sample a concentration of 1.61 gm./100 ml. Protein nitrogen levels in these samples were also determined by Kjeldahl analyses (Kabat, Mayer and Heidelberger, 1948, pp. 282-291) and were found to be comparable to those determined colorimetrically. The change, therefore, was attributed to loss of water during exposure and not to changes of measurable tyrosine or tryptophane content of the protein. Protein concentrations were adjusted for serological tests. The three irradiated and the non-irradiated hemocyanin samples were comparatively tested by turbidimetric serological tests 96 hours after irradiation. The first tube of each dilution sequence of antigens was adjusted to contain a protein concentration of 1:100; tests were performed at room temperature and timed for 60 minutes.

One test was made immediately after irradiation with the sample, which had received 4,000 r., and the results of this test were comparable to that obtained with the same sample tested at 96 hours postirradiation.

Aliquots of the above samples of hemocyanins were used in comparative tests employing the agar-diffusion method. The concentrations of antigen protein utilized were 1:100, 1:800 and 1:1600. Antiserum was placed in the center well and each of the four solutions of antigens, non-irradiated and the three irradiated materials, were separately put in one of the four peripheral wells. Photogram recordings of the plates were made at 36 hours, 7 days and 10 days (figure 2).

Figure 2. Photograms of plates of agar-diffusion serological tests with irradiated and non-irradiated hemocyanin. Legend: upper left well containing non-irradiated hemocyanin, upper right 4,000 r. irradiated sample, lower left 12,000 r. and lower right well 8,000 r. irradiated sample.

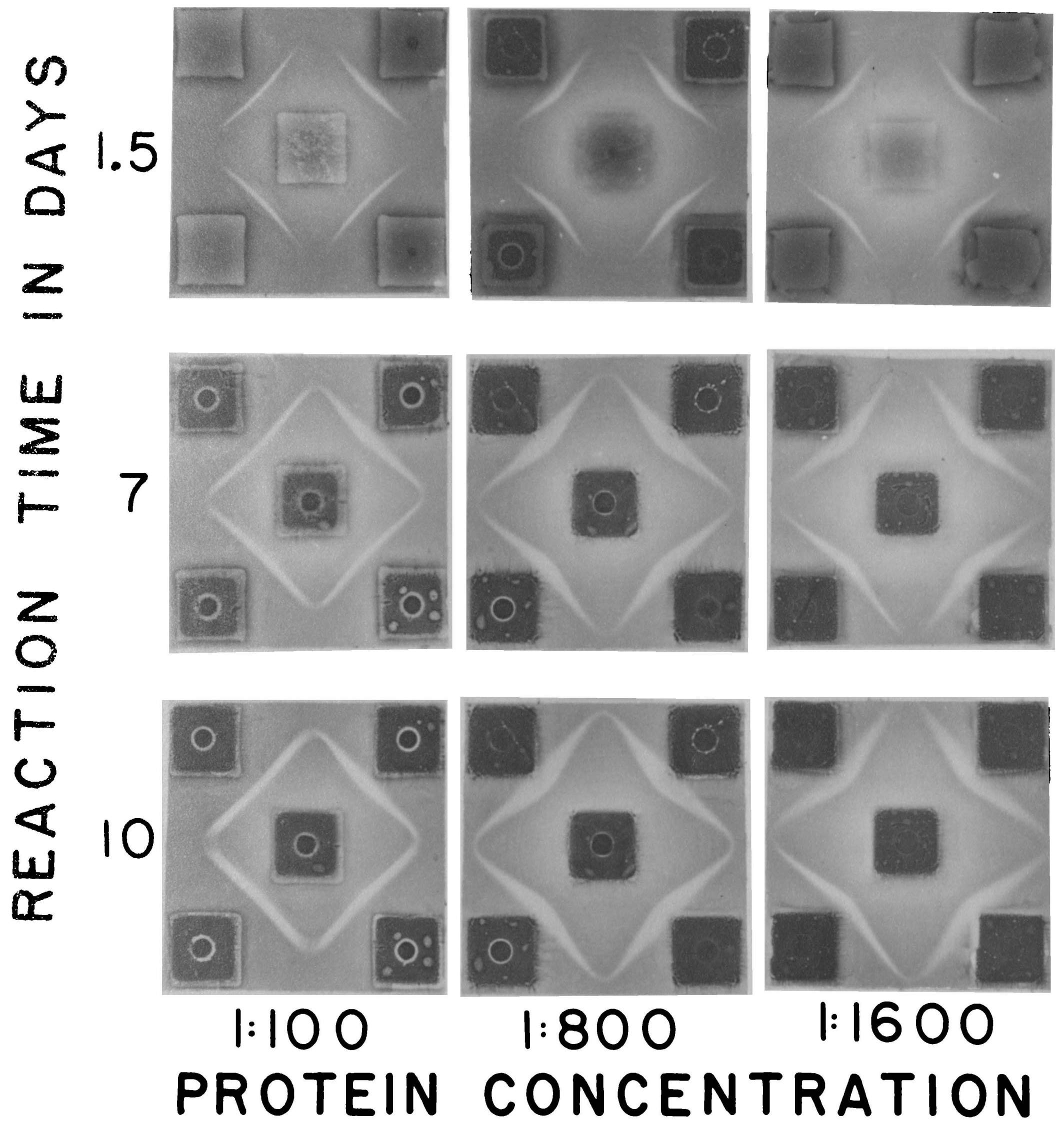


Figure 2.

Conclusions

The serological reactivity of hemocyanin is altered by X-irradiation (figure 1 and 2). When the curves of the irradiated samples are compared to that of the non-irradiated material several differences are discernible (figure 1, A). The curves of the irradiated samples possess a minor peak in addition to the principal peak demonstrable for the non-irradiated sample; there is an apparent shift of the principal peak toward higher protein concentrations. This shift is more apparent when the lines before the "breaks" in the curves of the irradiated samples are extrapolated so that they intersect and form a more smoothly peaked curve. In figure 1,B the shift of the peak is more readily seen, but only for the sample receiving 4,000 r. is an increase in the bimodal nature of the serological curve evident. The curve of the non-irradiated material shows a bimodality characteristic, which is seemingly due to the chemical characteristics of the antiserum. The shifts of the principal peaks on irradiation with 5,000 and 12,000 r. are greater than that obtained with 4,000 r. and this produces a masking of the bimodality intrinsic in the curve obtained with the non-irradiated sample.

Irradiation changed the protein; the characteristic serological turbidity curve had an additional peak suggesting that a new component, or components, have been produced during the absorption of the radiation. The new components probably are

dissociation products resulting from cleavages of the weaker peptide bonds of the giant hemocyanin molecule. These dissociation products, which may be considered as fractional components of the whole molecule, have serological characteristics which are different from those of the "parent" protein, and, in the serological titration of the irradiated antigen, the difference is expressed in the appearance of the additional minor peak. The peak of the serological curve is associated with optimal combining conditions for the antigen and antibody and in comparative tests ability of the dissociated products to react with the antiserum indicates a chemical similarity to the non-irradiated material, and the establishment of a new optimal proportion situation indicates a non-identical feature.

The supposition that some of the observed effects are caused by molecular dissociations is strongly supported by the findings of other investigators (Svedberg and Brohult, 1938 and 1939; Brohult, 1940; and Spiegel-Adolf, 1934a and 1934b). According to Svedberg and Brohult the irradiation of Helix hemocyanin with alpha rays or ultra-violet light produces a splitting of the molecules into fractional fragments and ill-defined low molecular compounds. Serum albumin and hemoglobin appear also to have been fractionated by irradiation. These workers utilized the ultracentrifugal characteristics of the proteins and the fragments as a means of studying radiation effects. Spiegel-Adolf exposed solutions of blood proteins

to ultra-violet irradiation and produced proteoses and peptones.

It would seem reasonable that because of differential diffusion properties the dissociation products would be resolved in tests using the agar-diffusion method. Seemingly this happened in tests performed here. Inspection of the data presented in figure 2 shows that in the test series utilizing a 1:100 concentration of hemocyanin, a precipitation zone, which is distinct from that of the principal zone of reaction for the 5,000 and 12,000 roentgen-irradiated samples, at seven days appears in the agar. These additional precipitation zones can be seen on the central side of the principal zones, which suggests that the dissociation products diffuse more rapidly than the non-dissociated hemocyanin. In agar-diffusion tests performed with samples which had received less than 5,000 r. of X-irradiation this second zone of reaction did not appear. This suggests an increase in products of dissociation with increasing levels of irradiation.

Progressive decrease in relative area under the principal portion of the curve (figure 1, A) with increasing levels of irradiation is a consequence of a shift of the peak and a change in relative concentration of undissociated hemocyanin. The shift of the principal peak of the serological curves, of irradiated hemocyanins, may be explained on the basis of molecular changes which favor larger antigen-antibody aggregates in situations of greater concentrations of antigen. In studying the effects of ultra-violet

light on the quantitative precipitin reaction of bovine serum albumin, Hanan (1952) concluded that irradiation caused an "unfolding" of the antigen molecules. His observed effects on the precipitin reaction resembled those shown here in that irradiation caused a progressive shift in the peaks to regions of high antigen concentration. His assumptions that unfolding of antigen molecules, which contains many combining sites, causes a decrease in number of these sites, explain the shift in the curves of irradiated hemocyanin. A decrease in number of combining sites on the antigen probably would make it necessary for more molecules of antigen to be incorporated into antibody-antigen aggregates of optimal size for precipitation. In the region of high antigen concentration the formation of this kind of aggregate would be favored. It would be expected that the antigen-antibody ratio of the precipitate would be increased under this condition. No data are given here regarding this ratio but it is reasonable to suppose that it has increased.

An effect of formaldehyde on proteins is an increase of the average molecular weight of proteins resulting from intermolecular reactions (Leone, 1953, p. 386). In studying the effects of formalin on the serological activity of hemocyanin of _Callinectes sapidus_, Leone (1953) found that addition of formalin to solutions of hemocyanin shifted the serological turbidity curve to regions of higher antigen concentration. These results are strikingly similar, in regard to the principal peak, to those obtained here on

irradiation of <u>Homarus</u> hemocyanin, and both results may be manifestations of increases in the ratio of antigen to antibody in the aggregates.

CHAPTER III

INFLUENCE OF CORPUSCULAR EMANATIONS FROM RADIOACTIVE PHOSPHORUS *IN VIVO* ON SERUM PROTEINS OF RATS

Some of the immunochemical properties of proteins are sensitive to *in vitro* X-ray irradiation (see Chapter II). Any agent which causes effects *in vitro* on this property, as does irradiation, is likely also to produce changes *in vivo*. The effect of *in vivo* beta-irradiation on the serological identity and electrophoretic nature of mammalian serum proteins was examined.

Serological Comparison of the Sera of Internally Irradiated and Normal Rats

Eighteen adult white laboratory rats were used in this experiment. Six were maintained as controls, and twelve were each given a single intraperitoneal injection of a sterile solution of $Na_2HP^{32}O_4$*, the specific activity of which was 870 μc./ml. An

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*This solution, which was physiologically normal and pyrogen-free, was obtained from the Abbott Laboratories, Chicago, Illinois.

injected activity of 2.4 μc./gm. body weight was the administered dose. This level of P^{32} activity was chosen based on the observations of Kligerman (1950), who reports that an injection of 1,200-3,600 μc./Kg. body weight produces a noticeable effect on the physical appearance and activity of rats. His report indicates that 3,600 μc./Kg. body weight is a MLD/112 days for forty-two day old rats. The 2.4 μc./gm. level is taken as being a highly injurious but sublethal dose and represents the mean of the range cited above. No selection as to sex of the animals was made. Injection of an amount of phosphate buffers in a non-radioactive state comparable to the administered dose was considered to be undisturbing physiologically. Fifty days after injection of the radioactive isotope all animals, experimentals and controls, were exsanguinated. The sera of the eighteen individuals were prepared from the bloods and stored at 4-6°C until used. Storage of these materials did not exceed seven days.

Antisera used in these tests were produced in chickens (Wolf, 1942, and Wolf and Dilks, 1946). A diluent of 2.0 per cent saline solution was used for preparing serial dilutions of the testing antigens.

Turbidimetric serological tests were performed using three different antisera, which had been produced against normal rat sera. Three series of comparative tests, therefore, were made. Each series consisted of tests made with the sera of four irradiated

animals and the sera of two normal control rats. In all of these tests, tube No. 1 was prepared to contain 1 part of protein to 100 parts solution, and the antiserum-antigen mixtures were kept at 4-6°C for 24 hours. All comparative tests involving any one antiserum were performed at the same time. The data are presented as serological turbidity curves in figure 3.

Since, with samples of normal sera from different individuals, considerable variation was obtained at each corresponding point, an analysis of the data by inspection was hindered. For this reason a statistical approach was attempted in hopes of revealing trends otherwise masked by seemingly normal, intraspecific variations of serum quality. In all instances the twelve corresponding points of the turbidity curves obtained by testing the sera of two normal animals were averaged and a mean curve considered. Individual turbidity curves of sera of irradiated rats were considered as well as the mean curve obtained from four sera (figure 3).

These curves show two distinct trends: (1) a positive displacement from normal curves in the region of low antigen dilution, and (2) a negative displacement from normal curves in the region of high antigen dilution (figure 3).

Although the three series of tests represent serological reactions using three different antisera an attempt was made to analyse these results simultaneously. Differences in specific

Figure 3. Serological turbidity curves obtained by comparatively testing sera of P^{32}-injected and sera of control rats. Irradiated rats are designated R-, and control rats are designated C- below:

(A) legend: —— mean curve of the sera of rats C-1 and C-2, — — —serum of rat R-1, ····· serum of rat R-2, — · —that of R-3, and — · · —that of R-4.

(AA) legend: —I—mean turbidity curve of the same four irradiated rats, whose curves are shown to the left, with the values of the ranges indicated at each point, and —⊓⊔— the mean curve of the sera of the two control rats tested with the same antiserum, with the ranges also indicated.

(B) legend: —— mean curve of the sera of rats C-3 and C-4, — — —serum of rat R-5, ····· serum of rat R-6, — · —that of R-7, and — · · —that of R-8.

(BB) legend: —I—mean turbidity curve of rats R-5 - R-8, and —⊓⊔— the mean turbidity curve of rats C-3 and C-4 with ranges indicated.

(C) legend: —— mean curve of the sera of rats C-5 and C-6, — — —serum of rat R-9, ····· serum of rat R-10, — · — serum of rat R-11 and — · · — that of R-12.

(CC) legend: —⊓⊔— mean turbidity curve of rats C-5 and C-6 and —I—the mean turbidity curves of the four irradiated rats, whose serum was used in this comparative test series, with ranges indicated.

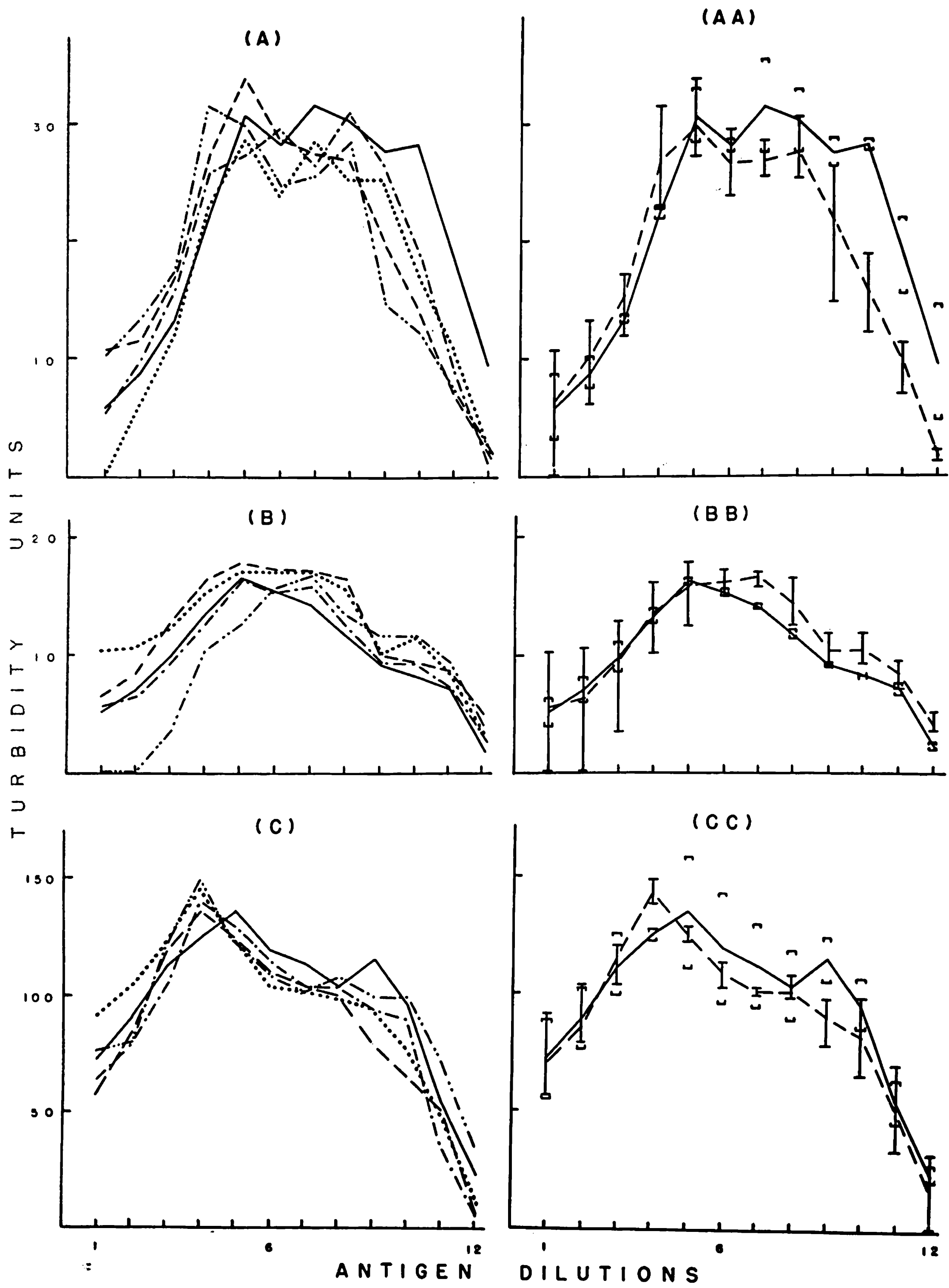

Figure 3.

qualities among these antisera do not, in my opinion, exclude the possibility of regional similarities in serological titration curves obtained by tests with them. On this assumption, the three groups of data were combined for analysis. For each test series the average deviations of irradiated sera from the normal, mean turbidity values at corresponding points were calculated and plotted against antigen dilution. This produced three curves, each curve representing the average deviation of the curves of the irradiated sera from the normal mean turbidity curve of a particular test series (figure 4, A). Corresponding points were averaged and the over-all average deviations were plotted against antigen dilutions (figure 4, B). The statistical curve obtained by grouping the data gives evidence of at least two regions of deviation of the irradiated values from the normal. One deviation was characterized by a positive displacement from normal in the region of low antigen dilutions. The peak of this deviation occurred in the curve of irradiated sera at the point corresponding to the antigen dilution contained in tube No. 4, (1:800). The second deviation was characterized by a negative displacement of the curve in the region of high antigen dilution, and its peak was situated at the locus on the serological curves represented by tube No. 9, which contained an antigen concentration of one part protein to 25,600 parts solution. These deviations in the serological properties of the sera of the irradiated animals are attributed to alterations in the chemical nature of the serum proteins induced by the irradiation.

Figure 4. Comparison of the serological nature of the sera of control and internally irradiated rats.

(A) Average deviations of the sera of the four irradiated rats from the normal mean values for each of the three series of comparative turbidimetric tests were calculated and plotted against antigen dilution. Legend: ——— from tests with R-1, R-2, R-3, and R-4; ····· with R-5, R-6, R-7, and R-8; and — — —from tests with R-9, R-10, R-11 and R-12.

(B) The over-all average deviations of the sera of irradiated animals from the normal mean values were calculated from the results of the three series of tests and plotted against antigen dilution.

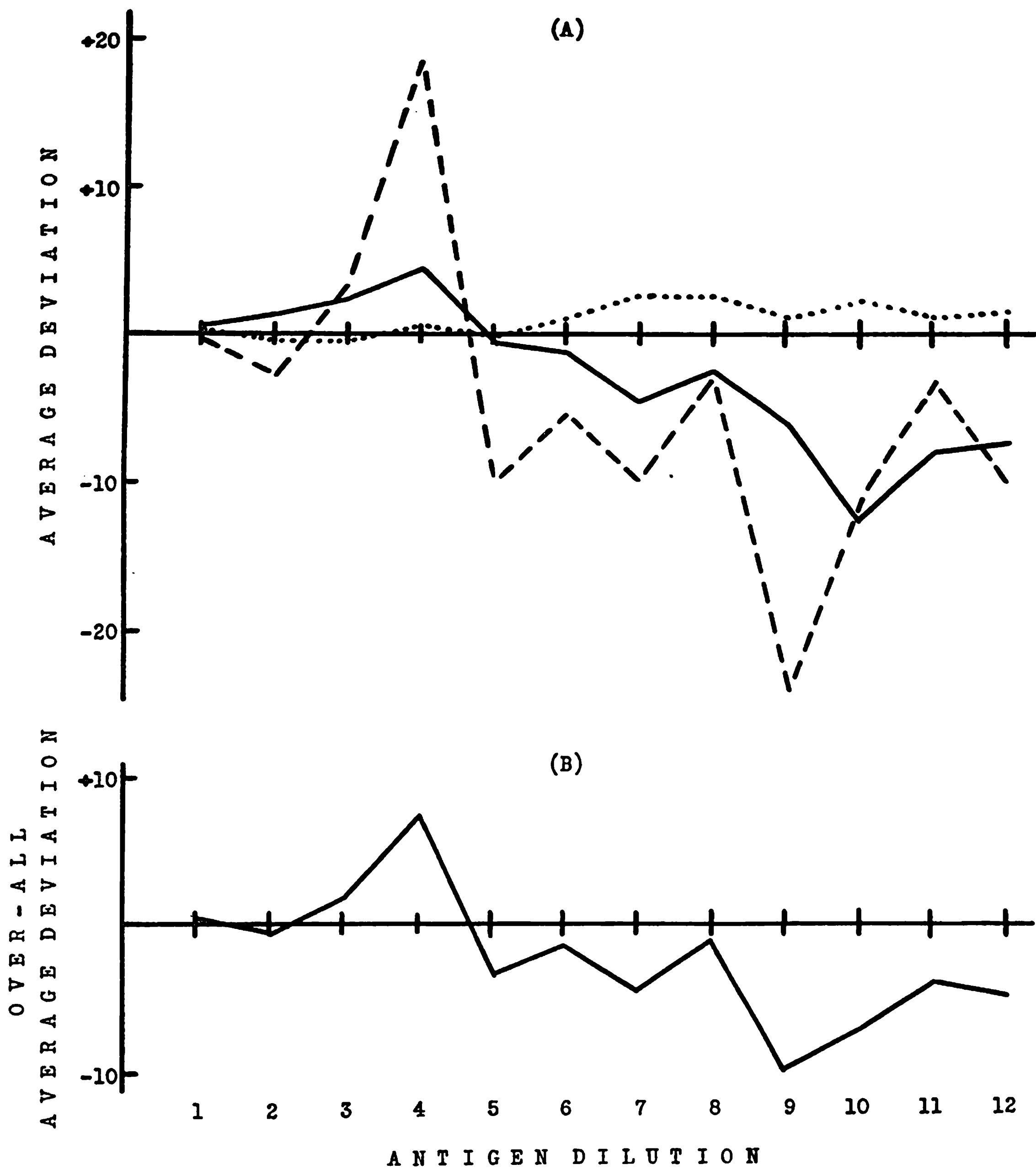

Figure 4.

A Comparison of the Extractibility of Proteins in the Tissues of the Normal and the Irradiated Rats

It was considered likely that alterations in the serological identity of the serum proteins induced by the P^{32} emissions would be accompanied by a change in the nature of some tissue proteins, particularly those of the liver, which is commonly accepted as being the site of production of serum proteins. To test this hypothesis the extractibility, under standardized condition, of the proteins of the organs of internally irradiated rats were compared to that of the organs of normal control rats.

Six of the irradiated rats (R1-R6) and four of the control rats (C3-C6), used previously to study the *in vivo* effect of P^{32} on the serological identity of the serum proteins of rats, were used also in this experiment.

In order to determine conditions of optimal extractibility, a hypothetical solubility curve for the proteins of rat liver in NaCl solutions (buffered with 0.0040 M Na_2HPO_4 and 0.0027 M KH_2PO_4) was established (see figure 5). Protein determinations were made immediately after the tissues were extracted. For standardized extraction a salinity of two per cent NaCl was chosen which, as indicated in the solubility curve, gives near optimal protein extraction of liver under the conditions employed.

Extractions were made of livers, lungs, hearts, kidneys, spleens and leg muscles. Immediately after their removal, the organs of the rats were washed in a stream of cold tap water for five minutes. All extraneous fascia, fat and blood vessels were removed;

Figure 5. A hypothetical solubility curve for the proteins of rat liver in NaCl solutions.

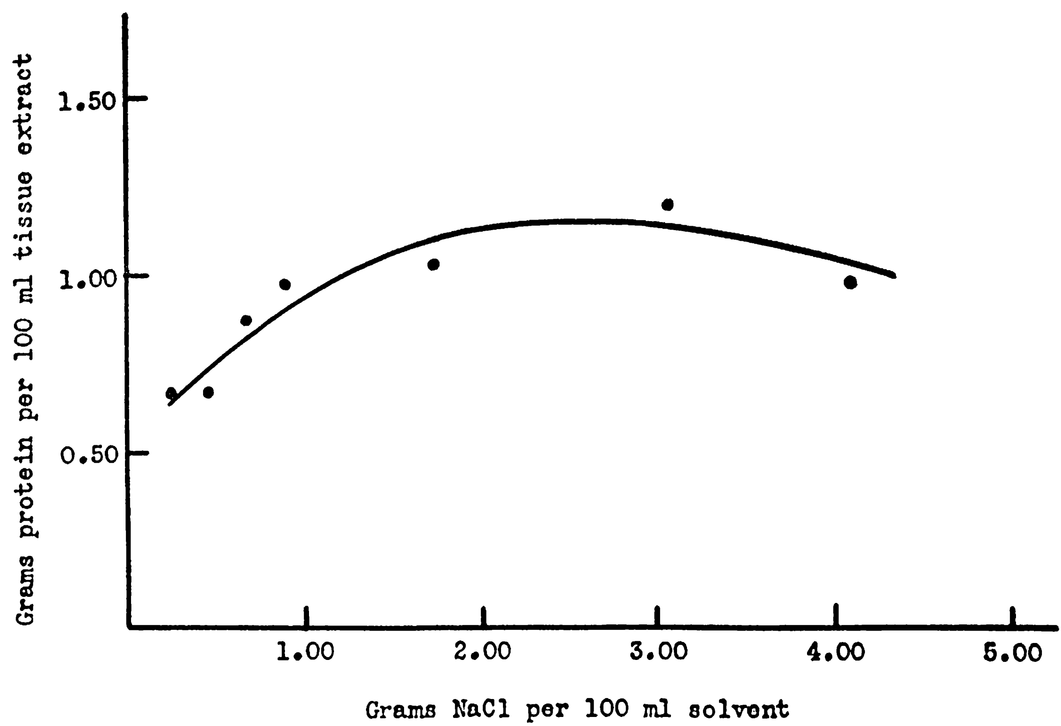

Figure 5.

the organs were semi-dried on blotting paper, wrapped individually in aluminum foil, frozen and stored at -20°C till used for extractions.

The tissues to be extracted were removed from storage, defrosted in a refrigerator at 0°C and weighed (accuracy of 0.01 gm.). The organs were then cut into small pieces and homogenized in a Waring Blender to which had been added twenty parts by weight of 2.0 per cent NaCl solution (buffered with 0.0040 M Na_2HPO_4 and 0.0027 M KH_2PO_4) for every part of tissue. The tissue-saline homogenate was transferred to a 250 ml. Erlenmeyer flask, the flask stoppered and the mixture allowed to remain in the cold, 0°C, for 48 hours to facilitate the desired dissolution. After the 48-hour period the homogenate was centrifuged at 7,500 gravities for fifteen minutes in a refrigerated centrifuge, in which the temperature was maintained at 0°C. Centrifugation produced a supernatant liquid free of visible tissue particles. The supernatant sol was poured off and filtered through a Seitz filter; the sterile filtrate was bottled and stored at 4-6°C. Within 24 hours total protein determinations were made colorimetrically on the extracts.

Every effort was made to carry on all operations of the extractions near 0°C. The only breach in maintaining this strict temperature control was in the sterilization by filtration. The supernatant liquid of the centrifuged homogenate was taken out of the cold and poured into a Seitz filter, the temperature of which was

approximately 25°C and which was fitted into a filter flask made tight by stoppering the tubulation of the flask. The filtration unit was then set in the cold, 0°C, and the cooling of the air in the flask reduced the pressure enough to induce a satisfactory filtration. The results of this experiment are given in table 2. It should be noted, by comparing the data in figure 5 and table 2, that approximately one-half of the protein was removed from the extracts by the filter.

Mean values of protein extractibilities were calculated from the observed results. In order to evaluate the statistical significance of the differences between the mean extractibility of tissues of control and irradiated animals, the *t* test of significance was utilized. The probabilities of chance differences between the mean values were obtained from a table of values of *t* (Croxton and Cowden, 1939, p. 875).

Assuming that "0.05 level of significance" is a satisfactory criterion, it is evident (table 2) that no significant statistical differences occurred between extractibilities of tissues of control and irradiated animals. It would appear, therefore, that the internal beta irradiation did not affect the solubility of proteins of liver, and the other tissues, for the solvent used here. Failure to demonstrate changes by this method does not, however, exclude the possibility of functional changes occurring in the tissues. Changes in enzymatic systems possibly have occurred and these changes may be accompanied by the production of altered serum proteins, the existence of which has been demonstrated by serological methods (see

Table 2.

A comparison of the extractability of proteins from the tissues of control and P^{32}-injected rats with 2% phosphate-buffered sodium chloride solution.

Tissue	Mean Protein Concentration gm./100 ml.		Std. Dev.	Std. Error	t value	Probability
	Controls (4 rats)	Irradiated (6 rats)				
Liver	0.42		0.073	0.037	0.28	0.80
		0.43	0.042	0.017		
Kidney	0.43		0.054	0.027	0.58	0.60
		0.40	0.093	0.038		
Spleen	0.44		0.208	0.104	0.89	0.40
		0.32	0.211	0.086		
Lung	0.29		0.030	0.015	1.50	0.20
		0.26	0.032	0.013		
Heart	0.18		0.066	0.033	--	--
		0.18	0.147	0.060		
Muscle	0.27		0.060	0.030	0.91	0.40
		0.24	0.045	0.018		

figure 3 and 4), but whose *in vitro* solubilities have not been altered.

Experiments were undertaken to attempt to demonstrate an autoantigenic property of irradiated tissues. Attempts were made to demonstrate the presence of autoprecipitins to tissue proteins in the sera of rats internally irradiated by injection of P^{32}, and an endeavor was also made to induce rats to produce autoprecipitins against X-irradiated rat tissues which had been intraperitoneally implanted or injected. The results of these tests were negative.

Electrophoretic Analyses of the Sera of Normal and Internally Irradiated Rats

Twelve adult, white laboratory rats (204-400 gms.) were used in this experiment. Six of these animals were maintained as normal controls. All 12 rats were kept in the laboratory under identical conditions. Each of the six experimental rats was given a single intraperitoneal injection of a $Na_2HP^{32}O_4$ solution, in the amount of 2.4 $\mu c./gm.$ body weight. The specific activity of the phosphate solution was 520 $\mu c./ml.$ Fifty days later, experimental and control animals were exsanguinated by cardiac puncture while in a lightly etherized state. Bleedings were preceded by a 24-hour period during which time the animals were deprived of feed. The sera were separated from the blood, and prepared for electrophoresis.

Concentrations of total serum proteins were determined colorimetrically by means of a Leitz Rouy-photometer according to

the method of Greenberg. The protein concentrations were adjusted to one gram per ml. using barbiturate buffer as the diluent. The buffer of ionic strength 0.1 and pH 8.6 at room temperature was made 0.02 N with respect to diethylbarbituric acid and 0.1 N with respect to sodium diethylbarbiturate as described by Longsworth (1942). Dialysis of the serum against the buffer was achieved in the cold (2°C) for a period of 24 to 36 hours with slight stirring of the buffer. One liter of buffer was used for dialyzing each 10 ml. sample of the diluted serum. The specific conductance of the dialysate was determined by means of a Wheatstone bridge, and used in calculating the electrophoretic mobilities of the various protein components of the rat sera. Electrophoretic determinations were made using an Aminco Portable Electrophoresis Apparatus with a current setting of 10 m.a. for 3 hours at 0°C. Protein mobilities were calculated from measurements of the descending boundaries; relative concentrations were calculated from measurements of ascending boundaries (Longsworth and MacInnes, 1940). The distances of the peaks of the various protein components from the protein boundary before migration were measured for calculating mobilities. Distances of migration and the component areas were estimated from tracings enlarged four times from the photographed electrophoretic picture obtained from the screen of the instrument. Globulin areas were divided, by the method of Tiselius and Kabat (1939), into alpha, beta and gamma fractions corresponding to those of human sera. The

areas under the component curves were estimated with a K & E Compensating Polar Planimeter.

The results of this experiment are recorded in table 3.

Lippman and Banovitz (1952), using a veronal buffer of ionic strength 0.1 and pH 8.6, have reported finding two alpha globulins in the sera of rats, but only one was resolved in my electrophoretic determinations. Alpha globulin is treated here as a single component of the sera of rats. Westphal, DeArmond, Priest and Stets (1952) and Gjessing and Chanutin (1947) have reported that the electrophoretic patterns of the globulins of rat sera and plasmas are not so distinct as those obtained with human sera. Their observations have been confirmed in my experiences with rat sera. Nevertheless, the diagrams do lend themselves feasibly to the separation of the components.

Mean values were calculated for the various electrophoretic characteristics of the sera of control and irradiated rats. To facilitate an evaluation of the data, the statistical significance of the difference of mean values of sera of control and irradiated animals were considered utilizing the <u>t</u> test of significance. The probabilities of chance differences between the mean values were obtained as previously described (see p. 27).

No significant statistical differences are demonstrable (table 3) between the mean values of total protein or the relative per cent composition of the proteins of the sera of six control and

Table 3.

Electrophoretic analyses of the sera of normal and internally irradiated rats.

Sample	Total Protein gm./100 ml.	Relative Per Cent Composition of Serum Proteins				A/G Ratio	Mobilities $cm^2/volt/sec \times 10^{-5}$			
		Albumin	Globulins				Albumin	Globulins		
			alpha	beta	gamma			alpha	beta	gamma
Control Rats (6 animals)										
Mean	5.94	57.3	10.6	20.5	11.5	1.36	5.70	4.34	2.57	1.56
Std. Dev.	0.51	3.6	1.7	2.3	4.0	0.20	0.20	0.51	0.17	0.22
Std. Error	0.207	1.45	0.69	0.93	1.61	0.083	0.083	0.207	0.069	0.091
P^{32}-injected Rats (6 animals)										
Mean	6.01	56.0	11.1	20.8	12.1	1.29	5.96	4.71	2.84	1.73
Std. Dev.	0.41	4.2	1.5	2.4	2.9	0.20	0.19	0.44	0.19	0.38
Std. Error	0.167	1.70	0.61	0.96	1.20	0.083	0.078	0.182	0.078	0.154
t values	0.26	0.58	0.55	0.22	0.30	0.59	2.28	1.34	2.73	0.95
Degree of Probability	0.80	0.60	0.60	0.90	0.80	0.60	0.05	0.30	0.05	0.40

six irradiated animals. During the course of this work it was necessary to determine the total serum protein levels of numerous animals. These data were grouped along with those obtained in the electrophoretic study. The determinations of total serum protein levels of 14 control rats and 18 P^{32}-injected rats when averaged showed a mean protein concentration (gm./100 ml.) of 6.35 ± 0.172 (Std. error) for controls and 6.28 ± 0.099 for internally irradiated animals. The estimated probability of a chance difference between these mean values was high, 0.60. These data indicate that no quantitative differences were evident between the total protein and protein component levels of the sera of irradiated and control rats.

From the electrophoretic mobility data in table 3, it is evident that there is a significant difference between the mobilities of the albumin and beta globulin components of sera of control and irradiated rats. The level of this significance of chance differences is about 0.05 for both components. It appears, therefore, that there are qualitative differences in the albumin and beta components of the sera of the normal and experimental rats. The mobilities of the two electrophoretic components of the sera of the irradiated animals are decidedly greater than those of the sera of the control animals (table 3).

The Relationship of the Components of the Sera of Rats to the Serological Turbidity Curve

Three lines of research were pursued to characterize the specific regions of reactivity of the proteins of the sera of rats.

The first entailed the chemical fractionation of proteins in a pooled sample of rat sera, and the comparative testing of these fractions and whole serum with an antiserum produced against whole, normal rat serum; the second involved the comparative serological testing of four samples of rat serum of known electrophoretic characteristics, and the third approach was by testing comparatively whole serum and the same whole serum to which had been added additional amounts of various serum fractions. All comparative serological tests were performed at 4-6°C for 24 hours. Different antisera were used for each of the three kinds of comparative tests.

The procedures of Howe (1921a, 1921b) as modified by Milne (1947) for the use of Na_2SO_4 in the quantitative determination of the proteins in blood were adapted here for the fractionation of the serum proteins of rats. Milne (1947), using human serum, identified the euglobulin precipitated by 19 per cent Na_2SO_4 as corresponding to the sum of the electrophoretic beta and gamma globulin fractions. In his electrophoretic analysis he used a veronal buffer of pH 8.6 and ionic strength 0.1. The pseudoglobulin fraction was identified as corresponding to the sum of the $alpha_1$ and $alpha_2$ globulins. The albumins determined by both methods were found to be comparable. Total globulin was precipitated by 26 per cent Na_2SO_4 solution. The identification of electrophoretic fractions with salted-out fractions of human serum were, for purposes of these experiments, considered to be approximately equal to those of rat serum.

To 10 ml. of pooled rat sera were added 300 ml. of 19.63 per cent Na_2SO_4 solution, which had been preheated to a temperature of 37°C. The mixture was allowed to stand for six hours. The resulting precipitate was then filtered off. With constant stirring 21.7 grams of anhydrous sodium sulfate crystals were added to the filtrate. This mixture was also allowed to stand for six hours. The resulting precipitate was separated from the supernatant solution by filtration. The first precipitate, euglobulin, and the second precipitate, pseudoglobulin, were each dissolved in 10 ml. portions of 0.01 N NaOH. These solutions, and the albuminous filtrate, were then dialyzed against distilled water for eight hours after which they were dialyzed against two per cent saline for 48 hours. Water was first removed from the solution of albumin by pervaporation at room temperature in a current of air produced by an electric fan, and the pervaporation activity was alternated with dialysis in saline. Water was removed by this method until the total volume of the albuminous solution was reduced to approximately 80 ml., and this final solution was dialyzed against two per cent saline for 48 hours.

In comparative tests performed with whole rat serum and serum fractions two different antisera were used. In these tests the concentration of protein in the first antigen dilution of the whole serum system in both series was 1:100. The concentrations of

protein in the serum fraction systems were so adjusted that corresponding tubes contained the same amount of the particular proteins relative to that found in whole serum, as determined previously by electrophoretic analysis of normal serum. For example, the euglobulin fraction, which contains the electrophoretic beta and gamma globulin components, represents about 32 per cent of the total protein concentration (see table 3, p. 32). The concentration of euglobulin in the initial dilution of one series, therefore, was adjusted to be 1/100 x 0.32, which is 1 part protein per 313 parts solution. By the same considerations the albumin system contained 1 part protein per 174 parts solution; the pseudoglobulin system, which contains the electrophoretic alpha component, contained 1 part protein per 943 parts solution.

The results obtained by testing sera of known electrophoretic characteristics with the same antiserum are presented in figure 6.

Results of comparative tests using whole serum and whole serum to which had been added additional amounts of the various serum fractions are presented in figure 7. Three normal sera were prepared so that each contained 10 per cent more of one of the three serum fractions relative to the average amount of those kinds of proteins found in whole serum, as determined previously by electrophoretic analyses of normal sera. For example, in the test series in which the euglobulin concentration was increased 10 per cent above the average normal level, the concentration of euglobulin

Figure 6. Comparative serological turbidity curves from tests performed with sera of known electrophoretic characteristics. Legend: —— curve of serum of rat C-15, — — — C-16, · · · · · C-17 and — · — of serum of rat R-21.

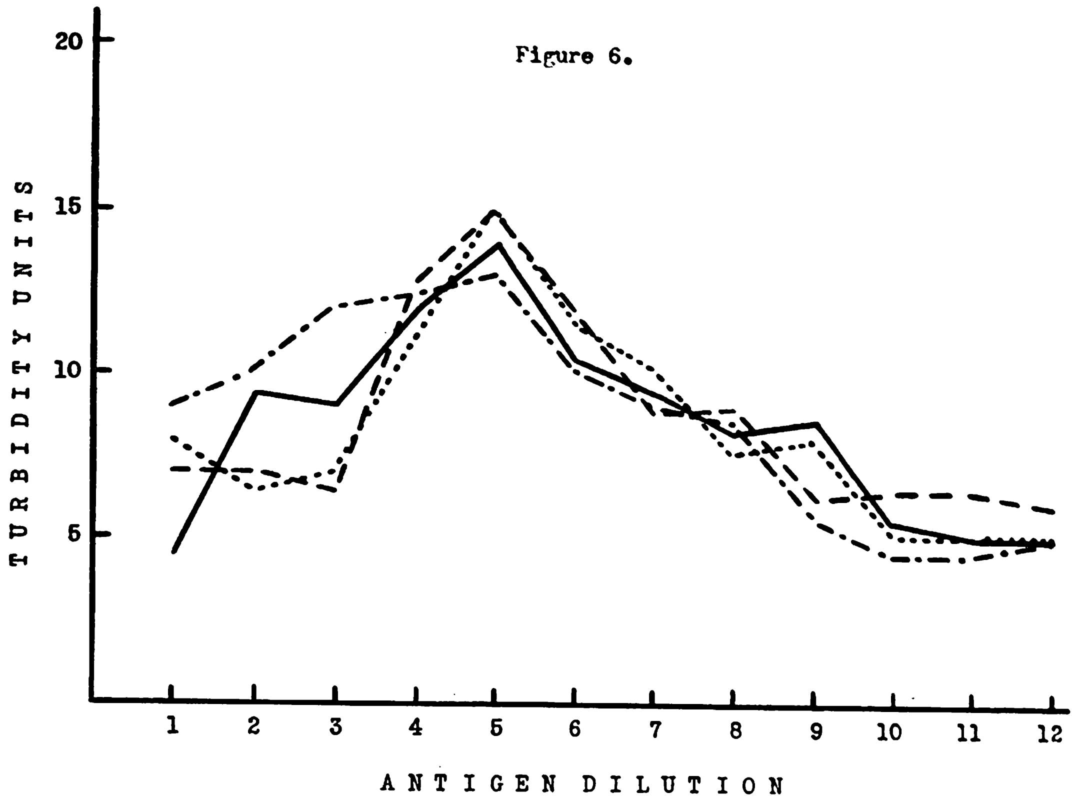

Table 4.

Electrophoretic mobilities
of the components of rat sera used in comparative serological tests.

Sample Number	Mobilities $cm^2/volt/sec \times 10^{-5}$			
	Albumin	Globulins		
		alpha	beta	gamma
C-15	5.92	4.76	2.65	1.44
C-16	5.52	3.60	2.75	1.41
C-17	5.45	4.04	2.27	1.49
R-21	6.01	3.85	2.94	2.39

Figure 7. Serological turbidity curves from comparative tests with serum of rat and the same serum to which had been added various salted-out protein fractions. Protein concentrations were adjusted so that the systems containing additional proteins had 10 per cent more of the particular components than the average level expected to be found in normal rat whole serum. Legend: ——— curve of whole serum, — — — whole serum plus 10 per cent increase of albumin, · · · · · whole serum plus 10 per cent increase of euglobulin, and — · — whole serum plus 10 per cent increase of pseudoglobulin.

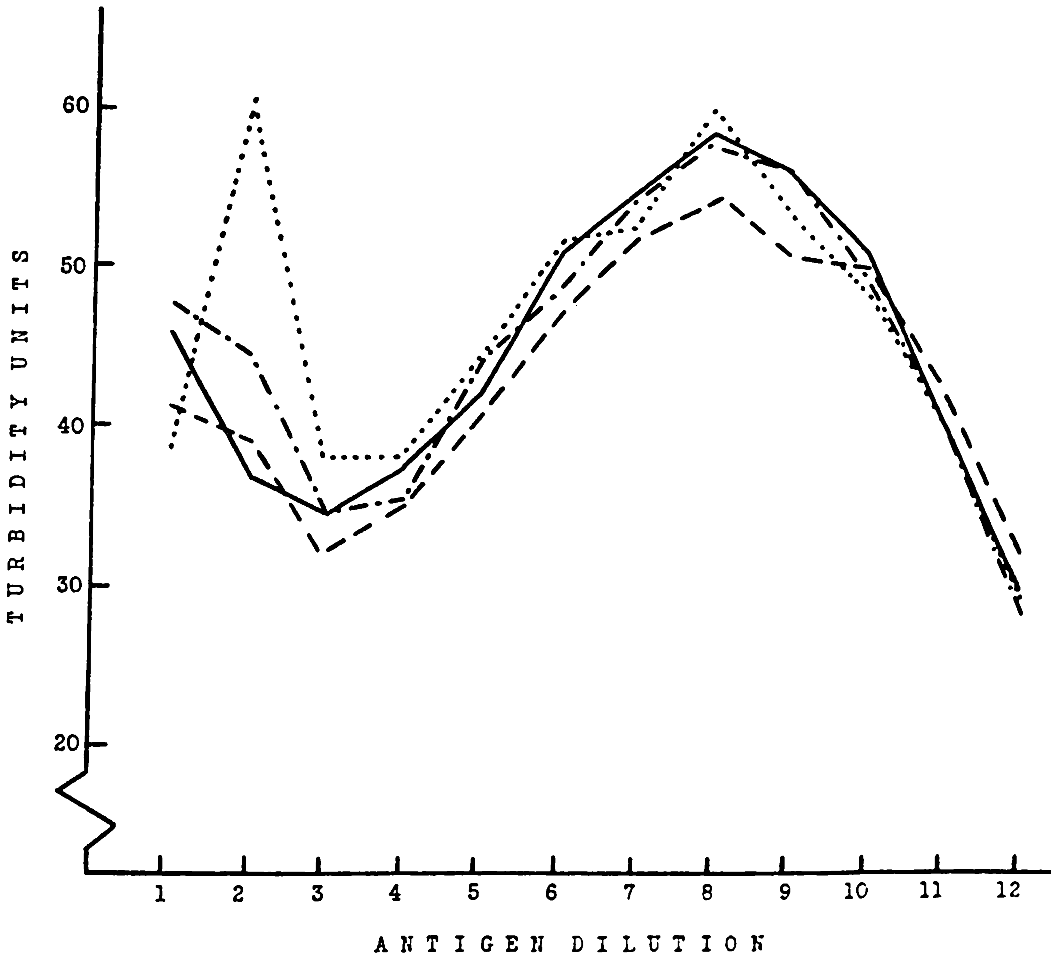

Figure 7.

in tube No. 1 was adjusted so that there was a ($0.02 \times 0.32 \times 0.10 \times 10^6$ gamma), 640 gamma, increase of that component. The increase in albumin was 1,140 gamma, and the increase in pseudoglobulin was 220 gamma above the average normal level.

The results obtained by the first line of approach gave no direct evidence of regional specificity of proteins in this heterogeneous serological system. Although the antisera were weak and the whole turbidity curves were not obtained, all three of the isolated, salted-out components were reactive over the full range of the antigen dilutions used. The euglobulins appear to be strongly antigenic to the chicken; the albumin behaves as a good antigen, and the pseudoglobulins are the least antigenic of the three fractions. It is interesting to note that the curves obtained with isolated euglobulins show that the serological reaction exceeds that of the homologous whole serum. The isolated euglobulins may give to the antigen-antibody complex a characteristic of particularly low solubility. In the absence of the other proteins of serum, which may act as protective colloids to stabilize the complex, the euglobulin-anti-euglobulin complex precipitates more readily. This kind of reactivity was lacking in tests in which the isolated albumin and pseudoglobulin fractions were used.

If the results obtained by comparatively testing sera of known electrophoretic characteristics are analysed solely on the basis of mobilities of albumin and beta globulin, some generalizations

may be made. Of the four samples of sera of known electrophoretic characteristics (table 4), sample R-21 possessed albumin and beta globulin components which had highest mobilities. In the serological turbidity curves this sample showed a high serological reactivity in the region of low antigen dilution and a low serological reactivity in the region of high antigen dilution. Sample C-17, which had the lowest albumin mobility, and sample C-16, which also had a relatively low albumin mobility demonstrated high reactivities at high antigen dilutions. Sample C-17, which also had the lowest beta globulin mobility, showed a low reactivity at high antigen concentrations. These observations suggest that high beta globulin mobility is associated with high serological reactivity in the region of low antigen dilution. They suggest also that high albumin mobility is associated with a low serological reactivity in the region of high antigen dilution. These are the directional changes and regions of change noted in turbidity curves of the sera of animals which had received internal beta irradiation (see figure 3 and 4). Also, these are the fractions whose mobilities were altered and the kind of change produced by the internal irradiation of rats (see table 3). These observations complement one another and permit the conclusion that the effects of <u>in vivo</u> beta irradiation of rat as revealed by electrophoretic and serological analyses probably are demonstrations of the same changes.

This kind of analysis appears to be justified for several reasons. The serological activities of euglobulins and of albumin

fractions probably dominate in the heterogeneous system of whole serum when the serum is tested with anti-whole serum. The antibody levels for these fractions, because they are good antigens, are probably particularly high in antiserum produced against whole serum. Slight changes, which would alter the serological activity of whole serum, would be expected to be associated with these fractions. Since, in the euglobulin fraction the electrophoretic beta globulin component is in higher concentration than the gamma globulin proteins, and the expected ratio of concentrations is 2:1 (table 3), it would seem that it is the beta globulin and albumin components that are highly determinate in the serological activity of whole serum. Also, the variability of the relative concentrations of the different electrophoretic components in the sera of the four rats used in these tests was not great. The effects of concentration on the serological nature of the whole sera probably were not great.

A 10 per cent increase in the level of euglobulin had the effect of markedly increasing serological reactivity in the portion of the turbidity curve characterized by low antigen dilution when the normal curve is used as a reference (figure 7). The salted-out proteins in this component and antibodies apparently form precipitable aggregates that are particularly unstable in suspension. A 10 per cent increase in albumin caused a decrease in serological reactivity at high antigen dilutions and particularly just to the right of the peak. Pseudoglobulin augmented serum showed a slight

increase in serological activity at high protein concentrations. These data also associate beta globulin reactivity with low antigen dilutions and albumin reactivity with high antigen dilutions in serological titrations employing whole serum of rat and the homologous antiserum. The increased concentration of a component causes a change in the position of the zone of optimal proportions for the particular antigen-antibody reaction which is involved, and a shift occurs in the position of various portions of the turbidity curves.

These observations support the conclusion that regional specificity does exist in the serological turbidity curve of mammalian serum for particular protein components. Such regional specificities have been demonstrated for the euglobulin and for albumin components of the serum of rat when whole serum is used. There is apparently a strong dependence on the presence of the different proteins in the reacting system because isolated components behave differently than the same proteins in the heterogeneous system. That is, the demonstrable serological reactivities of isolated components are different from those of the same components in whole serum. In the demonstration of regional specificities of proteins in the serological turbidity curve two different characteristics of proteins, mobility and relative concentration, were considered, but both properties seem to influence the same general regions of the curve. A qualitative change in one molecule of antigen protein may yield similar kinds of serological data as would a quantitative change involving several or many molecules of the protein.

CHAPTER IV

DISCUSSION

Effort has been made to study, at the molecular level, effects of *in vitro* X-irradiation and *in vivo* beta-irradiation on proteins. Changes were produced in serological reactivity of *Homarus* hemocyanin by X-radiation. These changes were marked and can be explained on the basis of molecular discociation, decrease in concentration of the parent protein and molecular modification (perhaps a partial denaturation) which alters the numbers of combining sites on the molecule. Denaturation of proteins in solution is commonly accepted as the most obvious effect of exposure to radiation (Arnow, 1936; Clark, 1939; Haurowitz and Tumer, 1948-1949; and Fricke, 1952). The mechanism causing splitting of molecules seems to involve the weaker peptide linkages of the protein, but the energy which causes the cleavage may not be directly absorbed at the locus of the -NHCO- linkage involved (Carpenter, 1939 and 1940; Svedberg and Brohult, 1939; and Brohult, 1940).

Although antigenicity studies were not made with irradiated hemocyanins, the changes demonstrable in serological reactivity suggest that the antigenic nature may also be changed. Studies which have considered the action of radiation on the potency of

proteins to produce passive and active anaphylaxis and on the antigenicity of non-homologous proteins show that both properties may be severely altered (Hanan, 1952; Smetana and Shemin, 1941; and others). The effect of X-rays on immunity has been reviewed by Taliaferro and Taliaferro, 1951.

The sera of rats that had received internal irradiation by means of a single injection of P^{32} and bled 50 days later, exhibited serological reactivities which deviated from those of normal sera. At least two regions on the turbidimetric curve were distinctly displaced. It can be postulated that these deviations are attributable to chemical modifications of the proteins serologically reactive in those regions of the titration curve. Electrophoretic analyses of the sera of rats subjected to the same levels of internal irradiation, as were used to study immunochemical changes, have disclosed a qualitative change in the electrophoretic mobilities of the albumin and beta globulin components of these sera. The serological findings have been correlated with the electrophoretic data by studying the relationship of the components of the sera of rats to the serological turbidity curve.

The serological data obtained with X-irradiated hemocyanin seems to elucidate an understanding of the serological data obtained with sera of rats subjected to <u>in vivo</u> beta irradiation. In contrast to hemocyanin, which is a relatively simple protein solution (Allison and Cole, 1940; and Tyler and Scheer, 1945),

mammalian serum is a heterogeneous mixture of several proteins. In figure 1 it was observed that radiation-induced changes of hemocyanin may modify the serological turbidity curve in various ways. Shifts of situations of optimal combining concentrations may cause a depression or elevation of various portions of the curve. The kind of change seen is a manifestation of the magnitude of the shift and the direction of the shift with the optimal combining situation as a reference. Increased reactivity is observed when the shift is such as to present conditions nearer the optimal combining situation and vice versa.

In considering the results of serological turbidity tests with mammalian serum it may be assumed that the serological reactivities of the various protein components are subject to the same kinds of shifts in combining conditions. The observed increase in a portion of the turbidity curves of sera of P^{32}-injected rats indicates a displacement of optimal combining conditions for beta globulin. The displacement is such as to favor an increase in observed reactivity for that component. On the other hand, beta irradiation has affected molecular modification of albumin, and this modification is demonstrable as a depression of a portion of the turbidity curve of whole serum. The differences in the observed results caused by alteration of albumin and of beta globulin are consequences of the nature of the system used to measure these changes. Actually, the alterations in serological nature of the two

components may be of the same type, but in the titrations alterations cause shifts in different directions with respect to the optimal combining situations of the two different components. The data presented in figure 7 demonstrates this feature of serological reactivity. Both euglobulin and albumin concentrations were increased and in the same relative amounts; however, the observed effects on the turbidity curves were different for the two components.

The mechanisms of protein synthesis are not known. That internal beta irradiation produces qualitative changes in some proteins of the sera of rats, may be a step forward in understanding the nature of the synthesis. The disparity in the quality of the serum proteins probably is not caused by direct actions on circulating proteins but on the systems concerned with their production. This view is supported by the report that about ten per cent of the plasma proteins are replaced in 24 hours.(Miller et al, 1949). It is tempting to suggest that the observed alterations are mediated by way of an effect on nucleic acids. Caspersson (1947) has suggested a close relationship between protein synthesis and ribonucleic acid. Administering labeled phosphorus to rats shows that the radioactive phosphorus is incorporated into the ribonucleic acid and desoxyribonucleic acid of liver (Brues, Tracy and Cohn, 1944; Hevesy, 1948). Since solubility of proteins in the tissues of rats is not chronically altered by beta irradiation, it

seems reasonable to suppose that the changes producing altered circulating proteins have only a subtle effect on structural proteins of organs, and that these slight alterations were not detected under the conditions of the experiment involving the extraction of organs, but did appear in the serological and electrophoretic data.

Numerous investigators have reported on the effects of irradiation on the soluble proteins in rat blood (Hauschildt and Supplee, 1949; Gjessing and Chanutin, 1950; Kohn, 1951a, 1951b; Volkin and Kohn, 1951; Supplee, Hauschildt and Entenman, 1952; Westphal, Priest, Stets, and Selden, 1953; Gabrieli and Chang, 1955; and others). These investigations have shown that the soluble proteins in rat blood are sensitive to X-irradiation. Quantitative changes in the protein composition of plasma and sera were demonstrated. It seems that the changes are not consistent but are usually manifested as a rise in A/G ratio and a decrease in the total protein level. No distinct qualitative changes have been shown for the soluble proteins in rat blood following *in vivo* X-irradiation. The adaptation of serological techniques to this area of study may also be fruitful in revealing qualitative changes in proteins.

SUMMARY

1. The serological activity of hemocyanins, as measured by the quantitative, turbidimetric precipitin reaction, was altered by *in vitro* roentgen-irradiation.
2. The effects of irradiation are explained on the basis of (1) production of new products by dissociation of the parent molecules, (2) decrease in the concentration of the parent molecules, and (3) by a physical change, an unfolding of the molecules, which reduces the number of combining sites on their surfaces.
3. Dissociation products of irradiated hemocyanin were detected in serological tests using the agar-diffusion method.
4. Corpuscular emanations from radioactive phosphorus, *in vivo*, produce chronic changes in the specificity of some proteins of rat serum, and these changes are serologically detectable as shifts in the position of portions of the turbidity curves. In addition, the electrophoretic mobilities of the albumin and beta globulin components of sera of irradiated rats are decidedly greater than those of the same components of control animals.
5. Study of the relationship of the components of the sera of rats to the serological turbidity curve reveals that regional specificity does exist in the serological turbidity curve of

mammalian serum for particular protein components. Sera with relatively high levels of albumin yield curves which are lower than normal in the region of low concentration of antigen. The increased concentration of a component causes a change in the position of the zone of optimal proportions for the particular antigen-antibody reaction that is involved, and causes a shift in the position of various portions of the turbidity curves.

6. Qualitative changes of the albumin and euglobulin in the sera of irradiated animals, and the influence of increased concentrations of these components in the sera of normal animals yield results which are serologically similar.

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