QUANTITATIVE STUDIES OF THE PHOTOCHEMICAL DESPECIATION OF HORSE SERUM*

An Approach to the Problem of Intravenous Foreign Protein Therapy

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The antigenicity of foreign protein solutions complicates their employment either as plasma substitutes, or as nutritive agents. Recently interest has been aroused in the possibility of doing away with antigenicity, while retaining the great molecular weight of the protein. The present paper deals with the effects of light energy in the reduction of the antigenicity of horse serum, thus rendering it tolerable to guinea pigs. The problem of sensitivity as a bar to the intravenous injection of foreign proteins is discussed incidentally, and the methods currently employed to reduce antigenicity are considered.

Salter (1) first demonstrated with experimental animals the nutrient value of serum when given parenterally. Among the larger domestic mammalian sera, he found normal horse serum the least likely to give rise to immediate symptoms, or to that delayed pyrexia, arthropathy, and exanthem which was frequent in all animals receiving large amounts of foreign serum. He determined that toxicity is associated with the globulin fraction. By heating the serum he greatly reduced both the immediate and delayed toxic effects. Correlating the available evidence, he stated "the serum from any given animal is practically innocuous towards other animals of the same species."

Rous and Wilson (2) found early in 1918 that in rabbits, homologous plasma was superior to both acacia and gelatin in restoring and maintaining the circulating fluid volume lost in hemorrhagic shock, and therefore suggested its use in human beings. Later in the year, Mann (3) arrived at exactly the same conclusion, working with anesthetized dogs, submitted to intestinal manipulation. He strongly recommended the use of homologous plasma, and suggested its storage for emergencies.

Rous and Wilson (2) noted further, that in non-sensitive animals, foreign serum was fully effective in the immediate treatment of shock. They followed the blood pressure in patients given antipneumococcus horse serum for pneumonia, and recorded a serious fall in blood pressure on injection of 2 cc. into a sensitive man, but no symptoms with amounts as large as 90 cc. in a non-sensitive man. They concluded that only in grave emergencies is the use of horse serum justifiable as a plasma substitute. Working on a larger scale, and with bovine, in place of horse plasma, Kremen *et al.*

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(4) have recently repeated and confirmed these results. It is interesting to note that in England in 1918, Ward (5) unaware of the results of Rous and Wilson, suggested the use of plasma for the treatment of hemorrhage, and proposed comparative studies, similar to those these authors had published. The use of homologous plasma for the combat of shock has followed as a logical sequel to these early studies.

Whipple has for many years studied the metabolic effects of homologous serum given intravenously (6), and his findings, with their emphasis upon the importance of plasma proteins in cellular nutrition, in the protection of the liver, and in resistance to infection, supplied a sound experimental basis for contemporary clinical exploitation of the plasma fraction of blood. Whipple's group attempted to use horse plasma in dogs, but reported that it could not be used owing to toxic symptoms (7). Salter had greater success with heated serum in rabbits, and was able to demonstrate a slight nitrogen sparing effect in some cases (1). Recently Wagensteen *et al.* (8) have shown that similar favorable changes in nitrogen balance can be demonstrated with bovine plasma when given to non-sensitive human beings, and Addis (9) has recorded similar results with normal horse serum when given to rats.

Much attention has been paid of late to the isolation of a non-antigenic fraction from the serum of the larger domestic mammals. The comprehensive studies of Doerr and Russ (10 *a*), Dale and Hartley (11), Hektoen and Welker (12), and Goldsworthy and Rudd (13), have proved that the crystalline serum albumen fraction obtained from horse serum by salting-out methods, has an antigenic specificity distinct from that of the globulins, and is less active in this respect. Hooker (14), and more recently, Janeway and Beeson (15), and Taylor and Keys (16), have demonstrated human sensitivity to non-crystalline foreign albumen fractions.

It is important to determine the degree of reduction of antigenicity that is necessary before a foreign protein solution can be safely used, without regard to the amount injected, or to the sensitivity of the recipient. In considering the use of foreign sera, it is not enough to avoid the toxic effects due to heterolysins and heteroagglutinins, and the discomforts of serum sickness: the problems of sensitization to subsequent doses of the protein, and anaphylaxis in those who are already hypersensitive must also be considered.

With regard to heterolysins and heteroagglutinins, the figures given by Salter (1), Weiss (17), and Uhlenhuth (18) for the relative immediate toxicity of horse and bovine serum have recently been confirmed, in principle, by Davis and Eaton (19). The respective lethal doses per kilo in the rabbit are in the proportion of 5 to 1, *i.e.*, 45 to 9 cc. per kilo. Sheep serum lies in an intermediate position. This ratio remains constant in various species, but the dosage in cubic centimeters per kilo varies. In man, the lethal dose of bovine plasma is probably higher. However, Kremen has shown that in spite of treatment by human red cells to adsorb heteroagglutinins, doses as low as 100 to 200 cc. gave frequent pyrexial reactions (3). It seems, therefore, that a reduction of such factors to 1/10th to 1/100th their original concentration is desirable.

Gerlough (20) has shown that serum sickness will develop in a percentage of recipients, which varies approximately as the square root of the amount administered. 5 cc. of horse serum will lead to symptoms of serum sickness in 10 per cent of the recipients, whence one may conclude that if a litre of protein solution is to be given,

it ought to contain less than this amount of unchanged horse serum, or its equivalent in active antigenicity.

Hooker (21) and Park (22) have shown that sensitization will follow remarkably small doses of antigen. Thus a majority of those who had received 1/100 cc. of horse serum years before, were found on skin testing to react positively. A protein solution intended for intravenous therapy in shock, or as a nutritive agent, will be given in doses of the order of 1 litre. Therefore, in order to avoid any danger of sensitization, there must be in 1 litre, less than the equivalent in active antigenicity of 1/100 cc. of normal horse serum. That is, if the material is obtained from bovine plasma, then the antigenicity of the derivative should be less than 1/100,000th the original. The significance of such a figure is realized on recalling that the albumen obtained by fractionation is probably not less than 1/100th as antigenic as the original serum (10 *a*).

However, Vaughan (23) has emphasized the difficulties of sensitizing an individual to a protein with which he is constantly in contact. The reason may be, as Walzer (24) has shown, that dietary antigens are constantly absorbed unchanged into the blood stream, and the evidence from post-prandial donor transfusion reactions (23, 25, 26) suggests that the amounts thus absorbed and circulating may be quite large, on occasion equivalent in antigenic activity to at least 1/10th to 1/100th cc. of normal horse serum. Vaughan (23) considers that the effect of this continued absorption of foreign protein may be, except in the case of certain allergic subjects, to hyposensitize the recipient to these antigens. If bovine serum is employed in place of horse serum (with which little contact is made), there may not be the same dangers of sensitization and shock when a solution is injected which contains small traces of the original antigen.

Hypersensitivity in the frankly allergic may be intense. 1/20th cc. of horse serum injected intradermally in a child weighing 50 pounds has been fatal (27). While it is true that such reactions are rare, and that one fatality occurs to every 50,000 cases treated with serum (22, 28), the dangers of spontaneous hypersensitivity are not to be underestimated. For every fatal reaction there will be dozens of dangerous ones, and many hundreds of minor disturbances which may prejudice the course of a critical illness. Nor can hypersensitivity be entirely avoided by anamnesis or skin testing. Rutstein *et al.* (29) and Kremen *et al.* (4) have shown that it is possible to obtain very serious reactions in persons who were negative on skin testing, and who gave no history of allergy. On the other hand, a litre of fluid is not often given in less than 1 hour, and the gradual administration (30) over this period of 1/10th to 1/100th cc. of normal horse serum, or its equivalent in active antigenicity, will probably not give rise to any uncontrollable reaction in a significant percentage of recipients.

Direction of Approach

If the foregoing analysis is correct, it can be accepted that hypersensitivity is the greatest bar to the use of foreign sera as plasma substitutes. Immediate toxicity, and even serum sickness may possibly be avoided by use of the albumen fraction, as suggested by Cohn (31), Davis and Eaton (32), and Keys, Taylor, and Savage (33). Sensitization of normal persons by small doses may perhaps be evaded if the plasma of a food animal is employed. Wolfe (34), Doerr (10 *b*), and Fleisher and Jones (35),

have shown that the antigenicity of sheep, pig, ox, and horse serum, is of much the same order. Simon (36) has shown that sensitivity to one of these products is accompanied by sensitivity to all of them in over one-third of the positive reactors. It is, therefore, probably not possible to find for all those who are sensitive to one particular protein, another to which they will not react.

An approach to this problem would be the development of a method of removing protein antigenicity, without at the same time reducing molecular weight. The immunological results of four such methods have been studied extensively. Heat, first employed by Salter (1), and later studied by Schmidt (37) and Furth (38), will reduce antigenicity to roughly 1/10th. The use of acidification and alkalinization, as studied by TenBroeck (39), Landsteiner (40), Wells (41), and more recently by Fleisher and Jones (42), and Davis and Eaton (43), will reduce antigenicity, to perhaps, 1/1,000th. The same is to be said of the controlled peptic hydrolysis, first studied by Michaelis (44), and more recently employed by Weil, Parfentjev, and Bowman (45), and later Pope (46), for the purification of antitoxins. Finally, the yeast ferment taka diastase, used by Coghill and Fell (47), would appear to give a similar reduction, based on experimental and clinical study (48, 49).

The effects of these agents upon the physical chemical characteristics of proteins have been studied in both the ultracentrifuge (50, 51) and the Tiselius apparatus (52–54). All give rise to increased dispersion, suggesting marked variations in molecular size, and also, probably in molecular length (75). Under their influence there is an increase of the beta globulin peak, at the expense of the other protein fractions. Similarly, the immunological changes show, in common, a deviation in specificity accompanying the reduction in antigenic activity of the treated product, with the possible exception of peptic digestion. Since there is usually left in such sera, a certain percentage of unchanged protein, a quantitative description of the activity of the mixture involves two estimates. The order of magnitude of the activity per cubic centimeter of the new specificity must be stated in terms of some convenient unit, *e.g.*, normal horse serum. Further, the residual content per cubic centimeter of material having the same activity as the normal unchanged protein must be determined.

It is possible to measure these changes by three methods. First, by use of quantitative anaphylactic studies of the guinea pig *in vivo*, patterned on those employed by Doerr and Russ (10 *a*) and by Wells (55), in their original studies. They based their estimates on the constancy of the minimal sensitizing dose of horse serum given subcutaneously to a young guinea pig, *i.e.*: 1/100,000 cc. The minimal shocking dose they found to be 1/100 cc. The incubation period, Doerr (10 *a*) has shown, depends on the dosage of antigen employed. That is, large doses of antigens of low activity, such as albumen, will give rapid sensitization, while small doses of active antigens will require up to 30 days. The minimum sensitizing dose mentioned refers to incubation periods of 2 to 3 weeks. Longer time intervals may decrease the dose required still further. An *in vitro* modification of this technique supplies a second and more sensitive method.

Dale (56) has shown that the isolated uterine horn is also capable of a quantitative response, and that if fully sensitized, it will respond to normal horse serum in concentrations of 1/10,000 to 1/100,000 cc. serum per cc. bath fluid. The activity of a

solution containing either an unknown quantity of normal horse serum, or an antigen of unknown activity, can then be titrated by determining the minimal sensitizing and shocking doses of the material.

A further method is the injection of equal amounts of materials of known and unknown antigenicity into two series of rabbits, and the titration to optimal proportions of the resultant precipitins. The general law governing the precipitins developed may be assumed to follow the pattern of that for serum sickness, and to vary as the square root of the antigen dosage. However, this is an assumption, and individual differences between animals are very marked. For these reasons it is not possible to correlate the results of this technique closely with those of the guinea pig studies. By using these three separate methods, and by applying them at various stages of any process employed to reduce antigenicity, a reasonable degree of conviction might be attained. Accuracy would be further increased by using large numbers of animals to smooth out individual variations. The quantitative aspects of the following studies were based on this assumption.

While the results are considered accurate to less than one decimal place, the actual reduction required in order to render a foreign serum innocuous is from at least four to six such orders of magnitude. It is as important to give estimates of the reduction attained by any method of despeciation as it is to have a clear conception of the order of reduction required in the first place. Therefore, emphasis has been placed throughout upon the quantitative aspect of the findings.

Methods and Materials

Study of the various denaturing agents available led to the choice of light energy. It offered many practical advantages, notably simplicity, sterility, possibility of operation at low temperatures, and of ready expansion to large scale production. In addition, certain theoretical considerations, to be outlined later, influenced the decision.

It has long been noted (57, 58) that ultraviolet light reduces the activity of protein antigens, in the sense that irradiated sera react poorly with antisera to normal serum. However, no studies had been made to determine whether the changes involve merely a deviation in specificity, or whether, in addition, a reduction in antigenic activity occurs. In 1935, Kallos (59) in Upsala, suggested that ultraviolet light might be employed to remove the serum sickness factor from therapeutic sera. Following up this suggestion, Stecher (60) and his coworkers studied the active antigenicity of irradiated serum and found that although specificity had been deviated, antigenicity was if anything, enhanced. They (61) warned against the casual adoption of Kallos' suggestion.

The use of visible light with a photosensitizer has also long been known to produce changes in antigenicity, but similarly the active antigenicity of these products have never been studied (62). In the course of his work on photo-oxidation, Smetana, at Columbia in 1938 (63), obtained evidence suggesting that photo-oxidised proteins were no longer antigenic, and in 1941 he confirmed the point (64). His statement that "photo-oxidation does not produce a different antigen, but destroys the antigenicity of the protein," contrasts sharply with Stecher's observations. This diametrical opposition resulting from the use of two methods so closely allied, can be resolved if

it is noted that Stecher irradiated whole serum incompletely, allowing it to stand under a lamp for a few hours only. Smetana used pure ovalbumen with a photosensitizer in high concentration in a modified Warburg apparatus. The protein solution was thoroughly agitated for 4 days, and tests for antigenicity undertaken only when photo-oxidation appeared complete. Analysis suggests that his tests would not have detected a reduction in antigenicity below 1/100th the original. It may be that the the sequence of events is the same as for other methods, such as alkali treatment. That is, while partial irradiation deviated specificity, prolonged irradiation reduced the activity of this new antigen to a point where Smetana's contention that it was virtually destroyed, was in essence correct. It was decided to attempt to reconcile these two points of view and to explore further the possibilities raised by Kallos of using irradiation as a practical method of reducing the antigenicity of foreign sera.

Methods of Irradiation

The low pressure mercury arcs recently developed for germicidal purposes (65) and now commercially available, were employed as a source of ultraviolet light. They

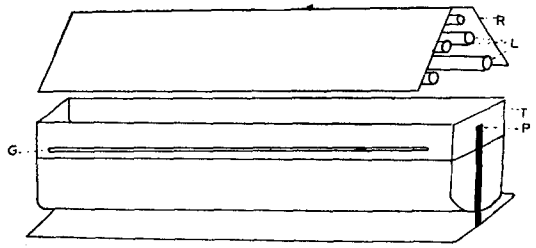

FIG. 1. Light bank and tank for irradiation. *R*, reflector; *T*, tank; *G*, glass rod; *L*, lamps; *P*, pivot.

are efficient, economical, and cool, running at 40°C. Designed to give a maximum output at the wave length desired, their radiation is as nearly monochromatic as is possible without the use of complex apparatus. That is, about 90 per cent of their energy is in the 2537 Å line (65). By using an aluminum reflector, together with five 15 watt General Electric Company[1] lamps, arranged in a "v" bank (Fig. 1), an intensity of about 600 microwatts per cm.^{2} of energy in the desired 2537Å line could be applied to the serum to be irradiated. The intensity of irradiation was measured by a standard selenium iron photoelectric photometer to which was attached a zinc silicate fluorescent screen. By this means the approximate constancy of output throughout the course of the experiments was established.

Visible light was obtained from three blue, and two green, fluorescent tubular lamps. Their design was the same as that of the ultraviolet lamps, except that to increase the wave length of the light to the visible range, the inside of the tube was

[1] I am indebted to the General Electric Company of Canada for supplying me with data on the output of the lamps employed.

coated with various fluorescent salts, such as zinc silicate (66). Their energy output and spectral distribution were approximately known from published data. Their actual output when in the bank was measured with a calibrated photometer and found to be 700 foot candles. The serum was placed in an enamel tray of dimensions 18 × 4 × 2 inches, under the lamps. The tray itself fitted into a tank which contained a refrigerating coil which provided a means of temperature control. The tank was mounted on pivots and connected by a rod to an eccentric which was driven at some 60 to 100 revolutions per minute by an electric motor. The speed of the motor was controlled by a series resistance. Within the tray was placed a mercury-loaded, sealed glass tube, which moved from side to side as the tray was tilted. By adjusting the speed of the motor and the diameter and loading of the tube, agitation could be made very complete without the development of frothing. The temperature of the irradiated solution did not rise above 25°C. Refrigeration was not employed. The sterility of solutions was assured by autoclaving, or Seitz filtering all materials, using precautions for asepsis throughout, and by the germicidal effect of the irradiation itself. Since there was some evaporation, sterile distilled water was added by a burette connected by a rubber tube to a fine capillary. Bacteriological tests were kindly performed on a sample of irradiated serum by Professor Frederick Smith of the Department of Bacteriology of McGill University, and the serum was found to be sterile.

The Serum and Sensitizer.—Sterile normal horse serum was obtained through the courtesy of the Parke Davis Company of Detroit. The asepsis employed permitted the omission of preservatives. It was considered important to employ normal horse serum since Doerr (67) has shown that the antigenicity of a serum may increase tenfold during the induction of the hyperglobulinaemia of immunity. Before subjection to irradiation, the serum was diluted 1:1 with either 0.85 per cent saline, or with M/15 phosphate buffer at pH 7. 150 cc. of the solution were placed in the large tray, or 50 cc. in a smaller tray, 8 × 3 × 1 inch, thus giving a layer only 3 to 4 mm. deep. As a photosensitizer, hematoporphyrin, a product of the Nordmark Chemical Company was employed in a 0.2 per cent solution in M/15 phosphate buffer. It was sterilized by Seitz filtration.

The Progress of Irradiation.—The degree to which the precipitin reaction to anti normal horse serum had decreased was chosen as an index of the progress or irradiation. In order to follow this reduction to the furthest extent possible, a high titre antiserum was obtained by giving a prolonged course of immunization to rabbits. This was then titrated against specimens of irradiated sera taken as various time intervals. On some occasions the proportions were held constant at 1:400, on others titration was to optimal proportions. The amounts used were 1/10 cc. of antiserum to 2 cc. of antigen dilution. The containing tubes were shaken, stood for 18 hours at room temperature, and the resultant turbidity read in a slightly modified Libby photronreflectometer (68). Thus a quantitative measure could be obtained of degrees of antigen-antibody combination, so slight that a turbidity was hardly visible, and the ring precipitating test indefinite. Calibration with barium sulfate showed that in the region of the opacities employed, the galvanometer readings were directly proportional to the amount of material in suspension. Over the range employed, the galvanometer readings were thus proportional to the amount of insoluble antigen-

antibody complex formed. A method sensitive to faint turbidity was considered especially desirable under the circumstances of this study, since the aim was to determine the point at which no further reactions occurred. Observations were controlled throughout, either by repetition of results with an antiserum of lower titre, or by running parallel estimates, substituting saline for irradiated serum, and normal rabbit serum for immune anti serum.

The precipitins which developed in a series of twelve rabbits receiving various sera were also followed. Each was given 20 mg., either of normal horse serum, or of irradiated protein, eight times, at 4 day intervals, three times intravenously and five times intraperitoneally, and bled, when fasting, 6 days later. These antisera were not pooled, but titrated separately to optimal proportions, and the ensuing opacities read with the photometer. In some cases a control serum was taken before the course began, in order to demonstrate by direct comparison, any minimal development of precipitin that might have occurred.

To study the changes in antigenicity resulting from irradiation, 180 to 250 gm. guinea pigs were sensitized by intraperitoneal injection of 1 mg. of protein material, some once, and some three times at 3 day intervals. Young, healthy animals of the same breed were used throughout the studies in order to obtain constancy of reaction and high sensitivity. The diet was kept constant and was of adequate vitamin C content. They were tested at intervals varying from 2 to 4 weeks by intravenous injections and observed for symptoms. The intravenous injections were usually made into the saphenous vein. In a few cases the ear, jugular, or cardiac route was employed. Immediately after injection the animal was liberated, his behavior noted, and his rectal temperature followed as an index of the severity of a reaction (10*c*). Autopsy was always carried out, and any cases receiving cardiac puncture observed for hemopericardium.

The Dale uterine horn technique was carried out in a smaller muscle bath than usual, *i.e.*, 2 cc. The calcium content of the Dale's solution was reduced to one-half in accordance with Dale's original observation (56) that spontaneous contractions were thereby reduced. Increasing concentrations of the test antigen were added, and the bath emptied and refilled between each addition until full anaphylaxis had occurred. The completeness of the reaction was confirmed by failure to respond to a second dose in spite of a full response to a standard dose of 0.5 gamma histamine per cc. This constant dose of histamine also served as a standard by which the responses of two differing uteri could be compared.

In order to study roughly the chemical changes developed, the xanthoproteic, Millon's, and Hopkins-Cole color reactions (69) were applied to the sera and to their dialysates. The effects of the various standard protein precipitants were noted, the pH changes observed roughly with nitrazine paper, and the histamine content estimated by the method of Barsoum and Gaddum (70). The total nitrogen, and also the nitrogen not precipitable by phosphotungstic acid, was estimated by the micro Kjeldahl technique.

RESULTS

Irradiation with Visible Light.—The effects, under varying conditions, of a series of irradiations with visible light were followed by use of the precipitin

reaction at constant proportions. Fig. 2 shows the results when diluted horse serum was irradiated with blue green light of 600 foot candles, both with and without hematoporphyrin. Curve 1 shows how slight are the changes to be obtained with the use of light alone, and curve 2 the effects of prolonged agitation in the dark. To do this, 5 cc. of serum were placed in a 4 × 1 inch vial and rolled in the tray for 72 hours. In curve 3 is seen the powerful effect

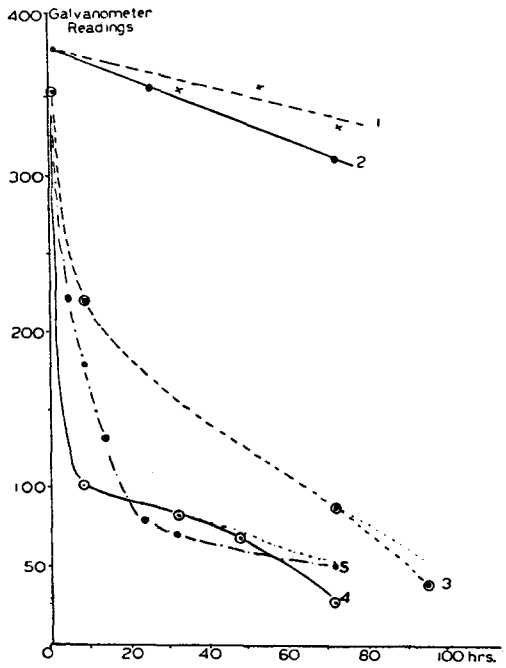

FIG. 2. Titration with 0.1 cc. of antiserum at constant proportions of 2 cc. of 1:400 dilution of irradiated sera. Curve 1, 600 foot candles, blue, green light without sensitizer. Curve 2, agitation in the dark. Curve 3, 600 foot candles, blue, green light and 2 mg. of hematoporphyrin per 100 cc. A further 4 mg. added at 72 hours. Curve 4, same light, but 20 mg. hematoporphyrin added per 100 cc. and a further 20 mg. added at 32 hours. Curve 5, same light, but 4 mg. hematoporphyrin added every 6 hours for 24 hours.

of the addition of 2 mg. of hematoporphyrin per 100 cc., and the additional effect when at 72 hours, 2 more mg. were added. Curve 4 demonstrates the effects with ten times the amount of hematoporphyrin. In this case, the second dose was added at 32 hours. Curve 5 demonstrates that the addition of 16 mg. of hematoporphyrin in four 6 hourly doses, has, if anything, a slightly greater effect than the larger single initial dose of 20 mg.

Since curve 4 showed the closest approach to extinction of the precipitin

TABLE I

Anaphylaxis of Guinea Pig to Serum A

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
NHS, 0.1 cc. given 3 times at 3 day intervals	21	Serum A 2 cc.	−	2 guinea pigs injected
Serum A, 0.2 cc. given 3 times	21	NHS 2 cc.	++++	1 pig injected
		NHS 1 cc.	+++	1 " "
Serum A, 0.2 cc. given once	27	Serum A		
		0.2 cc.	++++	1 " "
		2 cc.	++++	1 " "

NHS indicates normal horse serum.

Death, ++++; severe anaphylaxis, +++; dubious symptoms, ±; unaffected, −.

TABLE II

Minimal Lethal Shocking Dose of Horse Serum for Sensitized Guinea Pigs

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
NHS, 0.1 cc. given 3 times at 3 day intervals	28	NHS 1.5 cc.	++++	3 pigs injected
		" 0.2 cc.	++++	3 " "
		" 0.02 cc.	++++	1 pig injected
		" 0.05 cc.	++++	1 " "
				(Autopsy confirmatory on all 8 pigs)
" "	28	" 0.01 cc.	++	1 pig. Temperature constant
" "	28	" "	+++	1 pig. Temperature 101–96°F.
" "	28	" 0.005 cc.	+++	1 pig
" "	28	" 0.002 cc.	+	2 pigs. Temperature constant
NHS, 0.1 cc. given once	22	" 1.0 cc.	++++	1 pig
		" 0.5 cc.	++++	2 pigs
		" 0.1 cc.	++++	3 "
		" 0.01 cc.	++++	2 "
				(Autopsy confirmatory on all 8 pigs)

reaction, the irradiated material, which may be termed serum A, was employed to sensitize six guinea pigs. In Table I it is seen that 2 cc. of the 1:1 dilution with normal saline, containing 10 mg. of irradiated protein, failed to cause anaphylaxis in an animal fully sensitized to normal horse serum. Specificity has thus been deviated. That animals sensitized with serum A react to normal horse serum, would seem to confirm the results of the precipitin test, and show that sufficient unchanged horse serum antigens remain in the 3 mg. of serum A to sensitize to horse serum. The reaction of serum A sensitized animals when given serum A, proves that not only is specificity deviated, but the material is strongly antigenic.

In Table II are presented the control data obtained with guinea pigs sensitized with the same batch of normal serum. These animals developed fatal

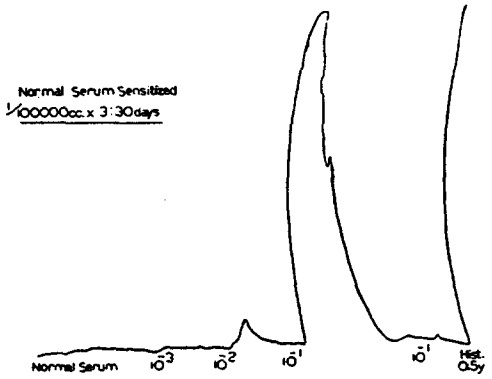

FIG. 3. Dale test with a guinea pig uterus sensitized to normal serum. The animal sensitized with 3 injections of 1/100,000 cc. of normal horse serum, at 3 day intervals, and tests made 30 days after the last injection.

shock with a minimal dose in the region of 1/100 cc., and symptoms with about 1/500 cc. Thus, the sensitivity of the animals was the same as those studied by Doerr (10*a*) and by Wells (55). The minimum sensitizing dose is the same as that found by these authors, *i.e.*, approximately 1/100,000 cc., as may be seen in the accompanying uterine tracing. In the case of Fig. 3 the animal received a sensitizing dose of three injections of 1/100,000 cc. of normal horse serum 4 weeks before testing.

Rough quantitative estimates of these changes can therefore be made. Since 1/500 cc. of normal serum will usually cause symptoms in a normal serum sensitive animal, the now altered proteins in 2 cc. of serum A were equivalent in antigenic activity to less than 1/100 cc. of the original unchanged horse serum per cc. The fact that 1/5 cc. of serum A killed serum A sensitized pigs suggests that the fresh antigenicity of serum A was at least 1/20th that of

normal horse serum, if the minimum lethal dose of normal horse serum is accepted as 1/100 cc.

Irradiation with Ultraviolet Light.—Diluted sera were irradiated with approximately 400 microwatts per cm.^{2} of 2537Å light from three 15 watt lamps. Fig. 4 shows the results of the study of the precipitin reaction at constant proportions. Curve 1 was determined for serum with 20 mg. of hematoporphyrin added, that had been irradiated with only the visible and long ultraviolet

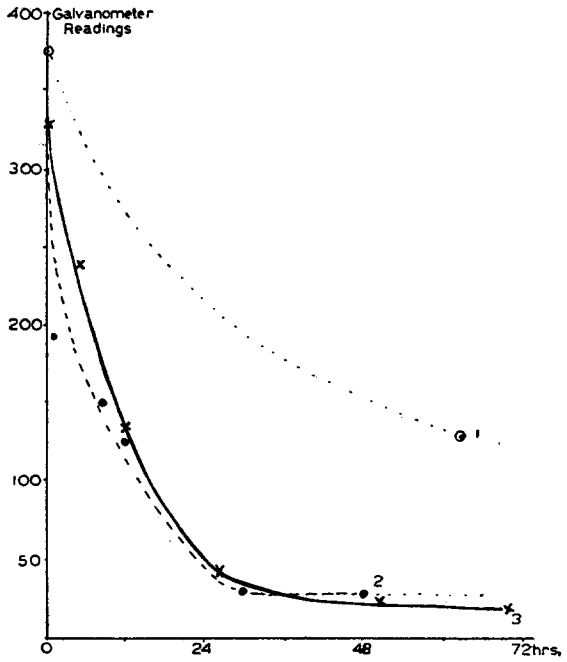

FIG. 4. Effects of ultraviolet light with and without sensitizer. Titration of 0.1 cc. high titre rabbit antiserum with 2 cc. of 1:400 dilutions of irradiated sera. Curve 1, visible light output only of ultraviolet lamps serum sensitized with 20 mg. hematoporphyrin per 100 cc. Curve 2, 400 microwatts per cm.^{2} 2537 Å ultraviolet (serum B). Curve 3, 400 microwatts per cm.^{2} ultraviolet with 20 mg. hematoporphyrin per 100 cc.

output of these lamps, the short ultraviolet being cut off by a plate glass filter. There is as marked a reduction in precipitin reaction in 72 hours as is obtained with the whole output in 14 hours. This shows that the light from the lamps is by no means all confined to the 2537 Å line. Curve 2 is for ultraviolet light alone without a sensitizer, and curve 3, that for ultraviolet light with 20 mg. of hematoporphyrin per 100 cc. Curve 3 suggests a slightly increased effect as the result of this addition.

To determine more accurately the changes occurring, a series of curves were derived from titration to optimal proportions of sera irradiated with 600 microwatts per cm.^{2} of ultraviolet light for varying periods. Fig. 5 shows the progressive changes in precipitin reaction up to 96 hours irradiation. This curve was derived from serum that had been irradiated for 96 hours with an initial addition of 20 mg. of hematoporphyrin per 100 cc. The parts of the 48, 72, and 96 hour curves in antigen concentration above 1/128 were corrected for

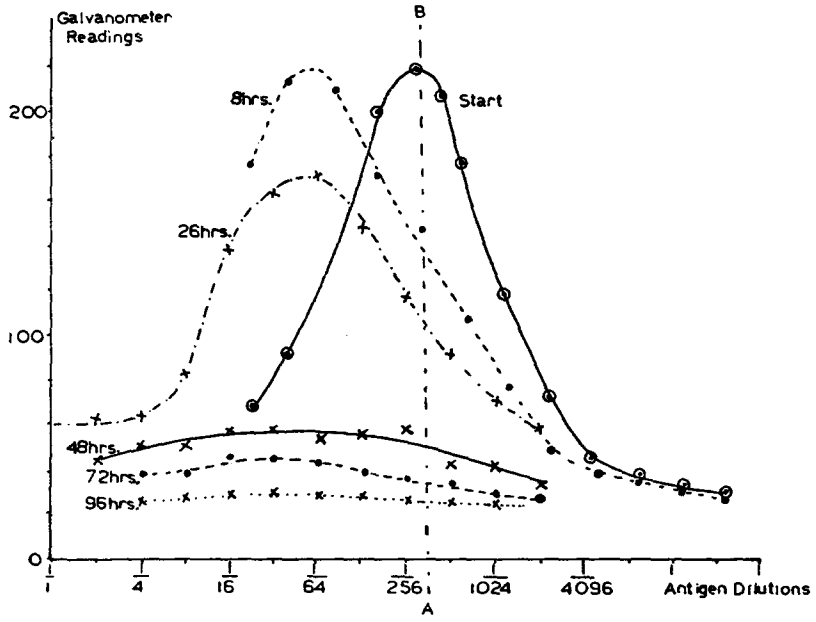

FIG. 5. Effects of irradiation of horse serum antigens with ultraviolet light. Titration to optimal proportions of 0.1 cc. horse antiserum with various dilutions of horse serum. Start, before irradiation. 8 hours, after 8 hours, 600 microwatts per cm.^{2}, light of 2537 Å. 26 hours, after 26 hours of same irradiation. 48 hours, after 48 hours. 72 hours, after 72 hours. 96 hours, after 96 hours of 600 microwatts per cm.^{2} of ultraviolet light of 2537 Å with addition of 20 mg. of hematoporphyrin per 100 cc.

the opalescence that develops in the more irradiated sera. This was accomplished by reference to the control titrations with normal rabbit serum which were set up in parallel with every titration against the anti-horse immune rabbit serum.

The physical characteristics of the serum which had been irradiated for 72 hours with 600 microwatts per cm.^{2} of ultraviolet light without photosensitizer, and which may be termed serum B, were as follows: It was of the

same color, but had become slightly opalescent. There was a very faint odor of burnt protein. The protein precipitants, *e.g.*, trichloracetic acid, phosphotungstic acid, and salts of the heavy metals, produced voluminous precipitates. Unlike normal serum, the addition of 0.2 cc. normal acetic acid in equal volume caused a heavy precipitation, but on the other hand, boiling failed to cause any turbidity or precipitation. Dialysis through No. 450 cellophane against normal saline gave a dialysate which became turbid on

TABLE III

Anaphylaxis of Guinea Pig to Serum B

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
Serum B, 0.2 cc. given 3 times at 3 day intervals	22	Serum B 2 cc.	++++	1 pig. Autopsy confirmatory
" "	22	0.7 cc.	+++	1 pig. Temperature 100–96°F.
" "	22	0.2 cc.	+++	1 pig. Temperature 101–99°F.
" "	22	NHS 2 cc.	++++	1 pig. Autopsy confirmatory
" "	22	" 0.4 cc.	++++	1 pig. Autopsy confirmatory
" "	22	Serum B 2 cc.	++++	1 pig. Autopsy confirmatory
" "	22	" " 2 cc.	+	1 pig. Temperature constant
Serum B, 0.2 cc. given once	22	" " 2 cc.	+	1 pig. Temperature constant
" "	22	" " 1.5 cc.	−	3 guinea pigs
" "	22	NHS 1 cc.	+	2 " "
NHS, 0.1 cc. given 3 times	22	Serum B 2 cc.	−	2 " "
NHS, 0.1 cc. gven once	22	" " 2 cc.	−	3 " "
		" " 5 cc.	−	1 " pig

Death, ++++; severe anaphylaxis, +++; definite, +; dubious, ±; unaffected, −.

adding tannic acid or heavy metals. The color tests for the benzene ring and for tyrosine were as strongly positive for the irradiated protein as for normal serum. That for tryptophane however was only doubtfully present. Intermediate stages in irradiation showed diminishing amounts of tryptophane. None of the tyrosine present in the dialysate was free, it was all incorporated in that material in the dialysate which was precipitable by tannic acid or by heavy metals. To control the possibility of adsorption, free tyrosine was added to the dialysate and was readily detectable.

Tests at intervals with nitrazine paper showed that unless the serum was buffered, the pH fell progressively during irradiation, from 7.0 to 4.5 at 72 hours. The histamine content increased from the normal value of 0.004 gamma per cc. only to 0.0080 gamma per cc. The total nitrogen content of the original serum was 11.7 mg. per 100 cc., and of this, 0.7 mg. was not precipitable by phosphotungstic acid. After irradiation, this fraction rose to 2.1 mg. per cc., showing that 1.4 mg. of the original 11.7 mg., or about 10 per cent, had been reduced to a form not precipitable by phosphotungstic acid.

In Table III are seen the summarized results of studies with serum B upon the guinea pig *in vivo*. Animals sensitized to normal horse serum do not react to serum B in amounts up to 5 cc. A single injection of 0.2 cc. of serum B

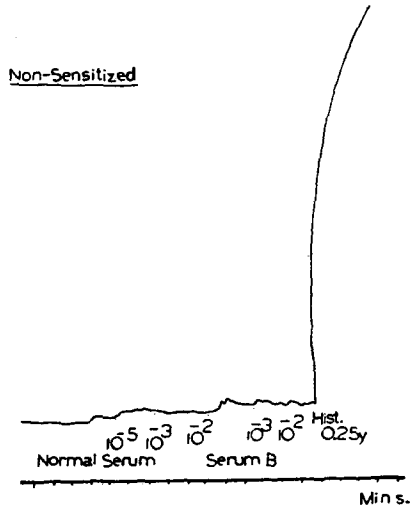

FIG. 6. Dale test with control non-sensitized guinea pigs.

does not sensitize, either to normal serum, or to serum B. On the other hand, three injections of 0.2 cc. sensitize both to normal serum and to serum B, and fatal shock was obtained with the latter with a dose of 2 cc., but not with a dose of 0.7 cc.

These results suggest that the proteins in 1 cc. of serum B are equivalent in antigenic activity to less than 1/5,000 cc. of normal horse serum, for serum B fails to shock normal serum sensitized animals, and 0.2 cc. fails to sensitize to normal horse serum (the minimal sensitizing dose of normal serum is of the order of 0.00001 cc.). Since it requires more than 1 cc. of serum B to shock fatally, serum B sensitized animals, the actual powers of shocking, haptenic powers of serum B are approximately 1/100th those of normal horse serum (Table III). The active antigenic powers of serum B may be even less than

this, since one dose, 1,000 fold the sensitizing dose of normal horse serum fails to sensitize to serum B.

In the Dale test, the non-sensitized uterus did not respond to concentrations of normal serum and of serum B up to 1:100 in the bath (Fig. 6). The normal serum sensitive uterus reacted to 1:100,000 of normal serum, but 10,000 times

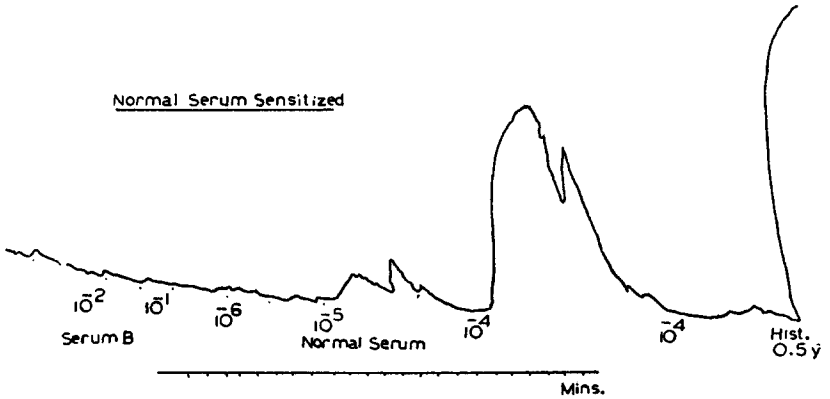

FIG. 7. Dale test with guinea pigs sensitized to normal serum. Sensitized with three injections of 1/10 cc. normal horse serum at 3 day intervals, and tested 3 weeks after the last injection.

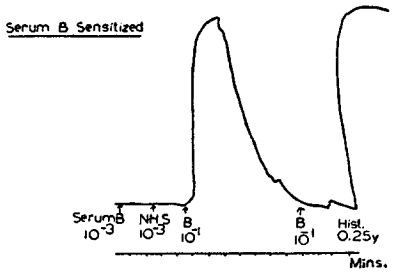

FIG. 8. Dale test with guinea pigs sensitized with serum B. 200 gm. pigs given 1.0 mg. protein intraperitoneally three times, at 3 day intervals, and tested 3 weeks after the last injection.

this concentration of Serum B failed to elicit a response (Fig. 7). In contrast, a three times serum B sensitized guinea pig uterus did not react to serum B until a concentration between 1:100 and 1:10 was reached (Fig. 8). These tracings, confirmed by three others not illustrated, support preceding approximations that the antigenic activity of serum B was reduced to 1:10,000 in terms of normal horse serum, and that the new active antigenicity of deviated

specificity of this serum is of the order of 1/1,000th that of normal horse serum.

Fig. 9 represents the average of the values of the individual titration of the four antisera to normal serum obtained after giving the standard course to

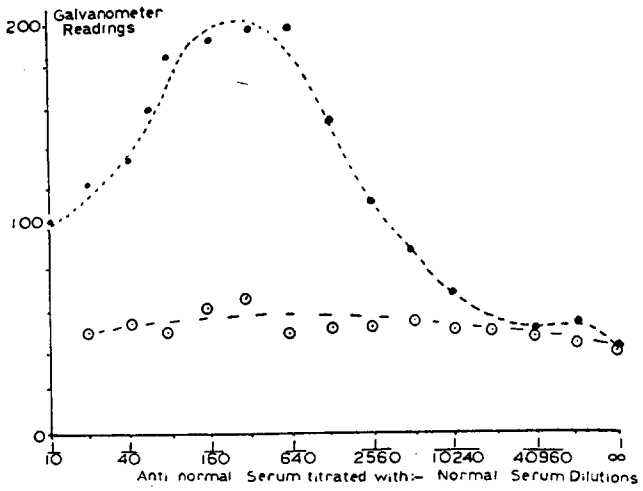

FIG. 9. Antiserum to normal serum. Circles, control titration of normal rabbit serum with normal horse serum. Dots, rabbit antisera to normal horse serum, titrated to optimal proportions with normal horse serum.

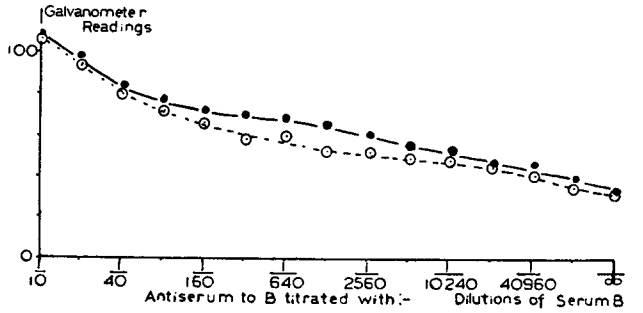

FIG. 10. Antisera to serum B. Circles, control titration with normal rabbit serum. Dots, rabbit antisera to serum B, titrated to optimal proportions 0.1 cc. with 2 cc. of varying dilutions of serum B. (Average of five curves.)

four control rabbits. The contrast with Fig. 10 is sharp. Here the averaged results of equivalent courses of serum B given five rabbits are shown. On averaging the titrations of the five antisera to serum B with normal serum, there was no detectable reaction, showing that any antibodies developed are

TABLE IV

Anaphylaxis of Guinea Pig to Serum C

Sensitizing dose	Elapsed time from last injection	Shocking dose	Result	Comment
	days			
NHS, 0.1 cc. given 3 times at 3 day intervals	27	Serum C 2 cc.	−	(1) 300 gm. pig
Serum C 0.2 cc.	19	" " 2 cc.	−	(3) 800 " pigs
" "	26	" " 2 cc.	−	(3) 200 " "
" "	"	" " 4 cc.	±	(1) 200 " pig. Temperature 100–102°F.
" "	19	NHS 2 cc.	−	(3) 800 gm. pigs
" "	27	" 1 cc.	+	(1) 180 " pig
" "	27	" 2 cc.	+	(1) 240 " "

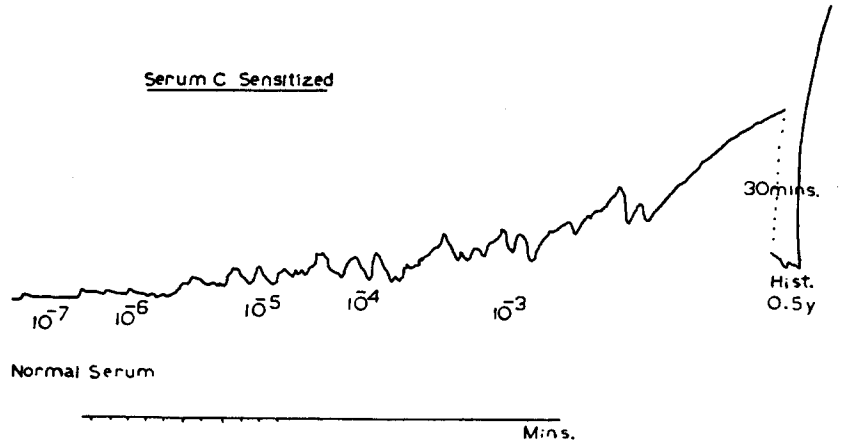

FIG. 11

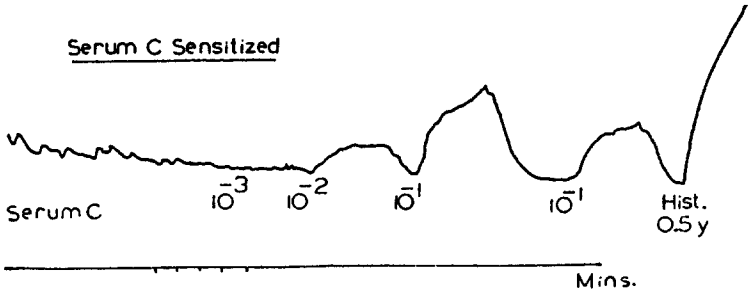

FIG. 12

FIGS. 11 and 12. Dale test with guinea pigs sensitized with serum C. 200 gm. guinea pigs given 1.0 mg. protein intraperitoneally three times at 3 day intervals, and tested 4 weeks after the last injection.

to the altered antigens in serum B, and that if any minute quantities of normal serum antigens do remain, they have given rise to no observable antibody concentration.

To find whether these results could be confirmed, serum was irradiated for 96 hours with 600 microwatts of ultraviolet light in the 2537 Å region. It was buffered with M/15 phosphate to pH 7 and treated with 20 mg. of hematoporphyrin per 100 cc. In this product the test for tryptophane was negative. It was expected that the antigenic activity of this serum, termed serum C, would be even lower than that of serum B. Table IV shows that in spite of three

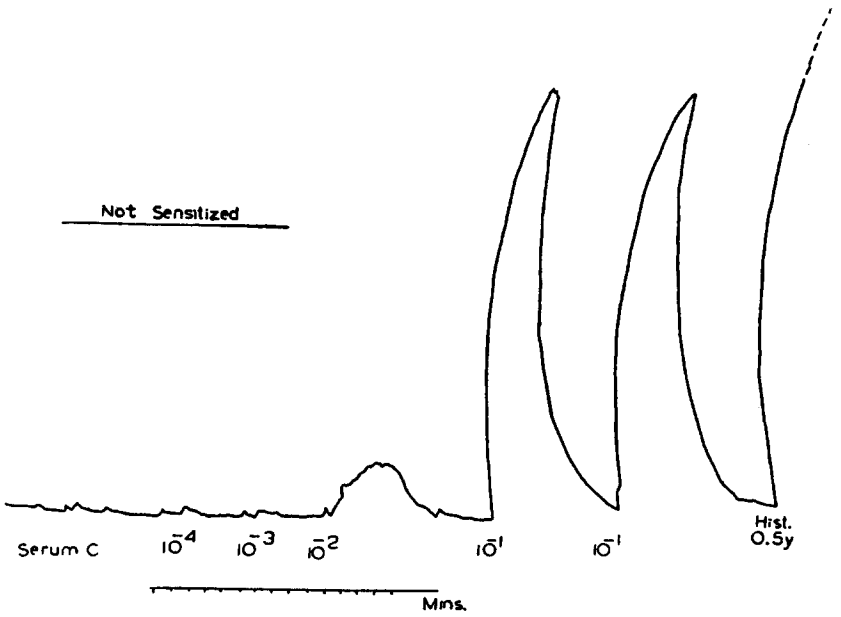

FIG. 13. Dale test with serum C upon the normal guinea pig uterus.

intraperitoneal injections of 1 mg. of protein, and an incubation period of 3 to 4 weeks, a shocking dose of up to 4 cc. of serum C failed to elicit symptoms. Nor were these animals affected by what would be in those sensitized to normal horse serum, two hundred times the minimal lethal dose of normal serum. The implication following the reasoning already employed is that the active antigenicity of serum C is of the order of 1/10,000th that of normal serum, and that the residual content of antigens having the specificity of normal horse serum may be as low as the equivalent of 1/100th cc. of horse serum per litre.

The uterine strip which is more sensitive than the tests *in vivo* confirms these results. Animals sensitized with three injections of 0.1 cc. of serum C reacted

after an incubation period of 1 month, very incompletely to doses one hundred times the full shocking value (Fig. 11). This suggests that in the three doses of 1/10th cc., there was less than the antigenic equivalent of 1/100,000 cc. of normal serum (Fig. 12), and demonstrates that in serum C sensitized uteri, a reaction occurs only at high concentrations of serum C. Since it can be repeated, and since it also occurs in the non-sensitized uterus, (Fig. 13) it is not anaphylactic, but may be due to the effect of break-down products. On titrating three antisera to serum C and averaging the results, a slight but definite reaction was observed when the graph was compared with the average

TABLE V

Effects of Irradiation upon the Antigenicity of Normal Horse Serum

	Normal serum	Serum A	Serum B	Serum C
Treatment	–	600 ft. candles blue, green 72 hrs. with 20 mg. Hp per 100 cc.	600 micro-watts per cm.^{2} 2537 Å 72 hrs.	600 micro-watts per cm.^{2} 2537 Å 96 hrs. with 20 mg. Hp per 100 cc.
pH	7.0		4.5	
Tyrosine	++++		++++	
Tryptophane	++++		±	–
Histamine	0.004 gamma per cc.		0.008 gamma per cc.	
Approximate active antigenicity	1	$\frac{1}{20}$	$\frac{1}{1,000–5,000}$	$\frac{1}{5–20,000}$
Residual unchanged horse serum antigenic equivalents, *cc./cc.*	1	$\frac{1}{1,000 \text{ cc.}}$	$\frac{1}{10,000 \text{ cc.}}$	$\frac{1}{100,000 \text{ cc.}}$

Hp, hematoporphyrin.

of the control titrations of serum C, with the sera taken before the immunising courses were started. As in the case of serum B, no antibody to normal horse serum was detected in the antisera to serum C. The reactions in all but one of these cases were so indefinite that they could be detected only with the photometer.

A summary of the more significant findings appears in Table V. The numbers of animals used, the variety of the techniques, and the controls employed throughout, serve to counterbalance the inaccuracies of the methods, and random errors of individual variations. Therefore, approximate values have been affixed for the order of magnitude of the antigenicity of the materials

studied; for without such an estimate it is not possible to assess their activity relative to each other, and to those materials, such as horse serum, whose effect upon human beings is now well known.

DISCUSSION

The masterly studies of Rideal and Mitchell (71 *a*) upon the photochemistry of the peptide link have shown that in surface films, the chief effect of light of energy content from 3000 Å to 2600 Å is to rupture those links in the polypeptide chain immediately adjacent to the aromatic residue. The photochemical threshold for this process lies at 3000 Å and is also the threshold for absorption by the aromatic nucleus (71 *b*). The benzene ring will not resonate to incident photons of a longer wave length. The energy of combination of the CO—NH link, is approximately one-fifth that of a photon of threshold wave length. If a means of transmitting the energy of photons of longer wave length were available, it would still be sufficient to rupture the peptide links. Photosensitizing dyes probably supply structures which, resonating to these longer wave lengths, perform this transmission of energy (72, 73).

Sanigar, Krejci, and Kraemer (74), studying the effects of ultraviolet light upon human serum albumen have obtained evidence suggesting the splitting off of low molecular weight fractions of high aromatic amino acid content. At the same time, their ultra-centrifugal studies suggest that the majority of the protein increases in size as a result of aggregation. The average resulting molecular weight they estimate as between two to three times the original; the protein is highly polydisperse, with probable marked variations in molecular length (75) as well as in weight. These findings have been confirmed by Svedberg and Brohult (76). Bernhart (77), using dialysis and nitrogen estimates as a gauge of molecular weight changes, has found marked increases in the dialysable low molecular weight fractions following prolonged ultraviolet irradiation.

The physical chemical findings after irradiation with visible light and a photosensitizer are similar to those with ultraviolet light. Smetana and Shemin (64) have observed aggregation and polydispersion in studies with the ultracentrifuge. Harris (78), Lieben (79), and Carter (80), have shown that the aromatic nucleus in the protein is selectively affected, and Lieben (79) has demonstrated a selective loss in tryptophane and tyrosine. Recently Smetana (64) has confirmed this loss of tryptophane, but not that of tyrosine. During photolysis, both by ultraviolet and by visible light, there is oxidation of sulfhydryl groups (81).

The findings with serum B and serum C showed a destruction of tryptophane, and dialysable fractions containing aromatic amino acids were also demonstrable. The opalescence obtained suggests the presence of some aggregates of very high molecular weight. Possibly this is to be anticipated in view of the marked polydispersion.

The effects of ultraviolet and of visible light upon proteins are of interest, for here is an agency which strikes directly at the aromatic rings. The greater susceptibility to light of antibodies, as compared with antigens, may be, because in order to function as an antibody, a protein must preserve exactly that pattern which enables it to fit, or neutralize, an antigen. On the other hand,

an antibody so denatured that it is stretched out (82 *a*, *b*) and otherwise modified so that it could not possibly function as an antibody, may still be highly antigenic, albeit with a deviated specificity. There are over seventy tyrosine radicles in a globulin molecule (83), arranged, according to Bergmann's hypothesis (84), in repeating sequence. If light energy disrupts the linkages holding these into the peptide backbone, varying stages of photolysis may be expected to give varying degrees of disorganization of the original pattern. The changes would not necessarily involve either liberation of the aromatic residue by the unlikely event of rupture of the link on each side, or a total photolytic destruction of the residue itself. Landsteiner (85) has emphasized that alkali-treated protein of reduced antigenicity could be made more antigenic by nitration of the aromatic residues. Since the addition of groups to the aromatic residues restores antigenicity, it is not unreasonable to venture that distortion from their normal relations may lead to a great diminution in antigenicity (86, 87).

The evidence presented has shown that under the conditions used, the photo-oxidation of serum with visible light will lead to a deviation in specificity, but to little diminution in antigenicity. Smetana has noted only a loss of antigenicity, however he was using pure ovalbumen and only studied the final stages of photo-oxidation. His evidence does not preclude a deviation in specificity during the course of the process.

The results with ultraviolet light point to a very marked diminution in the original horse serum active antigenicity. The group of curves in Fig. 5 are of interest in this respect. The early stages which show a progressive diminution in antigenic activity may be explained as associated with a shift in the constitution of the antigen-antibody complexes from the usual preponderance of antibody to one of antigen (88). It is interesting that after 8 hours there is a persistence of the optimal ratio at approximately constant proportions, and a decrease in the total precipitate, regardless of the amount of antigen added. Smetana's first published curve ((64), Fig. 1) with a pure antigen and the single antibody evoked by it, though not carried to such high antigen concentrations as were the data for Fig. 5, suggests a similar constancy of optimal proportions.

Haurowitz (89) has shown that several antibodies are elicited to even a single pure antigen. It may be permissible to postulate that even in so simple a system as irradiated ovalbumen/anti normal ovalbumen, irradiation may have had the effect of distorting the antigen molecules so that a decreasing percentage of the variously shaped molecules of the antibody complex will react with them. This might lead to the effect observed, of a steadily decreasing volume of precipitate at approximately constant optimal proportions.

Stecher's (60) conclusion that deviation of specificity follows ultraviolet irradiation is fully confirmed by the studies described. The viscosity of the solutions increased during irradiation. Neurath (90) has shown that changes

in viscosity of protein solutions can be directly related to the degree of extension of the molecule that occurs during denaturation. Thus there is indirect evidence of a change in molecular shape which may be associated with the deviation in specificity observed. The marked decrease in active antigenicity was as definite as the deviation in specificity. This may prove of significance in the problem of removing protein antigenicity.

It is not possible to estimate what part the addition of hematoporphyrin played in the further reduction in antigenicity noted with serum C, since the duration of irradiation was also increased to 96 hours. Harris (78) considered it probable that hematoporphyrin sensitized, not only to visible, but to ultraviolet light as well. Since his ultraviolet light was not free from longer wave lengths, proof was not clear. The decrease in precipitin reaction noted in Fig. 4, curve 1, with the plate glass filtered light from the ultraviolet lamps, may have been due in part to long ultraviolet waves of the 3600 Å to 3300 Å resonance bands, as well as to the even longer, visible wave lengths produced by these lamps. The effect is sufficient to prevent any clear cut decision that the hematoporphyrin increased the efficiency of the ultraviolet light.

The use of the purified albumen fraction of animal sera as a blood substitute has recently received much attention. If, as has been suggested by Taylor and Keys (16), the use of this fraction in large amounts in human beings is not practicable owing to its residual specific antigenicity, then some method of removing protein antigenicity would become necessary. Such a method could be applied either to serum or to the albumen fraction, as Davis and Eaton (43) have recently suggested, or even to other proteins such as those in milk.

Svedberg (91) has emphasized the value of the photon as a means of applying small and known amounts of energy to proteins and so obtaining a controlled manipulation of these complex delicate molecules. Stimulated by this and other observations, a study of the effects upon proteins of radiations in the range 6000 Å to 2537 Å has been commenced. It is felt that the first results have been sufficiently promising to suggest a careful consideration of this agent as a means of removing protein antigenicity.

Studies of the toxicity of irradiated protein solutions, and of their capacity to substitute for homologous plasma are in progress.

SUMMARY

1. Normal horse serum was irradiated for periods of 3 to 4 days, with visible light or with ultraviolet light of known intensity and wave length. The photosensitizer hematoporphyrin was employed in some instances. The serum was exposed to the air in thin layers, and thoroughly agitated throughout irradiation.

2. The irradiated sera were unchanged in color, and over 90 per cent of the original protein content remained precipitable by phosphotungstic acid.

3. Studies of the antigenicity of the sera were carried out on guinea pigs

and rabbits. Fresh antigenicities of deviated specificity and of an activity of the order of 1/50th, 1/1,000th, and less than 1/20,000th that of normal horse serum were obtained. The residual content of material having the same antigenic specificity as normal horse serum was estimated as approximately equivalent in activity to dilutions of normal horse serum of 1 cc., 1/10 cc., and less than 1/100 cc. per litre respectively.

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