[CONTRIBUTION FROM THE SLOAN-KETTERING INSTITUTE FOR CANCER RESEARCH]

The Relative Rates of Iodination of Serum Components and the Effect of Iodination on Antibody Activity[1]

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Immunochemical methods are unique in their sensitivity for the determination of a single protein component in a mixture of proteins, and can be useful in the study of the kinetics of various protein reactions. The reaction kinetics of various reagents with individual pure proteins can be measured.[4] However, it is possible by immunochemical methods to analyze the rate of alteration of one protein relative to the rates of alteration of other proteins in the same system. Such measurements of relative rates of reaction have the advantage that the comparison of the reactivities of the various proteins, which are present together in a mixture, must undergo reaction under identical conditions and thus there are no uncontrolled variables which may exist when the reactions of the individual proteins are run separately.

The relative alteration of various proteins can be determined by using the protein specific antibody to isolate the individual proteins; or the relative alteration of the antibody can be determined by using the specific antigen to isolate the altered antibody.

Since our research group has been interested in the iodination of antibodies to tissues,[5] we carried out the present investigation to determine the relative reaction rate constant[6] for iodination of antibody and other serum components, and the effect of iodination on antibody activity.

The iodination of proteins has been rather widely studied and it is known that iodine probably attaches itself to the tyrosine rests and perhaps the histidine rests, and can oxidize various parts of the protein molecule.[7a] The iodination of antisera was first carried out by Breinl and Haurowitz[7b] who showed that increasing degrees of iodination decreased the agglutinating power of horse antisera specific toward paratyphoid A, paratyphoid B, and atoxic dysentery. Later it was shown by Pressman and Keighley,[5] that when antisera are so iodinated that they still retain serological activity, the antibody molecules actually do contain iodine. Also, they have shown that it is possible to use antibodies thus labelled with radioiodine to study the *in vivo* localization of anti-rat-kidney serum in rats. Subsequently, Butement[8] showed also that antibodies containing iodine could be demonstrated in a radioiodinated antiserum to *Proteus vulgaris*.

We have extended our investigations to a determination of the effect of iodination on antibody activity of antiovalbumin serum and anti-beef-serum-albumin serum; and to a determination of the relative reaction rate constants for the iodination of antibody and other serum components, and the relative reaction rate constants for the iodination of the antibodies in these two different antisera. The results are reported here.

Experimental

Materials

Antigens.—Ovalbumin was the crystallized product from chicken eggs prepared by the method of Keckwick and Cannan.[9] Bovine serum albumin, hereafter referred to as BSA, was the crystallized product produced by Armour and Company.

Antisera.—All of the antisera used were prepared in rabbits. Some of the sera had been lyophiled before use. Most of the antiovalbumin sera used were prepared by injection of rabbits, intraperitoneally or intravenously, with one or two ml. portions of 0.5% ovalbumin solution. Several weekly courses of two to three injections were given and the rabbits were bled one week after the last injections. The rest of the antiovalbumin serum used was prepared from ovalbumin coupled with diazotized *p*-arsanilic acid and still retained ovalbumin specificity. The serum was prepared by injecting rabbits with the antigen combined with adjuvants according to the method of Freund, *et al.*[10] The antigen emulsion was prepared by grinding together 10 ml. of a 5% solution of the antigen with 10 ml. of Bayol F containing 10 mg. of heat-killed Tubercle Bacilli and with 5 ml. Falba. Five rabbits were injected intramuscularly and subcutaneously with 1 ml. portions of the emulsion in each of four locations along the back. The sera obtained from the rabbits were all of high titer one month after the injection and remained of high titer for several months without further injections.

The anti-BSA serum was prepared similarly by injecting bovine serum albumin coupled with diazotized *p*-arsanilic acid. This serum retained BSA specificity.

Iodine 131.—This was obtained from the United States Atomic Energy Commission, Oak Ridge Operations, Isotopes Division, Oak Ridge, Tennessee.

Borate Buffer.—This was prepared by mixing a solution 0.2 *M* in H_3BO_3 and 0.16 *M* in sodium chloride with a 0.16 *M* solution of sodium hydroxide to the appropriate *p*H.

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(3) Fellow of the Dazian Foundation.

(4) For reviews of the reactions of proteins with chemical reagents, see: (a) R. M. Herriott, "Advances in Protein Chemistry," Vol. III, Academic Press Inc., New York, 1947, p. 169. (b) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Rev.*, **41**, 151 (1947).

(5) D. Pressman and G. Keighley, *J. Immunol.*, **59**, 141 (1948).

(6) The term relative reaction rate constant is used to designate the ratio of the reaction rate constants for the iodination of the components under comparison. It is proportional to the relative alteration of the two components.

(7a) W. L. Hughes, Jr., and R. Strassle, THIS JOURNAL, **72**, 452 (1950).

(7b) F. Breinl and F. Haurowitz, *Z. Immun.*, **77**, 176 (1932).

(8) F. D. S. Butement, *Nature*, **162**, 731 (1948).

(9) R. A. Keckwick and R. K. Cannan, *Biochem. J.*, **30**, 227 (1936).

(10) J. Freund, J. Thompson, H. B. Hough, H. E. Sommer and T. M. Pisani, *J. Immunol.*, **60**, 383 (1948).

This procedure has the advantage that the combination of the two original solutions in any proportions results in an isotonic solution 0.16 molar in salt.

Analytical Methods

The Determination of Iodine.—The iodine content of various samples was determined from the radioactivity of the samples since the iodine used contained tracer amounts of radioactive I^{131} in known concentration. In some experiments the iodine contents and protein contents of the specific precipitates were determined on replicate precipitates, while in others the iodine was determined on a portion of the reaction mixture obtained after the determination of the protein content by the Folin reagent.[11] The Folin reagent did not interfere with the iodine determination. The samples which were free of Folin reagent were placed in metal bottle caps of 3.3 cm. diameter, diluted with water, made alkaline with two drops of 15% sodium hydroxide solution, combined with two drops of 0.5% aerosol solution, evaporated to dryness under a bank of infra-red heat lamps and counted under a thin window, bell shaped, Geiger-Müller tube. The samples were less than 3 mg./sq. cm. thick and there was no effect of self absorption. The samples containing Folin reagent were treated similarly. The samples were 5 ml. of the reaction mixture after the protein color was read. A 5-cm. diameter bottle cap was used so that the final sample thickness was less than 10 mg./sq. cm. The sample showed no pronounced self absorption.

The Determination of Protein.—The protein contents of the various solutions were determined by digesting the protein in sulfuric acid and hydrogen peroxide and determining the ammonia by the Nessler reagent according to the method of Lanni, Feigen and LeRosen.[12] The protein contents of the specific precipitates were obtained by washing them three times with 10-ml. portions of 0.85% sodium chloride solution and determining the protein by the Folin reagent according to the method described previously.[11] The iodination procedure did not reduce the Folin readings in the range of iodination used here, namely, up to 18 groups per antibody molecule. Higher iodination did lower the Folin reading somewhat. Kleczkowsky reported decreased readings but with proteins containing much more iodine.[13]

Method of Fractionating Sera[14a]

Serum was fractionated with ammonium sulfate by dialyzing it on a rocking platform against the volume of 3.0 *M* ammonium sulfate (at *p*H 7) calculated to give the desired equilibrium concentration. After several hours the mixture was centrifuged, and the precipitate washed with the dialysate. After a second centrifuging, the precipitate was made up to the desired volume with borate buffer of *p*H 8.0 and dialyzed against 17 liters of saline for four hours, and against a fresh 17-liter portion of saline for eighteen hours, and finally against 3 liters of borate buffer of *p*H 8.0 for six hours. The solution was then cleared by centrifugation. The globulin thus prepared was about 75% γ-globulin.[14a] The albumin was obtained from the supernatant from the ammonium sulfate precipitation by dialysis against saline as described for globulin above.

The separation into euglobulin and pseudoglobulin was carried out by dialysing the globulin solution against two 17-liter portions of distilled water. The euglobulin was centrifuged down, washed with chilled distilled water, and taken up in borate buffer of *p*H 8.0. The pseudoglobulin in the supernate was dialyzed against borate buffer.

These procedures were carried out in a cold room at 5°.

Method of Iodinating Proteins

The protein solution was dialyzed on a rotating dialyzer against borate buffer of *p*H 8.0 for at least three hours at 5°, then cleared by centrifugation and added at 2° to a mixture of 0.089 *N* iodine in 0.1 molar potassium iodide solution, borate buffer of *p*H 8, and a tracer amount of radioiodide.[14b] After fifteen minutes, 1 *N* sodium thiosulfate solution was added to reduce excess iodine. The *p*H value during iodination changed less than 0.1 unit during iodination. The samples were then dialyzed at 5° overnight against 17 liters of saline and then for twenty-four hours against another 17 liters of saline and finally against 6 liters of borate buffer of *p*H 8.0 for at least three hours. This procedure reduced the concentration of radioactivity, not precipitated with trichloroacetic acid, to below 1%.

The Reaction of Antisera with Antigens

The reactions were carried out in borate buffer at *p*H 8.0. The tubes were incubated for two hours at 37°, and then permitted to stand for forty to ninety hours in the refrigerator; the precipitates were centrifuged down, washed with two 8-ml. portions of saline and analyzed for protein by the Folin Reagent and for iodine by a determination of radioactivity. In some cases, replicate samples were analyzed for iodine and protein separately. In others the iodine determination was made on the mixture after the protein determination. The experimental procedure in each experiment is described in the corresponding table.

Results and Discussion

The Effect of Iodination of Whole Antiovalbumin Serum on Specific Precipitation.—The extent to which the specific precipitating power of whole antiovalbumin serum was affected by the number of attached iodine atoms is shown in Table I, and the effect is illustrated in Fig. 1 where the amounts of precipitate are plotted against the amount of antigen added. As the amount of iodine was increased, the amount of precipitable antibody was decreased with a shift of the zone of maximum precipitation to the region of lower antigen concentration, indicating a destruction of antibody.[15] Appreciable amounts of precipitate were obtained when even as many as 40 or 64 groups per molecule of average molecular weight, 160,000, were coupled. The amount of iodine required to destroy the precipitating power of the antiserum was different for different runs, being of the order of 4 to 8% iodine or 50 groups to 100 groups per serum molecule of average molecular weight, 160,000. This variation was probably due to the oxidation effects of the iodine which were not determined here.

The number of iodine atoms required to produce an appreciable change in the amount of precipitable antibody varied also for the different runs. In series B (Table I) the introduction of only six groups appreciably decreased the amount

(11) D. Pressman, *Ind. Eng. Chem., Anal. Ed.*, **15**, 357 (1943).

(12) F. Lanni, G. A. Feigen and A. L. LeRosen, *Archives of Biochemistry*, **8**, 251 (1945).

(13) A. Kleczkowsky, *Brit. J. Exptl. Path.*, **21**, 98 (1940).

(14a) H. N. Eisen, and D. Pressman, *V. Immunol.*, in press.

(14b) The I^{131} is incorporated into the free iodine and the triiodide ion through the rapid equilibrium existing between I^-, I_3^- and I_2. That actual exchange takes place was shown by the fact that the I_2 extracted from the iodination mixture by CCl_4 contained the amount of I^{131} calculated on the basis of complete exchange.

(15) The uniodinated antiovalbumin sera used in Table I gave antibody/antigen weight ratios greater than 20 for precipitates in the region of far antibody excess. Thus the antisera prepared using adjuvants (and azoprotein) act like multiple injection sera, since M. Heidelberger and F. E. Kendall, *J. Exptl. Med.*, **62**, 697 (1935), have shown that the antibody/antigen ratio in the precipitate increased as the number of injections given during the preparation of the serum was increased.

TABLE I

EFFECT OF IODINATION OF WHOLE ANTIOVALBUMIN SERUM ON SPECIFIC PRECIPITATION[a]

Iodine added, meq.	Composition of iodoserum: Concn. of protein, g./ml.	Gram atoms of iodine combined per ml. $\times 10^5$	Number of iodine atoms per protein molecule[b]	Specific precipitation, amount of ovalbumin added, μg: 12	16	24	36	50	247
				Amount of precipitate obtained, μg.[c]					
				Series A					
0	0.0122	0	0	321				826	456
0.09	.0125	0.053	7	434				936	466
.27	.0130	.19	23	275				736	173
.80	.0120	.48	64	296				151	49
2.4	.0115	.68	95	19				30	30
				Series B					
0	.1030	0	0	174				793	580
0.09	.1030	0.045	6	276				750	252
.27	.0130	.12	15	264				363	92
.80	.0130	.32	39	145				94	36
2.4	.0130	.38	47	25				20	0
				Series C					
0	.0202	0	0		432	(601)	832		
0.11	.0201	0.051	4		423	591	776		
.21	.0218	.14	10		420	588	781		
.86	.0201	.42	33		375	(445)	637		

[a] For series A and B, a lyophilized antiovalbumin serum preparation was regenerated by adding 6.0 g. of lyophilized preparation to 60 ml. of distilled water. It was dialyzed against buffer and then cleared by centrifugation. The protein concentration was 0.073 g./ml. in series A and 0.078 g./ml. in series B. To 10-ml. portions of this were added mixtures of iodine–iodide solution and radioiodide made up to 50 ml. with borate buffer. The control tube with no iodine was also made up to 60 ml. The iodinated solutions were dialyzed to remove iodide and the protein concentration of these are tabulated. The iodide concentrations as determined by radioactivity measurements are also tabulated. For series C, 150 ml. of antiserum was dialyzed against borate buffer and then centrifuged. The resultant solution had a protein concentration of 0.061 g./ml. Portions of 24 ml. of this solution were iodinated with a mixture of iodine–iodide solution and radioiodide made up to 72 ml. with borate buffer. Series C was set up in the region of great antibody excess. For the specific precipitation experiments, 2 ml. of antigen and 6 ml. of iodinated serum solution were used and the tubes were incubated two hours at 37° and then in the refrigerator for sixty-four hours in series A and B and 40 hours in series C. [b] Calculated on the basis of protein of molecular weight 160,000. [c] Averages of triplicate determinations with average deviation ±3%, duplicate analysis in parentheses. No correction was made for the amount of ovalbumin in the precipitate. This correction is small in most cases.

of precipitate obtained in the region of antigen excess, while in series A, no effect was observed in the region of antigen excess with seven iodine groups present. In general, the effect in the region of antibody excess was less than in the region of antigen excess. Series C, which was set up entirely in

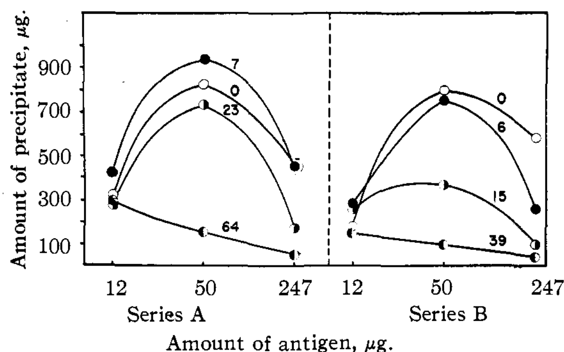

Fig. 1.—Effect of iodination of whole antiovalbumin serum on specific precipitation: plots of data in Table I of amount of precipitate obtained against the amount of ovalbumin added. The figures above curves indicate number of iodine atoms per average serum molecule.

the region of antibody excess showed but slight change, even with thirty added iodine atoms. However, the addition of only a few iodine groups brought forth the interesting effect of increasing slightly the amount of precipitate obtained in the equivalence zone and the region of antibody excess. This increased precipitation is clearly seen in series A and B. Actually, the iodine content of the precipitating antibodies is lower than that of the average serum molecule since antibody molecules iodinate at a slower rate than do other serum components.

The Relative Rates of Iodination of Antibody and Whole Serum.—In order to determine the extent of iodination of iodinated antibody, experiments were carried out with iodine of a specific activity high enough so that the precipitates had a radioactivity of at least three counts per minute per μg. of precipitated protein. The results for the iodination of whole antiovalbumin serum are shown in Table II. Whole antiovalbumin serum was iodinated with two different quantities of iodine so that the resulting solution contained molecules with 19 and 51 iodine atoms per protein molecule of mean molecular weight 160,-

TABLE II

THE IODINE CONTENT OF THE SPECIFIC PRECIPITATE OBTAINED FROM IODINATED ANTIOVALBUMIN SERUM[a]

Iodine added, meq.	Concn. protein, g./ml.	Gram atoms iodine combined per ml. × 10^5	Number of iodine atoms per protein molecule[b]	Amount of specifically precipitated antibody,[c] μg.	Iodine atoms per molecule of pptd. antibody	Ratio of iodine content of av. serum protein molecule to iodine content of pptd. antibody
Series A, Whole Serum						
0.09	0.0107	0.125	19	319	6	3.1
0.27	0.0110	0.35	51	222	18	2.8
Series B, Globulin Fraction (Insol. 1.75 m. $(NH_4)_2SO_4$)						
0	0.0032	0	0	298		
0.0045	.0033	0.0057	3	312	1.3	2.4
.009	.0036	.014	6	275	3.6	1.7
.018	.0031	.037	19	199	9.1	2.1
.027	.0031	.054	28	92	13	2.1

[a] Series A—A 17-ml. portion of serum was dialyzed against buffer and then cleared and 7-ml. portions were iodinated with a mixture of the iodine–iodide solution and the radioiodine solution made up to 35 ml. with borate buffer. After dialysis the protein and iodine concentrations were determined and antibody specifically precipitated by adding 25 μg ovalbumin in 2 ml. of solution to 6 ml. of the iodinated antiovalbumin serum solution. The mixtures were incubated for two hours at 37° and permitted to stand for sixty-four hours at 5°. Tubes were set up in sextuplicate for each reaction and three were analyzed for protein and three for iodine in the precipitate. Series B—The globulin fraction of antiovalbumin serum was prepared and solutions containing 0.067 g. of protein in 3.5 ml. were iodinated with a mixture of iodine–iodide solution and radioiodide made up to 18.5 ml. with borate buffer. The precipitin tests were carried out similarly with 25 μg ovalbumin in 1 cc. solution and 3.0 ml. of iodinated globulin solution. [b] Calculated on the basis of protein of weight 160,000. [c] The values for antibody precipitated were corrected for the antigen precipitated by assuming that all of the antigen added was precipitated since the tests were set up in the region of large antibody excess for uniodinated serum. Due to the shift of equivalence zone with iodination, this assumption may not be valid in the case of greatly decreased precipitation as in the last sample in Series B.

000. However, the specifically precipitated antibody obtained in the region of antibody excess contained only 6 and 18 atoms of iodine, respectively, per molecule. Thus, the whole serum contained 3.1 to 2.8 times as much iodine per unit weight of protein as did the precipitable antibody. This lower value for the amount of iodine in the precipitable antibody is due to a difference in rate constants for the iodination of the specific antibody and the other serum components. It is not due to the possibility that the more highly iodinated antibody molecules become denatured and therefore do not precipitate, while only those of low iodine content precipitate. This is shown by the fact that from the more highly iodinated solution, the precipitating antibodies had an average of 18 groups per antibody molecule and thus antibodies with even more than 18 groups can be specifically precipitated. However, from the solution which had an average of 19 iodine atoms per average whole serum molecule, the precipitable antibodies contained only 6 atoms of iodine. Since antibodies with an average of 18 iodine atoms do precipitate, and would be formed in this iodination if antibodies iodinate at the same rate as other serum components, the conclusion must be drawn that antibodies iodinate more slowly. Actually, the distribution of the number of molecules of antibody with respect to the number of iodine atoms per molecule is such that for an antibody solution having an average of 6 iodine atoms per antibody molecule, one would expect to find less than 1% with 18 atoms attached (see paragraph on distribution of molecules).

The Relative Rates of Iodination of Albumin, Globulin and Antibody.—In order to determine more closely the relative rate constants for the iodination of the various components of serum, antiovalbumin serum was separated at 1.75 molar ammonium sulfate concentration into albumin and globulin fractions. Both of these were iodinated under the conditions described in Table II for the globulin fraction, and it was found that the extents of iodination of the two fractions were the same when iodinated separately. Therefore, any difference in iodine contents of the globulin fraction and the albumin fraction in the iodination of whole serum was not due to a limitation of the extent of iodination possible in the globulin fractions under these conditions, but must have been due to a difference in rates of iodination of the albumin and globulin fractions.

The iodinated globulin fraction was analyzed for the composition of the precipitable antibody, and, here also, there was an effect of antibody protein being iodinated apparently more slowly than the rest of the globulin fraction. This greater rate of iodination of the globulin fraction may have been partially due to albumin contained in the globulin fraction, although this method of separation with ammonium sulfate is quite efficient in removing albumin,[14] or it may be due to the greater relative rates of iodination of the other globulins of the fraction.

The average iodine content of the specifically precipitable antibody was 1.3 to 13 atoms per antibody molecule for the experiments reported in Table II, part B, and was about half that

TABLE III

FRACTIONATION OF ANTIOVALBUMIN SERUM INTO ALBUMIN AND GLOBULIN FRACTIONS AFTER IODINATION[a]

	Iodine added, meq.	Protein in sepd. fractions, g./ml.	Iodine atoms per protein[b] molecule	Antigen added, μg 2 / Antibody pptd., μg[c]	4	25	Iodine atoms per molecule of pptd. antibody[b] (25 μg antigen added)	Ratios of iodine content of av. protein molecule to content of pptd. antibody
Albumin	0	0.032						
Globulin	0	.014		22	39	281		
Albumin	0.102	.029	3					
Globulin	.102	.0055	1.6	34	53	170	0.7	2.3
Albumin	.132	.024	8					
Globulin	.132	.0052	4	31	66	164	2	2.0
Albumin	.414	.021	15					
Globulin	.414	.0057	7	36	63	196	4.5	2.0
Albumin	.926	.029	29					
Globulin	.926	.0071	15	27	45	184	6	2.7

[a] A 140-ml. portion of antiovalbumin serum was dialyzed against buffer and cleared. Portions of 26 ml. were iodinated by a mixture of iodine–iodide solution and radioiodide made up to 26 ml. with borate buffer. The mixture was dialyzed and then fractionated at 1.75 molar ammonium sulfate at *p*H 7.0. The precipitates were taken up in 26 ml. solution. The supernates and dissolved precipitates were dialyzed and protein concentrations determined. The antibody was precipitated specifically by adding ovalbumin in 1 ml. solution to 2 ml. of globulin fraction. [b] Calculated on basis of protein of weight 160,000. [c] Average of triplicate determinations with average deviation ±4%.

for the corresponding content of the whole globulin fraction in each case. Since the ratio of the iodine content of the average serum protein molecules to the iodine content of the precipitated protein is essentially constant for this ten fold range of iodination, this experiment emphasizes the fact that this ratio is due to a difference in rates of iodination of the antibody molecules and the other components of the globulin fraction. Also, it emphasizes the previously reported fact that the lower iodine content of the precipitating antibody was not due to a denaturation of the more highly iodinated molecules so that they no longer precipitate specifically.

In order to measure the relative rates of iodination of the albumin fraction, globulin fraction and antibody, antiovalbumin serum was iodinated with a nine-fold variation in iodine concentration and then separated into albumin and globulin fractions, with 1.75 molar ammonium sulfate. The antibody was specifically precipitated from the globulin fraction by ovalbumin and the results are given in Table III. It can be seen that the albumin fraction iodinated about twice as fast as the globulin fraction over the range of iodine concentrations used, and the globulin fraction iodinated twice as fast as the specifically precipitable antibody fraction.

That the iodinated albumin fraction was not precipitated by ammonium sulfate of concentration 1.75 molar was shown by iodinating portions of an albumin solution containing 20 mg. protein per ml., which was the concentration of protein in albumin fraction of Table III, so that the final iodine content of the protein was 0.3, 3.5 and 12% iodine. In the three samples this corresponded to 4, 45 and 170 iodine atoms per protein molecule of molecular weight 160,000. These solutions were dialyzed against the volume 3.0 molar ammonium sulfate solution which would bring the final ammonium sulfate concentration to 1.75 molar. The small amount of precipitate formed was centrifuged down, dissolved in saline and dialyzed free of ammonium sulfate. Protein analysis by Nessler showed that only 0.1, 0.8 and 5% of the albumin precipitated under these conditions. This shows that the iodinated albumin remained in the solution under the conditions that iodinated globulin was precipitated above.

Some Properties of the Iodinated Albumin Fraction.—The greater iodine content in the albumin fraction was not due to the presence of iodine which accumulated in the supernate, since less than 2% of the radioactivity was not precipitable by trichloroacetic acid. That the iodine content in the albumin fraction was not due to the iodination of lipoid bound to the albumin fraction was shown by the following experiment. Two iodinated anti-BSA sera containing 9 and 27 iodine atoms, respectively, were precipitated by adding 6 volumes of acetone. The precipitates were then washed with 6 volumes of acetone, a mixture of 4 volumes of acetone and 2 volumes of ether, and finally 6 volumes of ether. The total amount of iodine removed by this procedure was 1.5 and 3.0% of the total originally present. In a second experiment the serum was again precipitated by 6 volumes of acetone and the precipitate was extracted in a Soxhlet for six hours. The total iodine extracted by this procedure was only 1.3%. These experiments show that the iodine in the fraction not precipitated by 1.75 M ammonium sulfate was not free iodide nor lipoid-bound iodine and must have been protein-combined iodine.

Relative Rates of Iodination of Antibody Molecules of Different Specificities.—In order to investigate the relative rates of iodination of antibody molecules of different specificities, antiovalbumin serum and anti-BSA serum were

TABLE IV

IODINATION OF GLOBULIN FRACTIONS[a]

Fraction	Concn. $(NH_4)_2SO_4$ used for fractionation, moles/liter	Iodine added, meq.	Iodinated protein: Sol. protein, g./ml.	Iodinated protein: Iodine atoms per protein molecule, Total fraction	Iodinated protein: Iodine atoms per protein molecule, Soluble fraction	Composition of specific ppts.[b] BSA 187 μg added: Antibody pptd. μg	BSA 187 μg added: Antigen pptd. μg	Ovalbumin 149 μg added: Antibody pptd. μg	Ovalbumin 149 μg added: Antigen pptd. μg	Iodine atoms per molecule of antibody, pptd. by BSA	Iodine atoms per molecule of antibody, pptd. by Ovalbumin	Ratio of iodine content of average protein molecule to content of pptd. antibody: Soluble fraction BSA	Soluble fraction Ovalbumin	Total fraction BSA	Total fraction Ovalbumin
Euglobulin	1.35	0	0.0089			726	119	1759	149						
		0.009	.0037	6.6	4.1	260	52	721	78	1.8	1.9	2.3	2.2	3.6	3.5
		.018	.0034	11	8.4	103	29	372	(51)	5.3	4.9	1.6	1.7	2.0	2.1
		.036	.0039	21	17			98	14		11.0		1.6		2.0
		.053	.0021	29	23										
Euglobulin	1.75	0	.0089			796	187	1417	149						
		0.009	.0063	5.2	3.5	628	(151)	1397	149	2.2	2.2	1.6	1.6	2.4	1.8
		.018	.0038	10	6.9	(295)	53	621	85	4.3	5.0	1.6	1.3	2.3	2.0
		.036	.0037	22	14	75	30	271	44	12.2	11.2	1.2	1.3	1.7	1.9
Pseudo-globulin	1.35	0	.0089				40	1397	149						
		0.009	.0046		4.8	105	49	(537)	100	4.0	(2.4)	1.2	(2.0)		
		.018	.0040	13	9.0	47	23	304	70		8.2		1.1		1.5
		.036	.0029	26	19	16	13	84	(21)		10.0		1.9		3.8
		.053	.0017	38	29	0	0	0	0						
Pseudo-globulin	1.75	0	.0089			376	82	1467	147						
		0.009	.0060	6.0	4.4	337	72	818	115	3.7	3.1	1.2	1.4	1.7	1.8
		.018	.0051	12	11	154	39	546	85	7.8	7.8	1.4	1.4	1.5	1.5
		.036	.0042	21	19	25	(16)	175	33		12.7		1.5		1.6
		.053	.0031	25	33	0	0	0	0						

[a] A pool of 100 ml. of anti-BSA serum and 300 ml. of antiovalbumin serum after dilution with 400 ml. saline was fractionated with ammonium sulfate at *p*H 7.0. Then the euglobulin fractions were separated as described in the experimental section. The protein solutions were made up to a constant concentration of 0.010 g./ml. Thirteen ml. of this solution was taken for iodination by a mixture of iodine–iodide and radioiodide made up to 1.3 ml. Specific precipitates were obtained by adding 187 mg. BSA or 149 mg. ovalbumin in 0.8 ml. borate to 1.6 ml. of iodinated protein solution in triplicate. The tubes were incubated for two hours at 37.5° and permitted to stand 64 hours at 5°. The iodine content of the precipitates was determined from the solutions following Folin protein determination. [b] Values are averages of triplicate determinations, duplicate determinations are in parentheses; average deviation ±2%.

pooled and the mixture was separated into two fractions by adding ammonium sulfate to a concentration of 1.35 molar, removing the precipitate and then bringing up to 1.75 molar and removing the second precipitate. Each of these precipitates was taken up in saline and dialyzed against distilled water yielding a water soluble pseudoglobulin fraction and a water insoluble euglobulin fraction. The resulting four fractions were adjusted to a protein concentration of 8.9 mg./ml. and iodinated to various degrees as shown in Table IV. After iodination and during subsequent dialysis the globulin fraction yielded an appreciable quantity of insoluble matter, although such non-specific precipitation did not take place during the iodination of whole serum or of the globulin fraction obtained at an ammonium sulfate concentration of 1.75 molar. The extent of destruction can be seen in the fourth column of Table IV. From columns 5 and 6, it can be seen that in general the insoluble material was composed of the more highly iodinated molecules in any one fraction since the supernatant solutions contained less iodine per protein molecule.

Replicate samples of iodinated proteins were then treated, some with BSA and others with ovalbumin, for specific precipitation. The amount of antigen used was that which gave the optimum precipitation with the whole serum. The amount of antigen precipitated was determined by analyzing the supernate of the precipitin reaction for unprecipitated antigen by adding an excess of the specific antibody and determining the amount of precipitate formed thereby. The amount of antigen required to give this amount of precipitate was then read from a curve for the test antiserum.

Values are listed for the iodine contents of the specifically precipitable antibodies of the two types and are seen to be essentially the same. Values are also listed for the ratios of the iodine contents of the average protein molecules of both the soluble fraction of the iodinated protein and the total iodinated protein to the iodine content of the precipitated antibody. These former values, averaging about 1.5, are somewhat less than those obtained with the whole globulin fraction, while the latter were closer to the value 2 obtained with whole globulin. This shift in ratio presumably is due to the partial denaturation of some of the more highly iodinated molecules in these purified systems.

In general no differences were observable in the properties of the iodinated antibodies from the four fractions or with the two different specificities. The antibodies in the different fractions iodinated to the same extent and at approximately the same rate.

The non-specific precipitation of radioiodinated proteins during the precipitation of iodinated antiovalbumin with ovalbumin was investigated and found not to be a contributing factor in the composition of the precipitate, since radioiodinated protein was not brought down non-specifically when antibody was brought down specifically in the presence of radioiodinated normal serum.

The Distribution of Molecules of Protein with Respect to the Number of Iodine Atoms per Molecule.—This distribution for systems of various iodine contents was calculated with the following assumptions: (a) only tyrosine rests are iodinated, (b) the rate of iodination of a protein molecule is proportional to the number of unsubstituted tyrosine rests, (c) all tyrosine rests have the same rate of iodination and (d) the introduction of a second iodine atom into a monoiodotyrosine rest is so rapid that it takes precedence over the initial substitution of an unsubstituted tyrosine rest.

The first assumption is valid since histidine probably does not iodinate at the levels of iodination used here (see Herriott[4]). The second and third assumptions are probably only approximations since Li[16] has shown that the tyrosine groups within individual proteins, pepsin and albumin, iodinate at different rates. The fourth assumption stems from the fact that Li[17] has shown that diiodotyrosine formation takes precedence over monoiodotyrosine formation in the iodination of tyrosine and proteins.[16,17] Herriott,[18] by iodinating pepsin with very minimum amounts of iodine, so that there was less than one iodine atom per molecule of protein in the iodinated solution, was able to isolate monoiodotyrosine from the protein. This result by Herriott merely indicates that the rate of a second iodine entering the monoiodotyrosine rest is not greater than the rate of substitution of all the remaining tyrosine rests in pepsin if we take into account the relative concentration of the total tyrosine and the total monoiodotyrosine present.

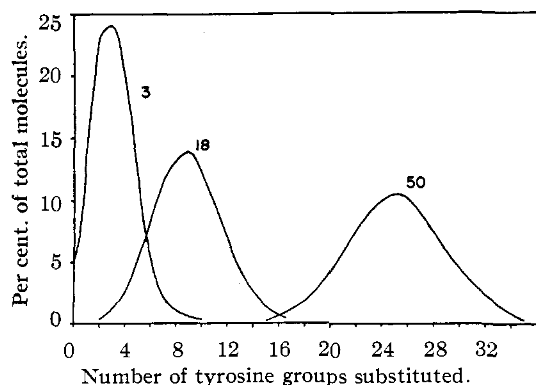

Fig. 2.—Theoretical distribution of iodinated antibody molecules with 6, 18 and 50 iodine atoms average according to the number of tyrosine groups substituted: note that each tyrosine group substituted represents two iodine atoms.

The rate expression for the increase in concentration of protein molecules with m iodinated tyrosine groups from a protein molecule with $(m - 1)$ iodinated tyrosine groups is given in equation 1

$$\mathrm{d}A_m/\mathrm{d}t = (n - m + 1)kA_{m-1} - (n - m)kA_m \quad (1)$$

where n is the total number of tyrosine groups in the molecule, A_m and A_{m-1} are the concentrations of protein molecules with m and $m-1$ substituted tyrosine groups, respectively, and k is the reaction rate constant which includes the instantaneous iodine concentration. Then the concentration of the molecules A_m at the time t is given by equation 2

$$A_m = \frac{n!}{m!\,(n - m)!} A_T e^{-nkt}(e^{kt} - 1)^m \quad (2)$$

where A_T is the total protein present and kt is actually the total exposure to iodine. The exposure, kt, can be calculated from the total combined iodine in the mixture as in equation 3.

$$\begin{aligned} \text{Total iodine} &= 2A_1 + 4A_2 + 6A_3 + \ldots + 2nA_n \\ &= 2A_T e^{-nkt} \sum_{m=1}^{n} \frac{(n - 1)!}{(m - 1)!(n - m)!} (e^{kt} - 1)^m \\ &= 2A_T n(1 - e^{-kt}) \quad (3) \end{aligned}$$

If we take n equal to 58 by assuming that rabbit γ-globulin has the same number of tyrosine rests as found by Brand and co-workers[19] for human γ-globulin, the distribution of the iodinated antibody molecules can be calculated for the systems containing different amounts of bound iodine. The results of such a calculation for systems having an average of 6, 18 and 50 iodine atoms (3, 9 and 25 iodinated tyrosine groups), respectively, is shown in Fig. 2.

From this figure it can be seen that antibody must iodinate at a rate lower than that of other components of serum. For example, in a system containing an average of 50 iodine atoms per protein molecule (Table II, series A) there was still an appreciable amount of precipitable antibody. However, less than 1% of the original molecules (if iodination to the extent of an overall average of 50 groups had taken place) would be available to give a distribution corresponding to the observed value of an average of 18 atoms of iodine for the precipitable antibody.

Significance of Different Rates of Iodination of Various Serum Constituents.—The results obtained here show that the albumin fraction iodinates twice as rapidly as the globulin fraction and the globulin fraction twice as rapidly as the antibody. These differences in the rates of iodination are probably due to the difference

(16) C. H. Li, This Journal, **67**, 1065 (1945).

(17) C. H. Li, *ibid.*, **64**, 1147 (1949).

(18) R. M. Herriott, *J. Gen. Physiol.*, **25**, 185 (1941).

(19) E. Brand, B. Kassell and L. V. Saidel, *J. Clin. Invest.*, **23**, 437 (1944).

in the availability of the various tyrosine groups in the different proteins, as observed in the cases of pepsin and albumin by Li.[16]

Since, at least in the case of human proteins,[19] the albumin contains less tyrosine, 4.6%, than does the γ-globulin, 6.7%, we would expect the γ-globulin to iodinate more rapidly on the basis of the tyrosine concentration alone. However, this is not the case here, since antibody iodinates more slowly than the albumin.

These differences in rates of iodination are especially of significance in connection with studies on antitissue sera.[5] It has been shown that when the radioiodinated globulin fraction of antiserum prepared against rat kidney was injected into rats, radioiodine localized in the kidney to the extent of about 1% of the radioactivity in the injected material. The localized radioactivity was apparently attached to antibody specific to rat kidney. Since the amount of antibody localized was taken to be equivalent to the amount of protein associated in the globulin fraction with the amount of iodine localized, we must now revise the figure by a factor of 2, to take into account the difference of iodination—thus: 2% of the injected protein was antibody which localized in the kidney.

The Effect of Iodination on Antibody Activity.—The decrease in precipitability of antibodies observed here was due to the iodination of the antibody and the oxidation of the antibody with either a decrease in the combining constant for the antigen or an increase in the solubility of the antigen–antibody complex. It is more probable that a decrease in combining constant was the reason for the reduction in the amount of precipitate. It is reasonable that any iodination or oxidation in the specific region would greatly affect the combination of antigen and antibody, while alteration elsewhere in the molecule would affect the combination but slightly.

Acknowledgment.—We wish to thank Miss B. Sherman and Messrs. R. Clark, R. Medina and A. Silverstein for assistance in this study.

Summary

It was shown that, by immunochemical methods, it is possible to determine relative rates of iodination of proteins in a mixture of proteins. Thus antibody to ovalbumin iodinates at one-third the rate of the proteins of whole serum and at one-half the rate of the proteins of the globulin fraction separated at 1.75 molar ammonium sulfate. The globulin fraction iodinated at one-half the rate of the albumin fraction. Antibody to beef serum albumin and antibody to ovalbumin iodinate at the same rate. The introduction of even 18 iodine atoms per antibody molecule does not destroy its ability to undergo specific precipitation.

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[CONTRIBUTION NO. 741 FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF PITTSBURGH]

Electric Moments and Structure of Substituted Thiophenes. III. Polysubstituted Thiophenes[1,2,3]

BY ROBERT G. CHARLES AND HENRY FREISER

The work described herein is an extension of a study of the effect of substitution on the character of the thiophene nucleus. Measurements described in the previous papers of this series have indicated the likelihood of resonance interaction of some substituents in the 2-position and the thiophene nucleus. A possible or partial explanation of the results was advanced which involved a decreased participation of the sulfur electrons in the thiophene nucleus resonance. Accordingly, compounds were chosen for study which might exhibit this change in the role of sulfur in the thiophene ring resonance to a more marked degree. Measurements were made of the dielectric constants, refractive indices and densities of benzene solutions of benzothiophene, dibenzothiophene, 2,5-dimethylthiophene, tetrachlorothiophene and tetraphenylthiophene at 30°. The dielectric polarization of dinaphthylenethiophene at 30° was determined from measurements in *p*-xylene solutions, since this compound was not sufficiently soluble in benzene. The moment values are of some significance in explaining certain aspects of thiophene chemistry.

(1) For Paper II, see Keswani and Freiser, THIS JOURNAL, **71**, 1789 (1949).

(2) This work is abstracted from a portion of the thesis to be submitted by Robert G. Charles to the University of Pittsburgh in partial fulfillment of the requirements for the Master of Science degree.

(3) This paper was presented before the Physical and Inorganic Division at the Atlantic City A. C. S. convention, September 22, 1949.

Experimental

Purification of Materials.—Jones and Laughlin reagent-grade benzene was used after purification *by careful fractionation* as described previously.[4] Paragon *p*-xylene was recrystallized and fractionally distilled to give a product that boiled at 136.4–137.6° at 733 mm. and had a melting point of 13.0°, $d^{30}{}_4$ 0.852116 g./ml. Its dielectric constant was determined at 30° by comparison with that of benzene and air and found to be 2.2497.

The 2,5-dimethylthiophene was prepared by ring closure

(4) Keswani and Freiser, THIS JOURNAL, **71**, 218 (1949).