[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Preparation and Properties of Serum and Plasma Proteins. XXIV. Iodination of Human Serum Albumin[1a,b]

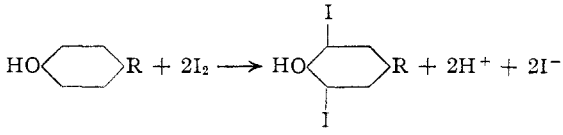
BY W. L. HUGHES, JR.,[2a] AND R. STRAESSLE[2b]

Introduction

These studies were undertaken to determine the utility of iodine as a specific reagent for the tyrosyl groups of human serum albumin. The distribution of the iodine, and the properties of the products were followed, with the object of preparing derivatives as homogeneous as possible, both in physicochemical properties and with respect to the positions substituted in the protein molecule. The iodination of human serum albumin was chosen, as one of several suitable reactions, as a part of a larger project to prepare modified crystalline proteins[3] containing heavy atoms in known positions of the molecule. It is hoped that valuable information concerning the structure of proteins may be obtained by comparing the X-ray diffraction pattern of such modified protein crystals with that of the original protein.

The iodination of human serum albumin has previously been studied by Li,[4] and that of horse serum albumin by Blum and Strauss,[5] Bonot,[6] Salter and Muus,[7] and Shahrokh.[8] The products obtained had variable iodine content, and some of them could be crystallized. Other studies on the iodination of proteins have been made on zein by Neuberger,[9] on pepsin by Herriott,[10,11,12] on casein by Ludwig and Mutzenbecher[13] and Abelin,[14] on horse serum globulin by Kleczkowsky,[15] on insulin by Harington and Neuberger,[16] and comprehensive reviews on the subject have been written.[17,18]

In a most careful study Herriott iodinated pepsin, at *p*H 5.0–6.0, without appreciable oxidation, although the activity was lost. The amounts of oxidation and of substitution were determined by comparing iodine consumed with iodine organically bound to pepsin. In a pure substitution reaction, such as the formation of diiodotyrosine, half of the iodine consumed should be bound



Consequently, any greater consumption of iodine than this is a measure of the amount of oxidative side reactions taking place. Upon hydrolysis, most of the bound iodine was found with the tyrosyl residues as diiodotyrosine (and with smaller amounts of iodine as monoiodo tyrosine). The titration curve of iodinated pepsin revealed the expected changes: diiodotyrosyl groups replaced tyrosyl groups. A partially iodinated pepsin was crystallized and its solubility behavior investigated.

Materials and Methods

The albumin preparation used, decanol-10, was crystalline human serum albumin, crystallized with the aid of small amounts of *n*-decanol, as prepared by Cohn, Hughes and Weare.[19] The albumin contained less than 0.1% of globulins as found by immunological assay. It was at least 95% homogeneous in the ultracentrifuge, the remainder being a faster sedimenting material.

The iodinating reagent used was iodine dissolved in 2 moles of potassium iodide. This was preferred to alcoholic iodine because of its greater solubility and weaker reactivity.

Iodimetry proved a convenient method of following the degree and the rate of the iodine uptake. Aliquots of the reaction mixture were removed at intervals and titrated either with 0.1 *N* arsenious oxide, if the mixture had a *p*H of 8

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(2a) Assistant Professor, Harvard Medical School.

(2b) Research Fellow, American-Swiss Foundation for Scientific Exchange, 1947-1950.

(3) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, THIS JOURNAL, **71**, 2476 (1949), reported the guanidination of human serum albumin.

(4) C. H. Li, THIS JOURNAL, **67**, 1065 (1945).

(5) F. Blum and E. Strauss, *Z. physiol. Chem.*, **112**, 111 (1920).

(6) A. Bonot, *Soc. chim. biol.*, **21**, 1422 (1939).

(7) J. Muus, A. H. Coons, and W. T. Salter, *J. Biol. Chem.*, **139**, 135 (1941).

(8) B. K. Shahrokh, *ibid.*, **151**, 659 (1943).

(9) A. Neuberger, *Biochem. J.*, **28**, 1990 (1934).

(10) R. M. Herriott, *J. Gen. Physiol.*, **20**, 335 (1937).

(11) R. M. Herriott, *ibid.*, **25**, 185 (1941–1942).

(12) R. M. Herriott, *ibid.*, **31**, 19 (1947).

(13) W. Ludwig and P. von Mutzenbecher, *Z. physiol. Chem.*, **258**, 195 (1939).

(14) I. Abelin, *Helv. Chim. Acta*, **25**, 1421 (1942).

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

(16) C. R. Harington and A. Neuberger, *Biochem. J.*, **30**, 808 (1936).

(17) R. M. Herriott, "Advances in Protein Chemistry," Vol. III, Academic Press Inc., New York, N. Y., 1947, pp. 177, 205.

(18) H. S. Olcott and H. Fraenkel-Conrat, *Chem. Revs.*, **41**, 182 (1947).

(19) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1735 (1947).

or higher, or with 0.1 *N* sodium thiosulfate solution after acidification of the mixture with 2 *N* sulfuric acid. Starch solution gave no color in the presence of this protein. No indicator was therefore added.

Estimation of organically-bound iodine was carried out according to Herriott[10] by alkali-fusion of the iodoprotein, oxidation of the solution of the melt with bromine water at *p*H 4, volatilization of the excess bromine, and, after addition of potassium iodide, titration of the iodine liberated with 0.01 *N* sodium thiosulfate. The method was tested by adding an aliquot of an aqueous potassium iodide solution to albumin, which then was subjected to the alkali fusion. Other aliquots of the potassium iodide solution were directly oxidized with bromine water and titrated in a similar way. The two methods agreed within 0.3%.

The Millon-Nasse reaction[20] was used to estimate the amount of 3,5-unsubstituted tyrosyl groups in the iodinated albumins. The protein was hydrolyzed for twenty-four hours with 4 *N* barium hydroxide, the barium was removed with sulfuric acid, and the clear filtrate was boiled with mercuric sulfate and then treated with sodium nitrite to develop the pink color. Diiodotyrosine gave a negative test under these conditions.

The presence of tryptophan in various preparations was followed by the method of Tauber,[21] which consists in the formation of a greenish fluorescence with perchloric acid and potassium dichromate. The test proved sensitive to 20γ of tryptophan in 3 ml. of solution.

Estimation of sulfhydryl groups was carried out by titration in guanidine–hydrobromide solution at *p*H 10 with CH_3HgNO_3 using sodium nitroprusside as indicator.[22]

Ultraviolet absorption spectra of iodinated proteins and amino acids at different *p*H values were measured in a Beckman spectrophotometer, using a hydrogen discharge lamp and 1-cm. quartz cells.

Electrodialysis.—A Brintzinger electrodialyzer with a rotating inner cell was used at a potential of 500 volts. Although the current was usually found to be constant after three hours, the dialysis was continued overnight.

Ultracentrifugal analyses were made[23] in an air-driven ultracentrifuge, equipped with a modified Philpot schlieren optical system. The speed was 54,000 r. p. m. and the average temperature 24°.[24]

Experimental

In attempting to control the degree and specificity of the iodination of a protein, there are several parameters which may be conveniently varied and controlled. In the following studies they have been varied as follows:

(1) The temperature has been maintained as low as possible (*i. e.*, in the neighborhood of 0°) on the premise that the rate of denaturation reactions increases more rapidly with rising temperature than simple chemical reactions.

(2) The protein concentration has been between 2 and 10% in order to avoid denaturing effects in more dilute and too high a viscosity in more than 0.3 unit in any experiment.

(3) The initial iodide concentration has usually been just sufficient to prepare the iodine reagent (*i. e.*, 4 moles of potassium iodide per mole of iodine). The concentration increased, to be sure, as the reaction proceeded.

(4) To prevent slowing of the reaction by accumulating hydrogen ions, the solutions have been well-buffered. The *p*H has not changed more than 0.3 unit in any experiment.

(5) In controlling the degree and specificity of the reaction, the *p*H has appeared to be the most useful variable as has been pointed out by Anson[25] and Li.[26,27] In our studies, the *p*H has been varied from 9 to 10.5. The limit on the alkaline side depends both upon the instability of the protein, and the reagent (see below); on the acid side, upon the rate of the reaction.

(6) It is well known that iodine reacts with hydroxyl ions to form iodate and iodide ions.[28,29] Since such a reaction would complicate stoichiometric studies of iodination, conditions were sought at which this reaction became negligible.

Mixtures of buffer and iodine–iodide solution were allowed to stand for a period of three days at 2°. Aliquots of the mixtures were then extracted exhaustively with carbon tetrachloride, and the iodine titrated by shaking the extract with 0.1 *N* sodium thiosulfate solution. The iodine-free aqueous solution was then acidified and the iodine liberated from the iodate + iodide titrated with thiosulfate.

No iodate was formed in phosphate buffer at *p*H 7.05. The mixture employed consisted of 6 vol. of *M* phosphate buffer + 1 vol. of 0.1 *N* iodine, 0.2 *M* iodide solution. Table I gives the results of a series of experiments carried out in 1 *M* mixtures of carbonate–bicarbonate buffers at various *p*H values. The final mixtures consisted of 3 vol. of buffer and 5 vol. of the above iodine solution.

TABLE I

EFFECT OF *p*H ON IODATE–IODIDE FORMATION AT 2°

Time, hr.	% of iodine as iodate at *p*H 9.46	*p*H 9.87	*p*H 10.30
1/4	0.2	0.8	18.8
3	.2	2.9	31.8
20	.8	5.6	44.4
42	.9	8.1	51.3
70	.9	11.5	56.0

(20) O. Folin and V. Ciocalteu, *J. Biol. Chem.*, **73**, 636 (1927).

(21) H. Tauber, *ibid.*, **177**, 337 (1949).

(22) W. L. Hughes, Jr., unpublished.

(23) Ultracentrifugal analyses were carried out by Mr. Charles Gordon and computed by Miss Patricia Baker and Mr. Frank Gordon under the supervision of Dr. J. L. Oncley.

(24) J. L. Oncley, G. Scatchard and A. Brown, *J. Phys. Coll. Chem.*, **51**, 184 (1947).

(25) M. L. Anson, "Protein Denaturation and the Properties of Protein Groups," in "Advances in Protein Chemistry," Vol. II, Academic Press, New York, N. Y., 1945, p. 361.

(26) C. H. Li, THIS JOURNAL, **66**, 225 (1944).

(27) C. H. Li, *ibid.*, **66**, 228 (1944).

(28) Gmelin, "Handbuch der anorganischen Chemie," 8. Aufl., Nr. 8: Jod, Verlag Chemie, Berlin, 1933, pp. 444, 552.

(29) C. H. Li and C. F. White, THIS JOURNAL, **65**, 335 (1943).

These experiments indicated that no measurable iodate–iodide formation took place below *p*H 9.5. Above this *p*H appreciable reaction occurred, increasing rapidly with increasing alkalinity. At *p*H 9.46 it was found that even considerable variation in the ratio of iodine to buffer did not result in iodate formation. This *p*H value was therefore tentatively considered safe for the iodination of human serum albumin.

(7) The initial iodine concentration was usually just sufficient to carry the reaction to the desired point. Under conditions where the reaction was proceeding so slowly that the stoichiometric amount would have required several days to react, an excess, considered suitable, was added and removed at the desired end-point. In general, three different degrees of reaction have been studied: (a) the reaction of the first few equivalents of iodine (b) the reaction of amounts approximately equivalent for the conversion of all the tyrosyl residues to the diiodo-derivative, and (c) the reaction with still larger amounts of iodine.

Oxidation of the Sulfhydryl Group.—Many observations indicated that the sulfhydryl groups of a protein are the first to be oxidized. From the amount of iodine added, and from the iodine content of the final product, as determined by microanalysis, the equivalents of iodine used for oxidation may be calculated and compared with the sulfhydryl content. For this purpose only a few equivalents of iodine per mole of albumin were added. These experiments are summarized in Table II.

TABLE II

IODINATION OF HUMAN SERUM ALBUMIN WITH SMALL AMOUNTS OF IODINE

	3 equiv./mole	6 equiv./mole
G. of albumin (dec. 10, moisture-free)	4.48	4.75
Dissolved in water, ml.	20	20
M Na_2CO_3 added, ml.	15	15
M $NaHCO_3$ added, ml.	5	5
*p*H of solution	10.16	10.16
0.1 *N* I_2, 0.2 *M* KI added, ml.	2.00	4.29
Time allowed for reaction, min.	15	15
Analysis		
Mg. of protein	164.2	126.1
Mg. of iodine	0.23	0.52
I_2 content in %	0.14	0.41
Stoichiometry (equiv./mole)		
Iodine content found	0.77	2.25
Iodine added	3.00	6.00
Consumed for oxidation	1.46	1.50

Since sulfhydryl analysis of the albumin indicated only two-thirds mole of SH per mole of protein, iodine consumed in oxidation amounted to 2.2 moles per SH group.[30] These experiments thus indicated that the sulfhydryl group was oxidized beyond the disulfide stage by even small amounts of iodine. This would seem reasonable, since one sulfhydryl group cannot form a disulfide linkage except intermolecularly, and this reaction would appear to be hindered, except under special conditions,[32] by the large size of the albumin molecule.

Crystallization of Iodinated Albumin.—Preparations which contained 1–15 atoms of iodine per mole of albumin have been crystallized from ethanol–water mixtures at low temperature and low ionic strength under conditions similar to those found successful for the crystallization of unmodified human serum albumin.[19] The conditions were

Albumin	17–18 g./100 ml.
Ethanol	22–23 ml./100 ml.
Ionic strength	0.20–0.25
*p*H (acetate)	5.1–5.3
n-Decanol	Approx. 0.2%

Yields of crystals of about 80% were readily obtained with preparations containing 3–7 atoms of iodine/mole. With preparations containing 10 atoms of iodine/mole, crystallization was more difficult and the yields were only 36–40%. Recrystallization was effected by using ethanol–water mixtures, which were adjusted to the conditions mentioned above. The shape of the crystals is similar to that of human serum albumin, crystallized at 25% ethanol, *p*H 5.5 and an ionic strength of 0.1 (see Fig. 1).

Preparation of Crystallized Iodinated Human Serum Albumin with 10 Atoms of Iodine/Mole.—Fifty grams of human serum albumin (46.1 g. moisture-free), which had been crystallized with decanol, was dissolved in 30 ml. of water with the addition of a mixed buffer, consisting of 120 ml. of *M* sodium carbonate and 40 ml. of *M* sodium bicarbonate solution, giving a final *p*H of 9.41. To this solution 150 ml. of 0.1 *N* iodine–0.2 *M* iodide solution (corresponding to 1.831 g. of iodine or 10.8 moles per mole of albumin) were added in two portions, with subsequent addition of 80 ml. of *M* sodium carbonate solution, which brought the *p*H to 10.06. The solution was left for two days at 2° and then dialyzed for six days with frequent changes and dried from the frozen state; yield 46 g. (about 44.5 g. moisture-free).

For crystallization, this iodinated albumin was dissolved at −5° in 120 ml. of 15% ethanol and 36.5 ml. of an acetate buffer containing 33.0 mmoles. of sodium acetate and 62.7 mmoles. of acetic acid, added slowly with stirring. A clear solution with a *p*H of 5.12 resulted. To this solution 55 ml. of 75% ethanol, containing 0.4 ml. of *n*-decanol, were added in small portions with careful stirring. A slightly turbid solution was obtained. The conditions were

Albumin	19 g./100 ml.	Ionic strength	0.19
Ethanol	24.3 ml./100 ml.	*p*H	5.12

The solution was seeded with crystals of iodinated albumin and left at −5° where crystallization soon started. After collecting the first crop of crystals, more 75% ethanol was added to the supernatant solution and a second crop of crystals obtained. This procedure was repeated giving an over-all yield of 56 g. of crystal paste with approximately 20 g. of dry protein or 45% of the starting material.

(30) In confirmation of this, mercaptalbumin[31] (the fraction of serum albumin containing a free SH group) consumed, under the same conditions, an average of 2.6 equiv. of iodine per mole of albumin.

(31) See W. L. Hughes, Jr., "Protein Mercaptides," *Cold Spring Harbor Symposium*, in press.

(32) W. L. Hughes, Jr., and R. Straessle, in preparation.

For recrystallization, the total paste was dissolved in 20 ml. of 15% ethanol with the addition of 5 ml. of *p*H 5.60 acetate buffer ($\Gamma/2 = 2.5$). The solution, which contained approximately 20 g. of protein in 85 ml. (23% wt./vol.), had an ethanol concentration of 17% by volume, and an ionic strength of 0.23. It was seeded and allowed to stand at 2°. Crystallization quickly started after one day. The crystals were removed by centrifugation at 0° and the clear supernatant solution, left at −5°, yielded another crop of crystals. Further amounts crystallized upon the addition of 75% ethanol to the separated solution. The total yield was almost quantitative.

Iodine Analysis of the Crystals.—1.037 g. of the first crop, representing the purest fraction of crystals, was dissolved in 5 ml. of water; 2 ml. = 115.3 mg. of protein = 2.0 mg. of iodine = 1.73% I; iodine content = 10 atoms/mole of iodinated albumin.

Iodination Equivalent to the Tyrosine Content.—In an effort to convert the tyrosyl residues quantitatively and specifically into diiodotyrosyl residues, iodination with the minimum practical quantity of iodine was attempted; since the rate of the reaction was increasingly slow at less alkaline *p*H, larger excesses of iodine were then used.

The experiments, which are listed in detail in Table III, were carried out as follows: The albumin was dissolved in water and mixed with a precooled buffer solution. The calculated amount of iodine was then added in the form of a 0.1 *N* iodine–0.2 *M* iodide solution and the mixture left at 0° until it was decolorized, or if a slight excess of iodine was used, until the color matched that of a blank, containing an iodine amount equal to the excess used in the experiment with albumin. After dialysis against water, the iodinated albumin was dried from the frozen state and analyzed for organically bound iodine.

TABLE III

IODINATION EQUIVALENT TO THE TYROSINE CONTENT

Experiment		1	2	3	4
G. albumin (moisture-free)		9.6	1.869	2.820	4.428
Ml. of 0.1 *N* I_2–0.2 *M* KI		300	26.3	33.5	50.0
Buffer	mmoles. phosphate	70	.....	.....	.....
	mmoles. $NaHCO_3$	.....	.....	75	5
	mmoles. Na_2CO_3	.....	.....	15	25
	mmoles. borate	.....	20	.....	.....
Final volume, ml.		523	100	123	128
*p*H		7.13	9.18	9.35	10.15
Reaction time, hr.		170	4½	98	5½
Ml. 0.1 *N* I_2 removed (titr.)		202	6.1	2.26	1.55
Eq. iodine/mole alb.	Consumed ÷ 2	34.0	36.0	36.9	36.6
	bound	28.8	34.0	34.4	33.1
	side reactions	10.4	4.0	5.0	7.0

Preparations obtained in this way were analyzed for unsubstituted tyrosyl residues by the Millon–Nasse reaction. It was found that approximately 30% of the tyrosyl residues of human serum albumin (or roughly 5 groups) had not been converted to diiodotyrosine.[33] The *p*H had no appreciable effect on the specificity of the tyrosine substitution.

Substitution with Amounts of Iodine Greater than the Tyrosyl Equivalents.—When the initial iodine concentration was increased, more iodine was consumed and the iodine content of the products increased. The substitution reaction might be expected to reach a maximum when all tyrosyl and histidyl residues were substituted. The following experiments indicate how far this substitution with iodine has been accomplished thus far, in the case of human serum albumin.

(33) A synthetic sample of monoiodotyrosine, kindly provided by Herriott, showed a more intense color than tyrosine itself in the Millon–Nasse test, the absorption maximum being concomitantly shifted to 540 mμ from the 500 mμ peak of tyrosine. Consequently, a part of the red color produced in this reaction with iodinated albumin may be due to monoiodotyrosyl residues so that more than 70% of the iodine may be bound to the tyrosyl groups.

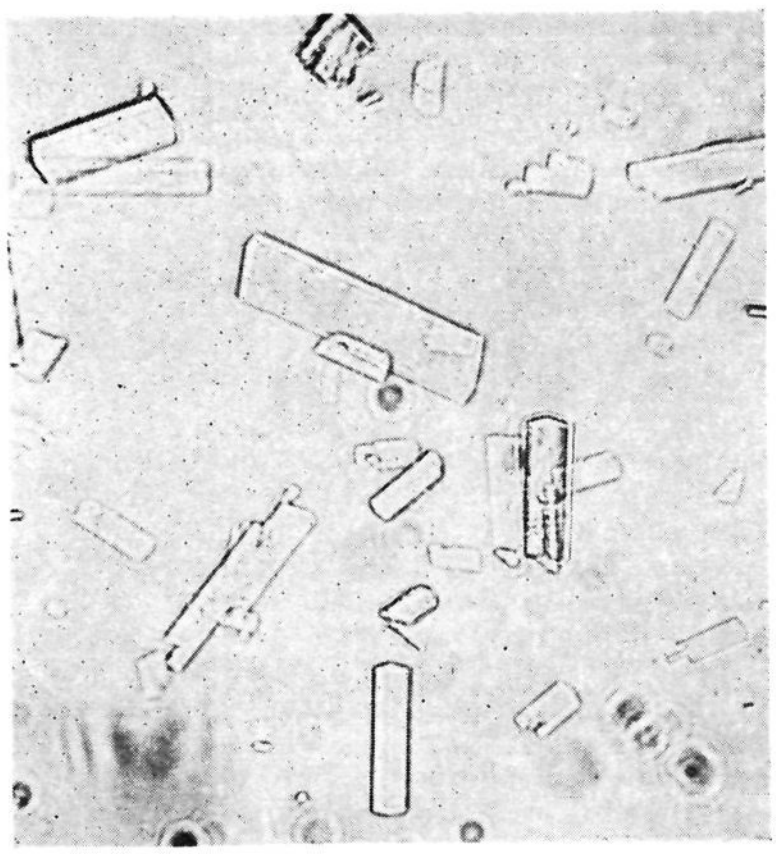

Fig. 1.—Crystals of iodinated human serum albumin: 10 atoms of iodine per mole of protein; 0.08 mole fraction ethanol; $\Gamma/2 = 0.2$; *p*H 5.1; −5° (magnification: 650 ×.)

Iodination with 142 Moles of Iodine/Mole at *p*H 9.23.—5 g. of albumin (4.7 g. moisture-free) was dissolved in a buffer mixture, containing 20 mmoles. of sodium carbonate and 100 mmoles. of sodium bicarbonate. A total of 200 ml. of 0.1 *N* iodine–0.2 *M* iodide solution was added in three steps with intervals of one and five hours, respectively. Following the final addition, the *p*H was 9.23 and dropped to 9.03 after a reaction time of seventy-one hours. The final volume was 323 ml. At this stage, the solution was decolorized with an amount of sodium thiosulfate corresponding to 51.9 ml. of the iodine solution used. The solution remained yellow even after addition of an excess of thiosulfate, and it was therefore dialyzed against 2 liters of 0.01 *M* sodium bisulfite solution buffered at *p*H 5 with acetate for a period of fifteen hours, finally against distilled water. Even this treatment did not result in removal of the yellow color of the solution. Two ml. of the dialysate, containing 20.0 mg. of protein, was analyzed for iodine: 2.01 mg. = 10.05% I.

Iodine consumed: 142–37.5 =	104.5 moles/mole alb.
Iodine content found	61.4 atoms/mole
For side reactions	86.2 equiv./mole

This preparation gave a negative tryptophan and Millon–Nasse test. Since under the conditions of this experiment no iodate formation occurred, the high amount of 86 equivalents of iodine consumed in side reactions per mole of albumin indicated that a considerable number of other groups had been oxidized.

Another experiment was carried out, under similar conditions, at *p*H 10.3. This brownish-yellow preparation had an iodine content of 64 atoms per mole, and, like the first experiment, gave negative Millon and tryptophan tests. In the ultracentrifuge, it appeared to consist of 2 components, 73% having the sedimentation constant characteristic of iodinated albumin and the remainder moving faster.

Results and Discussion

The experiments reported indicate that considerable amounts of iodine, varying from very small amounts, to amounts greater than the equivalent of the tyrosyl residues, may readily be introduced into human serum albumin without detectable degradative changes, as judged by ultracentrifugal analysis.

Preliminary studies of the rate of disappearance of the I_3^- color, indicated that several different reactions were proceeding simultaneously. Attempts to find conditions where tyrosyl substitution would proceed exclusively and quantitatively have thus far failed, since all of the factors studied which affected the rate of one of these reactions appeared to affect the rate of the other reactions in the same direction.[34]

However, the rates of these reactions were sufficiently different so that a fair degree of specificity was ultimately achieved (Table IV). Thus the sulfhydryl group may be completely oxidized, with simultaneous substitution reactions occurring to the extent of only a fraction of an iodine atom per mole. Following this, about 70% of the 3,5-positions in the tyrosyl residues may be substituted before other substitution reactions become appreciable. And, finally, the substitution reactions may be pushed to the complete coverage of the 18 tyrosyl residues (though not without concomitant iodination of other groups, presumably histidyl)[35] before further oxidation reactions become appreciable. The oxidation of cysteine to cysteic acid residues,[36] requiring 6 equivalents of iodine per mole, while not proven to occur, might account for most of the oxidative side reactions taking place during these substitution reactions. However, the iodination of a human serum albumin preparation, whose sulfhydryl group had been blocked by iodoacetamide, oxidatively consumed about the same amount of iodine (see Table IV).

(34) For more complete rate studies, including the effect of denaturation on iodination, see ref. 4.

(35) The preparation of mono- and diiodohistidine has been recently investigated by K. J. Brunings, THIS JOURNAL, **69**, 205 (1947).

(36) With small amounts of iodine, oxidation of sulfhydryl cannot go as far as cysteic acid formation, since 2.5 equivalents of iodine per sulfhydryl group were sufficient to abolish the nitroprusside reaction of serum albumin. At this stage the reaction was readily reversed by an excess of cysteine. Obviously in this case disulfide formation was impossible except as a bridge linking 2 albumin molecules, which ultracentrifugal analysis has shown does not occur under these conditions.

TABLE IV

IODINE CONSUMED FOR SUBSTITUTION AND FOR SIDE REACTIONS

*p*H	Moles I_2/mole alb. Added	Moles I_2/mole alb. Consumed	Org. bound atoms/mole	Side react. equiv./mole	Millon Nasse	Trypto-phan
10.16	1.5	1.5	0.77	1.46	Pos.	Pos.
10.16	3.0	3.0	2.25	1.50	Pos.	Pos.
10.15	37.8	36.6	33.1	7.0	Pos.	Pos.
9.00	46.8	36.0	34.0	4.0	Pos.	Pos.
9.35	36.0	35.9	32.3	7.2[a]	Pos.	Pos.
9.35	39.6	36.9	34.4	5.0	Pos.	Pos.
7.13	104.0	34.0	28.8	10.4	Pos.	Pos.
9.23	142.0	104.5	61.4	86.2	Neg.	Neg.

[a] In this experiment the sulfhydryl group had been blocked by previous treatment with iodoacetamide (negative nitroprusside test) before iodination.

Comparative experiments at *p*H 7.13 and 9.00 to 9.35 (see Table IV) would seem to indicate that oxidative side reactions occurred to a greater degree at lower *p*H for the same amount of substitution. This effect, which has been noted by previous workers,[17,18] may be due to the large excess of iodine required to effect substitution at the lower *p*H.

With still larger amounts of iodine, while the amount of substitution increased only slightly, many equivalents of iodine were consumed oxidatively. The disappearance of tryptophan would account for only a few of these and the fate of the remainder is still not explained. The ninhydrin test did not change. However, this stage of the iodination was characterized not only by the heterogeneity of reaction pathways, but also by the development of gross physico-chemical heterogeneity, so that the value of its further study was questioned.

In general the properties of iodinated human serum albumin changed progressively with increasing iodine content. Thus no insoluble material appeared upon electrodialysis when the content of iodine was less than 10 atoms per mole of albumin. In preparations containing more than 34 iodine atoms, more than 90% was insoluble under these conditions. Similarly the sedimentation constant increased by the amount expected on the basis of the increase in molecular weight and density (*i. e.*, 20% for samples containing 34 iodine atoms).

Ultraviolet absorption spectra of a series of iodinated serum albumins were studied and are presented in Fig. 2. *p*H 10.75 was chosen, since it is intermediate between the *pK* of the tyrosyl[37] and the *pK* of iodinated tyrosyl residues, in order that the maximum shift in the absorption spectra might be obtained. It will be noted that the shift from 279 to 312 mμ compares favorably

(37) Quantitative studies of this effect (similar to those of Crammer and Neuberger on insulin[38]) have led C. Tanford to assign average *pK* values of 11.7 for the tyrosyl residues in a serum albumin. This value thus appears to be displaced from that of the corresponding amino acid (10.1) by an amount assignable to electrostatic interaction.[39]

(38) J. L. Crammer and A. Neuberger, *Biochem. J.*, **37**, 302 (1943).

(39) C. Tanford, THIS JOURNAL, **72**, 441 (1950).

with that of tyrosine from 275 to 312 mμ as reported by Herriott.[9]

In addition to the maximum at 279 mμ, typical of tyrosine in proteins, a new maximum at 312 mμ characteristic of diiodotyrosine appeared, and the magnitude of this increased as the iodine content increased. At very low iodine contents, however, this new maximum shifted toward 305 mμ, the maximum for monoiodotyrosine reported by Herriott.[12]

The crystallizability of modified proteins not only gives one greater assurance that degradative changes have been minimized, but also permits further purification and characterization of the products. Crystallization of iodinated albumins has been only partially successful. Following the introduction of small amounts of iodine, the products have been crystallized under conditions quite similar to those used for the parent protein apparently as an isomorphous series (see Fig. 1). In this way products have been crystallized containing up to 15 atoms of iodine per mole of albumin (2.5% I). Shahrokh[8] reported crystallization with ammonium sulfate of horse serum albumin containing up to 4.4% of iodine, and Bonot,[4] of horse serum albumin containing 11.47% iodine.

The most favorable conditions for the substitution by iodine in the 3,5-position of tyrosyl residues of human serum albumin, that have been found, thus far have been at 0°, in solutions well buffered at *p*H 9.0 to 9.5 with bicarbonate–carbonate or borate, and a protein concentration between 2 and 5%. Under these conditions amounts of iodine only slightly in excess of theoretical need be used, and the reaction appears quite specific for the tyrosyl residues until most of the latter have reacted.

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Summary

1. Crystallized human serum albumin has been iodinated at low temperature and under variation of *p*H, iodine and iodide concentration.

2. Conditions have been found in which reaction occurs predominantly with the tyrosyl residues. Preparations containing 36 atoms of iodine per mole of albumin, the amount theoretically sufficient to saturate the 18 tyrosyl groups, contained 12 diiodotyrosyl groups. Iodine equivalent to this difference appears to be bound to histidyl groups.

3. Highly iodinated preparations, containing up to 64 atoms of iodine per mole have been obtained following treatment with large amounts of iodine (150 moles per mole of albumin). All of the 18 tyrosyl and presumably most of the 16 histidyl groups have been substituted under these conditions.

4. The following side reactions have been observed: (a) Oxidation of the cysteine residue to an extent varying with the iodine concentration; (b) oxidative destruction of other residues, such as tryptophan, with high iodine excess at *p*H 10.3; (c) iodate–iodide formation at *p*H values beyond 9.5.

5. Iodo albumin preparations with an iodine content of 1–15 atoms per mole have been crystallized from ethanol–water mixtures at low temperatures and low ionic strength under conditions similar to those for the crystallization of human serum albumin.

6. Properties of these iodoproteins are described.

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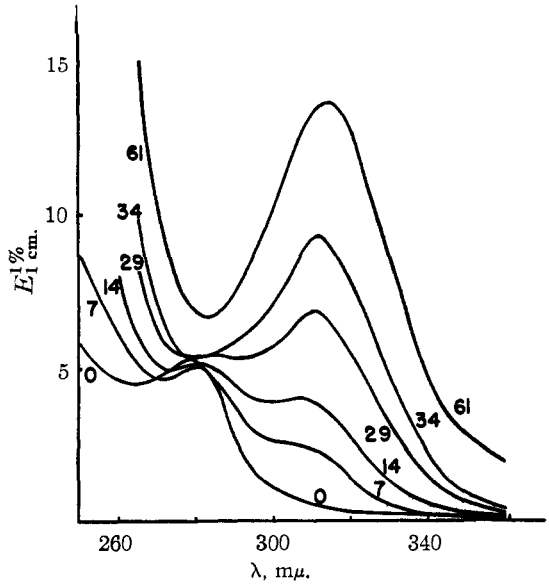

Fig. 2.—Ultraviolet absorption spectra (Beckman quartz spectrophotometer) of human serum albumin, at *p*H 10.75, as a function of iodine content. (Numbers on curves indicate atoms of iodine per mole protein.)