for one hour and thereafter analyzed. The amount of methane formed was the same as that produced under the same conditions and in the absence of toluene. Hence, the compound responsible for the formation of the additional quantity of methane is not toluene.

Bromination of the liquid which was obtained by decomposing the benzene solution of acetyl peroxide, furnished another piece of evidence supporting our mechanism of addition of methyl radicals to an aromatic hydrocarbon. Such a liquid has been shown to contain olefinic compounds. For example, decomposing 0.8 millimole of acetyl peroxide in 10 cc. of benzene (by heating this solution at vacuum and 65° for about 100 hours) produced a liquid which absorbed at 0° 1.5 cc. of 0.1 *N* solution of bromine. It was proved that neither the pure solvent, nor the unheated solution of acetyl peroxide in benzene, were absorbing bromine. Hence, the olefinic compound was produced by the decomposition of acetyl peroxide, and this fact points to the formation of compounds like, *e.g.*, dimethylcyclohexadiene.

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DEPARTMENT OF CHEMISTRY
COLLEGE OF FORESTRY
STATE UNIVERSITY OF NEW YORK
SYRACUSE, N. Y.

Crystallization and Ultraviolet Spectra of Iodinated Insulin

BY GERALD OSTER AND SYLVIA MALAMENT[1]

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Harington and Neuberger[2] in their studies on the iodination of insulin at alkaline *p*H showed that completely iodinated insulin contained 15.4% by weight iodine corresponding closely to the complete conversion of the tyrosyl residues to diiodotyrosyl residues. They were not able to crystallize the completely iodinated insulin although iodinated serum albumins[2-4] and pepsin[5,6] containing less iodine have been crystallized. In the present work the iodination of insulin was carried out under conditions which, for serum albumin, at least, are optimal for the iodination specifically of the tyrosyl residues.[4] Increasing iodination was carried out on different samples and the conditions for crystallizing the protein were determined at each stage of the iodination. Since iodinated tyrosine has a different ultraviolet absorption spectrum from that of tyrosine, the course of the iodination could be followed conveniently by spectrophotometry.

The sequence of amino acids along the polypeptide chains of insulin has been determined by Sanger.[7,8] It is not known, however, how the chains are rolled up in the protein. From the X-ray diffraction studies by Low[9] on crystalline insulin sulfate it appears that the polypeptide chains are straight rods. An X-ray analysis of the iodinated insulin crystals described in the present work might give information as to the relative dispositions of the tyrosine residues in the insulin molecule.

Experimental Procedure and Results

Iodination.—The insulin used in the present work is a sample of crystalline zinc insulin (calf), lot number W-1302, which was kindly supplied by Dr. W. W. Davis of the Eli Lilly Company. A weighed amount of insulin (dried over P_2O_5 for one week at room temperature) of about 150 mg., was dissolved in 5 ml. of a glycinate buffer made up as described by Clark[10] to be *p*H 9.5. The solution was placed in an ice–salt-bath and after the solution was cooled to 0°, the addition of iodine was begun. The iodine solution was added dropwise from a microburet over the course of an hour, the cold reaction mixture being stirred continuously. After the addition of the calculated amount of iodine (see below), the reaction mixture, still being cooled by the ice–salt-bath, was stirred for an hour to assure completeness of reaction. The solutions were then dialyzed against distilled water in seamless cellophane sausage casings at 4° over a period of one week with daily changes of distilled water.

To obtain samples of different degrees of iodination varying volumes of a 0.14 *N* iodine solution were added. The tyrosine content of insulin is reported by Brand[11] to be 12.3%, a figure which is equal to the average of that found by other workers.[12] Since two moles of iodine combine with one mole of tyrosine to form one mole of 3,5-diiodotyrosine and one mole of hydrogen iodide, then for complete iodination of 150 mg. of insulin 2.925 ml. of 0.14 *N* iodine is required. The actual amounts of iodine used are given in Table I. The iodine solutions were added dropwise so that the orange color of excess iodine disappeared before a further drop was added.

A chemical analysis for tyrosine by means of Millon reagent[13] was carried out on sample K which had been hydrolyzed for 24 hours in 4 *N* $Ba(OH)_2$. It was found that this sample contained 1.1% by weight of tyrosine, hence 91.3% of the tyrosyl residues in sample K had been iodinated.

Ultraviolet Spectra.—The ultraviolet spectra were determined in a Beckman spectrophotometer using fused quartz cells one centimeter in path length. All the spectra were determined on solutions in glycinate buffer at *p*H 9.74. The samples were diluted to give a maximum in spectra having an optical density in the neighborhood of 0.5.

At *p*H 9.74 tyrosine exhibits a maximum in its absorption spectrum at 277 mμ with a molecular extinction coefficient of 1383. Insulin also exhibits a maximum in its spectra at this wave length, the optical density at a concentration of 0.77 mg./ml. being 0.728. The tyrosyl content of insulin is then calculated to be 12.1% in good agreement with the value chosen by Brand.[11] The only other residue absorbing in this region of the ultraviolet is that of phenylalanine and although present in insulin to the extent of 7.9%,[11] it has a molecular extinction coefficient of less than 10 at 277 mμ[14] and hence makes a negligible contribution to the absorption of insulin at this wave length.

The spectra of the iodinated insulin samples resemble those obtained by Hughes and Straessle[4] for human serum albumin at various stages of iodination. The maximum at 305 mμ for the less iodinated samples, B, C and D are probably due to the presence of monoiodotyrosyl residue since monoiodotyrosine has a maximum at this wave length.[6]

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The maximum at 311 mμ for the more highly iodinated samples is due to the presence of diiodotyrosyl residues. The molar extinction coefficient of 3,5-diiodotyrosine at 311 mμ was found to be equal to 5815. Using this value and the absorption at 311 mμ for the various samples (Table I), the diiodotyrosyl content of the samples can then be calculated. Assuming a value of 12.3% tyrosine content in untreated insulin, we calculate values for the diiodotyrosine content of samples F-K which are lower by as much as 14% of that calculated stoichiometrically (Table I). Apparently the ultraviolet absorptivity of the 3,5-diiodotyrosyl group is slightly depressed when it is part of the protein molecule.

TABLE I

Sample	Insulin, mg.	Iodine, ml., 0.140 *N*	Optical density at λ 312 mμ for 0.77 mg./ml. initial insulin at *p*H 9.74	Calcd. % fully iodinated tyrosine from spectra	Calcd. % fully iodinated tyrosine from stochiometry	Optimum *p*H for crystallization
A	...	..	0.086	..	0	6.24
B	151.9	0.30	.320	..	10	6.21
C	159.1	.62	.598	..	20.0	6.36
D	151.1	.91	.787	..	30.0	6.72
E	147.4	1.14	1.12	..	40.0	6.92
F	154.5	1.29	1.12	41	43.1	6.94
G	150.8	1.46	1.39	46	50.0	6.87
H	148.0	1.65	1.64	54	57.5	7.40
I	161.5	2.02	1.72	57	64.5	7.49
J	151.1	2.10	1.98	65	71.7	7.51
K	151.0	2.94	2.65	87	100.0	7.58

Crystallization.—All the iodinated samples were crystallized by a modification of the method of Scott[15] as used to crystallize pure insulin. About 100 mg. of the dried dialyzed iodinated insulin was suspended in 5.0 ml. of distilled water. To this was added 135 ml. of a phosphate buffer (8.38 g. of $Na_2HPO_4 \cdot 12H_2O$ and 1.38 g. of KH_2PO_4 in 500 ml. of water) which had been adjusted to *p*H 2.32 with 1 *N* HCl to give a clear solution. Next 1.30 ml. of a 0.5% zinc chloride solution was added, followed by the addition of 13.0 ml. of acetone. The *p*H of the clear solution was in the neighborhood of *p*H 2.5. The *p*H was raised with ammonia to the appropriate value given in Table I. The optimal *p*H for crystallization of a given iodinated sample was determined by trial and error. The beaker containing the solution was scratched with a glass rod, left for 15 minutes at room temperature and then refrigerated for five days.

Crystals which fell to the bottom of the vessel were examined in the polarizing microscope. Samples which had been iodinated to 30% or less (samples A-D) had an appearance similar to that of ordinary zinc insulin, that is, they were of the trigonal system. More highly iodinated samples, on the other hand, crystallized in the form of flat plates, the edges of the plates being unequal in length and all mutually perpendicular. The crystals exhibited a feeble birefringence and appeared to belong to the orthorhombic system.

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DEPARTMENT OF CHEMISTRY
POLYTECHNIC INSTITUTE OF BROOKLYN
BROOKLYN, NEW YORK

Complexes of Alkaline Earth Cations Including Radium with Amino Acids and Related Compounds

BY JACK SCHUBERT

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As a supplement to some previously reported studies[1-3] measurements have been made of the interaction of alkaline earths with additional amino acids and related compounds. Most of the work reported here has been done under approximately physiological conditions, *i.e.*, ionic strength of 0.16 and *p*H 7.2–7.3. A few measurements at higher *p*H's and lower ionic strengths are included.

Experimentally, the ion-exchange technique[2] has been employed. The mass action formation quotient, K_f, for the 1:1 complexes, refers to the reaction

$$M + A \rightleftarrows MA \qquad (1)$$

where M represents the total stoichiometric concentration of the metal uncorrected for ionization, hydrolysis, binding by other ligands except the specific ligand A, etc., and MA the resulting complex. In many cases, such as for citric and tartaric acids, the parent acid, H_nA, of the ligand A is practically completely ionized at *p*H 7.2. However, for amino acids such as glutamic acid, H_3A, where one of the amino hydrogens is considered acidic, the predominant ion in solution at *p*H 7.2 is HA^- but as the *p*H is raised to 10 and above the ion $A^=$ becomes predominant (pk_1(COOH) = 2.3, pk_2(COOH) = 4.4, $pk_3(NH_3^+)$ = 9.7).[4] The over-all molar concentration of A as a basis for calculation of K_f was deliberately chosen because of its direct applicability to physiological problems. In many cases, it is very difficult, of course, to calculate true or intrinsic values of K_f because the degree and nature of the hydrolytic and other reactions involving the metal ions and ligands are not known.

The formation quotient, K_f, is calculated from the relation[1-3]

$$K_f = \frac{(K_d^\circ/K_d) - 1}{(A)^n} \qquad (2)$$

where K_d° and K_d are the distribution coefficients of M between the resin and solution phases in the absence and presence of the ligand A, respectively. The distribution coefficient for M is

$$K_d = \frac{\%M \text{ in resin}}{\%M \text{ in soln.}} \times \frac{\text{vol. of soln. (ml.)}}{\text{mass of resin (mg.)}} \qquad (3)$$

From plots of $1/K_d$ *vs.* A it was found that all of the complexes were of the 1:1 type, *i.e.*, $n = 1$. This assumes, as Toribara and Feldman have pointed out,[2] that the dissociated metal ion in solution is not polymerized. The results of the present study are summarized in Table I together with corresponding values of K_f reported in the literature for similar ionic strengths.[5-8]

Several points of interest present themselves. The values of K_f for the complexes of Ba and Ra are probably the most accurate available since previously reported values were preliminary ones. A good example is that of barium citrate where the literature except for reference 5 indicated a value of K_f nearly identical with that of strontium citrate an unlikely situation.

Values of log K_f for the calcium complexes of adenosine triphosphate and adenosine diphosphate

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