Biochemistry VOLUME 3, NUMBER 11

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December 7, 1964

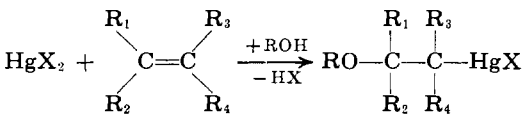
The Interaction of Mercuric Acetate with Indoles, Tryptophan, and Proteins

L. K. RAMACHANDRAN* AND B. WITKOP

From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Md.

The bathochromic shifts resulting from the interaction of mercuric acetate with indole derivatives, such as 2- and 3-substituted aryl and alkyl indoles, tetrahydrocarbazoles, indole propionic acid, and derivatives of tryptophan, including tryptophan-containing proteins, have been recorded. The average red shift is 5–10 mμ and the extinction is nearly doubled. The requirements for the shift are the presence of the nitrogen hetero atom, and the availability of the π-electrons of the 2,3- double bond of the indole nucleus. Electronegative substituents on both the 2 and 3 positions prevent this characteristic reaction. The 1,2-di- and 1,2,3-triacetoxymercury derivatives of indole and the di-(1,2-diacetoxymercuryindole propionic acid) mercury salt have been isolated and characterized. The indole nucleus of tryptophan in proteins provides a binding site for mercury, when ionizing mercury salts are used. The presence of excess mercuric acetate during *N*-bromosuccinimide oxidation of polypeptides and proteins, containing both tyrosine and tryptophan, makes possible the selective oxidation of tryptophan and cleavage of the adjacent C-tryptophyl peptide bonds.

The reaction of mercuric acetate with olefins results in the addition of the basic mercuric salt to the double bond (Wright, 1957; Chatt, 1951). Since the proper-



ties of the oxymercuration products permit classification either as addition or coordination compounds, the term "quasi-complex" has been applied (Nesmeyanov, 1945). Mercuric acetate and similar ionizable salts also react with acetylene derivatives, heterocyclic compounds, and aromatic hydrocarbons (Sidgwick, 1950). The reactions of mercury salts with alcohols, phenols, and amines are also well known. Recently, cleavage of olefinic model peptides and rufomycin A, under the conditions of oxymercuration, has been observed (Ramachandran *et al.*, 1964).

We have now examined the reaction of mercuric acetate with derivatives of indole and of tryptophan including tryptophan-containing peptides and proteins. In this study the spectrophotometric rather than preparative aspects have been emphasized. Previous published studies on the mercuration of aromatic compounds have been of a preparative nature, with little or no information on the absorption characteristics of the mercuration products. In one theoretical study the spectra of furan, thiophene, and their mercury derivatives have been examined (Leandri and Tundo, 1954).

* Visiting Scientist of the U. S. Public Health Service, 1963–64. Present address: Chemistry Department, University College of Science, Osmania University, Hyderabad-7. (A.P.), India.

EXPERIMENTAL

Materials.—Gelatin, ribonuclease, zinc-insulin, bovine serum albumin, glucagon, lysozyme, and gramicidin used in this investigation were commercial samples. Gramicidin was a mixture containing the known A, B, C, and D components. Tobacco mosaic virus protein, I-peptide from TMV[1] protein, human serum albumin, and β-casein were prepared by known methods. Most of the indole derivatives were commercial samples, and the tetrahydrocarbazole derivatives prepared in this laboratory during earlier investigations.

Measurements of Spectra.—All ultraviolet spectra were recorded with a Cary Model 14 or 15 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.). The NMR spectra were recorded with the Model A-60 instrument of Varian Associates, Palo Alto, Calif.

Mercuric Salts.—Mercuric acetate, mercuric nitrate, or mercuric chloride were dissolved in 50% acetic acid to a concentration of 0.5 M.

Changes in Absorption Characteristics on Mercuration.—All of the simple model compounds having an ϵ_M of 5–10 × 10^3 were dissolved in 50% acetic acid to give a concentration of 10 μmoles/ml (0.01 M). Proteins and other compounds which had lower or higher extinction coefficients were dissolved in the same solvent but at a higher or lower concentration.

In a typical experiment 50 μl (0.5 μmole) of a solution of L-tryptophan was mixed with 2.95 ml of 50% acetic acid and 1 ml of the mercuric acetate solution. The absorption characteristics of the treated sample

[1] Abbreviations used in this work: TMV, tobacco mosaic virus; NMR, nuclear magnetic resonance; NBS, *N*-bromosuccinimide.

were measured against a reference solution containing all the ingredients except the mercuric acetate (difference spectrum, curve C, Fig. 1) and separately against another reference containing all the ingredients except the sample (e.g., curve D, Fig. 1). The absorption characteristics of a solution of 0.5 μmole of L-tryptophan in 4 ml of 50% acetic acid were also recorded for purposes of comparison (curve A, Fig. 1). All curves in Figures 3–5 were obtained in this way, and the data recorded in Table I for various compounds were calculated from such spectra.

Preparation of Mercurated Indoles.—(A) To a stirred solution of 5 mmoles (0.59 g) of indole in 10 ml of ethanol was added a suspension of 1.59 g of mercuric acetate (5 mmoles) in 15 ml of ethanol. The precipitate, which was allowed to stand overnight, was collected, washed with alcohol, and dried (1.23 g). The pale-yellow sample turned brown above 190° and decomposed with shrinking and darkening above 270°, with the odor of indole being evident. The product contained more mercury (60.34%) than calculated (53.45%) for a monoacetoxymercury derivative.

(B) Indole was mercurated under exactly the same conditions as in the previous experiment, but with 3.17 g of mercuric acetate equivalent to a molar ratio of 2 mercury versus 1 indole. The product was isolated, washed, and dried (2.76 g). The colorless product turned purple above 205° and decomposed gradually with shrinking but not melting.

Anal. Calcd for the diacetoxy derivative $C_{12}H_{11}NO_4Hg_2$: C, 22.7; H, 1.75; N, 2.20; Hg, 63.25. Found: C, 22.66; H, 1.76; N, 2.29; Hg, 63.03.

(C) Indole was treated with mercuric acetate under the conditions of the two previous experiments but with a 4:1 molar ratio of mercuric acetate versus indole. A precipitate rapidly formed and then redissolved. The solvent was allowed to evaporate slowly, and the residue was triturated with water, filtered, and washed with water, alcohol, and ether to yield 1.56 g. From the filtrate another 0.36 g of material was recovered. The product became gray above 210° and turned dark purple and decomposed with shrinking over 270°. The sample contained 67.79% mercury and 1.45% nitrogen compared to 67.6% and 1.57%, respectively, calculated for a triacetoxymercuryindole.

Samples of A, B, and C were dissolved in glacial acetic acid to give concentrations of 3.96, 5.32, and 3.9 mg/ml, respectively. Aliquots (0.1 ml) were diluted with water to 4 ml and the ultraviolet absorption was recorded (Fig. 2). To each of the samples 0.1 ml of 8% thiolacetic acid was added, and the amount of indole present in the samples was calculated from the extinction at 280 mμ. These values for the content of indole were used as a basis for the calculation of the molar extinction coefficients (s = shoulder):

(A) $\lambda\lambda_{max}$ 305s, 287 (5.08), 280 (5.28), 260 (6.62), ~220 (8.9); $\lambda\lambda_{min}$ 284, 274, 248.

(B) (diacetoxymercury derivative): $\lambda\lambda_{max}$ 305s, 287 (5.24), 280s (5.0), 260 (6.88), ~230 (>8.65); $\lambda\lambda_{min}$ 284, 274, 249.

(C) (triacetoxymercury derivative): $\lambda\lambda_{max}$ 293–6 (5.78), 256–8s (~8.8), ~230; λ_{min} 273–4.

The NMR spectrum of B in trifluoroacetic acid (20% solution) showed the presence of the four benzenoid and the six acetoxymercury protons. The expected ratio is 0.67. The observed ratio, on integration, was found to be only 0.55, possibly owing to the presence of contaminating acetate or mercuric acetate in the sample or, alternately, errors in the integration of peak intensities. Signals corresponding to the indole protons at positions 2 and 3 (Cohen *et al.*, 1960) were, however, entirely absent.

Mercury-bis[1,2-diacetoxymercuryindole-3-propionate].—A solution of 5 mmoles of indolepropionic acid (0.95 g) in 50 ml of ethanol was treated with powdered mercuric acetate (30 mmoles). After the solution had remained overnight, the insoluble pale-yellow product was collected and washed with ethanol and diethyl ether to yield 3.4 g. The sample darkened above 260° and decomposed at 285–287°.

Anal. Calcd for $C_{30}H_{28}N_2O_{12}Hg_5$; C, 22.3; H, 1.75; N, 1.74; Hg, 62.3. Found: C, 22.1; H, 1.81; N, 1.81; Hg, 59.4.

In 20% solution in trifluoroacetic acid the product gave an NMR spectrum showing the presence of the protons in the benzene ring, the CH_2 groups of the carboxyethyl side chain in the 3 position of the indole nucleus, and the acetoxymercury groupings, and the absence of the proton at the 2 position of indole. Integration of the peak areas in the NMR spectrum gave a ratio of 1:1.1:1.36 for benzenoid protons versus side chain methylene protons versus acetoxymercury protons. The expected ratio is 1:1:1.5, while the ratio would be 1:1.33:2 for the presence of one of the acetoxymercury residues on the benzene nucleus. The acetoxymercury residues would presently be best assigned as substituting the protons at positions 1 and 2 of indole-3-propionic acid.

In dilute acetic acid (2.4%) the product showed λ_{max} 286–288 mμ (ϵ 7.61 × 10^3) and a shoulder at 210–230 mμ (ϵ 12.7 × 10^3), with λ_{min} at 260 mμ.

Mercurated N-Acetyl-L-tryptophanamide.—The mercuration with one molar equivalent of mercuric acetate in a medium of 15% acetic acid in ethanol led to the isolation of two fractions, *a* (decomp) 215°, and *b* (decomp) 258–260°. When excess mercuric acetate (4 moles) was used the product *c* was isolated, which turned dark over 160° and decomposed above 200° over a wide range.

In 2.5% acetic acid the ultraviolet-absorption characteristics, $\lambda_{max}(\epsilon)$, of the products were as follows:

(*a*) $\lambda\lambda_{max}$286–7 (7.87 × 10^3), 292–3s; ~230 (15.75 × 10^3); λ_{min}252

(*b*) $\lambda\lambda_{max}$286 (8.4 × 10^3), 292–3s, ~230 (15.9 × 10^3); λ_{min}254

(*c*) $\lambda\lambda_{max}$294 (9.25 × 10^3); ~230 (29.8); λ_{min} 267–8.

These extinction values were calculated from the 280 mμ absorption of the parent indole which was regenerated when the sample solutions were treated with excess thioacetic acid. With products *a* and *b* the original indole spectrum was regenerated almost immediately, while with *c* several hours were needed for reversion to the typical indole spectrum.

With excess mercuric acetate in ethanol 2,3-dimethylindole, 2,3-diphenylindole, and 1-methyl-2-acetylindole also yielded insoluble products.

Regeneration of Parent Indoles.—Passage of a solution of mercurated indolepropionic acid in 70% acetic acid through a column of Dowex-50 × 8 (H^+ form) led to retention of the mercury by the ion-exchange resin while indolepropionic acid was eluted in yields of 57–70%, based on recrystallized material. Similarly, the addition of excess thiolacetic acid to a solution of the mercurated compound in acetic acid led to the quantitative release of free indolepropionic acid. Passage of hydrogen sulfide into acetic acid solutions of the mercurial had the same effect. The same treatments released indole from 2,3-diacetoxymercuryindole.

The Effect of Mercuric Acetate on the Oxidation of Tyrosine by N-Bromosuccinimide (NBS).—To a stirred solution of tyrosine (2 μmoles) in 3.95 ml of water was added 50 μl of a 0.5 M solution of mercuric acetate in 50% acetic acid. A solution of NBS (10 μmoles/ml) was added in small portions until a total of 6 μmoles of

NBS had been added. The final absorption spectrum is shown in Figure 6D. There was no evidence for the formation of dienone (λ_{max} 260 mμ), although there was an appreciable increase in absorption in the peak region part of which is explained by absorption of NBS itself.

In a similar experiment mercuric acetate, but not the equivalent amount of acetic acid, was omitted. Figure 6B depicts the spectral changes caused by normal oxidation and disappearance of the tyrosine chromophore; the final spectrum shown (with 6 μmoles of NBS) corresponds to that of the dibromo-*spiro*-dienone lactone. Free tyrosine consumes more NBS for the completion of oxidation than *N*-acylated derivatives.

NBS Oxidation of an Equimolar Mixture of Phloretylglycine and N-Acetyl-L-tryptophanamide.—*N*-Acetyl-L-tryptophanamide (0.2 μmole) and phloretylglycine (*i.e.*, *p*-hydroxyphenylpropionylglycine) (0.2 μmole) were dissolved in 3 ml of 50% acetic acid containing 50 μmoles of mercuric acetate. One μmole of NBS (about 2.5 moles per mole of phenol and indole) was added. The spectrum was scanned before and after the addition of NBS. The initial spectrum corresponded to that of the sum of the spectra of phloretylglycine and mercurated acetyltryptophanamide, and, after NBS oxidation, corresponded to the sum of the spectra of unchanged phloretylglycine and the oxindole-*spiro*-lactone from acetyltryptophanamide (Fig. 7). The absorption at 260 mμ changed but little (allowing for the absorption of excess NBS present), indicating the absence of significant amounts of dienone lactone from phloretylglycine.

Under similar conditions, the mixture was treated with NBS equivalent to 2, 4, 6, and 8 moles per mole of indole present (leaving an excess of at least 5 moles of NBS per mole of phenol). The addition of 2 moles of NBS released the maximum amount of ammonia (25–35%), as estimated, after removal of Hg^{2+}, by ninhydrin assay (Rosen, 1957). There was no free glycine present as was ascertained by paper chromatography. Addition of more oxidant led to the destruction of the amminia released.

The Effect of Excess NBS on Phloretylglycine in the Presence of Excess Mercuric Acetate.—Phloretylglycine (2 μmoles) was dissolved in 0.5 ml of 0.5 M mercuric acetate in 50% acetic acid. Aliquots of this solution were oxidized by (2, 4, 6, and 8 moles) of NBS which was added as a 0.1 mM solution in acetic acid. After 15 min the solutions were diluted to 2 ml and mercury was precipitated as mercuric sulfide by hydrogen sulfide. Excess hydrogen sulfide was removed by a stream of nitrogen. The solutions were diluted to 4 ml and mercuric sulfide was removed by centrifugation. Aliquots of 0.5 ml were evaporated to dryness, and the amino nitrogen content in each was determined by the ninhydrin reaction. The liberation of free glycine, if any, under the above conditions amounted to less than 4%. Paper chromatography of aliquots did not show the presence of free glycine. When not protected by mercuric acetate, phloretylglycine is cleaved by 3 moles of NBS releasing 82–96% of free glycine (Schmir *et al.*, 1959). After the removal of mercury ions the ultraviolet absorption shown by the supernatant indicated that phloretylglycine remained essentially unaltered and that the excess mercuric acetate had protected it from oxidation by NBS.

Discussion

When indole, indolepropionic acid, indolepropionylglycine, tryptamine, L-tryptophan, L-tryptophol, and *N*-acetyl-L-tryptophan react with excess mercuric acetate in 50% acetic acid solution the absorption maximum of the indole chromophor is shifted from 280 mμ to about 284–288 mμ, and the extinction nearly doubles. Figure 1 depicts altered absorption for L-tryptophan, and Table I summarizes these changes. The changes in absorption could also be followed conveniently as "difference spectra" by measuring the absorption of the mercuric acetate–treated sample with the untreated sample in the reference cell. For the group of substances mentioned (group I) the difference spectra show a minor peak at 284–288 mμ and a major one at 293–305 mμ, whose intensities, after correction for the absorption of mercuric acetate, are given in Table I.

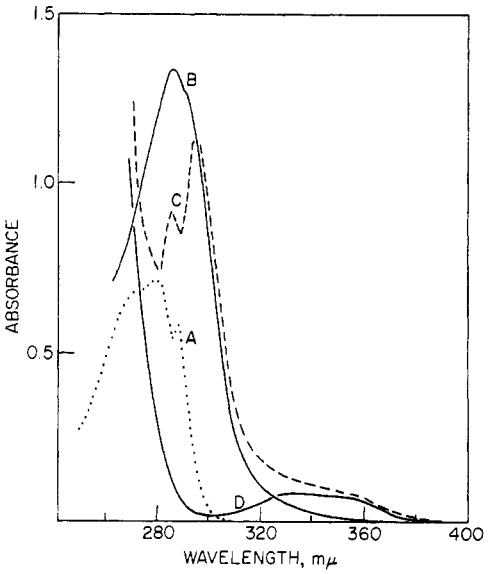


FIG. 1.—Altered absorption for L-tryptophan. (A) L-Tryptophan, 0.5 μmole in 4 ml 50% AcOH; (B) in the presence of 0.125 M $Hg(OAc)_2$; (C) difference spectrum of B with A in the reference cell; (D) $Hg(OAc)_2$ (0.125 M) in 50% AcOH.

The spectral changes are noticed immediately after the addition of mercuric acetate, and thus imply that the reactions involved are fast ones. However, the further storage of such treated samples, either at room temperature for a day or heating at 80–100° for a few minutes, causes the major peak in the difference spectrum to shift to longer wavelengths by 4–5 mμ with a small increase in extinction, while the minor peak at 284–288 mμ stays the same with a slight decrease in intensity (Table I). At room temperature urea (10 M) did not accelerate these time-dependent secondary spectral changes.

Ionized mercuric salts, such as the nitrate, cause exactly the same changes as the acetate, while nonionizing salts, e.g., the chloride, are ineffective. The presence of chloride ions *before* the addition of mercuric acetate completely prevents the described spectral changes. The addition of sodium chloride *after* the reaction with mercuric acetate slowly reverses the change in the spectrum. But the original spectrum is not restored, even after several hours at room temperature. Methylmercuric hydroxide, known to cause hyper- and hypochromic shifts on reaction with nucleosides and the nucleic acid bases (Simpson, 1964), caused none of the characteristic spectral changes when added (3×10^{-2} M) to indolepropionic acid in neutral or acidic solution. A drop in intensity of the indole absorption by about 6–7% was noticeable above 260 mμ.

While alterations in the spectra of tryptophan and indoles had hitherto not been noticed, the formation of mercurated products is known. Thus tryptophan is precipitated from a mixture of amino acids in sulfuric

TABLE I

CHANGES IN ABSORPTION CHARACTERISTICS OF INDOLES, TRYPTOPHAN MODELS, AND PROTEINS ON TREATMENT WITH MERCURIC ACETATE[a]

Absorption in 50% AcOH	Absorption in 0.125 M $Hg(OAc)_2$ in 50% AcOH	Difference Spectrum
Group I		
L-Tryptophan—λ_{max}^{272-3s} 5.24; λ_{max}^{279-80} 5.63; λ_{max}^{288-9} 4.57	λ_{max}^{286-8} 9.99; λ_{max}^{293-8} 9.96; after heating 10 min at 70°, λ_{max}^{290} 9.6; λ_{max}^{295-6s}	λ_{max}^{285-8} 6.73; λ_{max}^{294} 8.24; after heating 10 min at 70°, λ_{max}^{284-5} 4.72; λ_{max}^{297} 8.24; after 2 days at room temp, λ_{max}^{286} 4.56; $\lambda_{max}^{299-300}$ 9.12
Indolepropionic acid—γ_{max}^{263-4s} 4.88; λ_{max}^{280} 5.21; λ_{max}^{288-9} 4.36	λ_{max}^{285} 10.44; λ_{max}^{292s} 10.14; λ_{max}^{323-s}	λ_{max}^{286} 5.6; λ_{max}^{294-5} 9.12; λ_{max}^{320-s}
Indolepropionylglycine—λ_{max}^{275s}; λ_{max}^{280} 5.38; λ_{max}^{290} 4.52	λ_{max}^{289} 9.94; λ_{max}^{293-4} 9.97	λ_{max}^{287-8} 5.10; λ_{max}^{298} 8.06; after 21 hr at room temp, λ_{max}^{288} 4.56; λ_{max}^{303} 9.65
N-Acetyl-L-tryptophanamide—λ_{max}^{273s}; λ_{max}^{280} 5.8; λ_{max}^{289} 4.8	λ_{max}^{2} 12.46; λ_{max}^{294} 12.36	λ_{max}^{286-7} 7.32; λ_{max}^{296-7} 10.57; after 21 hr at room temp, λ_{max}^{286-7} 4.56; λ_{max}^{300} 11.52
Tryptamine·HCl—λ_{max}^{272-3s} 5.46; λ_{max}^{279-80} 5.70; λ_{max}^{288} 4.68	λ_{max}^{288s} 10.14; λ_{max}^{296-7s} 8.64	λ_{max}^{284-5} 5.94; λ_{max}^{293-4} 7.08
Tryptophol—λ_{max}^{273-4s}; λ_{max}^{280} 6.08; λ_{max}^{289-90} 5.04	$\lambda_{max}^{310(broad\ 290-325)}$ 8.0; spectra with $Hg(OAc)_2$ variable	λ_{max}^{286-7} 2.24; $\lambda_{max}^{310,322(broad\ 300-325)}$ 7.78
Indole—λ_{max}^{270} 5.92; λ_{max}^{277-8} 5.67; λ_{max}^{287-8} 4.37	λ_{max}^{292-3} 9.12	λ_{max}^{284-5} 1.44; λ_{max}^{305} 9.05
Group II		
Gramicidin, assuming 4 Try residues—λ_{max}^{273-5s}; λ_{max}^{279-82}; λ_{max}^{290s}	λ_{max}^{296-7} (9.75)	λ_{max}^{286-7} (4.54); λ_{max}^{298-9} (8.97)
Glucagon—λ_{max}^{280}; λ_{max}^{290s}	λ_{max}^{283-4} 12.4; λ_{max}^{296-7s} 10.9	λ_{max}^{285-7s}; λ_{max}^{295-8} 7.47
TMV protein—λ_{max}^{279-80}; $\lambda_{max}^{289-90s}$	λ_{max}^{284-5} (11.48); λ_{max}^{293-4s} (10.65)	λ_{max}^{287-8} (8.89); λ_{max}^{296-7} (10.97)
Lysozyme chloride—λ_{max}^{273s}; λ_{max}^{279-81}; λ_{max}^{289}	λ_{max}^{287-8} (12.3); λ_{max}^{295} (11.45)	λ_{max}^{287-8} (7.47); λ_{max}^{296-7} (9.24)
β-Casein—λ_{max}^{276-9}; λ_{max}^{290s}	λ_{max}^{277s} (8.6); λ_{max}^{283-4s} (8.3); λ_{max}^{294-5s} (6.0)	λ_{max}^{286-7s}; λ_{max}^{295} (4.2)
Group III		
Bovine serum albumin—λ_{max}^{276}; λ_{max}^{283s}	shoulder ~280 High increasing absorption below 330 mμ	shoulder ~293.6
Human serum albumin—λ_{max}^{276-7}; λ_{max}^{283s}	shoulder ~280 High increasing absorption below 330 mμ	shoulder ~293–6
Group IV		
Zinc-insulin—λ_{max}^{276-7}; λ_{max}^{283-4s}	$\lambda_{max}^{\sim 260}$ 17.8; $\lambda_{max}^{270-85s}$ Increasing absorption below 320 mμ	No shoulder or peak 280 mμ and above
Bovine ribonuclease—λ_{max}^{2750-6}; λ_{max}^{283s}	$\lambda_{max}^{\sim 260}$; λ_{max}^{280-3s}	No peaks or shoulder 280 mμ and above. High increasing absorption below 320–330 mμ
Others		
Gelatin	Practically no absorption between 250–330 mμ with or without added $Hg(OAc)_2$	
2,3-Diphenylindole—λ_{max}^{307-8} 15.9	λ_{max}^{307-8} 16.8	No significant difference spectrum
Oxindole—λ_{max}^{249} 8.97; λ_{max}^{280s} 1.32	λ_{max}^{280s} 1.92; no peak above 260	Slight difference spectrum, with no peaks between 263–340 mμ
Indoline—$\lambda_{max}^{292,285s}$	λ_{max}^{292-9} ~0.3; λ_{max}^{350-3} ~0.3	$\lambda_{max}^{299-300}$ ~0.3; λ_{max}^{283s}; λ_{max}^{350-6} ~0.3
Benzofuran—λ_{max}^{282}	Very slight increase in absorption between 280 and 310 mμ	
Indene—$\lambda_{max}^{\sim 250}$	$\lambda_{max}^{283s,277s}$ Increased absorption below 300	No difference spectrum above 280
2-Methylindole—λ_{max}^{270} 6.5; λ_{max}^{275-7s}; λ_{max}^{287} 4.8	λ_{max}^{303-6} 8.6; λ_{max}^{260-1} 12.2	λ_{max}^{286-7s} ~0.3; λ_{max}^{306} 8.44
2,3-Dimethylindole—λ_{max}^{280} 6.36; λ_{max}^{288s} 5.36	λ_{max}^{306-11} 7.8; λ_{max}^{261-2} 12.3	λ_{max}^{287s}; λ_{max}^{309-14} 7.02
3-Methylindole—λ_{max}^{273s}; λ_{max}^{280} 5.16; λ_{max}^{289} 4.32	$\lambda_{max}^{296-300}$ 8.48; λ_{max}^{320s} 6.64	λ_{max}^{304-5} 7.36; λ_{max}^{289} ~3.44; λ_{max}^{320s}
2-Phenylindole—λ_{max}^{247} 14.4; λ_{max}^{304-6} 24.6	λ_{max}^{321-2} 10.3; $\lambda_{max}^{\sim 260}$ 11.85	λ_{max}^{343-4} 8.45; λ_{min}^{303-7} −20.2
3-Phenylindole—λ_{max}^{268} 14.15	$\lambda_{max}^{265-85(broad)}$ 11.94; λ_{max}^{294s} 11.2	λ_{max}^{314} 3.04; $\lambda_{max}^{288s,298s}$
2-Biphenylindole—λ_{max}^{257} 17.4; λ_{max}^{331} 37.4	λ_{max}^{263} 21.5; λ_{max}^{320} 16.35	λ_{max}^{373} 4.23; λ_{max}^{270-5} ~12.6; λ_{min}^{333} −24.2
N-Methylindolepropionic acid—λ_{max}^{280s}; λ_{max}^{290} 5.44	λ_{max}^{292-3} 9.12	λ_{max}^{296-7s}; λ_{max}^{310} 5.6
N-Methyl-2-acetylindole—λ_{max}^{309} 21.7	λ_{max}^{316} 15.2	λ_{max}^{336-52} 2.1; λ_{min}^{304-5} −7.83
Carbazole—λ_{max}^{246} 18.2; λ_{max}^{254} 17.3; λ_{max}^{291} 15.7; λ_{max}^{321} 3.44; λ_{max}^{333} 2.96	$\lambda_{max}^{\sim 270}$; λ_{max}^{295} 12.7; λ_{max}^{325} 3.07; λ_{max}^{336} 3.04	λ_{max}^{297} 7.45; λ_{max}^{328-9} 0.88; λ_{max}^{342-3} 1.6
Tetrahydrocarbazole—λ_{max}^{280-3} 7.04; λ_{max}^{290s} 5.92	$\lambda_{max}^{\sim 260}$; λ_{max}^{306-8} 8.0	λ_{max}^{286-7s}; λ_{max}^{311-2} 7.28; λ_{min}^{283} −1.72
1-Keto-tetrahydrocarbazole—$\lambda_{max}^{\sim 240}$ ~6.7; λ_{max}^{310} 22.85	λ_{max}^{313} 19.45	λ_{max}^{330} 2.44; λ_{max}^{357-9} 1.6; λ_{min}^{295} −4.4

TABLE I (*Continued*)

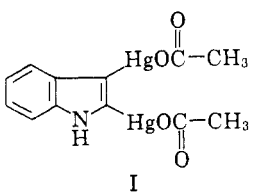
Absorption in 50% AcOH	Absorption in 0.125 M $Hg(OAc)_2$ in 50% AcOH	Difference Spectrum
7-Acetyltetrahydrocarbazole—$\lambda_{max}^{261\text{-}2}$ 13.0; λ_{max}^{313} 11.6; λ_{max}^{354} 10.78	$\lambda_{max}^{261\text{-}2}$; λ_{max}^{313} 10.23; $\lambda_{max}^{354\text{-}5}$ 8.73	$\lambda_{max}^{273\text{-}4}$ 4.4; λ_{min}^{313} −1.6; $\lambda_{min}^{352\text{-}3}$ −2.4
N-Methyl-7-acetyltetrahydrocarbazole—$\lambda_{max}^{\sim 260}$; λ_{max}^{315} 11.85; $\lambda_{max}^{365\text{-}6}$ 10.25	$\lambda_{max}^{305\text{-}6,315,\sim 260}$ Extinctions almost unchanged	Almost no difference spectrum above 260 mμ
N-Benzoyltetrahydrocarbazole—$\lambda_{max}^{262\text{-}3}$ 16.2; $\lambda_{max}^{310\text{-}3}$ 4.34	λ_{max}^{270s} 11.6; $\lambda_{max}^{310\text{-}3}$ 4.34	No difference spectrum above 280–90 mμ
2,3-Cyclooctenoindole—$\lambda_{max}^{\sim 245}$; λ_{max}^{290} 6.4; λ_{max}^{282} 7.05	λ_{max}^{263} 13.9; λ_{max}^{309} 8.3	$\lambda_{max}^{285\text{-}90s}$; $\lambda_{max}^{313\text{-}4}$ 7.2
2-Keto-cyclooctenoindole—λ_{max}^{317} 18.7	λ_{max}^{323} 17.3	λ_{max}^{337} 3.6; λ_{max}^{370s} 1.64

[a] All molar extinction coefficients are recorded as $\epsilon \times 10^{-3}$; wavelength given in mμ; s = shoulder; values in parentheses apply to proteins and are extinctions calculated per tryptophan.

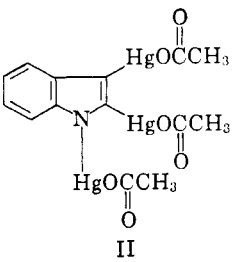
acid solution by mercuric sulfate (Hopkins and Cole, 1901) and the red color, which is formed when this mercurated product is treated with sodium nitrite, has been the basis for one of the older analytical assays for tryptophan (Lugg, 1937). Also, compounds of the indole series have been reported to yield, on treatment with mercuric acetate, products from which mercury is readily split off (German Patent, 1912). One published account (Mingoia, 1930) of the mercuration of indoles is worth special mention for possible value in synthesis of indole derivatives. Mercuration of indole was reported to yield a 2,3-diacetoxymercury derivative (complete analyses were not given) which on treatment with chloride ions was converted to a 2,3-dichloromercury derivative and subsequently, by reaction with excess potassium iodide, to 2,3-diiodoindole. Analogous reactions were reported for the preparation of 2-iodoskatole and 3-iodo-2-methylindole. Earlier efforts in this laboratory (W. B. Lawson and B. Witkop, unpublished data) failed to substantiate the above reports. During the present study, indole reacted with 1, 2, and 4 moles of mercuric acetate in ethanolic solution to yield three different products, each of which gave a characteristic ultraviolet spectrum (Fig. 2). Indoles treated with 2 and 4 moles mercuric acetate yielded mercuration products which analyzed well for diacetoxymercury and triacetoxymercury derivatives of indole, respectively. The NMR spectrum of the diacetoxymercuryindole indicated the absence of peaks at 6.78 and 6.1 ppm corresponding to the protons at positions 2 and 4 (Varian Associates, 1962). The peaks present are due to the aromatic and acetoxy protons. There is the possibility of the presence of polymeric material containing C-Hg-N bonds. The NMR spectrum excludes an oxymercuration product resulting from addition. On the basis of both the NMR spectrum and the elementary analysis, the reaction between indole and 2 moles of mercuric acetate in ethanol solution yields a product in which two acetoxymercury groups substitute for the protons at positions 2 and 3 in indole. The NMR spectrum of the product of reaction between indolepropionic acid and mercuric acetate similarly shows the absence of the proton originally present at position 2. Thus reactions of indoles with mercuric acetate are substitutions while the well-known oxymercurations of olefins are additions. The above observations would also warrant a reinvestigation of synthetic possibilities in the indole series via acetoxymercury derivatives.

The data in the experimental section lead us to postulate structures I and II for the diacetoxymercury and triacetoxymercury derivatives, respectively, of indole, and structure III for the mercurated indolepropionic acid.

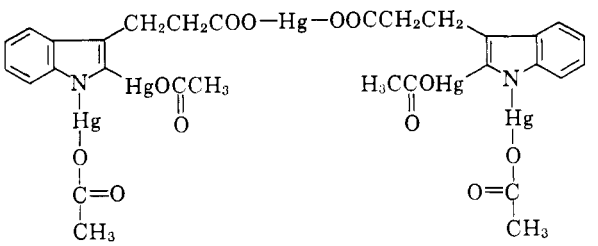
With acetoxymercury derivatives of both indole and



I

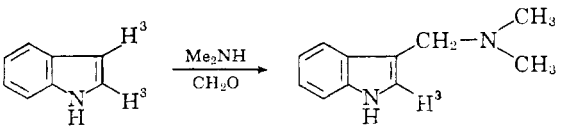


II



III

indolpropionic acid regeneration of the parent indole proceeds with high yields (70–100%), on treatment with either strong cation exchanges (Dowex-50 × 8, H^+ form) or thiols (H_2S and thioacetic acid), analogous to the demercuration of mercurated olefins (Wright, 1957; Chatt, 1951) and heterocyclic compounds (Challenger and Miller, 1939). In addition 2,3-diacetoxymercuryindole is reductively cleaved to indole by lithium aluminum hydride. When 2,3-diacetoxymercuryindole is reduced with $LiAl^3H_4$ one obtains a *tritiated indole which loses half its activity on conversion to gramine.* This proves structure I and provides a convenient approach to indoles and tryptophan metabolites selectively tritiated in the diagnostically important 2 position. The regeneration of indoles, together with the



spectral data, exclude the possibility of mercuration in the 3 position of the tautomeric indolenine. Indole, as an enamine, is capable of reacting in the tautomeric indolenine form. Analogous to enamines (Brodersen *et al.*, 1964) indoles undergo *N*-mercuration.

The indole chromophor of tryptophan bound in peptide linkage in gramicidin (4 Try), glucagon (1 Try), TMV protein (3 Try), I-peptide (1 Try) from the N-terminal region of TMV protein, β-casein (2 Try), and lysozyme (6 Try) (group II) undergo spectral changes with excess mercuric acetate analogous to the indole

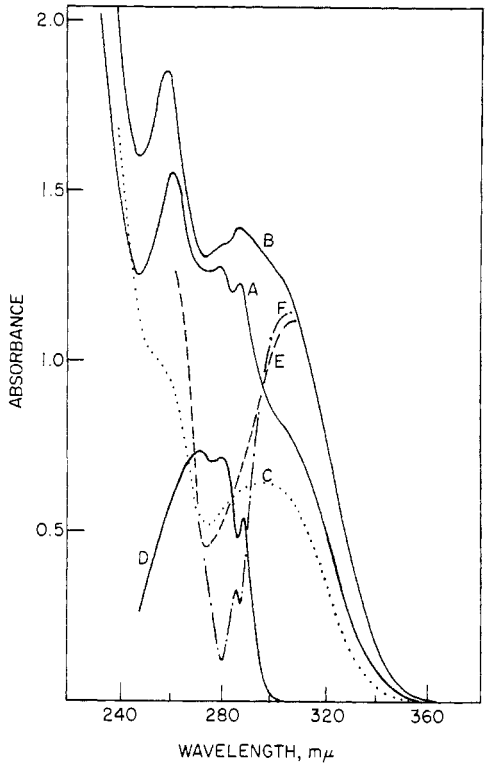


FIG. 2.—Mercurated indole derivatives in 2.5% AcOH. (A) Prepared with equal ratios of reactants (equivalent to about 0.968 μmole of indole); (B) prepared with 2-fold amount of $Hg(OAc)_2$ (equivalent to about 1.08 μmole of indole); (C) prepared with 4-fold amount of $Hg(OAc)_2$ (equivalent to about 0.45 μmole of indole); (D) 0.5 μmole of indole in 4 ml of 50% AcOH; (E) 0.5 μmole of indole in 4 ml 0.125 M $Hg(OAc)_2$ in 50% AcOH; (F) difference spectrum of E with D in reference cell.

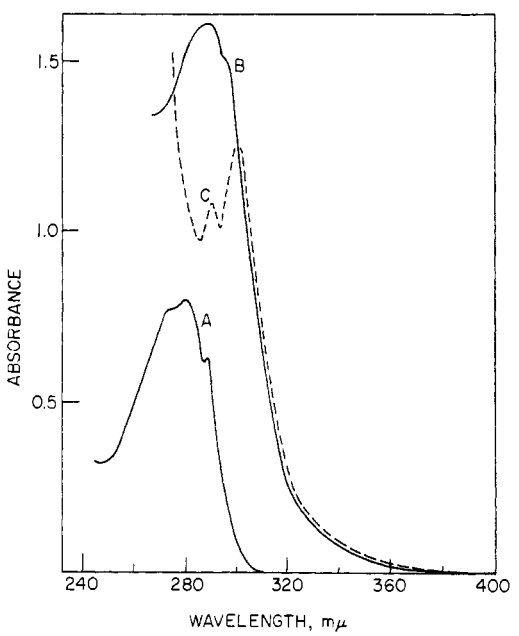


FIG. 3.—Spectral change of indole chromophor of lysozyme. (A) With lysozyme hydrochloride (~0.088 μmole); (B) with $Hg(OAc)_2$; (C) difference spectrum.

derivatives in group I. The spectra obtained with lysozyme hydrochloride are illustrated in Figure 3. The typical peaks at 284–288 mμ and at 293–305 mμ in the difference spectrum reflect the presence of the indole nucleus in the materials. In separate experiments

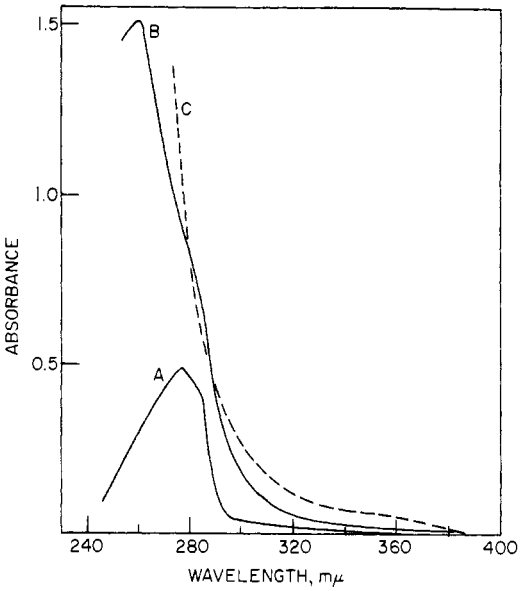


FIG. 4.—Spectra with zinc-insulin. (A) Zn-insulin (1.94 mg, uncorrected for moisture or Zn content); (B) with $Hg(OAc)_2$; (C) difference spectrum.

it was found that, within experimental error, the presence of excess mercuric acetate caused no appreciable changes in the absorption characteristics of phenylalanine and tyrosine above 265 mμ. Other proteins tested included bovine serum albumin (2 Try) and human serum albumin (1 Try) (group III). In the presence of mercuric acetate, the spectra of these proteins fall in a class by themselves with a shoulder at 270–285 mμ, increasing absorption at lower wavelengths, and no peaks of absorption. However, the difference spectra are characterized by a slight shoulder at 293–296 mμ and increasing absorption and no peaks at lower wavelengths. The shoulder at 293–296 mμ appears related to the presence of tryptophan. Two proteins tested which contained no tryptophan were zinc-insulin and bovine ribonuclease (group IV). In the presence of mercuric acetate a peak of absorption at 260 mμ was seen with both proteins, although in the difference spectrum neither shoulders nor peaks were evident above 275 mμ. The spectra with zinc-insulin are shown in Figure 4. The source of the 260 mμ absorption in the presence of mercuric acetate remains obscure at the moment. Experiments done with cystine, histidine, glycinamide, alanylphenylalanine, and amines only reveal that SH groups, disulfide bonds, amino groups, imidazole groups, and peptide or amide bonds in the presence of mercuric ions have no absorption at 260 mμ. Mercury salts are known to react with various functional groups in proteins (Gurd and Wilcox, 1956). But the present demonstration that the spectral shift found with simple indole derivatives also extends to tryptophan-containing proteins implicates indole groups in proteins as possible binding sites for mercury. Unlike substances in groups I, II, III, and IV, gelatin which contains little or no tryptophan, tyrosine, and cystine shows no change in absorption, in the presence of mercuric acetate, above 250 mμ.

The simple indole models in 50% acetic acid with even limited amounts of mercuric acetate show large spectral change of the type shown in Figure 1. Lysozyme even with several moles of mercuric acetate shows only a very gradual spectral shift indicating that the interaction of mercuric acetate with the indole nucleus in lysozyme occurs gradually along with other known interactions.

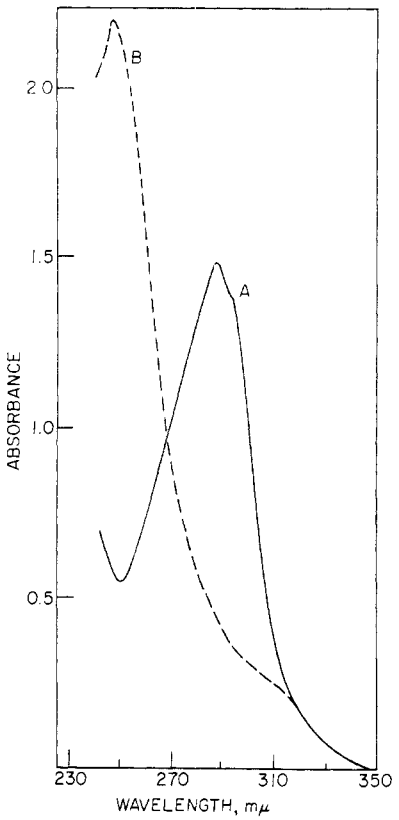


FIG. 5.—Spectra of indolepropionic acid. (A) Indolepropionic acid (0.51 μmole) and 4 ml of water containing 100 μl 50% AcOH and 25 μmoles $Hg(OAc)_2$; (B) final spectrum (uncorr) after addition of 1 μmole NBS (0.1 ml) to A.

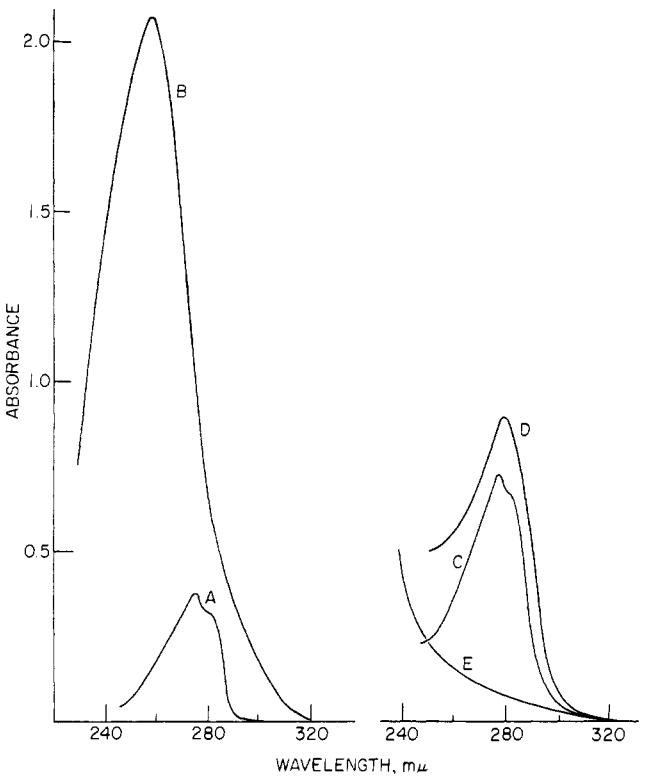
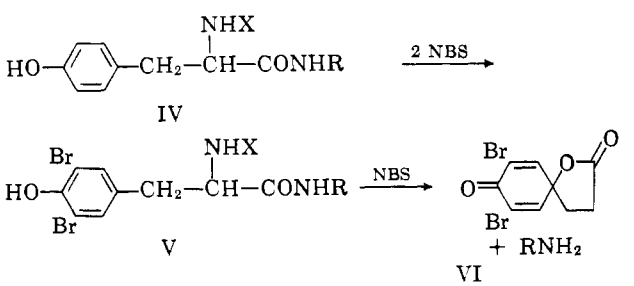


FIG. 6.—NBS oxidation of tyrosine. (A) Tyrosine (1 μmole) in 4 ml 0.5 M AcOH; (B) final spectrum of A after oxidation with 6 μmoles of NBS (not corrected for volume change); (C) 2 μmoles of tyrosine in 4 ml 0.5 M AcOH containing 25 μmoles $Hg(OAc)_2$; (D) final spectrum of C after treatment with 6 μmoles of NBS (not corrected for volume changes, absorption of NBS, or interaction between NBS and $Hg(OAc)_2$); (E) *N*-bromosuccinimide (1.1 mM solution).

As a consequence of the mercuration reactions, certain interesting differences in reactivities of phenols and indoles toward oxidizing agents have also been noticed. *N*-Bromosuccinimide is known to oxidize tryptophan and its derivatives to a *spiro*-oxindolelactone (Witkop, 1961). This lactonization reaction occurs with participation of the peptide bond in tyrosine and tryptophan peptides, with release of the amine component in varying yields. We find that with the model substance, indolepropionic acid, the oxidation by NBS in the presence of mercuric acetate proceeds smoothly as evidenced by the changes in ultraviolet absorption; the final spectrum with 1.5 moles of NBS corresponds to that of an oxindole (Fig. 5). However, tyrosine, which normally undergoes facile oxidation to the dibromo-*spiro*-dienone lactone, is not attacked in the presence of excess mercuric acetate even by an excess of NBS as large as 6 moles. Figure 6 depicts the spectral changes during normal oxidation and the inhibitory effect of excess mercuric acetate. The explanation for this effect resides in the ability of phenols to mercurate with great ease, leading first to the attachment of mercury to the oxygen and subsequently to mercuration of the *ortho* and *para* positions (Sidgwick, 1950). With tyrosine peptides, the position *para* to the phenolic group being already substituted, probably only *o*-mercuration occurs. This *o*-mercuration probably prevents *o*-dibromination, and formation of compound V, which is a precursor of the lactone IV. Consequently, when a mixture of *N*-acetyl-L-tryptophanamide and phloretylglycine is oxidized with a slight excess of NBS (6 moles) in the presence of excess mercuric acetate, the changes in absorption corresponds to formation of *spiro*-oxindole only but not dienone lactone, and ammonia, but not glycine is liberated (Fig. 7).

In a separate experiment the treatment of phloretylglycine with NBS (up to 8 moles; 3 moles required), in the presence of mercuric acetate, caused no significant spectral changes and no glycine was found on



paper chromatography. A direct ninhydrin reaction for free amino groups showed the presence of less than 3–4% of 1 mole, whereas in the absence of mercuric acetate the amine component was released in yields of 82–96% of theory (Witkop, 1961). In the oxidative cleavage of C-tryptophyl bonds in proteins a considerable excess of reagent has often been necessary to obtain optimum cleavage yields (Witkop, 1961; Ramachandran, 1962). Even slight participation of C-tyrosyl bonds in oxidative cleavage would vitiate the selectiveness of the cleavage reaction. In an oxidation medium of 8.0 M urea nonspecific cleavage of tyrosyl bonds is reduced or suppressed (Funatsu *et al.*, 1964). In 8.0 M urea NBS is converted to *N*-bromourea, an oxidant less active than NBS. Its reactivity versus tyrosyl groups is too slow, while indole groups are oxidized at a fast rate. Likewise, the presence of excess mercuric acetate

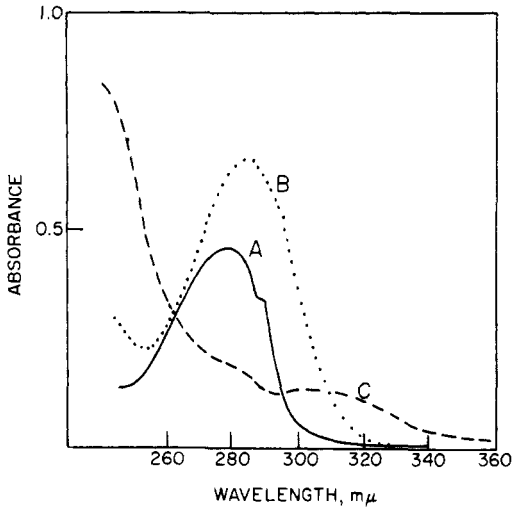


FIG. 7.—Action of excess NBS on an equimolar mixture of phloretylglycine and acetyl tryptophanamide. (A) Phloretylglycine and acetyl tryptophanamide (0.02 μmole each) in 3 ml of 50% AcOH; (B) same as in A but with 50 μmoles $Hg(OAc)_2$ present; (C) same as in B but with 1 μmole NBS (0.1 ml) added.

during NBS oxidation of proteins with C-tryptophyl and C-tyrosyl bonds would assure selective cleavage of the C-tryptophyl bond.

Of other indole compounds studied 2,3-diphenylindole showed almost no changes in absorption with mercuric acetate. Strong electron-withdrawing groups on both the 2 and 3 positions of the indole nucleus thus prevent the interaction with mercuric acetate, characterized by the typical spectral changes seen with compounds of groups I and II. Similarly, oxindole yields none of the typical difference peaks although there is slightly increased absorption between 265 and 320 mμ. Indoline yields only very weak peak of absorption (ϵ 0.3 $\times$ 10^3) at 292–299 and 350–353 mμ in contrast to the very high intensities (about 10 $\times$ 10^3) at 284–288 and 293–305 mμ found for substances of groups I and II. Thus the presence of the 2,3 double bond of the indole nucleus is essential for the typical bathochromic shifts. Benzofuran, in which the nitrogen of the indole nucleus is replaced by an oxygen atom, shows only negligible increase in absorption between 280 and 310 mμ. Hence the described spectral shifts require the presence of the indole NH grouping in addition to the 2,3 double bond. The role of the NH function is evident also from experiments with indene, in which CH_2 replaces the NH function of indole. No difference spectrum exists above 290–300 mμ, though at lower wavelengths there is somewhat increased absorption. A 1-methyl substitutent on the indole nitrogen (*N*-methylindolepropionic acid) causes a shift of λ_{max} from 285 mμ (indolepropionic acid) to 292–293 mμ, and loss of the low-intensity peak at 285–286 mμ in the difference spectrum.

2-Methyl- and 2,3-dimethylindole in the presence of mercuric acetate show λ_{max} 306–311 mμ, while 3-methylindole shows λ_{max} 296–300 mμ a difference spectrum more similar to compounds of groups I and II. 3-Phenylindole gives spectral shifts similar to, though much smaller than those of groups I and II. 2-Phenylindole shifts to longer wavelength and has a difference peak at 343 mμ, but considerable extinction is lost in the peak region (304–306 mμ) of the parent compound. This behavior is also shown by 2-biphenylindole, where a loss of extinction of 24.2 $\times$ 10^3 at the peak of absorption (331–333 mμ) is found as a result of mercuration, and a difference peak is found at 373 mμ. One consequence of mercuration of the above type of compounds is a considerable loss of absorption of parent chromophor. *N*-Methyl-2-acetylindole shows the long-wavelength shift on reaction with mercuric acetate, but again the shift is accompanied by a substantial loss in extinction at the λ_{max} of the parent compound.

Included in this study were some indoles containing fused rings at the 2,3 position such as carbazole and tetrahydrocarbazoles derivatives. With carbazole there is a red shift of 3–4 mμ in the wavelengths of absorption, and the maximum extinction difference is at 297 mμ. But the similarity between carbazole and the indoles is small. Acetoxymercuration of carbazoles at positions 3 and 6 is known (Elderfield, 1952). Tetrahydrocarbazole, on the other hand, shows changes in spectrum and a difference spectrum similar to that of many indole derivatives. With 1-keto-tetrahydrocarbazole there is the shift to longer wavelength of the spectrum, but the difference spectrum shows no similarities to that of indoles of groups I and II. With 7-acetyltetrahydrocarbazole there is no change in wavelength of absorption, but extinction is lower for the peaks at 353–355 and 313 mμ. With *N*-methyl-7-acetyltetrahydrocarbazole there were no noticeable spectral changes. A benzoyl substituent on the tetrahydrocarbazole nitrogen also leads to the loss of the difference spectrum above 285–290 mμ, although a slight increased absorption below this wavelength is noticed. 2,3-Cyclooctenoindole (Witkop *et al.*, 1951) shows a spectral change similar to 2,3-dimethylindole. However, 2-ketocyclooctenoindole, though showing the long-wavelength shift, has a difference spectrum different from the indoles.

In this study attention has been drawn to the changes in absorption characteristics which ensue when the indole chromophor interacts with excess mercuric acetate in aqueous acetic acid solution. The variety of different absorbing species in solution (Fig. 2) poses a challenging preparative problem. Not only are there differences in absorption between 1,2-diacetoxymercury (curve B) and 1,2,3-triacetoxymercuryindole (curve C), but also between these derivatives and indole in the presence of excess mercuric acetate (curve E). This additional interaction with excess mercuric acetate may involve π-complexing or the formation of mixed complexes.

The observations recorded are now being examined for preparative and analytical applications, e.g., for the preparation of selectively tritiated indoles or for the exploration of the reactivity of tryptophan units in protein molecules.

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Cleavage of Olefinic Peptides under the Conditions of Oxymercuration

L. K. RAMACHANDRAN,* H. IWASAKI,† AND B. WITKOP

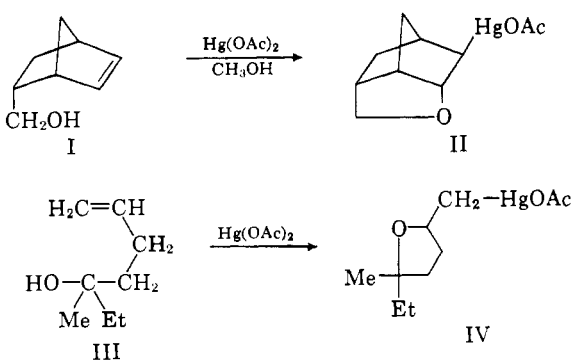
From the National Institute of Arthritis and Metabolic Diseases, National Institutes of Health, Public Health Service, U. S. Department of Health, Education, and Welfare, Bethesda, Md.

Received June 24, 1964

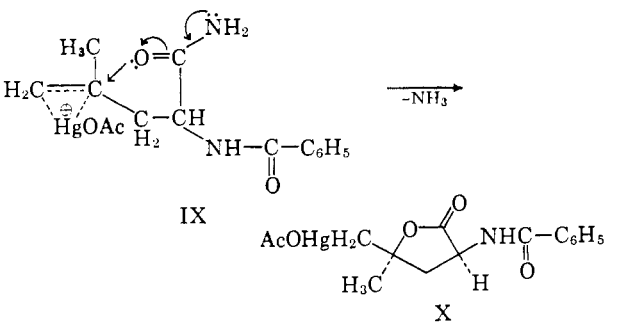
Under the conditions of oxymercuration, i.e., mercuric acetate in ethanolic buffer solution at 75° for 15–100 minutes, the model peptides DL-2-benzamido-4-methyl-4-pentenoic acid amide (V), DL-2-*p*-toluenesulfonamido-4-methyl-4-pentenoylglycine (VI), and DL-2-benzamido-4-pentenoic acid amide (VII) liberated ammonia or glycine in yields up to 30%. DL-3,4-Dehydroprolinamide (VIII) showed cleavage only to the extent of 2–7%. *O*-Methyldihydrorufomycin A (XI), a cyclic heptapeptide containing the unsaturated amino acid Δ^4-norleucine, liberated the adjacent tryptophan unit only to a minor extent, and nonspecific cleavage catalyzed by mercuric ions up to 46% of one peptide bond was observed instead. It is concluded that the extent of peptide cleavage under conditions of oxymercuration depends on the environment and substitution of the double bond.

Nonhydrolytic, nonenzymatic methods for cleavage of peptides involve participation of amide carbonyls in internal nucleophilic displacement reactions (Witkop, 1961). Among the reagents and methods used there have been *N*-bromosuccinimide, *N*-bromoacetamide, *N*-bromocarbamide (Funatsu *et al.*, 1964), anodic oxidation (Iwasaki *et al.*, 1963) and solvolytic cleavage (Iwasaki and Witkop, 1964). We have now investigated the use of mercuric acetate for cleavage of peptide bonds.

Participation of neighboring hydroxyl groups in the oxymercuration of norbornene (I → II) (Henbest and Nicholls, 1959) or of acyclic olefins (III→IV) (Sand and Singer, 1903) suggests an extension of this principle (Traylor and Baker, 1963) to the cleavage of peptides.



Cleavage of Amides and Peptides of 2-Acylamino-(4-methyl)-4-pentenoic Acids.—The model amides and peptides used in this study had been previously synthesized (Izumiya *et al.*, 1962). They were DL-2-benzamido-4-methyl-4-pentenoic acid amide (V), DL-2-*p*-toluenesulfonamido-4-methyl-4-pentenoylglycine (VI), DL-2-benzamido-4-pentenoic acid amide (VII), and DL-3,4-dehydroprolinamide (VIII). Participation of the amide group would best be formulated through a mercurinium (Lucas *et al.*, 1939) intermediate (IX), going to the iminolactone and lactone (X) (Izumiya and Witkop, 1963), analogous to the assumption of reactive



bromonium intermediates for the cleavage of peptide bonds by *N*-bromosuccinimide. The yields achieved in this cleavage depended on the conditions and on the type of peptide. They did not exceed 30%.

Under the conditions of oxymercuration the γ,δ-unsaturation in tetrahydrophenylalanine peptides (Wilchek and Patchornik, 1962) participates and undergoes cleavage in excess of 50% (A. Patchornik, personal communication). Table I summarizes the observations made so far on the influence of double bond substituents on the cleavage yield and the conditions of oxymercuration.

Unspecific Cleavage of Rufomycin A by Mercuric Ions.—Naturally occurring olefinic amino acids are comparatively rare. The antibiotic cyclopeptide rufomycin A (Ueyanagi *et al.*, 1963), whose *O*-methyldihydro derivative is pictured in structure XI (Iwasaki and Witkop, 1964), contains 2-amino-4-hexenoic acid whose double bond reacts easily with *N*-bromosuccinimide leading to oxidative cleavage and up to 48% liberation of the

* Visiting Scientist of the U. S. Public Health Service, 1963–1964.

† Associate in the Visiting Program, 1963–1964.