was eluted with 150 ml. of benzene and identified by its infrared absorption in carbon disulfide solution, which was void of any carbonyl absorption and which showed strong terminal methylene absorption at 887 cm.$^{-1}$.

A mixture composed of 63 mg. of crude ursa-12:20(30)-dien-3β-ol, 1.5 ml. of absolute dioxane, 5.0 ml. of ethylenediamine and 134 mg. of lithium was stored 13 hours at room temperature and was then heated to 58° for 3 hours. The mixture was diluted with water, and the product was extracted into ether and worked up in the usual way. The semi-solid product was treated with a mixture of 1.0 ml. of pyridine and 1.0 ml. of acetic anhydride for 45 min. on a steam-bath and then allowed to stand for 48 hours at room temperature. Methanol was added to the reaction mixture to decompose the excess acetic anhydride, and the mixture was concentrated to dryness. The residue was dissolved with a few drops of benzene and placed on a 2-g. column of alumina. Elution with 30 ml. of benzene afforded 54.5 mg. of crude α-amyrin acetate. One recrystallization from methylene chloride–methanol gave 30 mg. of colorless crystals, m.p. 209.5–212.5° (hot-stage). The melting point was raised to 217–221° with two more recrystallizations from the same solvents. A sublimation followed by a recrystallization from *n*-heptane raised the melting point to 223.5–225° (hot-stage). A mixed melting point with an authentic specimen of α-amyrin acetate (m.p. 225–226°) was 223.5–225° (hot-stage). The infrared absorption spectra of the authentic specimen and the synthetic product in carbon disulfide were identical. X-Ray powder diffraction patterns were prepared for both the synthetic product and the authentic specimen. The *d*-values are given in ångström units with the chromium radiation weighted wave length of 2.28962 Å. used as the basis of spacing calculations. Rotatory dispersion curves were run on both the synthetic and authentic specimens of α-amyrin acetate. The curves were identical.

Authentic specimen	Synthetic product	Authentic specimen	Synthetic product
15.41	15.41	4.35	4.34
9.18	9.29	3.80	3.82
7.74	7.74	3.47	3.47
6.45	6.50	3.31	3.32
5.76	5.76	3.07	3.07
5.40	5.42	2.98	2.99
4.65	4.60		

A second preparation of α-amyrin acetate from 55 mg. of 20-norurs-12-en-20-on-3β-yl benzoate (m.p. 250–252° on a hot-stage) afforded 43 mg. of crude ursa-12:20(30)-dien-3β-ol and 45 mg. of crude α-amyrin acetate. The product after one recrystallization from methylene chloride–methanol melted from 202–212° (hot-stage). A sublimation followed by two recrystallizations from *n*-heptane gave α-amyrin acetate, m.p. 223–225°.

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Reaction of Anhydrous Formic Acid with Proteins[1]

BY KOZO NARITA[2]

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The effect of anhydrous formic acid on proteins and on serine containing peptides at 25° has been studied. In contrast to the action of concentrated mineral acids and in contrast to the interpretation of Josefsson and Edman, formic acid transforms the aliphatic hydroxyl groups into formate ester groups but does not cause any significant N → O-peptidyl shift.

Introduction

Elliott[3,4] clearly demonstrated that under the influence of concentrated sulfuric acid the peptide bonds involving the nitrogen of hydroxyamino acid residues in silk fibroin and lysozyme were transformed to O-ester linkages (N→O-peptidyl shift), and the amino groups of serine and threonine became free. Later McConnell and co-workers[5,6] applied this method to gluten and gliadin. Lucas and co-workers[7] showed that the peptidyl shift of serine residues in silk fibroin took place to almost the same extent in anhydrous phosphoric acid as in concentrated sulfuric acid. Recently Josefsson and Edman[8,9] reported reversible inactivation of lysozyme and ribonuclease due to N→O-peptidyl shift which resulted from incubating the proteins with anhydrous formic acid.

Since of the three acid reagents anhydrous formic acid seemed to be the mildest, the action of this acid on several proteins was tested. Contrary to expectation almost no increase of α-amino groups could be observed by the ninhydrin reaction after formic acid treatment of proteins. Furthermore, it was found that the action of formic acid on N-acetylseryltyrosine which was isolated from tobacco mosaic virus protein[10,11] did not produce an amino group in this peptide as would be expected if the acetyl shift had taken place and O-acetylseryltyrosine had been formed.

Consequently, in order to elucidate the reaction between formic acid and hydroxyamino acid residues in protein, the action of formic acid on N-acetylserine and on several proteins has been studied. The results which are described in the present paper show that formic acid does not act primarily as a catalyst for the N→O-acyl shift but as a formylating agent of the hydroxyl group of serine (and threonine) residues in peptide linkage.

Experimental

Formic Acid.—98–100% formic acid (Merck) was dried over boric anhydride for a week and distilled over anhydrous copper sulfate *in vacuo*. Formic acid-C[14] was pre-

(1) Aided by United States Public Health Service Training Grant CRTY-5028.

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(3) D. F. Elliott, *Biochem. J.*, **50**, 542 (1951).

(4) D. F. Elliott, in "Ciba Foundation Symposium on the Chemical Structure of Proteins" (Eds., G. E. W. Wolstenholme and M. P. Cameron), J. and A. Churchill, Ltd., London, 1953, p. 129.

(5) L. Wiseblatt, L. Wilson and W. B. McConnell, *Can. J. Chem.*, **33**, 1295 (1955).

(6) L. K. Ramachandran and W. B. McConnell, *ibid.*, **33**, 1638 (1955).

(7) F. Lucas, J. T. B. Shaw and S. G. Smith, *Biochem. J.*, **66**, 468 (1957).

(8) L. Josefsson and P. Edman, *Biochim. et Biophys. Acta*, **25**, 614 (1957).

(9) L. Josefsson, *Acta Chem. Scand.* (*Proc. Danish Biochem. Soc.*), **11**, 1080 (1957); *Arkiv Kemi*, **12**, 183 (1958).

(10) K. Narita, *Biochim. et Biophys. Acta*, **28**, 184 (1958).

(11) K. Narita, *ibid.*, **30**, 352 (1958).

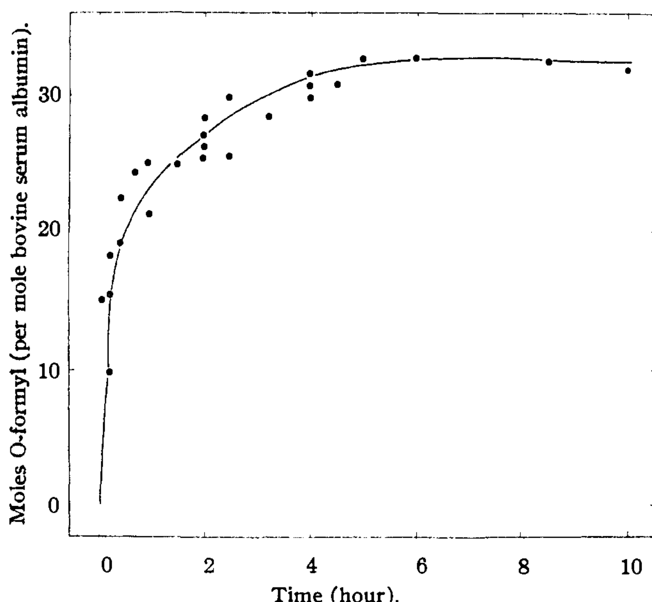

Fig. 1.—Formyl hydroxamate formation by the reaction of formic acid treated bovine serum albumin with the hydroxylamine reagent. The lyophilized anhydrous formic acid treated bovine serum albumin (about 15 mg.) was dissolved in 1 ml. of water (by adding one drop of acetic acid) and shaken vigorously with the hydroxylamine reagent at room temperature for various periods of time. For the color development with ferric chloride see text.

pared by dissolving 50 μC. of sodium formate (specific activity, 2.0 mC./mmole) in 5 ml. of anhydrous formic acid and used without further treatment.

Proteins.—Bovine serum albumin (Armour and Company, Chicago 9, Illinois, lot 128-176), lysozyme (Worthington Biochemical Sales Co., Freehold, New Jersey, lot LY 551), β-lactoglobulin (Pentex Incorporated, Kankakee, Illinois, lot B 4803), ovalbumin, sericine and polyglutamic acid (all supplied by Dr. H. Fraenkel-Conrat) were used in the present experiments without further purification.

O-Acetyl-DL-serine synthesized by the method of Sheehan and co-workers,[12] m.p. 151-152° (with dec.) (reported[13] 143-144°). *Anal.* Calcd. for $C_5H_9O_4N$: C, 40.9; H, 6.17; N, 9.52. Found: C, 41.0; H, 6.12; N, 9.31.

N-Acetyl-DL-serine prepared as reported previously.[11] Alternatively N-acetyl-DL-serine was prepared from O-acetyl-DL-serine by O → N-acetyl shift. One g. of O-acetylserine was dissolved in 3 ml. of 10% NH_4OH and the solution was held overnight at room temperature. Then it was dried *in vacuo* at room temperature in a desiccator over concentrated sulfuric acid. The partially crystallized sticky material solidified upon scratching of the wall of the vessel. This ammonium salt was dissolved in 2 ml. of water, applied to the acid form of a Dowex 50 column (50 ~ 100 mesh, 1 × 5 cm.), and washed with water until the effluent became neutral. The effluent was then lyophilized and the material recrystallized from ethanol-ether; yield 0.95 g., 95%; m.p. 130-131°. No melting point depression occurred upon mixing this with the previous preparation[11] (m.p. 129-130°). *Anal.* Calcd. for $C_5H_9O_4N$: C, 40.9; H, 6.17; N, 9.52. Found: C, 41.0; H, 6.08; N, 9.34.

N-Acetyl-O-formyl-DL-serine.—0.3 g. of N-acetyl-DL-serine was dissolved in 1 ml. of anhydrous formic acid and the solution was kept overnight at room temperature. On evaporation of the excess formic acid in a desiccator over sodium hydroxide pellet, a colorless residue was obtained and was recrystallized from ethanol; yield 0.38 g., 90%; m.p. 172°. *Anal.* Calcd. for $C_6H_9O_5N$: C, 41.1; H, 5.18; N, 8.05. Found: C, 41.0; H, 5.09; N, 8.11.

Reaction of Formic Acid with Protein.—About 50 mg. of the protein was suspended in 1 ml. of anhydrous formic acid and the solution (or suspension) was kept in a closed vessel at room temperature for 24 hr. The material was then transferred into a cellophane bag and dialyzed against distilled water at 5°. Ovalbumin and sericine gelled during the reaction and polyglutamic acid did not go into solution. During the dialysis ovalbumin and polyglutamic acid stayed without change and sericine precipitated. Bovine serum albumin and β-lactoglobulin precipitated in the dialysis bag after 24 hr., but these became soluble by the addition of one drop of acetic acid. Consequently, one drop of acetic acid was added into the dialysis bag every 24 hr. and dialysis was continued. The *p*H value of the final dialysate was about 5 in every case. The dialysate was lyophilized and used by dissolving or suspending in appropriate amount of water for the various experiments. Some of the preparations of formic acid treated bovine serum albumin were obtained directly by lyophilization without dialysis.

Measurement of Base Consumption.—Base consumptions at constant *p*H, due to the O → N acetyl shift of O-acetylserine or to hydrolysis of O-formyl groups of N-acetyl-O-formylserine and formic acid treated proteins, were estimated in a *p*H-stat[14] (Difunctional Recording Titrator, International Instrument Co., Canyon, California). 0.1-0.2 μmole of samples were placed in the *p*H-stat vessel (content, 10 ml.) and then adjusted to, and maintained at, the desired *p*H by means of 0.1 *N* sodium hydroxide. For the ninhydrin analysis, 0.1-ml. aliquots were pipetted from the *p*H-stat vessel and analyzed by the method of Moore and Stein.[15]

Estimation of O-Formyl Group by Hydroxylamine Method.[16]—The amount of O-formyl group in the formic acid treated protein was estimated by the hydroxylamine method. N-Acetyl-O-formyl-DL-serine was used for the preparation of a standard curve and the reaction conditions were examined. 1.5 ml. of the freshly prepared neutralized hydroxylamine solution (3 volumes of 40% hydroxylamine hydrochloride was mixed with 2 volumes of 3.5 *N* sodium hydroxide, *p*H 6.4) and 1.0 ml. of 0.1 *N* acetate buffer (*p*H 5.4) were added to 1.0 ml. of the sample solution. The mixture was allowed to stand for various periods of time at room temperature. 1.0 ml. of 12% trichloroacetic acid and 0.5 ml. of 3 *N* hydrochloric acid were then added to the reaction mixture, and the *p*H was adjusted to 1.0. The optical density at 540 mμ was measured after the addition of 1.0 ml. of 5% ferric chloride in 0.1 *N* hydrochloric acid. Maximal color was obtained after 10 minutes reaction with the hydroxylamine reagent. No absorption at 540 mμ was observed for N-acetyl-DL-serine under the above conditions. Surprisingly, only a faint color was obtained with formic acid treated bovine serum albumin under the above conditions. It was necessary to keep the reaction mixture for at least 5 hours before addition of the acids and ferric chloride, in order to obtain the maximum color for the formic acid treated protein as is shown in Fig. 1. Protein samples (10 ~ 20 mg.), in solution or suspension, were therefore shaken vigorously with the hydroxylamine reagent for 5 hours at room temperature, and the precipitate was filtered off after addition of the acids and ferric chloride solution. The color intensity of the filtrate was measured within 30 minutes. The untreated protein gave no absorption at 540 mμ under the above conditions.

Measurement of Radioactivity.—About 1 ml. of the formic acid-C^{14} treated protein solution or suspension which contained about 10 mg. of modified protein was pipetted on the cup for radioactivity measurement, lyophilized and weighed. Radioactivities in the radioactive formic acid treated proteins ranged from 500 to 1,000 counts per minute (2,000 ~ 10,000 counts per minute per μmole), with the exception of the case of polyglutamic acid. Specific activity of radioactive formic acid used was 120 counts per minute per μmole.

Results and Discussion

The ninhydrin color values of bovine serum albumin, β-lactoglobulin and tobacco mosaic virus

(12) J. C. Sheehan, M. Goodman and G. P. Hess, THIS JOURNAL, **78**, 1367 (1956).

(13) W. Sakami and G. Toennies, *J. Biol. Chem.*, **144**, 203 (1942).

(14) J. B. Neilands and M. D. Cannon, *Anal. Chem.*, **27**, 29 (1955); L. Leonis, K. Linderstrøm-Lang and M. Ottesen, in "Methods of Biochemical Analysis," Vol. IV, Interscience Publishers, New York, N. Y., 1957, p. 171.

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(16) F. Lipman and L. C. Tuttle, *ibid.*, **159**, 21 (1945).

TABLE I

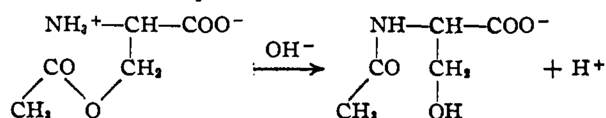
R_f VALUES OF THE PRODUCTS OF THE REACTION OF N-ACETYLSERINE AND SERINE WITH ANHYDROUS FORMIC, ACETIC, CONCENTRATED HYDROCHLORIC AND CONCENTRATED SULFURIC ACIDS

Material		R_f value (1-butanol-acetic acid-water, 4:1:1, vol.)						
Formic acid treated	N-Acetylserine							*0.60*
	Serine				0.13			
Acetic acid treated	N-Acetylserine					0.26 (trace)	*0.56*	
	Serine				.13			
Concd. HCl treated	N-Acetylserine				.12	.25	*.54*	
	Serine				.13			
Concd. H_2SO_4 treated	N-Acetylserine	0.044	0.087	0.11 (trace)	.13	.24~0.42[a]	*.56*	
	Serine	.042	.085	.10	.13			
Standard	N-Acetylserine						*.56*	
	O-Acetylserine					.26		
	Serine				.13			

Italic figures indicate ninhydrin negative and brom thymol blue revealed spot. [a] Streaked spot with strong sulfuric acid spot inside.

were not changed, within experimental error, by incubation with anhydrous formic acid for periods up to 20 hr. When N-acetyl-DL-serine was incubated with formic acid, no ninhydrin color was observed after 10 hr. Thus, no N→O-acyl shift seemed to have occurred under these conditions. N-Acetylserine and serine were then treated for 24 hr. with anhydrous formic, acetic, concentrated hydrochloric and concentrated sulfuric acids. The reaction products were isolated by lyophilization with the exception of the sulfuric acid treated one, which was isolated by precipitation with anhydrous ether in a Dry Ice bath and was washed several times with ether. The products were then analyzed by paper chromatography in 1-butanol-acetic acid-water (4:1:1, vol.). The R_f values of the products revealed either by ninhydrin or brom thymol blue[17] are listed in Table I. These results indicated that N-acetylserine was converted to O-acetylserine by the action of concentrated sulfuric, hydrochloric and acetic acids. The amount of O-acetylserine produced was very slight in the case of acetic acid. A large amount of serine was observed in the case of hydrochloric acid and, presumably, was due to hydrolysis of the O-acetylserine derived from the N-acetyl derivative by N→O-acetyl shift as was suggested by Desnuelle, *et al.*[18] Besides large amounts of O-acetylserine, three unknown spots were observed in the sulfuric acid treated N-acetylserine. These might be derived from small amounts of serine produced by hydrolysis because the same spots were detected in the reaction mixture from serine. In contrast, not even trace amounts of O-acetylserine could be found after formic acid treatment, and only one new spot appeared which was ninhydrin negative and revealed by brom thymol blue. Therefore, the amino group of this product must remain blocked. The isolation and identification of this product was then attempted, and it was crystallized from the reaction mixture as was described already in the experimental section. Elementary analysis suggested that it corresponded to acetylformylserine. Semiquantitative paper electrophoretical analysis[19] of the concentrated ammonia hydrolysate (10 hours, at 100°) of the product showed that it contained approximately each one mole of formic and acetic acids. Hydroxylamine treatment showed that the compound contained a rather labile acyl group. Consequently it must be N-acetyl-O-formylserine. On the other hand no O-formylation was produced from the reaction of serine with formic acid.

In the next stage, the reverse reaction with O-acetylserine, the O→N-acetyl shift, was studied. During O→N-acetyl shift of O-acetylserine, the free amino group will disappear and at the same time a titrable proton will be released.



Upon incubating O-acetylserine in alkaline solution, the ninhydrin color of reaction mixture rapidly decreased (Fig. 2). However, the rate of decrease of

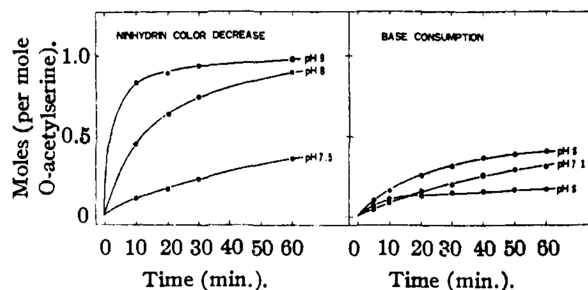


Fig. 2.—The rate of base consumption and ninhydrin color decrease of O-acetyl-DL-serine during O → N-acetyl shift at different pH's.

the ninhydrin color value did not agree with that of the base consumption. The ninhydrin color decrease during the reaction was the faster the higher the pH value of the solution, but the base consumption reached a maximum rate at pH 8. This phenomenon can be explained by the compensation of the increase in reaction rate at higher

(17) F. Brown, *Biochem. J.*, **47**, 598 (1950).

(18) P. Desnuelle, M. Rovery and G. Bonjour, *Biochim. et Biophys. Acta*, **2**, 134 (1948).

(19) D. Gross, *Nature*, **181**, 264 (1958). In contrast to Gross who used a high voltage apparatus, low voltage (450 volts, 60 minutes) has been employed in the present studies.

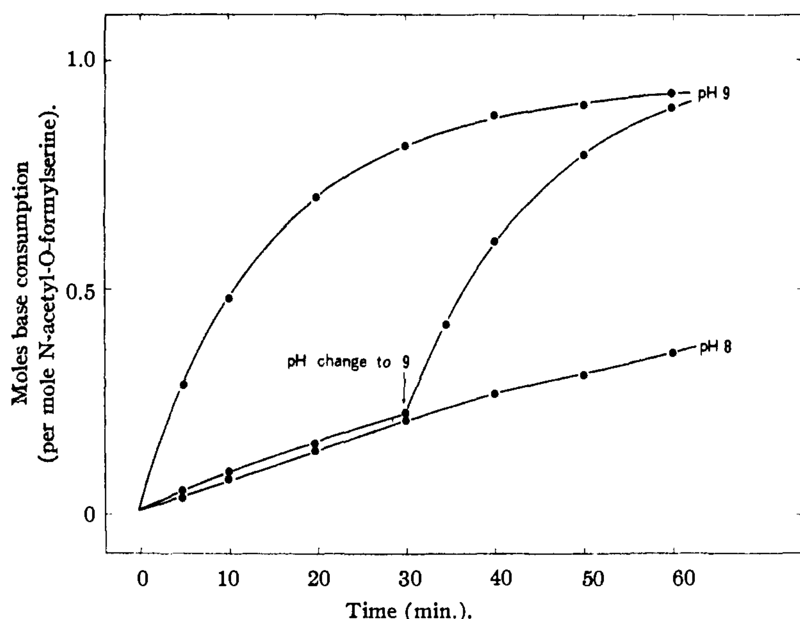


Fig. 3.—The rate of the hydrolysis of O-formyl group of N-acetyl-O-formyl-DL-serine at *p*H 8.0 and 9.0. In one experiment the *p*H was changed from 8.0 to 9.0 during the reaction. The base needed for this *p*H change was subtracted.

*p*H's by the supression of the dissociation of the amino group under these conditions. The evidence that the product of O→N-acyl shift was N-acetylserine was obtained by its identification on a paper chromatogram and by its isolation, in crystalline form, as described in the Experimental section.

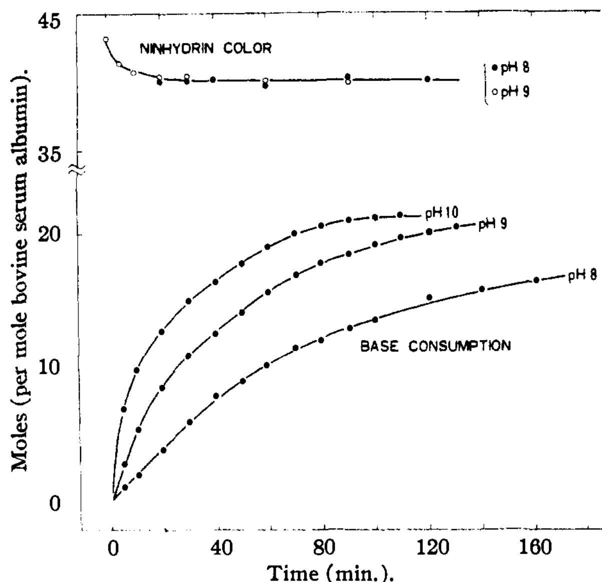


Fig. 4.—The rate of base consumption and ninhydrin color change during incubation of the formic acid treated bovine serum albumin at several *p*H's.

In contrast to O-acetylserine, the base consumption of N-acetyl-O-formylserine became more rapid in higher *p*H medium (Fig. 3). In this case, the base was required for neutralization of the formic acid which was released hydrolytically. N-Acetylserine was detected in the reaction mixture by paper chromatography.

If N→O-peptidyl shift occurred in formic acid treated proteins, the base consumption during the reaction in the *p*H-stat should decrease at higher *p*H. Actually, the base consumption of formic acid treated bovine serum albumin became more rapid and greater in more alkaline solution and the ninhydrin color was constant after the decrease of 2~3 leucine equivalents at the beginning of the reaction (Fig. 4). The total base consumption of this modified protein was approximately 20 moles per mole protein.[20] Therefore, if N→O-peptidyl shift had occurred during the formic acid treatment, it corresponded to only 10% of the base consumption. It is probable that most of the alkali uptake was due to hydrolysis of O-formyl group of serine (and threonine) residues in the protein. The relation between the base consumption and the ninhydrin color decrease of the formic acid treated bovine serum albumin during the reaction was compared with that of the concentrated sulfuric acid treated protein (24 hours, at room temperature) which was assumed to be largely in the O-peptidyl form. Figure 5 shows the large

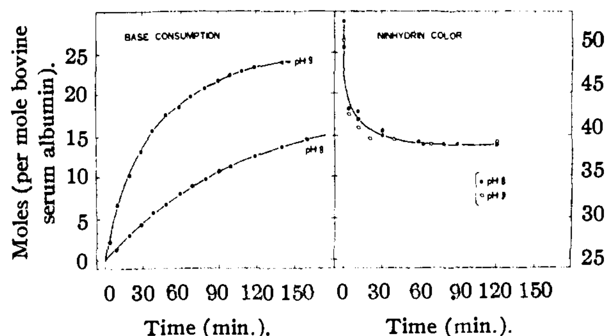


Fig. 5.—The rate of base consumption and ninhydrin color change during incubation of the sulfuric acid treated bovine serum albumin at *p*H 8.0 and 9.0.

decrease of the ninhydrin color (approximately 13~15 leucine equivalents per mole protein) during the incubation at constant *p*H, but the base consumption did not decrease in higher *p*H media, contrary to the author's expectation. This fact may be explained by the following consideration: the sulfuric acid treated protein contains large amounts of sulfate esters of hydroxyl groups of serine, threonine[22] (assumed to be an intermediate in N→O-acyl shift reaction) and tyrosine residues. The rate of hydrolysis of these sulfate esters may be more rapid and greater in more alkaline solution. The above results suggested that N→O-peptidyl

(20) It was reported that bovine serum albumin contained 28 serine and 34 threonine residues per molecule.[21] Assumed molecular weight used for calculation was 70,000.

(21) W. H. Stein and S. Moore, *J. Biol. Chem.*, **178**, 79 (1949).

(22) H. C. Reitz, R. E. Ferrel, H. Fraenkel-Conrat and H. S. Olcott, THIS JOURNAL, **68**, 1024 (1946).

TABLE II

THE NUMBER OF O-FORMYL GROUP AND BASE CONSUMPTION OF FORMIC ACID TREATED BOVINE SERUM ALBUMIN AFTER VARIOUS DIALYSIS PERIODS

Sample	O-Formyl group,[a] mole/mole protein	Base consumption at pH 9.5,[b] mole/mole protein	O-Formyl group[a] after alkali titration and dialysis, mole/mole protein
Lyophilized without dialysis	32.1	28.3	3.4
3 days dialyzed	17.0	13.9	4.3
6 days dialyzed	15.4		
8 days dialyzed	14.2		
15 days dialyzed	11.1	9.1	*ca.* 2

[a] Estimated by hydroxylamine method. [b] Corrected for blank titration value of bovine serum albumin without formic acid treatment.

When radioactive formic acid-C^{14} was used, prolonged dialysis was employed to remove unbound formic acid completely. After 5 days dialysis almost no radioactivity was found in the outside of the dialysis bag. Further dialysis brought slight loss of radioactivity from the protein sample. The radioactivity of the protein was measured after lyophilization of aliquots of the dialysate in the bag at various times. To eliminate the possibility of ionic binding of formate, the 10 days' dialysate of the formic acid treated bovine serum albumin was half saturated with ammonium sulfate, the precipitate was dissolved in water and dialyzed for 6 days. An equal volume of non-radioactive formic acid was added to this dialysate and dialyzed for 6 days further. Even after these treatments the radioactive carbon bound was not greatly decreased.

TABLE III

EFFECT OF ALKALI ON FORMIC ACID-C^{14} TREATED PROTEINS[a]

Protein	Dialysis time (days)	Base consumed (pH 9.5)	Formate carbon content (by C^{14}) Initial[b]	Final[b]	O-Formyl cotent (by hydroxylamine) Initial[b]	Final[b]
Bovine serum albumin	5		33.2			
	10	13.5	28.5	10.7	15.1	3.3
Ser 28[21]	$(NH_4)_2SO_4$ ppt.[c]		23.1		13.4	
Thr 34[21]	Addition of formic acid[d]	10.6	18.5	9.0	11.0	4.0
Lysozyme	10	3.4	23.7	8.8	6.5	0.9
Ser 10[24]	14	*ca.* 2	19.0	10.3		
Thr 7[23]	18		17.0		4.3	
	22		14.8			
β-Lactoglobulin	10	10.5	28.0	17.9		4.2
Ser 16[21]	14		23.5			
Thr 17[21]	18		20.0		4.1	
	22		14.6		3.5	
Ovalbumin	10	45.4	59.0	11.9		6.0
Ser 37[23]	14		50.0		22.5	
Thr 16[24]	18		40.1		22.1	
	22		32.8			
Sericine	10	159	278	159		91
Ser 322[25]	14		307		160	
Thr 75[24]	18		244			
	22		222			
Polyglutamic acid	10	5.1	5.5	0.8		0.7
	14		4.6		4.2	
Ser ..	18		4.8			
Thr ..	22		2.1			

[a] Figures are expressed as mole/mole protein and assumed molecular weights of bovine serum albumin, lysozyme, β-lactoglobulin, ovalbumin, sericine and polyglutamic acid used for calculation are 70,000, 15,000, 42,000, 45,000, 100,000 and 100,000, respectively. [b] Initial and final means that the values of the material before and after alkali titration and prolonged dialysis. [c] The 10 days' dialysate was half saturated with $(NH_4)_2SO_4$, the precipitate obtained was dissolved in water and was dialyzed for 6 days. [d] Equal volume of non-radioactive formic acid was added to the above dialysate and the solution was dialyzed for 6 days.

shift with concentrated sulfuric acid was not complete in this experiment under the conditions used.

The amount of base consumption of the formic acid treated protein was variable from preparation to preparation. This fact was attributed to the hydrolysis of some formyl groups in the protein during dialysis to remove formic acid. The same phenomenon was observed in the O-formyl estimation by the hydroxylamine method; namely, lower values were obtained on longer dialysis. In 3 days of dialysis one third of the O-formyl groups were apparently lost (Table II).

(23) J. C. Lewis, N. S. Snell, D. J. Hirschmann and H. Fraenkel-Conrat, *J. Biol. Chem.*, **186**, 23 (1950).

(24) B. H. Nicolet and L. S. Saidel, *ibid.*, **139**, 477 (1941).

Therefore radioactive formic acid must be bound to the protein by covalent linkages, such as O-formyl linkage. However, the amounts of radioactivity, O-formyl estimated by the hydroxylamine method and base consumption did not agree with each other in every protein examined. These discrepancies may be attributed to the difficulty of complete removal of traces of unbound radioactive formic acid, to the differences of optimal conditions of O-formyl estimation for different proteins and to the fact that some O-formyl groups in the modified proteins were hydrolyzed before the adjustment of the constant pH in the pH-stat vessel. After alkali titration and prolonged dialysis, appreciable amounts of radioactivity still remained

in the proteins. The possibility that contaminating traces of formaldehyde in the radioactive formic acid were bound to the protein was ruled out by the finding of negative results on formaldehyde estimation.[25] Since all radioactivity in the 25% sulfuric acid hydrolysate of the radioactive protein sample were recovered in the distillate in alkaline solution, the protein bound radioactive material must be formic acid. The amounts of radioactive carbon bound by various proteins seemed to be in parallel with their total hydroxyamino acid content (serine + threonine) as summarized in Table III. Therefore the main reaction of formic acid with proteins must be O-formylation of their hydroxyamino acid residues. O-Formylation of the tyrosine residue appears not to occur in anhydrous formic acid, since the paper chromatographic behavior and ultraviolet absorption spectra in both acid and alkali solution of the formic acid treated N-acetyl-L-tyrosine were the same as for the untreated compound.

Since serine did not give O-formylserine by reaction with anhydrous formic acid, O-formylation of the hydroxyl group of serine (and threonine) probably takes place with the assumed intermediate oxazoline or hydroxyoxazoline compound

```
—NH—CH—CO—NH—CH—CO—
     |         |
     R       HO—CH2
        | H+
        v
[ —NH—CH—C==N—CH—CO—   or
       |   |     |
       R   O-----CH2
                 OH
                 |
         —NH—CH—C—NH—CH—CO— ]
              |  |      |
              R  O------CH2
        | H·COOH
        v
—NH—CH—CO—NH—CH—CO—
     |         |
     R   HCO—O—CH2
```

Owing to the great differences in the stability of the O-formyl groups of proteins observed in the present experiments, it was not possible to estimate the exact number of formyl groups introduced into the protein by the reaction between formic acid and protein. Formic acid may nevertheless prove to be useful as a specific reagent for the hydroxyl group of serine (and threonine) residues in protein, since it appears to be milder and possibly more specific than concentrated sulfuric acid.[22]

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5-Bromodeoxycytidine and 5-Chlorodeoxycytidine[1]

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Two new deoxynucleoside derivatives, 5-chlorodeoxycytidine and 5-bromodeoxycytidine, have been prepared by a photocatalytic process. The inhibitory effect of these derivatives on the turbidity and viability of a thymine requiring mutant of *Escherichia coli* and a pyrimidine-requiring mutant of Neurospora are described.

Introduction

Several 5-halopyrimidines, their ribosides and deoxyribosides, have interesting inhibitory effects upon growth of bacteria, bacteriophage, molds and tumors. Some of these antimetabolites inhibit thymine synthesis, others prevent thymidine utilization, and depending to some extent on the size of the halogen,[2,3] the abnormal pyrimidine may be incorporated into either RNA or DNA. Shive and Skinner[4] have summarized these activities in a recent review. The unusual activities of the above derivatives suggested the synthesis of two new derivatives, 5-chlorodeoxycytidine and 5-bromodeoxycytidine, by a photocatalytic method similar to that reported for the synthesis of 5-halocytidine.[5] In addition to the possible usefulness of these derivatives in studies of nucleic acid metabolism, genetics and as chemotherapeutic agents, the new compounds are potential replacements for 5-methyldeoxycytidine found in some plant and animal DNA and 5-hydroxymethyldeoxycytidine in the coliphage DNA of the even varieties.

(1) This investigation was supported by the National Institutes of Health, Grant No. C2372.

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Experimental

5-Bromodeoxycytidine.—Deoxycytidine (2.15 g., 0.0095 mole), was dissolved in 155 ml. of anhydrous acetic acid, 110 ml. of anhydrous pyridine was added and the solution chilled to −10°. The cold solution was exposed to ultraviolet light at a distance of 9–10 cm. and 1.53 g. (0.0097 mole) of bromine in 10 ml. of cold anhydrous carbon tetrachloride was added with constant stirring. The ultraviolet source was a Lamp Projector, model 93LS, manufactured by the Keese Engineering Co. of Hollywood, Calif. The amber-colored solution was removed from the ultraviolet light and allowed to stand at room temperature overnight. The solvents were removed at temperatures not exceeding 40° using reduced pressure until a thick sirup was obtained. This material was dissolved in 30 ml. of absolute methanol and again reduced to a sirup. This process was repeated five more times or until a semi-crystalline white solid was