TABLE V

BUOYANT DENSITIES OF SUBSTITUTED AND NORMAL DNA IN CESIUM CHLORIDE

Organism	DNA density, g./cm.[3] Normal deuterium substituted	
Bacillus tiberius	1.694	1.737
Hemophilus influenzae	1.698	1.736
Bacillus subtilis	1.703	1.745[a]
Escherichia coli	1.710	1.751
E. coli phage T7	1.710	1.750
B. subtilis phage SP-8	1.743	1.787
[b]*Lemna gibba*	1.700	1.731

[a] The density of deuterium and N^{15} labelled *B. subtilis* DNA is 1.758 g./cc. [b] Grown in the presence of deuterioglucose[9] plus 50% D_2O, which is the maximum concentration compatible with rapid growth at this time.

and Doty.[14] Table V lists the densities of several representative samples of DNA isolated from bacteria, bacteriophages and *Lemna gibba* (duckweed). The buoyant density increase of the DNA due to the incorporation of non-exchangeable deuterium is approximately 0.042 ± 0.002 g./cc. This density increase is great enough under most circumstances to allow sufficient resolution in the interaction studies carried out between homologous DNA samples.[15] Denaturation of deuteriated *B. subtilis* DNA resulted in a density increase of 0.017 g./cc. By adding P^{32} to the DEAG medium described in Table I, it is possible to prepare nucleic acids whose fate can be followed in genetic and other studies. The ionic fraction from the acid hydrolysis of *C. vulgaris* cell wall[9] shows particular promise as a P^{32} labelling medium, as its phosphorus content is quite low. This ionic material can be substituted for the algae extracts described here.

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[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, BOSTON, MASS.]

The Reaction of Imidoesters with Proteins and Related Small Molecules[1]

BY M. J. HUNTER[2] AND M. L. LUDWIG[3]

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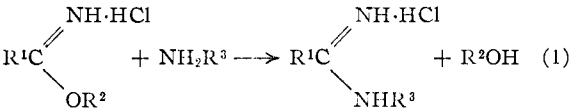
The possible use of imidoesters as group specific reagents for the modification of protein amino groups was investigated. By the use of model compounds it was established that, of the reactive groups present in proteins, only the amino group reacted with imidoesters in aqueous solvents. Balance studies of the reaction between glycine and methyl benzimidate at *p*H 9.5 indicated that the amino group was converted to a single reaction product, the corresponding monosubstituted amidine. The rates of reaction of methyl benzimidate and methyl acetimidate with glycyl-glycine and ϵ-NH_2 caproic acid were measured over the *p*H range 7–11. The rate was found to be strongly *p*H-dependent, passing through a maximum whose position on the *p*H scale was a function of the nature of the amine and the imidoester. Insulin–imidoester reactions were performed at room temperature and at *p*H values >7 but <10. Reaction with excess reagent resulted in a complete loss of the protein α- and ϵ-amino groups, the lysyl peptide bond being resistant to trypsin after reaction. Both the lysyl and the N-terminal glycyl residue appeared to have been completely converted to the corresponding amidine, but some minor side-reaction at or near the N-terminal phenylalanyl residue occurred during the reaction of the protein with aliphatic imidoesters. In general, it appears that the reaction of proteins with imidoesters does afford a useful means for the specific modification of protein amino groups to any of a variety of amidino groups under relatively mild conditions.

Introduction

Of the reagents in general use for the modification of proteins, few react with only one type of side-chain residue. Moreover, many of the available reagents yield derivatives whose solubility in water is very limited, largely due to the conversion of charged protein reactive groups to uncharged substituted groups. The search for a new group-specific reagent which would not *per se* alter the charge on the protein led to a re-investigation of the formation of amidines by the well-known imidoester–amine reaction



The reaction is formally analogous to that between O-methylisourea and amines.[4]

The preparation and reactions of imidoesters were studied many years ago by Pinner[5] who referred to them as "imidoäther" (iminoethers). More recent reviews have proposed to name these compounds imidates, or imidoates, as they are esters of the hypothetical imidic acids, RC(=NH)OH.[6,7] In this paper the class of compounds will be referred to as imidoesters; specific compounds will be called imidates, *viz.*, methyl benzimidate.

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(2) Biophysics Research Division, Institute of Science and Technology, University of Michigan, Ann Arbor, Michigan.

(3) Department of Chemistry, Harvard University, Cambridge, Mass. The work described in this paper was carried out while this author was the recipient of a post-doctoral fellowship (#CF-6611) from the National Cancer Institute.

(4) W. L. Hughes, Jr., H. A. Saroff and A. L. Carney, *J. Am. Chem. Soc.*, **71**, 2476 (1949).

(5) A. Pinner, "Die Imidoäther und ihre Derivate," Oppenheim, Berlin, 1892.

(6) R. L. Shriner and F. W. Neumann, *Chem. Revs.*, **35**, 351 (1944).

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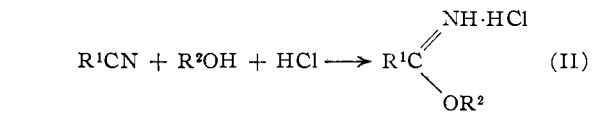
Pinner prepared a variety of imidoesters from nitriles by treatment with HCl and anhydrous alcohol. Though variations of his original method



$$R^1CN + R^2OH + HCl \longrightarrow R^1C(=NH\cdot HCl)(OR^2) \quad (II)$$

have since appeared and alternative methods for the preparation of imidoesters are available (*cf.* ref. 7), Pinner's method is still the one generally employed in the preparation of these weak bases.

R^1 can be varied considerably without preventing reaction. Among aliphatic nitriles, only those substituted at the α-carbon with a nitro group or with two or more halogens have been found unreactive; of the aromatic nitriles studied, only benzoyl cyanide and certain O-substituted benzonitriles are known to resist conversion to imidoesters.[5-7]

Amidines can be prepared by any of several general methods but many of them have been synthesized by the reaction of imidoesters with amines.[6] Considerable information about their physical and chemical properties is available.[6] Although amidines are not quite as strong bases as guanidines, they are stronger bases than typical amines (pK_a acetamidine = 12.5, pK_a benzamidine = 11.6[8,9]).

Whereas monosubstituted amidines have been prepared by the reaction of imidoesters with amines under certain reaction conditions, other products can be obtained by varying the conditions or by introducing other reactive substituents into the amine. Thus while Tietze and Petersen[10] prepared monosubstituted amidines by the reaction of imidoesters with amino acids, N,N'-disubstituted amidines have been prepared at higher temperatures in the presence of excess amine.[5]

Amino acid esters,[11] amino nitriles[12] and amino amides[13] have reacted with simple imidoesters to form N-substituted imidoesters and thence cyclic products (imidazoles, etc.). Oxazolines have been prepared by the reaction of hydroxy-amino acids with benzimidoesters at low *p*H.[14]

Provided that the imidoester reaction could be restricted to monosubstituted amidine formation, the general nature of reactions I and II suggested that they might be utilized in the development of a simple versatile method of preparing protein derivatives in which the modified amino groups remained basic. Meanwhile the R^1 group introduced might be varied readily with respect to size, shape and charge.

The preparation of protein derivatives predicates the use of aqueous solutions and demands that the reaction be performed at low temperatures and at *p*H values not too far removed from neutrality. The reactions of imidoesters with amines have generally been performed in non-aqueous media since imidoesters are subject to hydrolysis. The nature of the products (nitrile, ester, amide) depends on the structure of the imidoester, the *p*H and the temperature. Only a few amidine preparations have been carried out in mixed solvents containing water.[15]

Since amidine formation had not been studied under the conditions required for protein modification and since the course of the reaction in the presence of the other reactive groups occurring in proteins had not been determined, studies on the reaction of imidoesters with amino acids and other related small molecules seemed a necessary precursor to any investigations of the reaction with proteins. Accordingly, this paper is concerned with model studies involving small molecules, with imidoester–insulin reactions in some detail and with a few imidoester reactions involving other proteins.

Experimental

Materials. **Benzonitrile** was obtained from Eastman Organic Chemicals. It was aniline-free and was used directly from the bottle.

Acetonitrile, spectroscopic grade, was obtained from Matheson, Coleman and Bell and was used without further drying.

β-Dimethylaminopropionitrile was obtained from Eastman Organic Chemicals.

ω-Aminocapronitrile was obtained from Aldrich Chemical Co. Inc.

Trypsin was obtained from Worthington Biochemical Corp. In some experiments salt-free 2x recrystallized enzyme was used and in others the enzyme contained 50% $MgSO_4$.

Carboxypeptidase A was a suspension of 3 times crystallized protein, di-isopropyl fluorophosphate (DFP) treated, from Worthington Biochemical Corp.

Carboxypeptidase B was an amorphous suspension obtained from Worthington Biochemical Corp. It was dissolved by adjusting the *p*H to about 8, and then treated with 0.01 *M* DFP. The activity was determined to be 10,000 units/mg. using hippuryl-arginine as substrate.[16]

Ribonuclease III, chromatographically pure, was purchased from Sigma Chemical Co. and was oxidized by the procedure of Hirs.[17]

Insulin.—Three different beef insulin preparations were used; Lots W-1302 (amorphous powder) and 535664 (crystalline) from Eli Lilly and Co., and crystalline beef insulin, Batch No. 2189, from the British Drug Houses Ltd. Some of the latter material was oxidized by the procedure of Hirs.[17]

α-Chain of human globin was a gift from Drs. M. Naughton and V. M. Ingram, Department of Biology, Massachusetts Institute of Technology, and was likewise oxidized by the procedure of Hirs.[17]

Sephadex G-25 was obtained from Pharmacia Laboratories, New York, N. Y.

Elementary analyses were performed by Dr. S. M. Nagy, Massachusetts Institute of Technology.

Preparation of Methyl Benzimidate·HCl.—The method employed was basically that of Pinner.[5] Five grams (0.05 mole) of benzonitrile and 2.4 g. (0.075 mole) dry methanol were cooled in an ice bath and 2.8 g. (0.075 mole) of dry HCl added. Care was taken to maintain anhydrous conditions in the reaction vessel. After 3 hours the reaction mixture had crystallized to a solid mass. The crystals were washed well with ether, dried and stored in a vacuum desiccator over sodium hydroxide flakes; m.p. 105° with decomposition and evolution of gas, lit.[18] m.p. 105–106°. Titration (see below) of the dried benzimidate hydrochloride

(8) A. Albert, R. Goldacre and J. Phillips, *J. Chem. Soc.*, 2240 (1948).

(9) G. Schwarzenbach and K. Lutz, *Helv. Chim. Acta*, **23**, 1162 (1940).

(10) E. Tietze and V. S. Petersen, *Ann.*, **623**, 166 (1959).

(11) E. Schmidt, *Ber.*, **47**, 2545 (1914).

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(13) G. Shaw, *et al.*, *ibid.*, 1648 (1959).

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(15) H. J. Barber and R. Slack, *J. Am. Chem. Soc.*, **66**, 1607 (1944).

(16) J. E. Folk, *et al.*, *J. Biol. Chem.*, **235**, 2272 (1960).

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(18) J. T. Edward and S. C. R. Meacock, *J. Chem. Soc.*, 2009 (1957).

indicated that it was over 95% pure when freshly prepared. On standing for several months, even when stored in a vacuum desiccator, some decomposition did occur.

The molar extinction coefficient of methyl benzimidate (free base) in chloroform was determined by use of a Beckman DU spectrophotometer. The optical density showed no maximum in the region studied (245–290 mμ) but continually increased with decreasing wave length. Arbitrarily, 265 mμ was chosen as the standard wave length and a value of 644 calculated for the molar extinction coefficient in a 1-cm. cell at this wave length.

The apparent dissociation constant (pK_a') of methyl benzimidate in 30% dimethylformamide as determined from titration data was 6.0 at both 22° and 39°. Edward and Meacock[18] and Stieglitz[19] have reported values of 5.8 and 5.68 for the apparent dissociation constant in aqueous solution.

Preparation of Methyl Acetimidate·HCl.—The hydrochloride was prepared by a modification of the method described in ref. 20 for the preparation of ethyl acetimidoester·HCl. Dry methanol (40 ml., 1.0 mole) was cooled in a Dry Ice–Cellosolve-bath during the addition of 40–50 g. of HCl gas (1.1–1.4 moles). Precautions were taken to maintain anhydrous conditions. Dry acetonitrile (40 ml., 0.75 mole) was quickly added to the methanolic HCl after it had cooled to the temperature of the Dry Ice–Cellosolve mixture. After the acetonitrile had frozen out of the reaction mixture, the flask was allowed to warm to 0° in an ice-bath. The reaction mixture was kept in an ice-bath in a cold room at +1° overnight. Crystals separated out during this time and gradually increased in quantity. After 48–72 hours, 1–2 volumes of dry ether were added with mixing. About 1 hour later, the solid was filtered from the reaction mixture, washed with methanol–ether (1:2), then with ether and stored over H_2SO_4 in an evacuated desiccator. The filtering operations were carried out as rapidly as possible, preferably in a cold room; m.p. 93–95° with decomposition and gas evolution.

The Preparation of Methyl β-Dimethylamino-propionimidate·2HCl was based on the method of Baksheev and Gavrilov.[21] Under anhydrous conditions 6.0 g. of dry HCl gas (0.16_5 mole) was bubbled into 9 ml. of methanol (0.22 mole) cooled in an ice-bath. The methanolic HCl was transferred to another flask in which 0.5 g. of β-dimethylaminopropionitrile hydrochloride had been dried *in vacuo*. The transfer was effected through glass connections without opening either flask to the air during the addition. After the reaction mixture had been kept for 30–40 minutes in the cold room, 35 ml. of dry ether was added. White plates separated out in 2–3 hours. The crystals were collected by filtration and washed with ether; m.p. 100° with decomposition and gas evolution. A second crop of crystals (m.p. 100°) could be obtained by a further addition of ether.

Preparation of ω-(N-2,4-Dinitrophenyl)-aminocapronitrile.—One gram of $NaHCO_3$ was suspended with stirring in a solution of 1.5 ml. of ω-aminocapronitrile in 5 ml. of ethanol. Three grams of dinitrofluorobenzene (DNFB) in 30 ml. of ethanol was added to the nitrile solution and the reaction mixture stirred at room temperature for 30 minutes. Water was then added and a dark gum collected. The gum was dissolved in acetone, filtered, and water added to the heated solution until it became opaque. Dark yellow crystals of the product were obtained after cooling.

Preparation of Methyl ω-(N-2,4-Dinitrophenyl)-aminocaproimidate·HCl.—The above nitrile (100 mg.) was dissolved in 4 ml. of dry ethyl acetate and 4 ml. of anhydrous methanol. The ice-cooled reaction mixture was saturated with dry HCl gas and the reaction mixture stored at +1° for 24 hours. Ether was then added until the solution became turbid (*ca.* 40 ml.). After 36 hours, yellow crystals precipitated which were collected under anhydrous conditions; m.p. 113–115° with decomposition and gas evolution.

Preparation of Benzamidinoacetic Acid.—Glycine (150 mg.) was dissolved in 5 ml. of water, and 1 ml. of 33% trimethylamine added to the solution. Methyl benzimidate free base (500 mg.) was dissolved in the minimal amount of chloroform and added to the glycine solution. Methanol was added to the resultant two-phase system until there was no phase separation. The reaction then was allowed to proceed for 3 hours at 37°. Since the reaction mixture still contained some glycine at the end of this time, a further 250 mg. of the free base of the imidoester was added and the reaction allowed to proceed for an additional 2 hours at 37°. The reaction mixture now contained only trace amounts of glycine as determined by ninhydrin-sprayed chromatograms. Ether was added to the solution until two phases separated. The organic phase was discarded and the aqueous phase lyophilized. The lyophilized powder was crystallized from an ethanol–water mixture. The product was chromatographed in a buffered salt-saturated 2-propanol–water system.[22] Spraying with aged nitroprusside reagent revealed a single spot, R_f 0.3. Lint-free recrystallized material, m.p. 192° dec., was analyzed. *Anal.* Found: C, 60.72; H, 5.89; N, 15.80. Calcd. for $C_9H_{10}O_2N_2$: C, 60.67; H, 5.66; N, 15.72. The optical density of a standard benzamidinoacetic acid solution was measured in the ultraviolet. The absorption spectrum had a maximum at 230 mμ; the molar extinction coefficient at this wave length in a 1-cm. cell was determined as 1.3×10^4.

Preparation of β-Dimethylaminopropionamidinoacetic Acid·2HCl.—β-Dimethylaminopropionimidate·2HCl (500 mg.) was added to 100 mg. of glycine in 3–4 ml. of water. The *p*H of the solution was raised to about 9 by the addition of Dowex-1 on the hydroxide cycle. The reaction mixture was decanted from the resin and stirred for 1 hour; final *p*H 10.0. It was then shaken with ether to remove contaminating amide, ester or nitrile, and the ethereal layer discarded. After adjustment of the *p*H of the aqueous phase to 5–6 with HCl, the solution was passed through an IRC-50 column buffered at *p*H 5.5 with ammonium acetate. Any unreacted glycine was washed off the column with water. The resin, to which the amidine was still absorbed, was removed from the column and suspended in water. Ammonium hydroxide (1 *N*) was added to the suspension until the *p*H rose to 9.5. At this *p*H, the amidinoacetic acid was eluted from the resin, but any contaminating β-dimethylaminopropionamidine remained bound. The solution was freed of resin by filtration and the filtrate lyophilized (130 mg.). The dried powder was dissolved in a minimal volume of concentrated HCl. About 5 ml. of glacial acetic acid was added with warming followed by a dropwise addition of dioxane to turbidity. Needles separated on cooling. Lint-free recrystallized material, m.p. 194–197° with decomposition and gas evolution, was analyzed. *Anal.* Found: C, 34.63; H, 7.14; N, 17.27; Cl, 28.68. Calcd. for $C_7H_{17}O_2N_3Cl_2$: C, 34.15; H, 6.96; N, 17.07; Cl, 28.81.

Estimation of Methyl Benzimidate by Titration.—Although benzimidate hydrochlorides are water soluble, the free base of methyl benzimidate (pK_a' 6.0) is a water-insoluble oil which can be quantitatively extracted into organic solvents such as benzene or chloroform. Even the water-soluble free base of methyl acetimidate can be extracted in this manner. The addition of chloroform to a reaction mixture of imidoester, amino acid and amidine at *p*H 10 thus results in the extraction of the imidoester into the chloroform layer, the charged amino acid and amidine remaining in the aqueous layer.

This separation procedure was utilized in estimating imidoester concentrations in reaction mixtures. The concentration of the extracted imidoester could be determined from titration or optical density measurements. The details of the titration procedure follow.

A 1-ml. aliquot of imidoester containing not more than 0.0043 g. of methyl benzimidate·HCl (0.25×10^{-4} mole) was added to 1 ml. of 2% Na_2CO_3 and 1 ml. of chloroform in a test-tube and the test-tube well shaken. The chloroform layer was removed to a 20-ml. test-tube and the aqueous layer re-equilibrated with 1 ml. of chloroform. This process was repeated three times. The combined chloroform extract was washed three times with 2-ml. aliquots of distilled water and the washes discarded.

Five milliliters of standard 0.01 *N* HCl was added to the washed chloroform extract and the contents of the test-tube well shaken. The aqueous layer was removed to a 50-ml.

(19) J. Stieglitz, *Am. Chem. J.*, **39**, 29, 166 (1908).

(20) "Organic Syntheses," Coll. Vol. I, 2nd edition, edited by A. H. Blatt, John Wiley and Sons, Inc., New York, N. Y., 1944, p. 5.

(21) A. N. Baksheev and N. I. Gavrilov, *J. Gen. Chem., U.S.S.R.*, **22**, 2077, 2085 (1952); English translation.

(22) R. H. McMenamy, C. C. Lund and J. L. Oncley, *J. Clin. Invest.*, **36**, 1672 (1957).

erlenmeyer flask and the remaining chloroform layer washed three times with 1 ml. of distilled water. The washings were added to the erlenmeyer flask which thus contained all the benzimidoester hydrochloride and free HCl. The amount of free HCl in the solution was measured by titrating the solution to just above *p*H 4 with standard (*ca.* 0.01 *N*) NaOH, using brom phenol blue as an indicator. (Since the pK_a of methyl benzimidate is 6.0 it will remain as the hydrochloride and will not be titrated at this *p*H.) The difference between the amount of HCl originally added and the amount determined as above is thus equal to the amount of benzimidoester present in the solution.

Control studies in which known amounts of HCl were added to methyl benzimidate·HCl powder showed good agreement between the amount of HCl added and the amount titrated and that this somewhat arbitrary end-point did give a satisfactory estimate of the free HCl present.

This procedure was also applicable to methyl acetimidate. Some other titration procedures have been reported in the literature.[23,24]

Reaction of Imidoesters with Amines.—The reaction rate studies were carried out at constant *p*H by the use of a Radiometer TTT-I Titrator with a combination electrode, GK 2021 (Radiometer, Copenhagen, Denmark). The electrode vessel was a water-jacketed glass container. The temperature of the circulating water was controlled to ±1° by a thermostat and the solutions magnetically stirred during reaction.

For rate determinations, amine solutions of known molarity were adjusted to approximately the desired *p*H by the addition of 1 *M* NaOH or 1 *M* HCl. Solid imidoester hydrochloride was then added and the *p*H readjusted to the desired value. The total volume of titrant required for the initial *p*H adjustments was recorded and the titrator set to maintain a constant *p*H. At appropriate times, aliquots of the reaction mixture were removed for determination of imidoester concentration by titration. The volume of titrant which had been added to the reaction mixture to maintain a constant *p*H was recorded whenever an aliquot was removed for analysis.

Ninhydrin determinations were performed according to Stein and Moore[25] using citrate buffer and $SnCl_2$ in the reagent.

"Aged" Nitroprusside Stain.—As described by Block,[26] 2 ml. each of 10% NaOH, 10% potassium ferricyanide and 10% sodium nitroprusside were mixed, allowed to stand 20 minutes and diluted with 100 ml. of water.

Electrophoresis.—Analytical paper electrophoreses were performed using a modified Durrum type apparatus[27] in which the strips were air-cooled, large tanks in which the papers were immersed in Varsol[28] or on plates.[29]

Histidine and tyrosine were detected on paper chromatograms by means of the Pauly reaction.[30]

Results

I. Studies with Model Compounds.—Qualitative studies on the reaction of amino acids with methyl benzimidate and methyl acetimidate were carried out in aqueous solution at *p*H 9–10. The reaction mixtures were chromatographed in salt-saturated 2-propanol–water.[22] Aged nitroprusside reagent was used to identify amidines; unsubstituted amidines were found to produce an orange color whereas substituted amidines like benzamidinoacetic acid reacted to form a pink color. Imidoesters bleached the nitroprusside background color and appeared as white spots.

The chromatograms of all reaction mixtures gave an orange spot on treatment with aged nitroprusside. These spots had the same R_f values as benzamidine or acetamidine, which are formed in aqueous solutions of the imidoesters (see Discussion).[7] No nitroprusside-staining spots other than benzamidine and benzamidinoacetic acid were seen on chromatograms of glycine–methyl benzimidate reaction mixtures even after prolonged reaction.

A 1:1 lysine–methyl acetimidate reaction mixture yielded three bands which stained pink with nitroprusside and had R_f's greater than lysine; presumably these were α-substituted, ε-substituted and α,ε-disubstituted lysines. Histidine formed a single amino acid derivative with an R_f different from that of the parent amino acid. The derivative gave a positive diazo reaction,[30] and stained pink with nitroprusside reagent but did not react with ninhydrin. Arginine and tyrosine likewise formed only one ninhydrin-negative, nitroprusside-positive derivative. α-N-Acetylhistidine formed no nitroprusside-positive derivative.

Reaction of Methyl Benzimidate with Glycine.—A balance study was performed on the reaction between glycine and methyl benzimidate at both 37° and 20°. Solid methyl benzimidate·HCl was added to a solution of 0.05 *M* glycine in 30% dimethylformamide to give a final concentration of 0.045 *M*. The *p*H was maintained at 9.5 and aliquots of the reaction mixture removed at suitable time intervals. One series of aliquots was used to determine imidoester concentrations by titration as described in the Experimental section. Another series of aliquots was extracted with chloroform and the concentration of imidoester in the extract calculated from optical density measurements on the chloroform solutions at 265 mμ. The aqueous phase remaining after chloroform extraction was suitably diluted for determination of the optical density at 230 mμ; the amidine concentration was calculated from these readings. Aliquots of the aqueous phase also were used for ninhydrin determinations to measure the remaining glycine. Table I shows that the appearance of amidine closely paralleled the disappearance of reactants.

TABLE I

BALANCE STUDY OF GLYCINE–METHYL BENZIMIDATE REACTION AT pH 9.50

Time, min.	Glycine reacted (ninhydrin)	Amidine formed (230 mμ)	Imidoester reacted (265 mμ)	Imidoester reacted (Titration)
		$T = 20°$[a]		
15	0.012	0.009	0.011	0.008
62	.018	.019	.019	.019
90	.023	.027	.025	.024
123	.026	.029	.028	.027
		$T = 37°$[a]		
20	0.013	0.014	0.015	0.013
60	.026	.025	.025	.025
120	.032	.030	.031	.031
180	.034	.033	.032	.033

[a] Results are reported in moles per liter.

Reaction of Methyl Benzimidate with ε-NH_2-Caproic Acid and Glycylglycine.—Sufficient solid methyl benzimidate·HCl was added to a solution

(23) J. Stieglitz, *J. Am. Chem. Soc.*, **35**, 1774 (1913).
(24) J. B. Cloke and F. A. Keniston, *ibid.*, **60**, 129 (1938).
(25) W. H. Stein and S. Moore, *J. Biol. Chem.*, **176**, 367 (1948).
(26) R. Block, "Paper Chromatography," Academic Press, Inc., New York, N. Y., 1952, p. 142.
(27) E. L. Durrum, *J. Am. Chem. Soc.*, **72**, 2943 (1950).
(28) A. M. Katz, W. J. Dreyer and C. B. Anfinsen, *J. Biol. Chem.*, **234**, 2897 (1959).
(29) V. M. Ingram, *Biochim. et Biophys. Acta*, **28**, 539 (1958).
(30) D. Bolling, R. J. Block and H. A. Sober, *Federation Proc.*, **8**, 185 (1949).

of 0.1 M ϵ-NH_2-caproic acid or glycyl-glycine in 30% dimethylformamide to give a concentration of approximately 0.025 M. Aliquots (1 ml.) were removed at suitable time intervals for imidoester analysis as described in the Experimental section.

To find the optimal conditions for the reaction and to investigate the selectivity for ϵ- *versus* α-amino groups, rate studies were performed at a series of pH values. From the titration data, over-all second-order rate constants were calculated according to the expression

$$k_{(app)} = \frac{1}{t(B - A)} \ln \frac{A(B - x)}{B(A - x)} \qquad (III)$$

in which B is equal to the initial molar concentration of amine, A the initial molar imidoester concentration, t the time in minutes and x the molar concentration of product at time t. Initial amine concentrations were corrected for the volume increase due to the initial pH adjustment. The addition of titrant to maintain a constant pH during the reaction resulted in a progressive dilution of the reaction mixture. Although the volume of titrant was always small relative to the solution volume, all calculated imidoester concentrations were corrected by the appropriate dilution factor. Initial imidoester concentrations were obtained by extrapolation of the graph of log (imidoester) *versus* time to zero time, using early points when the reaction was pseudo first order.[31] The resulting $k_{(app)}$ values for the reaction of methyl benzimidate with ϵ-NH_2-caproic acid at 22° and 39° and with glycyl-glycine at 39° are given in Table II. The values of $k_{(app)}$ calculated in this manner were constant throughout each experiment, consistent with the assumption that the reaction was second order. The dependence of $k_{(app)}$ on pH, however, suggested that total concentrations of reactants should not be employed in the calculation.

TABLE II

REACTION OF METHYL BENZIMIDATE WITH ϵ-NH_2-CAPROIC ACID AND GLYCYL-GLYCINE AT VARIOUS pH VALUES[a]

ϵ-NH_2-caproic acid, T = 22°			ϵ-NH_2-caproic acid, T = 39°			Glycylglycine, T = 39°		
pH	$k_{(app)}$[b]	k^*[b]	pH	$k_{(app)}$[b]	k^*[b]	pH	$k_{(app)}$[b]	k^*[b]
7.02	0.0062	480				6.63	0.20	26
7.55	.0067	490				6.80	.35	41
8.00	.016	1100				6.95	.40	48
8.45	.020	1400	8.45	0.099	2,800	7.30	.69	71
8.82	.032	2300	8.60	.12	3,500	7.85	.55	96
9.05	.051	3600	8.65	.15	4,300	8.45	.39	150
9.50	.077	5700	8.91	.21	6,000	9.75	.053	300
9.55	.079	5900	9.27	.22	6,500	9.75	.057	320
10.00	.079	6500	9.70	.34	11,000			
10.61	.079	8800	10.65	.21	15,000			
10.75	.061	7800						
10.80	.058	7700						

[a] These rates are uncorrected for hydrolysis of imidoester. [b] Liters mole^{-1} minute.$^{-1}$

Assuming that the actual reactive species were the charged conjugate acid of the imidoester and the amine free base, the effective concentrations of imidoester and amine would depend on the pKs of these compounds and on the pH of the reaction. Another second-order rate constant, k^*, was therefore calculated from the expression

$$k^* = \frac{1}{\alpha\beta t(B - A)} \ln \frac{A(B - x)}{B(A - x)} \qquad (IV)$$

in which α denotes the fraction of imidoester in the charged form and β the fraction of amine present as the free base at a particular pH. The following pKs were determined in 30% dimethylformamide and used in calculating α and β: methyl benzimidate, 6.0 at 22° and 39°; glycylglycine, 8.00 at 39°; ϵ-NH_2-caproic acid, 10.45 at 39° and 10.85 at 22°.[32]

As can be seen from Table II, k^* was also pH-dependent. If the reaction proceeded by two paths according to a rate equation

$$-\mathrm{d(Im_{total})}/dt = k_1(\mathrm{Im^+})(\mathrm{NH_2R}) + k_2(\mathrm{Im})(\mathrm{NH_2R}) \qquad (V)$$

one would obtain the integrated expression

$$k^* = k_1 + k_2\left(\frac{\alpha}{1 - \alpha}\right) = \frac{1}{\alpha\beta t(B - A)} \ln \frac{A(B - x)}{B(A - x)} \qquad (VI)$$

A plot of k^* *versus* $\alpha/(1 - \alpha)$ should give a straight line whose slope is k_2 if this mechanism were correct; the experimental data however did not give a straight line when plotted in this manner.

Failure to fit the results by any of these comparatively simple assumptions about the reaction mechanism indicated that more detailed studies under somewhat different experimental conditions would be necessary before the reaction mechanism could be determined. This was not attempted.

The above reaction rate studies, however, were adequate to determine the pH-dependence of the reaction with amino groups. For ease of comparison, the $k_{(app)}$ values have been converted to half-times of disappearance of imidoester. These results are shown in Fig. 1. The concentrations of

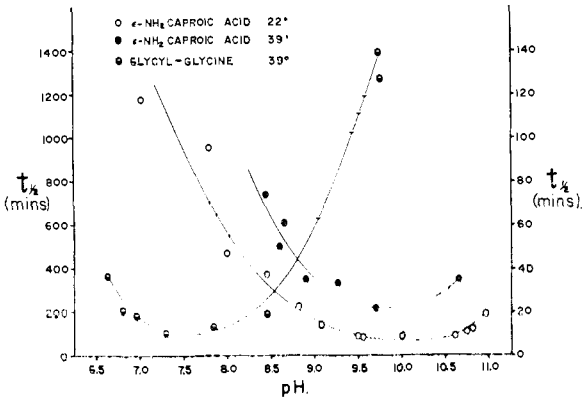


Fig. 1.—Half-times of reaction of ϵ-NH_2 caproic acid and glycyl-glycine with methyl benzimidate at various pH values. The left-hand ordinate applies to the data at 25°, the right-hand ordinate to the data at 39°.

reactants varied slightly from run to run and were corrected to 0.02 M in imidoester and 0.1 M in amine for calculation of the half-time of disappearance of imidoester. Since

$$\frac{1}{B - A} \ln \frac{A(B - x)}{B(A - x)} = tk_{(app)}, \; tk_{(app)} \text{ would equal}$$

$$\frac{1}{0.08} \ln \frac{0.02(0.09)}{0.1(0.01)} = 7.35$$

(31) The instability of the imidoester hydrochlorides to moisture necessitated rapid weighing of the solid hydrochlorides; the weight of imidoester hydrochloride therefore varied slightly from run to run.

(32) These values were obtained from titration data by the authors.

when the imidoester concentration was half its original value. The half-time ($t_{1/2}$) under these conditions is therefore equal to $7.35/k_{(app)}$ in minutes.

Hydrolysis of Methyl Benzimidate.[33]—Hydrolysis rates were determined in the same fashion as the rates of reaction with amine. Apparent first-order rate constants, k_h, and half-times of hydrolysis are given in Table III. The hydrolysis rates of methyl benzimidate were very small between *p*H 7 and *p*H 9.6 giving half-times of 2000 minutes or more at 39°. Acid or alkaline to this *p*H range, the hydrolysis rates did increase but in most cases were so small compared to the amine reaction rates that they could be ignored in calculation of the amine rate constant.

TABLE III

HYDROLYSIS OF METHYL BENZIMIDATE AT 39°

*p*H	$k_{(h)}$, min.$^{-1}$	$t_{1/2}$, min.	*p*H	$k_{(h)}$, min.$^{-1}$	$t_{1/2}$, min.
3.10	0.0073	95	9.27	0.00028	2500
6.65	.0012	590	9.55	.00033	2100
7.45	.00035	2000	10.40	.0016	420
8.50	<.0001	>3000	11.50	.012	59

Reaction of Methyl Acetimidate with ε-NH_2-Caproic Acid and Glycylglycine: Hydrolysis of Methyl Acetimidate.—Solutions (0.05 *M*) of ε-NH_2-caproic acid or glycylglycine were allowed to react with methyl acetimidate (0.0125 *M*) at 25° in water. Aliquots (2 ml.) were removed for imidoester analysis. Prior to the calculation of rate data, amine and imidoester concentrations were corrected for volume changes as previously described. Initial imidoester concentrations were obtained by extrapolation of pseudo first-order graphs to zero time. No corrections were made for concurrent hydrolysis. The results are summarized in Table IV. The second-order rate equation III was used to calculate $k_{(app)}$, and the half-times were corrected to initial concentrations of 0.05 *M* in amine and 0.01 *M* in imidoester. For direct comparison with the methyl benzimidate half-times, the acetimidate values should therefore be divided by two.

TABLE IV

REACTION OF METHYL ACETIMIDATE WITH ε-NH_2-CAPROIC ACID AND GLYCYLGLYCINE AT VARIOUS *p*H VALUES (25°)

ε-NH_2-caproic acid			Glycylglycyl			Hydrolysis		
*p*H	$k_{(app)}$[a]	$t_{1/2}$[b]	*p*H	$k_{(app)}$[a]	$t_{1/2}$[b]	*p*H	$k_{(h)}$[c]	$t_{1/2}$
8.00	2.2	6.8	6.95	4.2	3.5	7.15	0.030	23
9.30	5.7	2.6	7.85	7.6	2.0	8.00	.025	27
10.08	9.4	1.6	8.95	23.0	0.65	8.98	.018	39
10.59	9.8	1.6	9.17	13.0	1.2	10.55	.024	29
11.25	4.8	3.1				11.26	.026	26

[a] Liters mole^{-1} min.$^{-1}$. [b] Minutes. [c] Minutes^{-1}.

The hydrolysis rates were determined in a manner identical with that employed with methyl benzimidate. In these experiments, however, the methyl acetimidate concentration was 0.0125 *M* and the solvent water since methyl acetimidate free base is water-soluble at these concentrations. First-order hydrolysis rate constants (k_h) and half-times also are shown in Table IV.

Reaction of Imidoesters with other R-Groups Present in Proteins.—Various small molecules were chosen as models of the reactive groups of proteins. The half-time of disappearance of methyl benzimidate and methyl acetimidate in the presence of these small molecules was determined and compared with the hydrolysis rate. The results are shown in Table V. The rate of disappearance of imidoester in the presence of these model compounds was not significantly different from the hydrolysis rate.

TABLE V

HALF-TIMES OF DISAPPEARANCE OF IMIDOESTER IN THE PRESENCE OF VARIOUS SMALL MOLECULES[a]

Compound (*M*)	*p*H	$t_{1/2}$[b]	$t_{1/2}$(hydrol)[b]
Mercaptoethanol (0.1)	10.40	520	420
*p*OH phenylacetic acid (0.1)	10.40	480	420
*p*OH phenylacetic acid (0.1)	7.05	1650	1700
N-Acetylglycylglycine (0.1)	9.50	>5000	>2000
N-Acetylhistidine (0.05)	7.60	2500	2000
Triethanolamine (0.1)	9.60	>5000	>2000
N-Acetylhistidine (0.05)	7.4	48[c]	44[c]
Sarcosine (0.05)	8.55	56[c]	62[c]
*p*OH phenylacetic acid (0.05)	10.4	32[c]	30[c]
Hippurylarginine (0.025)	10.4	33[c]	30[c]

[a] Experiments were performed at 39° with methyl benzimidate (0.02*M*) unless otherwise indicated. [b] Minutes. [c] 25°, methyl acetimidate (0.01*M*).

Reaction of Methyl Benzimidate with Glycine and Phenylalanine.—The two N-terminal amino acids of insulin are glycine and phenylalanine. Since modification of this protein was planned, the rate of reaction of methyl benzimidate with these two amino acids was studied at two *p*H values. The reactions were performed at 39° as previously described. The data are shown in Table VI.

TABLE VI

REACTION OF GLYCINE AND PHENYLALANINE WITH METHYL BENZIMIDATE AT 39°

*p*H	Amino acid	$k_{(app)}$[a]	$t_{1/2}$[b]	k^*[b,c]
9.50	Glycine	0.958	7.7	6810
	Phenylalanine	.129	56.5	580
8.60	Glycine	.383	19.2	1680
	Phenylalanine	.090	82	160

[a] Liters mole^{-1} min.$^{-1}$. [b] $t_{1/2}$ (min.) was corrected to 0.02 *M* methyl benzimidate, 0.1 *M* amine. [c] Calculated using pK_a' phenylalanine = 9.13 and pK_a' glycine = 9.60.

II. Reaction of Imidoesters with Proteins.—Most of this work was performed with insulin. This protein was allowed to react with methyl acetimidate, methyl β-dimethylaminopropionimidate, methyl benzimidate and with methyl ω-(N-2,4-dinitrophenyl)-aminocaproimidate. Reactions employing the first two imidoesters were carried out in aqueous systems, whereas the water insolubility of the other imidoester free bases required the use of 30–50% ethanol or dimethylformamide. In general, insulin was modified at *p*H 8.5 to 9.25. Although this *p*H range is below the optimum for

(33) The decomposition reactions of imidoesters in aqueous solution are not all hydrolytic in nature (e.g., imidoester → nitrile). The use of the term hydrolysis is therefore not strictly correct to describe the over-all decomposition of these weak bases. For want of a better term, however, it is used throughout this paper to describe the total decomposition of imidoesters in water.

reaction of the ε-amino group, a higher *p*H was avoided to lessen the chance of denaturation, disulfide exchange and oxidation.

One of the authors (M.L.) in collaboration with Mr. Raymond Byrne[34] has also modified performic acid-oxidized ribonuclease, oxidized insulin and oxidized α-chain of human globin. Since precautions to avoid denaturation and oxidation were unnecessary in modification of these proteins, the *p*H of the reaction mixtures was raised to 9–10.

Typically, 1 to 2% protein solutions were allowed to react at 25° with an excess of imidoester (15–20 moles per mole of amino group). The *p*H of the protein solution was first adjusted to approximately the desired value and the imidoester hydrochloride converted to its free base by the addition of an equivalent amount of Na_2CO_3 or NaOH solution to the hydrochloride powder. The imidoester solution then was added rapidly to the protein solution and the *p*H readjusted to the desired value. This procedure prevented gross changes in *p*H on addition of the imidoester to the protein solution. The *p*H of the reaction mixture was maintained by automatic addition of titrant (HCl) whenever necessary. During the reactions of aliphatic imidoesters with proteins, considerable amounts of acid were consumed. Such large excesses of reagent were used that the rate of acid consumption was essentially a measure of the rate of imidoester hydrolysis. From the rate of acid uptake, it was possible to determine when most of the added imidoester had disappeared. Further additions of reagent then were made, in order to obtain extensive reaction. The reaction could finally be terminated by lowering the *p*H, but in most instances any remaining reagent or other small molecules were removed by dialysis or by passage through columns of G-25 Sephadex without adjustment of *p*H. The preparations were finally lyophilized.

Three insulin derivatives which were analyzed in considerable detail are described as follows: Preparation I: insulin (300 mg.) treated for two periods of 30–40 minutes each with methyl β-dimethylaminopropionimidate (each addition, 300 mg.), *p*H 8.6, final volume 20 ml.; preparation II: insulin (500 mg.) treated for two periods of one hour each with methyl acetimidate (each addition, 500 mg.), *p*H 9.25, final volume 35 ml.; preparation III: performic acid-oxidized insulin (75 mg.) treated for three periods of 40 minutes each with methyl acetimidate (each addition, 80 mg.) at *p*Hs 9.5, 10.0 and 8.8, and for a final 40 minutes at *p*H 9.5 with 160 mg., final volume 5 ml.

Analysis of Insulin Derivatives.—Insulin contains two α-amino groups (glycine and phenylalanine) and one ε-amino group per 5740 g.[35] The protein derivatives were examined by several methods to estimate the extent of reaction at each of these three sites:

1. Amino Acid Analysis Following Treatment with Dinitrofluorobenzene (DNFB).—Model studies had shown that benzamidinoacetic acid and acetamidinoacetic acid could be quantitatively hydrolyzed to glycine under the conditions normally employed for protein hydrolysis (6 *N* HCl, 110°, 18–20 hours) but that ε-amidinocaproic acid was only partially converted to ε-aminocaproic acid under these conditions. It was therefore not possible to determine the extent of reaction by direct analysis of amidino acids in the protein hydrolysates. An estimate of the extent of amidination could be made however by treatment of the modified protein with DNFB which does not react with the amidino group. Thus any ε-DNP-lysine present in the hydrolysate must be derived from unmodified lysyl residues and any DNP-phenylalanine or DNP-glycine from unmodified N-terminal residues. Modified N-terminal residues will appear in the hydrolysate as glycine or phenylalanine.

Insulin derivatives and control insulin samples were allowed to react with DNFB in the usual manner,[36] hydrolyzed and the amounts of DNP-glycine, DNP-phenylalanine, ε-DNP-lysine, glycine and phenylalanine estimated after chromatography.[22] Destruction of DNP-glycine during hydrolysis and the presence of three other glycines in the molecule made it difficult to obtain an estimate of the extent of reaction at the N-terminal glycyl residue. The derivatives formed by reaction with methyl β-dimethylaminopropionimidate and methyl acetimidate (20-fold excess of imidoester for 1 hour, *p*H 8.5–9.0) were found to contain about 20% unreacted N-terminal phenylalanine and almost no unmodified lysine.

2. Titration.—Since the *pK* of the amidino group is above 11, it is possible to measure the number of modified α-amino groups by titration. The equivalents of base required to raise the *p*H of the modified and native insulin solutions from 7.0 to 9.5 should be less for the derivative than for the control insulin to the extent that the α-amino groups of the modified insulin have reacted. The parent insulin and acetamidino insulin (preparation II) were dialyzed twice against 400 volumes of 0.1 *N* KCl at +1° to remove titratable small molecules. The resulting suspensions were dissolved by the minimal addition of 0.1 *N* NaOH and made up to volume (10 ml.) with 0.1 *N* KCl. Aliquots (8 ml.) of both protein solutions and 8 ml. of 0.1 *N* KCl were titrated with 0.05 *N* NaOH. The corrected titration curves of the proteins are shown in Fig. 2. The number of insulin groups titrated between *p*H 7 and *p*H 10.5 was 5.15, in good agreement with the value of 5.25 obtained by Tanford.[37] From Fig. 2 it can be seen that about 1.8 insulin groups which titrated between *p*H 7 and 9.5 were no longer present in the modified protein. Substantial modification of the two N-terminal amino acid residues had thus been accomplished.

The ε-amino groups in a protein can be estimated by titration in the presence of formalde-

(34) Department of Biology, Massachusetts Institute of Technology, Cambridge 39, Mass.

(35) An insulin molecular weight of 5740 has been used throughout this paper.

(36) F. Sanger, *Biochem. J.*, **39**, 507 (1945); H. Fraenkel-Conrat, J. I. Harris and A. L. Levy, "Methods of Biochemical Analysis," Vol. 2, Interscience Publishers, Inc., New York, N. Y., 1955, p. 359.

(37) C. Tanford and J. Epstein, *J. Am. Chem. Soc.*, **76**, 2163 (1954).

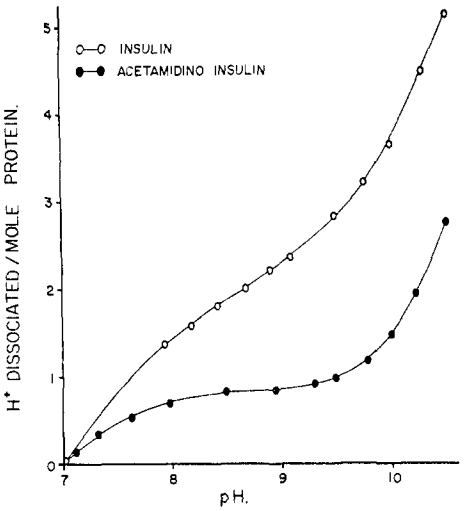


Fig. 2.—Hydrogen ion titration curves of insulin and acetamidino insulin at 25°, $\Gamma/2 = 0.1$.

hyde.[38] Model studies with glycine, ϵ-NH_2-caproic acid and acetamidinoacetic acid showed that, in the presence of 10% formaldehyde, glycine and ϵ-NH_2-caproic acid were completely titrated by *p*H 9. Acetamidinoacetic acid, however, did not begin to titrate until this *p*H had been reached (Fig. 3). Below *p*H 9, therefore, protein amidino groups will not be titrated even in the presence of formaldehyde. It is thus possible to determine by titration the ϵ-amino groups remaining after protein modification.

Insulin and its acetamidino derivative (preparation II) were dissolved in 0.1 *N* KCl and 6-ml. aliquots titrated to *p*H 9.2 with 0.05 *N* NaOH. The protein solutions were then titrated back to *p*H 9.2 after the addition of 4 ml. of 35% formaldehyde solution (final concentration *ca.* 12%). In a control experiment, 6 ml. of 0.1 *N* KCl was brought to *p*H 9.2, 4 ml. of formaldehyde added and the solution titrated back to *p*H 9.2. The results are shown in Table VII. Modification of the lysyl residue appeared complete within the limits of accuracy of the measurements.

TABLE VII

ϵ-AMINO GROUP CONCENTRATION AS DETERMINED BY FORMALDEHYDE TITRATION

	$\Delta NaOH - \Delta NaOH_{control}$[a] (mole)	Insulin[b] (mole)	ϵ-Amino group titrated, %
Insulin	0.605×10^{-5}	0.608×10^{-5}	99.5
Modified insulin	0.028×10^{-5}	0.800×10^{-5}	3.5

[a] $\Delta NaOH - \Delta NaOH_{control}$ represents the number of moles of NaOH required to bring the *p*H back to 9.2 after the addition of formaldehyde minus the number of moles of NaOH required to bring the control sample back to *p*H 9.2. [b] Insulin concentrations were determined by optical density measurements using an $E_{1\,cm}^{1\%}$ of 10.7 (at 277 mμ).

3. Tryptic Digestion.—Trypsin splits the C-terminal sequence of the B chain of insulin, -arg-gly-phe-phe-tyr-thr-pro-lys-ala-COOH, at arginine and lysine, releasing alanine and a heptapeptide.[39,40]

(38) D. French and J. T. Edsall, "Advances in Protein Chemistry," Vol. 2, Academic Press, Inc., New York, N. Y., 1945, p. 278.

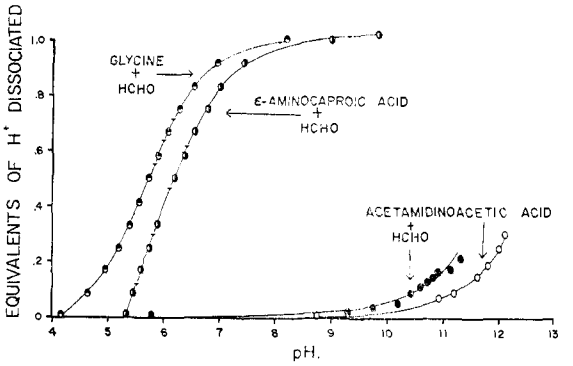


Fig. 3.—Hydrogen ion titration curves of glycine, ϵ-NH_2 caproic acid and acetamidinoacetic acid in the presence of 10% formaldehyde at 25°.

Amidination of the lysyl residue was found to block the action of trypsin, preventing the release of alanine. Digests obtained by treatment of the β-dimethylaminopropionamidino derivative (preparation I) with trypsin (18 hours, enzyme:substrate ratio of 1:25) were examined by chromatography as described by Harris.[39] Free alanine was absent from the digests, although a ninhydrin and diazo-positive peptide was found. Alanine was also absent from tryptic digests of the yellow ω-(N-2,4-dinitrophenyl)-aminocaproamidino derivative of insulin; in this case the release of a yellow peptide confirmed the presence of a modified lysyl residue. Qualitative amino acid analysis of the peptide obtained after tryptic digestion of preparation I demonstrated that it contained alanine in addition to the amino acids present in the insulin heptapeptide.

Resolution of the products of tryptic digestion was subsequently improved by the use of electrophoresis in 2% $(NH_4)_2CO_3$, *p*H 9.[41,42] In this buffer the modified octapeptide derived from acetamidino oxidized insulin (preparation III) was found to have a mobility different from that of the insulin heptapeptide. The absence of alanine and the presence of only modified octapeptide in the tryptic digests provided evidence for the completeness of amidination at the lysyl residue of preparation III.

4. Carboxypeptidase Digestion.—When insulin was digested for 7 hours at 25° with carboxypeptidase A (1:100) and carboxypeptidase B (1:200), alanine and lysine were released, the lysine in much lower yield.[43] Digestion of acetamidino insulin (preparation II) released alanine and smaller amounts of a basic amino acid with a mobility less than that of lysine. This new amino acid had a mobility identical with one of the products of the reaction of one equivalent of methyl acetimidate with lysine.

5. Electrophoresis.—Commercial insulin samples are known to be heterogeneous with respect to

(39) J. I. Harris and C. H. Li, *J. Am. Chem. Soc.*, **74**, 2945 (1952).

(40) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 481 (1951).

(41) The work done in the remainder of these studies on the tryptic digests was in collaboration with Mr. Raymond Byrne.[44]

(42) M. A. Naughton and F. Sanger, *Biochem. J.*, **78**, 156 (1961).

(43) The amino acids were identified by their positions after high voltage electrophoresis in *p*H 1.9 buffer.[44]

(44) G. N. Atfield and C. J. O. R. Morris, *Biochem. J.*, **81**, 606 (1961).

amide content[45,46] and minor components of different mobility can be resolved by electrophoresis at neutral or alkaline *p*H values. In 33% acetic acid, however, the protein carboxylic acid groups are protonated and amide heterogeneity is not reflected in the electrophoretic patterns. All insulins were found to be homogeneous in this solvent.

The reaction of insulin with β-dimethylaminopropionimidate resulted in the introduction of an additional basic group, the β-dimethylamino residue, $pK \sim 7$, into the modified protein. The rate and extent of the reaction could therefore be followed by electrophoretic analysis in 33% acetic acid. Aliquots of a reaction mixture of this imidoester (0.1 *M*) and insulin (1%) were acidified to stop the reaction, electrophoresed in 33% acetic acid and the paper strips stained with Wool Black.[47] After 5 minutes of reaction 90% of the insulin had been modified at one or more amino groups; about 10% of material with the mobility of insulin remained. After reaction for 25 minutes several faster-moving bands (mono-, di- and tri-derivatives) but no material with the mobility of the parent insulin could be observed.

All the insulin derivatives which were prepared, except the one containing the β-dimethylamino residue, have the same charge as the original insulin at low *p*H and should be indistinguishable from the unmodified protein on electrophoresis in 33% acetic acid. Various aliphatic amidino derivatives, however, all showed the presence of a second, slower-moving, minor component in this solvent. From the difference in mobility the minor component appeared to have one less positive charge. The concentration of this minor product varied with the preparation from about 5–15%. When the major and minor bands were eluted and re-electrophoresed in the same solvent, each migrated as a single band of mobility equal to that observed in the first electrophoresis. The appearance of the minor component was thus apparently not due to the acid conditions under which the electrophoresis had been performed.

To determine the extent of modification in each of the separated chains and to investigate the location and nature of the alteration which resulted in the appearance of the minor component described above, the amidino insulins were subjected to electrophoresis in the presence of thiols and performic acid-oxidized insulins were amidinated and examined by electrophoresis. The following solvent systems were employed: 2% $(NH_4)_2CO_3$, *p*H 9, with or without urea and mercaptoethanol[42]; 6.5 *M* urea, 0.1 *M* thioglycolate, *p*H 5.2[48]; and 7.5 *M* urea, 33% acetic acid, *p*H 3.3. Since amidinated amino groups gave little or no color on treatment with ninhydrin, the components were detected by the use of a stain specific for tyrosine[49] or by dyeing with Wool Black.[47] The insulin B chain, which contains both histidyl residues, could be distinguished from the A chain by the use of Sanger's modification of the diazo reaction which is specific for histidine.[50] Although the parent insulins were heterogeneous in the above buffers, comparison of band intensities and locations after electrophoresis of both modified and unmodified proteins permitted reliable estimates of the extent of reaction to be made.

As far as could be determined by analysis of the electrophoretic patterns of the separated chains, modification of the N-terminal residue of the A chain was essentially complete in preparations I, II and III. The β-dimethylaminopropionamidino derivative (preparation I) was electrophoresed in urea–thioglycolate buffer at *p*H 5.2; in this solvent the reduced A and B chains move in opposite directions.[48] The β-dimethylaminopropionamidino A chain, expected to have one less negative charge than the insulin A chain at this *p*H, migrated as a band of similar width but slower mobility than the insulin A chain. Acetamidino insulin (preparation II) and acetamidino oxidized insulin (preparation III) were electrophoresed at *p*H 9 in 2% $(NH_4)_2CO_3$; for the analysis of preparation II, the carbonate buffer also contained mercaptoethanol (0.5%) and urea (6.5 *M*). Since at *p*H 9, amidino and ε-amino groups are fully charged while unreacted α-amino groups are almost completely discharged, the mobility of α-amidino A and B chains will differ from that of unmodified α-amino chains thus enabling the extent of reaction at the α-amino groups to be determined. A major and a minor A chain band were found on electrophoresis of the unmodified insulin at this *p*H. Preparation II likewise gave a major and minor band, neither of which had mobilities corresponding to the two A chains of insulin. Similar results were obtained with preparation III as indicated in Fig. 4. In both cases the heterogeneity of the modified A chains appeared to be similar to that of the unmodified chain.

The electrophoretic behavior of the modified B chains was examined with preparations in which titration and/or tryptic digestion had shown that modification of the lysyl residue was complete. The modified B chains could therefore be examined electrophoretically without complications due to partial reaction of the lysyl residue. In all cases, the heterogeneity of the modified B chain was considerably greater than that of native B chain. Broad or multiple B chain bands were observed both with β-dimethylaminopropionamidino insulin (preparation I) electrophoresed in *p*H 5.2, urea–thioglycolate and with acetamidino insulins electrophoresed in *p*H 9 buffer. A preparation of acetamidino oxidized insulin, modified in the same manner as preparation II, was found by electrophoresis at *p*H 9 to contain three B chain bands: one ninhydrin-positive, two ninhydrin-negative. From the presence of the ninhydrin-positive material it appeared that reaction at the N-terminal phenylalanyl residue had been incomplete. To simplify interpretation of the electrophoretic patterns, oxidized insulin was treated with large excesses of methyl acetimidate (preparation

(45) F. Carpenter, *J. Biol. Chem.*, **237**, 404 (1962).

(46) E. J. Harfenist and L. C. Craig, *J. Am. Chem. Soc.*, **74**, 3083 (1952); **75**, 5528 (1953).

(47) Wool Black: Naphthol blue black, Color Index No. 246, Hartman-Leddon Co., Philadelphia, Pa.

(48) H. Lindley, *J. Am. Chem. Soc.*, **75**, 5673 (1953).

(49) R. Acher and C. Crocker, *Biochim. Biophys. Acta*, **9**, 704 (1952).

(50) F. Sanger and H. Tuppy, *Biochem. J.*, **49**, 463 (1951).

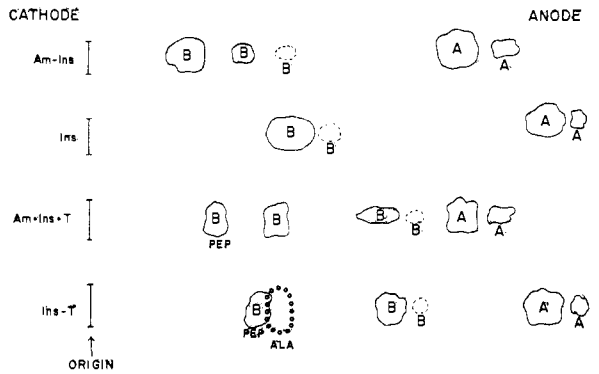


Fig. 4.—A composite representation of the electrophoretic patterns of modified and unmodified oxidized insulins after electrophoresis in 2% $(NH_4)_2CO_3$. A voltage of 2 kv. was applied across a 36-inch sheet of Whatman 3MM paper for 2.5 hours: Ins = oxidized insulin; Am-Ins = acetamidino oxidized insulin (preparation III); Ins-T = tryptic digest of oxidized insulin; Am-Ins-T = tryptic digest of acetamidino oxidized insulin; "A" denotes A chain bands which are tyrosine positive but histidine and arginine negative; "B" denotes B chain bands containing tyrosine, histidine and arginine; BPEP denotes ninhydrin and tyrosine positive peptide released from the B chain by trypsin; O O O O O O indicates the position of alanine as observed after staining with ninhydrin; - - - - - - denotes very minor components.

III). No ninhydrin-staining bands were found after electrophoresis of this preparation at *p*H 9; modification thus presumably was complete. Staining with hypochlorite[51] and with reagents specific for tyrosine[49] and histidine[50] demonstrated the presence of two main B chain components corresponding to the two ninhydrin-negative bands obtained with the less extensively modified preparation. The distribution of material in these bands is illustrated in Fig. 4.

Electrophoresis of tryptic digests of preparation III at *p*H 9 (Fig. 4) indicated the presence of only one octapeptide but two histidine-positive bands derived from the B chain. Electrophoresis of the tryptic digest of preparation III in the urea–acetic acid solvent gave one extra histidine-containing band which had no counterpart in the insulin digest. Since the N-terminal phenylalanyl–arginyl sequence of the B chain contains both histidyl residues of insulin, the heterogeneity appeared to have been introduced into this region of the molecule. The precise location and the nature of this secondary alteration have not been further studied.

In contradistinction to the results obtained with insulin which had been modified by reaction with aliphatic imidoesters, insulin preparations which had reacted with methyl benzimidate appeared homogeneous and had the same mobility as the parent insulin on electrophoresis in 33% acetic acid. Modification of performic-oxidized insulin with methyl benzimidate (two additions of a twenty-fold excess of imidoester, twelve hours apart, *p*H 8.8–9.3) yielded a preparation which could not be distinguished from the original oxidized insulin on electrophoresis in 33% acetic acid, 7.5 *M* urea. At *p*H 9, however, the electrophoretic patterns showed that the N-terminal glycyl residue had been completely amidinated.

Reaction of Methyl Acetimidate with Other Proteins.—The acetamidino derivatives of oxidized ribonuclease and α-chain of globin were digested with trypsin and the digests compared electrophoretically with those of the corresponding unmodified protein. The number of major components was greatly reduced in the digest of the modified proteins and the appearance of the electrophoretic patterns completely altered. The patterns showed the presence of only a few more peptides than would have been expected from the arginine composition. Ribonuclease, for example, gave 8–9 peptides rather than the predicted 5–6, but 4 of the major peptide spots contained arginine. Three of the arginine-containing peptides have been related to known sequences of ribonuclease; their amino acid compositions indicated that they were sequences between successive arginine residues.

Discussion

I. Studies with Model Compounds. The Preparation of Imidoesters and their Decomposition in Water.—The imidoester preparations have been described in some detail since the reaction conditions under which imidoesters can be prepared from nitriles in good yield vary considerably depending on the substituent groups of the nitrile and the alcohol. For example, although large excesses of alcohol usually are avoided to prevent orthoester formation, inert diluents being employed if extra solvent is necessary,[52] methanol has been successfully used as a diluent in the preparation of methyl β-dimethylaminopropionimidate·2HCl.[21] In general, as Roger and Neilson point out in their excellent discussion of imidoester reactions,[7] hydrolysis by moisture, orthoester formation and elevated temperatures are the principal causes of poor yields of imidoester.

The stability of imidoesters to heat and hydrolysis also depends on the substituents. Imidoester salts decompose to the corresponding amide and alkyl halide on pyrolysis or heating in inert solvents, but form esters and ammonium chloride in the presence of water.[5] The ammonia so produced reacts in turn with another molecule of imidoester to form an unsubstituted amidine.[53] All substituted amidines prepared in aqueous solution will thus be contaminated by the corresponding unsubstituted amidine. At higher *p*H values, however, the (uncharged) imidoester is also converted to nitrile and alcohol.[54]

The rate of hydrolysis of imidoesters has been studied by several workers.[19,55–57] Cloke and Keniston determined a hydrolysis constant of 0.0078 min.$^{-1}$ at 25° for γ-chloropropyl benzimi-

(51) S. C. Pan and J. D. Dutcher, *Anal. Chem.*, **28**, 836 (1956).

(52) J. N. Ashley, H. J. Barber, A. J. Ewins, G. Newberg and A. D. Self, *J. Chem. Soc.*, 103 (1942).

(53) H. G. Rule, *ibid.*, **113**, 3 (1918).

(54) H. I. Schlesinger, *Am. Chem. J.*, **39**, 719 (1908).

(55) J. T. Edward and S. C. R. Meacock, *J. Chem. Soc.*, 2009 (1957).

(56) J. B. Cloke and F. A. Keniston, *J. Am. Chem. Soc.*, **60**, 129 (1938).

(57) R. H. Hartigan and J. B. Cloke, *ibid.*, **67**, 709 (1945).

date·HCl and Hartigan and Cloke calculated a value of 0.00562 min.$^{-1}$ for the hydrolysis constant of methyl benzimidate at 25° using data obtained by Derby.[58] The value of 0.0073 min.$^{-1}$ which was obtained for the hydrolysis constant of methyl benzimidate at 39° and *p*H 3.1 in 30% dimethylformamide (*cf.* Table III), is close to the value that would be expected from the work of these earlier investigators.

Monosubstituted Amidine Formation from Amines and Imidoesters.—As indicated in the Introduction, the reactions of amines with imidoesters can yield any of several products depending on the other substituents in the reactants and on the reaction conditions. The formation of N-substituted imidates, disubstituted amidines and various heterocyclic compounds is discussed in some detail by Roger and Neilson in their review.[7] To determine whether the amine–amidine conversion in aqueous solution at *p*H 9–10 was complicated by appreciable formation of such side-products, imidoester–amine reaction mixtures were chromatographed and balance studies were performed. No evidence was found for the presence of products other than the expected monosubstituted amidines. The data obtained on the glycine–methyl benzimidate reaction showed that benzamidinoacetic acid was the predominant reaction product (>95%) at *p*H 9.5 in 30% dimethylformamide. Other measurements (Table III) had shown that the rate of hydrolysis at *p*H 9.5 was so slow relative to the reaction rate that the loss of imidoester due to hydrolysis would be insignificant and hence not complicate the results of the balance study. N-Substituted imidate formation can occur, however, in aqueous solution at a somewhat lower *p*H in the presence of excess amine.[59]

Reaction of Imidoesters with Other R Groups Present in Proteins.—The specificity of the reaction of imidoesters with amines was established by comparing the rates of disappearance of methyl benzimidate or methyl acetimidate in the presence and absence of appropriate model compounds (Table V). Small molecules (0.05–0.10 *M*) containing sulfhydryl groups, *p*-hydroxyphenyl groups, aliphatic hydroxyl groups, carboxylic acid groups, imidazole or guanidyl residues did not measurably change the rate of disappearance of imidoester (0.01–0.025 *M*). Either reaction failed to occur in aqueous solution in the *p*H range studied or the reaction rate was so slow as to be indistinguishable from the hydrolysis rate. Although 10-fold greater imidoester concentrations were employed in the imidoester–protein reactions, the concentration of any particular type of side-chain residue was 10–20-fold less; rates of reaction great enough to be of concern in protein–imidoester reactions should therefore have been detected by these model studies.

The failure of the peptide bond to catalyze the hydrolysis of the imidoesters, at least between *p*H 7 and *p*H 11, gave some assurance that the hydrolysis rate in the presence of protein would approximate that in water. The failure of triethanolamine to catalyze the imidoester hydrolysis was taken as evidence that general base catalysis of the hydrolysis reaction by amines did not occur under these experimental conditions.

The reaction of some of these groups with imidoesters under different reaction conditions has been reported. For example, ethereal solutions of imidoesters react with dry hydrogen sulfide to give thioesters and thioamides,[60] with dry alcohols to form orthoesters[61] and with secondary amines to form N,N-disubstituted amidines.[5] Nonetheless, under the conditions employed in the experiments described in this paper, the only protein reactive group which was found to compete successfully with the hydrolysis reaction was the amino group. The reagent should therefore be group-specific in protein modification reactions.

Reaction of Imidoesters with Amines.—ε-NH_2-Caproic acid and glycyl-glycine were selected as models for the ε- and α-amino groups of proteins. The kinetic experiments were not designed to obtain accurate rate constants but rather to investigate the *p*H-dependence of the reaction rate of these two types of amino groups. It was recognized that the volume changes which occurred during the reaction due to the addition of titrant did, to be exact, invalidate the use of second-order rate equations like equation III or IV to describe the reaction since the concentration of reactants changed during the reaction not only due to product formation but also due to dilution of the solution by titrant. As the volume changes were always small relative to the solution volume, however, the reaction rate constants calculated by the use of equations III and IV should approximate the true values.

From the kinetic data it was apparent that the rate of reaction was strongly *p*H-dependent and had a maximal value. Optimal apparent rate constants, $k_{(app)}$(max), and *p*H values, *p*H(max), are shown in Table VIII. As can be seen from the table, both $k_{(app)}$(max) and *p*H(max) varied with the imidoester group, R^1, and with the amine group, R^3. With any given imidoester, the difference in *p*H(max) for the two amines was a reflection of the different *p*H profiles of the reaction rates of these two amines.[59] The *relative* rates of reaction of α- and ε-amino groups with a given imidoester were thus also *p*H-dependent. For example, at *p*H 8.5 and 39° with equimolar concentrations of α- and ε-amino groups, the α-amino group reacted about four times faster with methyl benzimidate than did the ε-amino group (see Fig.

TABLE VIII

OPTIMAL APPARENT RATE CONSTANTS AND *p*H VALUES FOR CERTAIN IMIDOESTER–AMINE REACTIONS

	Methyl benzimidate[a] and ε-NH_2-Caproic acid	Methyl benzimidate[a] and Glycyl-glycine	Methyl acetimidate[b] and ε-NH_2-Caproic acid	Methyl acetimidate[b] and Glycyl-glycine
$k_{(app)}$ (max.)[c]	0.34	0.73	10	25
*p*H (max.)	9.5–10.0	7.6	10–10.5	9.0

[a] 39°. [b] 25°. [c] Liters mole^{-1} min.$^{-1}$.

(58) I. H. Derby, *Am. Chem. J.*, **39**, 437 (1908).

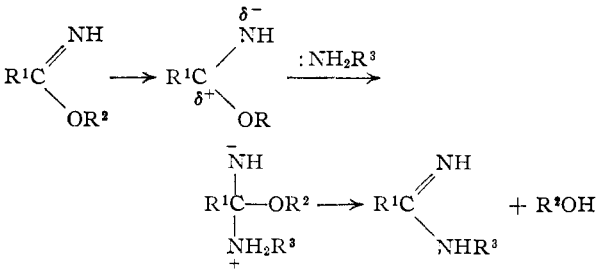
(59) E. Hand and W. P. Jencks, *J. Am. Chem. Soc.*, **84**, 3505 (1962).

(60) J. B. Jepson, A. Lawson and V. D. Lawton, *J. Chem. Soc.*, 1791 (1955).

(61) A. Pinner, *Ber.*, **16**, 1643 (1883).

1). At *p*H 9.7, however, the ϵ-amino group reacted seven times faster than the α-amino group. A similar *p*H-dependence of the rate constant was seen in the reactions of methyl acetimidate with ϵ-NH_2-caproic acid and glycylglycine. The reaction of a given imidoester in the presence of two amines of differing *pK* could therefore be controlled by a proper choice of *p*H so that one or the other of the amine reactions would predominate.

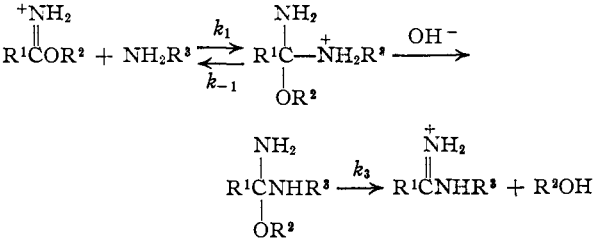
A few preliminary attempts were made to explain the kinetic data in terms of a reaction mechanism since at the time of the work reported in this paper, the mechanism of amidine formation had not been elucidated. In 1892, Pinner[5] had suggested that the reaction occurred *via* the free base of the imidoester and the free base of the amine. About 1913, Stieglitz,[23] as the result of detailed rate studies, postulated that the reactive species were the imidoester cation and the free base of the amine. In 1917, Knorr[62] suggested that the imidoester base reacted with the amine salt. In their review in 1944, Shriner and Neumann[6] proposed a mechanism similar to that of ester hydrolysis



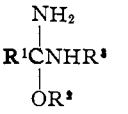
If the reaction mechanism involved a nucleophilic attack like that proposed by Shriner and Neumann, the observed *p*H-dependence of the apparent rate constants (Table II) would imply that the reactive species were the cationic form of the imidoester and the uncharged form of the amine. Recalculation of the data using the concentrations of these forms of the reactants (eq. IV), however, gave rate constants, k^*, which were still *p*H-dependent. An attempt was made to see whether the addition of an alternative reaction pathway between the uncharged imidoester and the uncharged amine would lead to two *p*H-independent rate constants (eq. VI). The resultant constants, k_1 and k_2, were however still *p*H-dependent. Reaction orders greater than one in amine did not fit the data which thus could not be explained in terms of general base catalysis by amines.

As none of these simple reaction mechanisms gave rise to *p*H-independent rate constants, it was apparent that the mechanism was complex and that more extensive rate studies would be necessary before the detailed mechanism could be ascertained.

The imidoester–amine reaction is formally analogous to the aminolysis of esters and may be classed with the nucleophilic substitution reactions at carboxylic groups recently discussed by Bender.[63] Jencks, who had studied the reaction mechanism of ester aminolysis,[64] undertook further investigations on the imidoester–amine reaction in collaboration with Hand.[59] Their results are published in the following paper. In brief, they have concluded that the reaction mechanism is indeed a nucleophilic substitution similar to that occurring in ester aminolysis. The major components of the system can be described as



Hand and Jencks concluded that the rate-determining step could be either the addition of the free base of the amine to the cationic form of the imidoester, denoted by k_1, or the formation of product, denoted by k_3. When breakdown of the intermediate becomes rate limiting, one of the consequences is that the *p*H may affect the reaction rate by controlling the concentration of the intermediate



According to this mechanism, a graph of $k_{(app)}$ *versus* *p*H for any pair of reactants passes through a maximum whose magnitude and position on the *p*H scale are complicated functions of the reactivities and *pK*s of the several components.

The kinetic data of the present paper can be accounted for in terms of such a mechanism. The k^*-values shown in Table II are, in fact, rate constants for the addition reaction (step I of the above mechanism). If, however, the reaction denoted by k_3 becomes the rate limiting step as the *p*H decreases, the calculated k^* values should decrease with decreasing *p*H. It would also be predicted from the above mechanism that the substituent R^1 of the imidoester would influence $k_{(app)}$ both by an effect on the *pK* of the imido group and by a direct influence on the electrophilic character of the central carbon. The finding that the reaction is very sensitive to the structure of the imidoester, the optimal rate increasing about thirty-fold when the R^1 phenyl group was replaced by a methyl group, is therefore not surprising. In the reaction of methyl benzimidate with different amino groups at high *p*H, where the addition step (step I) is rate limiting, the relative magnitudes of the k^* rate constants were ϵ-NH_2-caproic acid > glycine > glycylglycine, the compound with highest *pK* being the best nucleophile.

II. Reactions of Imidoesters with Proteins.—From the first preliminary studies with proteins, it was evident that imidoesters reacted readily with protein amino groups. In further work the aim was to attain extensive or complete modification of these groups. Complete reaction was emphasized for two reasons: firstly, the desirability of complete modification in many investigations; and secondly, the fact that any deleterious side-re-

(62) A. Knorr, *Ber.*, **50**, 229 (1917).
(63) M. L. Bender, *Chem. Revs.*, **60**, 53 (1960).
(64) W. P. Jencks and J. Carriuolo, *J. Am. Chem. Soc.*, **82**, 675 (1960).

actions were more likely to be detectable when the reaction had been conducted in the presence of a large excess of reagent. Most of the investigations on the protein–imidoester reaction were concerned with analysis of the derivatives of one protein, insulin, which had been prepared by treatment with large excesses of reagent.

In the amidination of proteins larger than insulin, the reaction of the ϵ-amino group is of major consequence. It was thus particularly important to establish that the ϵ-amino group of insulin could be stoichiometrically replaced by the amidino group. As judged by titration in formaldehyde and/or by the failure to release alanine during tryptic digestion, the loss of the lysyl ϵ-amino group was complete in preparations I, II and III. Electrophoretic analysis of tryptic digests showed the presence of only one modified octapeptide. Finally, the appearance of a basic amino acid, tentatively identified as ϵ-acetamidino-α-aminocaproic acid, after digestion of acetamidino insulin (preparation II) with carboxypeptidase A and B afforded proof that the amino group had indeed been converted to a monosubstituted amidino group.[65]

The several methods used to measure the extent of reaction at the lysyl residue of insulin are general and can be applied to other proteins. Treatment of the derivatives with DNFB and subsequent analysis of the hydrolysates have been developed by Cherry, Wofsy and Singer[67] into a quantitative method by the use of an amino acid analyzer. Titration in the presence of formaldehyde to determine the loss of ϵ-amino groups is rapid, technically easy and relatively accurate. Finally, it should be feasible to estimate the extent of the lysyl reaction by comparing base uptake during the tryptic digestion of modified and unmodified proteins.

Whereas the lysyl residue of insulin and most of the lysyl residues of oxidized ribonuclease reacted with 0.1–0.4 M methyl acetimidate in a few hours at room temperature, the presence of a few more than the predicted number of peptides in tryptic digests of acetamidino oxidized ribonuclease suggested that a few lysyl groups of this protein were less reactive than the majority and had been incompletely amidinated. However, Wofsy and Singer[66] have found that bovine serum albumin and rabbit γ-globulin can be totally amidinated by the use of more prolonged periods of reaction and somewhat greater concentrations of reagent.

As determined by titration data and electrophoretic analysis, extensive modification of the α-amino groups of insulin had also been achieved. In all preparations which were studied, it appeared that the α-amino group of the N-terminal glycyl residue had been completely modified. Reaction of the N-terminal phenylalanyl residue, however, seemed to be comparatively slow, since electrophoretic analysis showed the presence of an appreciable amount of **B** chain containing unreacted N-terminal phenylalanyl residues in preparations in which the A chain N-terminal glycyl residues had completely reacted.

The N-terminal phenylalanyl amino group of insulin is known to react relatively slowly with other reagents. Andersen[68] found that phenyl isocyanate reacted with the N-terminal glycyl residue much faster than with the N-terminal phenylalanyl residue, and Evans and Saroff[69] similarly noted that O-methylisourea reacted more readily with the glycyl than with the phenylalanyl residue. This lack of reactivity has been attributed to the structural conformation of the protein in the vicinity of the N-terminal phenylalanyl residue. However, the tenfold lower value of k^* which was obtained for the reaction of methyl benzimidate with phenylalanine as compared with glycine, suggests that at least some of the unreactivity of the insulin phenylalanyl residue was due to an intrinsic lower reactivity of the phenylalanyl amino group. Phenylalanine has a lower pK than glycine and would be expected to be a somewhat poorer nucleophile; steric hindrance might also contribute to the low order of reactivity, especially with the benzimidoester.

Although detailed kinetic studies were not performed on the imidoester–insulin reaction, the available data suggested that the reaction of proteins with imidoesters proceeded much as predicted from the studies with model compounds. Electrophoretic analysis of the reaction between insulin (1%) and β-dimethylaminopropionimidate (0.1 M) indicated that after five minutes of reaction, 90% of the insulin had been modified at one or more sites. The predicted half-times of reaction with methyl acetimidate under these conditions, calculated from the data in Table IV, are one minute for α-groups and about two minutes for ϵ-groups. The effect of pH on the protein reactions was not systematically investigated. However a single study of the reaction of the lysyl residue of insulin demonstrated that, as predicted, the reaction was much more rapid at pH 9.5 than at pH 6.7.

It is therefore expected that the reaction between proteins and imidoesters can, in general, be controlled in much the same way as the reactions involving smaller molecules. Only partial reaction of the ϵ-groups should take place at lower pH values where, per unit concentration of amine, the reaction of the α-group is favored. Decreasing the temperature from 25° to 1° decreases $k_{(app)}$ by about a factor of five; control of the reaction can thus be effected by temperature as well as by reaction time, imidoester:amine ratio and pH.

Investigation of the extent of reaction of the insulin amino groups was complicated by the appearance, after modification, of a new component first noticed as a minor slower-moving band (5–15%) in electrophoretic patterns of acetamidino insulins in 33% acetic acid. Whereas the mobility of this minor component in 33% acetic acid differed from that of insulin and the major reaction product by an amount corresponding to the loss of one

(65) This basic amino acid has also been found in hydrolysates of other acetamidino proteins and has been identified as ϵ-acetamidino-α-aminocaproic acid by other workers.[66]

(66) L. Wofsy, Ph.D. Thesis, Yale University, June, 1961; L. Wofsy and S. J. Singer, to be published.

(67) M. Cherry, L. Wofsy and S. J. Singer, to be published.

(68) W. Andersen, *Compt. Rend. Trav. Lab. Carlsberg, Ser. Chim.*, **30**, 104 (1956).

(69) R. L. Evans and H. A. Saroff, *J. Biol. Chem.*, **228**, 295 (1957).

positive charge per 6000 g., the minor band migrated ahead of the main reaction product at *p*H 9 and was thus more negatively charged at this *p*H. Unlike the unmodified B chain band, neither the major nor minor modified B chain bands reacted with ninhydrin. Electrophoretic analysis of tryptic digests of the separated chains indicated that the observed heterogeneity involved the N-terminal phenylalanyl–arginyl sequence in the B chain. It therefore seemed that the new component was due to a side reaction resulting in the loss of a positive charge by some group in this sequence of the molecule.

With the information available, one can only speculate as to the nature of this side-reaction. On the basis of the present specificity studies and from the failure of Cherry, Wofsy and Singer[67] to find any evidence for reaction of protein residues other than those containing amino groups, it appears most likely that some secondary product was formed by a reaction of the phenylalanyl amino group; whether this side-reaction was due to some very particular conformation at the phenylalanyl residue or whether the reaction is of a more general nature is as yet undetermined. Wofsy and Singer[66] have reported that some material of greater negative mobility than the parent albumin was present after electrophoresis of extensively reacted acetamidino bovine serum albumin in alkaline solution. This material could have been produced by a side-reaction similar to that discussed above. It is of interest that no heterogeneity was introduced by the modification of insulin with methyl benzimidate and that no side-reaction appeared to occur at the insulin A chain upon modification of the N-terminal glycyl residue by reaction with aliphatic imidoesters.

The solubilities of the amidino insulins did not differ appreciably from those of insulin. Quantitative solubility studies were not undertaken, but from such information as is available the behavior of the acetamidino derivatives was similar to that of comparable guanidinated proteins.

The observation that trypsin did not split the peptide bond adjacent to an amidinated lysyl residue, even though the residue still carried a positive charge, might have been expected since trypsin does not split peptide bonds adjacent to homoarginyl residues.[70] Imidoesters can thus be used for the limited degradation of proteins to large peptides, the usual first step in sequence analysis.

Reaction of the aliphatic imidoesters with proteins proceeds very much faster and more completely than does the reaction of O-methylisourea. Even benzimidoester reactions seem somewhat faster. Isoureas analogous to methyl benzimidate (*e.g.*, N-phenyl-O-methylisourea)[71] are almost completely unreactive. More importantly, amidination will proceed at relatively low *p*H values (8.5–9.5) whereas O-methylisourea reacts very slowly and incompletely with proteins below *p*H 10. The reaction of proteins with imidoesters is therefore much more likely to proceed without denaturation, an important consideration when the effect of reaction on physico-chemical or enzymatic properties is to be measured. In this regard the data on the physical properties of certain amidinated proteins reported by Wofsy and Singer[66] would indicate that extensive amidination had caused only minor, if any, conformational changes in these proteins.

The ease with which a variety of substituted imidoesters can be prepared, in contrast to the difficulties encountered in synthesizing substituted isoureas, permits the preparation of a variety of derivatives. Nitriles labeled with C^{14} can be readily obtained and may be used to introduce labeled carbon into proteins for metabolic or enzymatic investigations. Di-imidoesters, which can be prepared from dinitriles, are potential cross-linking agents. Moreover, colored reagents such as the one described in the present account can be extremely useful in structural studies. Derivatives in which the size, shape and charge of the substituent group can be varied systematically should assist in the correlation of physical measurements with structural parameters.

Acknowledgments.—Almost all the work reported in this paper was done in the laboratory of Dr. J. L. Oncley, and the authors would like to acknowledge a long-standing debt of gratitude to him for his continued help and guidance in all aspects of protein chemistry. They would also like to thank Dr. D. Waugh for permitting M.L. to continue some of this work in his laboratory and to thank Dr. W. P. Jencks for his help in the evaluation of the kinetic data.

(70) L. Weil and M. Telka, *Arch. Biochem. Biophys.*, **71**, 473 (1957).

(71) Various substituted O-methyl and S-methylisoureas were prepared by the authors. These were much less reactive than O-methylisourea and most of the compounds were tedious and difficult to prepare.