effect on the body weight stimulating effect of the androgen (Table II). The maximum daily nitrogen retention and total nitrogen retained also were not significantly changed ($P < 0.05$). Furthermore, an increase in the dose of the androgen (Table II) also did not produce any greater anabolic effects at either the 30 or 50% levels of protein intake.

Discussion. Once an adequate protein intake is provided a further increase in the protein intake of the rat by the replacement of part of the carbohydrate of the diet by an isocaloric amount of protein (casein) does not enhance the over all protein anabolic activity of testosterone propionate. Stimulation by androgen of the retention of nitrogen or growth processes apparently is regulated by inherent properties of the various cells.

Summary. The protein anabolic activity of testosterone propionate in adult male castrated rats was not altered by increasing the protein content of the diet from 18 to 28 and 43% by replacement of carbohydrate.

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New Inhibitors of Enzymatic Proteolysis.*† (19278)

OTTO SCHALES.

From the Chemical Research Laboratory, Alton Ochsner Medical Foundation, and the Department of Biochemistry, Tulane University School of Medicine, New Orleans.

Earlier reports from this laboratory(1,2) described the inhibition of pepsin, trypsin, papain, and cathepsins by several carbonyl group reagents. Since then, additional inhibitors of proteolytic processes have been found. The new inhibitors (guanidine, arginine, ethylenediamine, lysine, and others) show certain structural similarities to the compounds tested before, but they are not carbonyl group reagents.

Most of the new inhibitors were effective only when egg-white served as substrate and would not have been discovered if the current preference for synthetic substrates had been strictly adhered to. The observation that certain inhibitors of the proteolysis of natural substrates fail to interfere with the enzymatic hydrolysis of synthetic substrates, demonstrates that results obtained with well-defined synthetic systems are not necessarily applicable to more complex and more natural systems.

A. Experiments with egg-white as substrate. The results of these experiments are listed in Table I. Tests with a few aldehyde reagents and with pyrophosphate were carried out simultaneously for purposes of comparison. The substrate was prepared as described before(1) and the progress of hydrolysis was followed photoelectrically(1).

B. Experiments with casein as substrate. (a) Substrate for tests with pepsin—same as described before(1). *(b) Substrate for tests with trypsin and chymotrypsin*—a mixture of 2 g casein and 200 ml phosphate buffer (*M*/15; pH 8.30) was heated in boiling water for 15 minutes. After cooling, 20% NaOH was added dropwise until pH 8.4 was reached. *(c) Incubation mixtures*—to 4 ml substrate was added 1 ml water or 1 ml inhibitor solution, which had been adjusted to the pH of the incubation mixture with HCl or NaOH. The mixture was kept at 37°C for 10 minutes.

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TABLE I. Effect of Inhibitors on Enzymatic Hydrolysis of Egg-White.

Inhibitor	Mole/l	% inhibition after 30 min at 23°		
		Pepsin	Trypsin	Chymotrypsin
Guanidine	.10	62	94	84
Creatine	.04	48	25	—
Arginine	.04	58	75	64
Diethyldithiocarbamate	.02	52	50	—
Lysine	.10	71	88	66
o-Phenylenediamine	.02	—	75	74
	.04	79	—	—
Hydroxylamine	.02	26	55	27
Hydrazine	.02	81	65	38
	.10	—	96	90
Phenylhydrazine	.02	38	61	70
	.10	—	100	95
Ethylenediamine	.02	—	68	—
	.10	77	94	84
Tetracarboxymethyl-ethylenediamine	.02	—	85	65
	.10	—	99	91
Aniline	.02	33	—	—
	.10	—	27	—
Sulfanilic acid	.02	17	—	—
	.04	—	72	37
Sodium pyrophosphate	.01	—	75	62
	.10	60	96	95

Each inhibition test was performed together with a test in absence of inhibitor at the same pH (1.4-1.5 was the range for pepsin, 7.5-7.7 for trypsin, and 8.1-8.2 for chymotrypsin). The results from 6 readings each at 5 min intervals were plotted as pairs of time-hydrolysis curves. The degree of inhibition was then calculated from the inverse time-activity relationship. This method of evaluation was based on the finding that the times required to reach a given degree of hydrolysis were inversely proportional to the amounts of enzyme present. Enzyme concentrations (per 100 ml incubation mixture) were: cryst. pepsin .33 mg, cryst. trypsin 9 mg, and cryst. chymotrypsin 3.6 mg. The trypsin content of the cryst. trypsin preparation (Armour) was found to be about 40% by weight (protein nitrogen determination and measurement of ultraviolet absorption).

One ml enzyme solution (containing respectively 0.008-0.036 mg cryst. pepsin, or 1 mg cryst. trypsin, or 0.4 mg cryst. chymotrypsin), which had also been warmed to 37°C, was then added. The pH of the incubation mixtures was 1.61-1.63 (pepsin), and 8.1-8.4 (trypsin, chymotrypsin). *(d) Measurement of hydrolysis and calculation of degree of inhibition.* After 30 minutes incubation at 37°C, 10 ml 0.3 *N* trichloroacetic acid were added to each tube. After 10 minutes standing, the mixtures were filtered, and 1 ml portions of each filtrate were pipetted into colorimeter tubes containing 10 ml 0.3 *N* NaOH and 4 ml water. Three ml diluted (1:3) phenol reagent(3) were then added and the intensities of the resulting blue colors were measured using a Lumetron Colorimeter Model 402-E and filter 660. The degree of inhibition (expressed as % decrease in tyrosine containing, trichloroacetic acid soluble fragments) was calculated from the extinction measurements obtained in parallel experiments with and without inhibitors. Preliminary tests, made to ascertain that the inhibitors alone did not react with phenol reagent, removed hydrazine, hydroxylamine, o-phenylenediamine, phenylhydrazine, and Versene (tetrasodium salt of N,N,N′,N′-tetracarboxymethylethylenediamine) from consideration in this procedure. The inhibition of the peptic hydrolysis of casein by hydrazine had been determined previously(1) with the aid of ultraviolet absorption measurements in protein-free filtrates. The same technic was now used to determine the effect of hydroxylamine and of bisulfite on the peptic hydrolysis of this substrate. *(e) Results with pepsin.* Previous data showed(1) that the peptic hydrolysis of casein was inhibited by hydrazine, but that higher concentrations of this inhibitor were needed for this substrate than for egg-white

to give the same degree of inhibition. A similar observation was now made with guanidine as inhibitor. In presence of a guanidine concentration of 0.1 Mole/liter there was observed a 20% inhibition of the peptic hydrolysis of casein. The same concentration of guanidine inhibited the peptic hydrolysis of egg-white about 60% (Table I). Hydroxylamine (0.02 *M*) inhibited the hydrolysis of casein 32%, as compared to 26% after 30 minutes incubation time, when egg-white was used as substrate. Sodium bisulfite (0.002 *M*) inhibited 12%, whereas 8% inhibition were observed with egg-white as substrate. *(f) Results with trypsin and chymotrypsin.* In contrast to the observations with egg-white as substrate, no inhibition was caused by arginine (0.04 *M*), ethylenediamine (0.10 *M*), guanidine (0.10 *M*), lysine (0.10 *M*), and sulfanilic acid (0.10 *M*). Sodium pyrophosphate (0.018 *M*) produced a slight but reproducible inhibition of about 8% of trypsin as well as of chymotrypsin.

C. Experiments with synthetic substrates. (a) Pepsin and carbobenzoxy-L-glutamyl-L-tyrosine. It had been reported previously(1) that hydrazine (0.009 *M*) inhibited the effect of cryst. pepsin (7.3 mg/ml incubation mixture) on this substrate by 23%. Since then, the suggestion has been made that a cathepsin, contained in pepsin preparations, rather than pepsin itself might be inhibited by this agent (4). The experiments were therefore repeated using two different fresh preparations of cryst. pepsin. In 6 experiments, with the same enzyme and inhibitor concentrations as stated above, the inhibition varied from 0-4% (average 2.2%) and cannot be considered significant. Guanidine (0.1 *M*) under otherwise identical conditions, inhibited slightly (9-14%). *(b) Trypsin and benzoyl-L-arginineamide.* The incubation mixtures were prepared by adding 0.5 ml trypsin solution (20 mg cryst. trypsin dissolved freshly for each test in 5 ml water) to a mixture of 1 ml substrate solution (containing 0.05 millimole benzoyl-L-arginineamide in *M*/15 phosphate buffer, pH 7.86) and 0.5 ml inhibitor solution or water. The progress of hydrolysis at 37°C was followed with the Grassmann and Heyde procedure(5). In experiments with lysine and Versene interference by these substances with the observation of a sharp endpoint necessitated the use of an aeration method, similar to that described by Greenstein and Leuthardt(6), for the determination of the splitting of the substrate. In this method, the ammonia liberated by the action of trypsin was trapped in boric acid and measured by titration with HCl.

TABLE II. Inhibition of Tryptic Hydrolysis of Benzoylarginineamide by Guanidine, Lysine, and Arginine.

Inhibitor	Mole/l	pH	% inhibition after 30 min	60 min	2 hr
Guanidine	.05	7.48	23	15	23
	.10	7.45	47	42	41
	.15	7.40	55	55	54
	.20	7.42	60	58	56
Lysine	.10	7.57	19	18	19
Arginine	.04	7.52	7	4	15

First order reaction constants were calculated from the hydrolysis data and used to determine the degree of inhibition. The "constant" found after 2 hours incubation time was markedly lower than the corresponding values after 30 minutes and 1 hour, due to the inhibitory effect of benzoylarginine. The value for the "constant" at a given incubation time was, however, quite reproducible in numerous experiments with and without inhibitors.

The following substances did not inhibit the tryptic hydrolysis of benzoylarginineamide: hydrazine (0.10 *M*), phenylhydrazine (0.017 *M*), hydroxylamine (0.025 *M*), creatine (0.025 *M*), and Versene (0.030 *M*). Inhibition was observed in presence of guanidine, lysine, and arginine as shown in Table II.

D. Search for stable inhibitor-enzyme complexes. (a) Recrystallization experiments. Several samples of cryst. pepsin were recrystallized from either 25% alcohol or from 25% alcohol containing various concentrations of hydrazine (0.02-0.16 *M*). The time-hydrolysis curves obtained with the resulting products and casein as substrate were identical, indicating that the crystalline material was unchanged pepsin. *(b) Dialysis experiments.* Mixtures of enzymes (pepsin or trypsin) and inhibitors (hydrazine, guanidine, ethylenediamine) were dialyzed at pH 2.5-3.0 and samples were tested periodically against egg-white

as substrate. After 23-41 hours dialysis the mixtures, which had contained inhibitors at the beginning of the test, showed full activity, identical with that of enzyme solutions dialyzed at the same pH and 4°C in absence of inhibitors.

Discussion. It was found that, in addition to carbonyl group reagents, other compounds, which are structurally related to these reagents, interfere with enzymatic proteolysis. The diamines hydrazine, ethylenediamine, o-phenylenediamine, and Versene, for example, were found to inhibit egg-white hydrolysis. The effectiveness of Versene illustrates that free amino groups are not essential for inhibitory activity.

Since semicarbazide inhibited pepsin and trypsin(1), other carbamic acid derivatives were tested. Urea (0.10 *M*) was ineffective, but guanidine, and its derivatives creatine and arginine surpassed semicarbazide in inhibitory activity. Thiourea (0.10 *M*) showed no activity, but diethyldithiocarbamate (0.02 *M*) interfered markedly with the peptic and tryptic hydrolysis of egg-white.

As the effectiveness of the inhibitors described in this report depends quite obviously on the nature of the substrate used, one is inclined to assume that not the enzyme but the substrate seems to be influenced by the inhibitors in such a manner that it becomes more resistant to enzymatic attack. It has been shown by a number of investigators that the chemical manipulation of various proteins, such as benzoylation(7), reduction by sodium in liquid ammonia(8), or heating with glucose(9) resulted in resistance toward hydrolysis by one or several proteolytic enzymes. It is possible that some of the inhibitors described in this report act through a similar mechanism, which amounts, essentially, to the change of proteolysis facilitating side chains into less effective derivatives, such as addition products.

Summary. (1) The hydrolysis of egg-white by pepsin, trypsin, and chymotrypsin was inhibited by aniline, arginine, creatine, diethyldithiocarbamate, ethylenediamine, guanidine, lysine, o-phenylenediamine, pyrophosphate, sulfanilic acid, and a variety of carbonyl group reagents. The hydrolysis of this substrate by trypsin and chymotrypsin was also inhibited by N,N,N',N'-tetracarboxymethylethylenediamine. (2) The peptic hydrolysis of casein was inhibited by guanidine, and by several carbonyl group reagents. The hydrolysis of casein by trypsin and chymotrypsin was not inhibited by arginine, ethylenediamine, guanidine, lysine, and sulfanilic acid. (3) The hydrolysis of carbobenzoxy-L-glutamyl-L-tyrosine by pepsin was not influenced by hydrazine, but was slightly (9-14%) inhibited by guanidine. The effect of trypsin on benzoyl-L-arginineamide was inhibited by arginine, guanidine, and lysine, but not by creatine, hydrazine, hydroxylamine, phenylhydrazine, and Versene. (4) Dialysis and recrystallization experiments with enzyme-inhibitor mixtures led to the recovery of fully active enzymes. The experimental evidence suggests that not the enzymes but the substrates are influenced by the inhibitors listed above, in such a manner that they become more resistant to enzymatic attack.

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