not known from which part of the stomach they originate. Study of porcine fundic mucosal extracts in analogy to our studies (Seijffers *et al.*, 1963b) may reveal the presence of a fourth porcine pepsin and could more exactly reveal the quantitative relationship of these pepsins in different parts of the stomach. At present it can only be noted that parapepsin I is relatively alkali stable and that it is eluted before the other porcine pepsins from DEAE-cellulose (Ryle and Porter, 1959); in these respects it resembles human pepsin I. As most work pertaining to characterization of pepsins has been carried out with what has been designated as "crystalline porcine pepsin," it becomes clearly important to carry out studies which will establish the identity of "crystalline porcine pepsin" and its relationship to the human pepsins described in this report.

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Structural Studies of Ribonuclease. XIV. Tryptic Hydrolysis of Ribonuclease in Propyl Alcohol Solution*

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The unfolding of ribonuclease occurs at a lower temperature in aqueous *n*-propyl alcohol solution than in water, presumably because of the effect of the alcohol on hydrophobically bonded regions of the ribonuclease molecule. Tryptic digestion of ribonuclease in 25% propanol at 35° was employed to determine the region of the ribonuclease molecule which unfolds in aqueous alcohol. The digestion products were purified by IRC-50 chromatography. Two major components (*a* and *b*) were obtained. On the basis of amino acid composition and N- and C-terminal-group data, component *a* was found to be identical with component 1, a species obtained by digestion of component IV by trypsin in water at room temperature (Ooi and Scheraga, 1964a); component *b* is unreacted ribonuclease plus a small amount of component 5, obtained along with component 1 from component IV. Therefore the same regions of the ribonuclease molecule (between Lys 31 and Lys 37) are involved in the thermal transitions in 25% propanol at 35° and in water at 60°, respectively. Both transitions presumably involve the disruption of the same hydrophobically bonded regions of ribonuclease.

Even though trypsin and chymotrypsin do not digest native ribonuclease at room temperature, they do attack it at elevated temperatures, where the substrate undergoes a thermal transition (Rupley and Scheraga, 1963; Ooi *et al.*, 1963; Ooi and Scheraga, 1964a,b). In fact, it has been possible to identify the regions of the ribonuclease molecule which are involved in the thermal transition by determining the sites of initial proteolysis by trypsin and chymotrypsin, respectively.

The thermal-transition temperature of ribonuclease is lowered by the addition of alcohols at neutral *p*H (Schrier and Scheraga, 1962). This result has been attributed to the breaking of hydrophobic bonds in ribonuclease. It is thus conceivable that the parts of the chain which are exposed upon heating in the presence of alcohol might be different from those exposed when the heating is carried out in the absence of alcohol. If this were the case, then differences might be found in the sites of initial proteolysis by trypsin. Therefore experiments were carried out to determine whether trypsin is active in *n*-propyl alcohol and, if so, to identify the regions of the ribonuclease molecule which are attacked by trypsin in the presence of alcohol.

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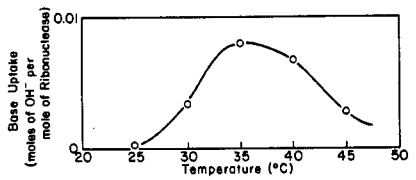

FIG. 1.—Temperature dependence of the degree of digestion after one addition of 28 μg of trypsin to 5 mg of ribonuclease in 5 ml of 25% propyl alcohol, 0.01 M KCl, pH 7.3. The value of the "base uptake" was taken as the asymptotic limit where digestion ceased.

EXPERIMENTAL

Materials.—Essentially all the materials and procedures used in this investigation have been described previously (Rupley and Scheraga, 1963; Ooi *et al.*, 1963; Ooi and Scheraga, 1964a).

N-Benzoyl-L-arginine ethyl ester (BAEE)[1] was obtained from Mann Research Laboratories, Inc. Other chemicals such as *n*-propyl alcohol were AR grade where available.

Method.—Trypsin activity in propyl alcohol was measured by a spectrophotometric method (Schwert and Takenaka, 1955). Digestion of ribonuclease with trypsin was carried out in a *p*H-stat (Radiometer TTT1) at constant temperature. The ribonuclease concentration was in the range of 1–10 mg/ml in 0.01 M KCl at *p*H 7–8.

In analytical experiments, chromatograms of the reaction mixtures on an IRC-50 column were monitored with a Technicon Autoanalyzer at a flow rate of 0.35 ml/minute. In preparative-scale experiments, the effluent was collected with a fraction collector, and the optical density of each tube was measured at 278 mμ with a Beckman DU spectrophotometer; the ninhydrin color of each tube was developed and recorded with a Technicon sampler device. In both cases gradient elution was employed, varying from 0.15 M sodium phosphate, 1 mM Versenate buffer, *p*H 6.40 to 1 M sodium phosphate, 1 mM Versenate buffer, *p*H 6.47. The column sizes were 1 × 30 cm for analytical-scale experiments and 2 × 40 cm for preparative-scale work.

Gel filtration (Sephadex G-25) was used for desalting the fractions obtained from the IRC-50 column. Amino acid analyses and C- and N-terminal-residue determinations were carried out by the same procedures described in the previous papers (Rupley and Scheraga, 1963; Ooi *et al.*, 1963).

RESULTS

Trypsin Activity in Alcohol.—The activity of trypsin at *p*H 8.0 toward the synthetic substrate BAEE in 25% (vol) *n*-propyl alcohol was measured at half-minute intervals over a period of 5 minutes after the addition of trypsin. The activity was expressed as the slope of the straight line resulting from the plot of the change in optical density at 253 mμ against time. Expressed in this way, the activity in alcohol at room temperature during the first 3 minutes was about 70% of that in the absence of alcohol. However, the optical density–versus–time plots showed a gradual loss of trypsin activity after 3 minutes.

Digestion and Chromatography of Ribonuclease.—Recognizing that trypsin loses its activity in alcohol, we might expect that the digestion of ribonuclease would begin upon addition of trypsin, but that the rate of digestion would decrease, ultimately to zero, as the tryptic activity decreased. In order to choose the optimum conditions for the digestion, the temperature dependence of the degree of digestion, caused by one addition of a given amount of trypsin to a given amount of ribonuclease in 25% propyl alcohol, was investigated. The degree of digestion, taken as the asymptotic, or final, value of the amount of base consumed before the trypsin lost all its activity, is plotted against the temperature in Figure 1. The resulting curve is bell-shaped with a maximum around 35°. The shape of this curve arises from two effects in alcohol solution: (1) the decreasing activity of trypsin as the temperature is raised, and (2) the increasing susceptibility of ribonuclease to digestion (owing to unfolding) as the temperature is raised. Even though a higher concentration of alcohol (e.g., 30%) increases the degree of unfolding of ribonuclease at a given temperature, it also increases the rate of denaturation of trypsin, and thus slows down the digestion. The optimum conditions for obtaining a reasonable rate of digestion appear to be 25% propyl alcohol and 35°; therefore all experiments were carried out under these conditions.

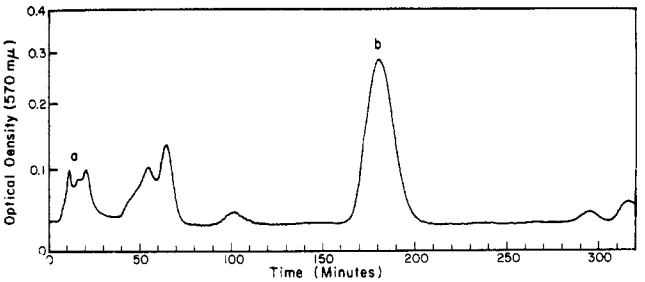

FIG. 2.—Chromatogram of analytical scale digestion mixture (after lyophilization to remove alcohol) on an IRC-50 column. The ordinate represents the ninhydrin color value in arbitrary units at 570 mμ, and the abscissa is the effluent volume on a time scale on which 0.35 ml corresponds to 1 minute.

The analytical-scale experiments were performed by successive addition of trypsin to a ribonuclease solution to obtain a degree of digestion where new components could be observed by chromatography of the digestion mixture on an IRC-50 column. When the reacted material was chromatographed on the IRC-50 column, the propyl alcohol was somewhat retarded, i.e., it did not move with the front, perhaps due to a hydrophobic interaction between the resin and the hydrocarbon group of the alcohol (Steinberg and Scheraga, 1962). This produced a disturbance of the chromatographic pattern which could be avoided either by dilution of the reaction mixture by a factor of 10 or by removal of the alcohol by lyophilization before applying the material to the column.

The chromatogram (from lyophilized material) of the analytical scale digestion mixture is shown in Figure 2. Ribonuclease itself normally appears approximately in the middle of the chromatogram (near the position of peak *b*). Apparently several new peaks, other than ribonuclease, appear in the chromatogram. As will be shown, these new peaks correspond both to large components and to small peptides. It is interesting to note that no peaks corresponding to components III and IV, obtained by tryptic digestion in water at high temperature (Ooi *et al.*, 1963), could be observed in Figure 2. These peaks normally appear after that of native ribonuclease.

In order to follow the course of the digestion, the chromatographic analysis was carried out at several stages of digestion. Patterns similar to that of Figure 2 were obtained, the only difference being in the relative sizes of each peak, i.e., the size of peak *b* decreased while those of the other peaks increased with increas-

[1] Abbreviations used in this work: BAEE, *N*-benzoyl-L-arginine ethyl ester; FDNB, 1-fluoro-2,4-dinitrobenzene.

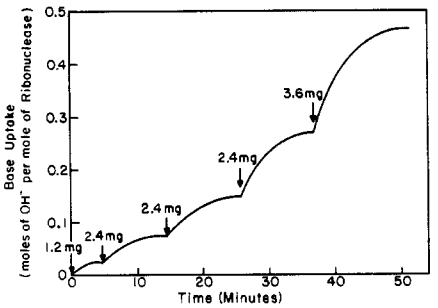

FIG. 3.—Time course of the digestion of 270 mg of ribonuclease in 24 ml of 25% propyl alcohol, 0.01 M KCl, *p*H 8.1 at 35°. The amount of trypsin added at various time intervals is also indicated.

ing time of digestion. Therefore the new peaks in the chromatogram of Figure 2 arise from products of the tryptic digestion of ribonuclease in alcohol solution.

The preparative-scale experiments were carried out in a manner similar to that used in the analytical-scale experiments. Figure 3 shows the time course of the digestion of 270 mg of ribonuclease in 24 ml of 25% propyl alcohol, 0.01 M KCl, *p*H 8.1 at 35°, by successive additions of trypsin, as indicated in the figure. Because of the gradual denaturation of trypsin, it was necessary to add trypsin at frequent intervals to obtain the desired degree of digestion of ribonuclease. It is interesting to note that the degree of digestion obtained by each addition of trypsin increased with each successive addition. This probably indicates that the digestion of ribonuclease proceeds more readily once the first one or two peptide bonds are cleaved.

It is also worth noting that the initial degree of digestion in alcohol at 35° is much less than that in water at 60° (Ooi *et al.*, 1963). Under the former conditions the amount of base consumed in 3 minutes after addition of trypsin is 0.0167 that under the latter conditions, even though a 10-fold excess of trypsin was used in the presence of alcohol. Even taking into account the loss of tryptic activity in alcohol, it appears that the conformation of ribonuclease in alcohol may be such as to make the molecule less susceptible to tryptic attack at 35° than it is in water at 60°.

To avoid the appearance in the chromatogram of a peak corresponding to trypsin, the total amount of trypsin used in the digestion was limited to about 0.05 that of ribonuclease.

An IRC-50 chromatogram of the preparative-scale digestion mixture is shown in Figure 4. Two major peaks (*a* and *b*) of high optical density at 278 mμ appeared, suggesting that the new components are of large molecular weight. The other peaks of low optical density at 278 mμ but of high ninhydrin color value are assumed to correspond to small peptides.

After separation of the two major components (*a* and *b*) and desalting by gel filtration, then amino acid analyses and N- and C-terminal-residue determinations were carried out as described.

Amino Acid Analyses.—The amino acid compositions of components *a* and *b* are given in Table I. It can be seen that component *a* is missing 22 amino acids, compared to the native molecule. Keeping in mind the results of the tryptic digestion of material previously designated component IV, component *a* appears to be identical in amino acid composition with material which was designated component 1 in the previous paper (Ooi and Scheraga, 1964a), i.e., component *a* appears to be lacking the peptides: Lys 1–Arg 10, Ser 32–Lys 37, and Thr 99–Lys 104.

Component *b*, which appeared in the same position in the chromatogram as does native ribonuclease, has the same amino acid composition as ribonuclease. However, the accuracy of the amino acid composition data does not exclude the possibility that component *b* may be a mixture of native ribonuclease and a very small amount of a digestion product of different amino acid composition which elutes at the same position as native ribonuclease. Specifically, component 5, missing the Ser 32–Lys 37 hexapeptide (Ooi and Scheraga, 1964a), appears in the position in the chromatogram normally occupied by native ribonuclease, and could be present in small amount as part of component *b* (*vide infra*).

N-Terminal Group Analyses.—The N-terminal residues, obtained by the FDNB method, are listed in Table II. Component *a* has N-terminal Glu, Asp,

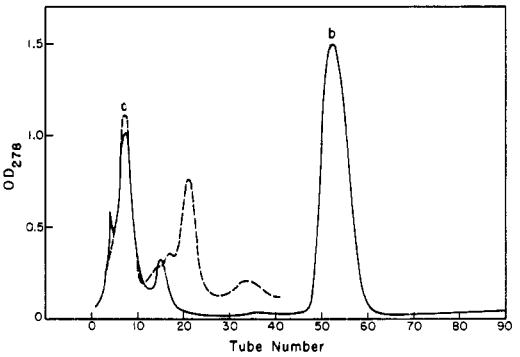

FIG. 4.—IRC-50 chromatogram of preparative-scale digestion mixture after removal of alcohol by lyophilization. The ordinate represents the optical density at 278 mμ (solid line) and the ninhydrin color value (broken line) in arbitrary units at 570 mμ. The flow rate was 1.2 ml/minute, and 6 ml was collected in each tube. The digestion mixture was obtained by the addition of a total of 12 mg of trypsin (in the increments shown in Fig. 3) to 270 mg of ribonuclease. The ninhydrin color was developed only until tube number 40.

TABLE I
AMINO ACID COMPOSITIONS OF COMPONENTS *a* AND *b*[a]

	Component *a* (moles amino acid/mole protein)			Component *b* (moles amino acid/mole protein)	
	Theoretical	Exptl	Amino Acids Missing	Theoretical	Exptl
Aspartic acid	13	12.9	2	15	15.0
Threonine	6	6.2	4	10	9.8
Serine	14	14.5	1	15	14.8
Glutamic acid	9	8.8	3	12	12.0
Proline	4	4.5		4	4.3
Glycine	3	3.4		3	3.1
Alanine	8	8.2	4	12	11.7
Half-cystine	8	8.2		8	7.8
Valine	9	8.7		9	8.8
Methionine	4	3.4		4	3.5
Isoleucine	3	2.9		3	1.9
Leucine	1	1.4	1	2	1.9
Tyrosine	6	5.6		6	5.7
Phenylalanine	2	2.0	1	3	2.7
Lysine	6	6.3	4	10	10.4
Histidine	4	4.3		4	4.5
Arginine	2	1.8	2	4	4.0

[a] The same hydrolysis losses were assumed as described in the previous paper (Rupley and Scheraga, 1963). An average value of all the amino acids was used as the basis for the calculations. The theoretical values were taken as those which best fitted the experimental values. The analyses were carried out on unoxidized material.

TABLE II
N-TERMINAL GROUP ANALYSES

	Component *a* (moles DNP–amino acid/mole component)	Component *b* (moles DNP–amino acid/mole component)
Aspartic acid / Glutamic acid	1.5[a]	0.12
Lysine		0.98
Histidine	0.2[b]	

[a] The ratio of aspartic to glutamic acid was not determined. [b] Not corrected for hydrolysis and chromatographic losses.

and His, probably corresponding to Glu(NH_2) 11, Asp 38, and His 105. This supports the tentative conclusion, based on the amino acid–composition data, that component *a* is identical with component 1 of the previous paper.

Component *b* has the N-terminal lysine of ribonuclease and about 0.1 this amount of N-terminal aspartic acid, suggesting that component *b* is primarily ribonuclease, but containing a small amount of another component.

C-Terminal-Group Analyses.—The C-terminal residues were obtained by digestion of unoxidized material with a mixture of carboxypeptidase A and B. The data are listed in Table III.

TABLE III
C-TERMINAL AMINO ACIDS OF COMPONENTS *a* AND *b* RELEASED BY CARBOXYPEPTIDASE A AND B

	Component *a* (moles amino acid/mole component)	Component *b* (moles amino acid/mole component)
Valine	0.35	0.30
Serine	0.35	0.28
Alanine	0.42	0.40[a]
Lysine	2.00[b]	1.00[b]
Methionine	0.98	0.50
Tyrosine	0.56	

[a] It is not clear why this value is greater than those for serine and valine. [b] The data of Table III were computed relative to assumed values for lysine (2.00 for component *a* and 1.00 for component *b*).

The presence of Lys, Met, and Tyr in component *a* (in addition to Val, Ser, and Ala, the C-terminal residues of native ribonuclease) is compatible with the conclusion that component *a* is identical with component 1 of the previous paper, i.e., these additional residues presumably correspond to Lys 31, Met 30, Met 29, Lys 98, and Tyr 97.

The new C-terminal residues of component *b* probably correspond to Lys 31, Met 30, and Met 29. Had oxidized instead of unoxidized protein been used for this determination, additional residues from the C terminus of native ribonuclease would have been obtained; these would have made it difficult to detect the lysine and methionine from the new component present in small amount in component *b*. Carboxypeptidase does not release significant amounts of C-terminal amino acids from unoxidized native ribonuclease (Sela *et al.*, 1957), whereas it does from tryptic components of ribonuclease (Ooi and Scheraga, 1964a). Therefore the presence of new C-terminal amino acids in component *b* indicates that it contains a new component other than ribonuclease A.

Identification of Components.—Combining the information from the amino acid analyses and the N- and C-terminal-group analyses, we can identify components *a* and *b*. Component *a* is missing 22 amino acid residues, its N-terminal residues are Glu, Asp, and His, and its C-terminal residues are Lys, Tyr, and Met, in addition to Val, Ser, and Ala. This serves to identify component *a* as component 1 of the previous paper, i.e., one which is missing the peptides Lys 1–Arg 10, Ser 32–Lys 37, and Thr 99–Lys 104. Further, component *a* appeared near the front in the chromatogram from the IRC-50 column, similar to component 1.

From the amino acid composition data, component *b* appears to be primarily unreacted ribonuclease. However, the amino acid analytical data are not sufficiently precise to exclude the possibility that this component is contaminated by a small amount of a digestion product. Indeed, the end-group analyses suggest that component *b* contains about 10% of a contaminant which may be identified as component 5 of the previous paper, i.e., one missing the hexapeptide Ser 32–Lys 37. In agreement with this conclusion is the observation (Ooi and Scheraga, 1964a) that component 5 eluted from IRC-50 in the same position as does ribonuclease.

Thus the sites of tryptic attack of ribonuclease in alcohol at 35° are Arg 10–Glu(NH_2) 11, Lys 31–Ser 32, Lys 37–Asp 38, Lys 98–Thr 99, and Lys 104–His 105, the same positions as found for the tryptic digestion of component IV at room temperature (Ooi and Scheraga, 1964a).

DISCUSSION

The results indicate that trypsin attacks ribonuclease in 25% propyl alcohol at 35° in the same positions that it attacks component IV in water at room temperature. Thus, the thermal transition in alcohol at 35° involves the region from Lys 31 to Asp(NH_2) 34, the same as is involved in water at 60°. Presumably the hydrophobic bonds in the region between Cys 26 and Cys 40, which are weakened in alcohol (providing the lower thermal-transition temperature in this solvent), are also involved in the thermal transition in water. The weakening of the hydrophobically bonded regions of ribonuclease by the alcohol still does not loosen the structure sufficiently to allow digestion at room temperature; heating to 35° is required for unfolding.

However, even though the sites of tryptic attack are the same in alcohol and in water, certain differences appear. Specifically, components IV and III, obtained in the digestion in water at 60°, do not appear in the chromatograms shown in Figures 2 and 4. Based on the appearance of component 5 as a minor constituent of component *b*, the first large fragment detectable in the proteolysis in alcohol is the one missing the hexapeptide Ser 32–Lys 37. Presumably components IV and III *are* formed, but have a conformation in alcohol that renders them susceptible to tryptic attack to produce component 5. Further digestion of component 5 leads to component *a*, i.e., component 1. This result is similar to that obtained previously with component IV (Ooi and Scheraga, 1964a).

Based on studies with chymotrypsin (Rupley and Scheraga, 1963), the region near Tyr 25 is also involved in the thermal transition in water at 60°. We cannot make any statement about the involvement of this portion of the molecule in the thermal transition in alcohol, since our experiments with chymotrypsin *in alcohol* indicated that this proteolytic enzyme becomes denatured too rapidly to be effective in attacking ribonuclease.

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Experiments on the Modification of Myosin Nucleosidetriphosphatase*

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In the sense that they activate the 25° Ca^{2+}-adenosine-5′-triphosphatase, inhibit the 25° Ca^{2+}-inosine-5′-triphosphatase, and inhibit the 0° activity of both nucleosidetriphosphatases, dioxane and hydrogen peroxide are true "modifiers" of myosin nucleosidetriphosphatase. Modification by peroxide, as by *p*-mercuribenzoate, clearly involves reaction of certain myosin sulfhydryl groups, but no compelling evidence for sulfhydryl involvement was found in modification by dioxane, and there was found some direct evidence against sulfhydryl involvement in modification by 2,4-dinitrophenol. On the other hand, the groups on the myosin molecule *directly* responsible for either activating or inhibiting phases of "modification" are probably the same for all modifiers. This hypothesis, expressed quantitatively in a stochastic model, was affirmatively tested in the case of *p*-mercuribenzoate and 2,4-dinitrophenol. It was found incidentally that histidine protects against modification which involves oxidation because histidine itself is easily oxidized in a reaction involving its amino group.

We are concerned in this paper with interpreting experiments in which the nucleosidetriphosphatase activity of Ca^{2+}-myosin is modified by various reagents. Such an objective presumes that the activity of *un*modified enzyme is understood, and this is not really the case. However, as a result of the work of many laboratories, there is a hypothesis about unmodified and modified myosin catalysis into which many observations seem to fit, and it is useful to state this hypothesis at the outset and in some degree to justify it *a posteriori*.

A Hypothesis about Myosin Nucleosidetriphosphatase.—In most experiments with substrate-saturated and Ca^{2+}-saturated myosin, the substrate is ATP, the temperature is *ca.* 25°, and the *p*H is near neutral. These conditions have come to define the "normal" or reference behavior of the enzyme, and one can discuss consequences of changing conditions; for example, at 25° a partial titration of the sulfhydryl content activates ATPase and inhibits ITPase,[1] but at 0° the same titration inhibits both. We (see Gilmour, 1960; Morales and Hotta, 1960) have felt it helpful to focus on more general properties of the enzyme and of the reagents which affect it. In our terms, the crucial properties of myosin catalysis are as follows (see also Figs. 1 and 2).

The *p*H-dependence of myosin NTPase is sigmoidal (Fig. 1), but at 25° the curve of ATPase has an "aberration," viz., a depression near *p*H 7–8. The rate-modifying substances (e.g., SH reagents) inhibit the NTPase of a "normal" sigmoidal curve. However, if one starts with the "aberrant" 25° ATPase, the modifiers first return its *p*H-dependence to the normal sigmoid shape (this involves an "activation" in the neutral range), and then on further addition they inhibit, just as they inhibit any other "normal" system.

Because near neutrality ATP and ITP have similar ionization behavior, and because the temperature coefficient of disappearance of the ATPase "aberration" is so high, it is natural to assume (as have Koshland, Blum, and Gilmour: Levy, Sharon, and Koshland, 1959; Blum, 1960; Gilmour and Griffith, 1957) that the relatively slow ATPase at 25° and neutral *p*H are due to a conformation of the enzyme (or of the water near the enzyme, Klotz (1963), which disappears toward either *p*H extreme or with lowered temperature. We may call this hypothetical inhibitory conformation α, and call the normal sigmoidal conformation obtaining elsewhere β. Furthermore we must assume that the passage from α to β and on to a state of total inhibition (γ) is a continuum, and that potentially all modifiers, in appropriate increments or in concert, are capable of effecting any portion of the $\alpha \rightarrow \beta \rightarrow \gamma$ transition. Now it has to be assumed that ITP is not only a substrate but also a modifier,[2] so that even at 25° and neutral *p*H, ITP in-

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[1] The following abbreviations are used: AET, *S*-β-aminoethylisothiuronium bromide; NTP, nucleosidetriphosphate; NTPase, nucleosidetriphosphatase; ITPase, inosine-5′-triphosphatase; DNP, 2,4-dinitrophenol; GSH, glutathione; IAA, iodoacetamide.

[2] One may alternatively construct a hypothesis in which ITP is "passive" and ATP induces the α form. However, this hypothesis contains two undesirable features: modifiers and ATP now "pull" in opposite directions, and either an ATP-induced rearrangement of reagent (reagent added first, then ATP) or an ATP protection of deactivating sites from reagent (ATP added first, then reagent) must be invoked to explain why 25° ATPase is not inhibited by initial additions of modifier.