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Different Allosteric Properties of Nucleoside Diphosphatase Isoenzymes from Rat Liver*

R. Parvin and Roberts A. Smith

ABSTRACT: The nucleoside diphosphatase activity of the rat liver microsomal fraction behaved differently from the nucleoside diphosphatase activity of the rat liver supernatant fraction. The microsomal enzyme was effectively inhibited by adenosine triphosphate; several other nucleoside phosphates were much less inhibitory. This adenosine triphosphate inhibition, was found to be competitive with respect to substrate inosine diphosphate, as it decreased the affinity of the enzyme for substrate without affecting the maximum velocity. The plot of percentage inhibition against adenosine triphosphate concentration was sigmoidal showing cooperative effects in the inhibition. The nucleoside diphosphatase activity of the supernatant fraction, on the other hand, was markedly stimulated by adenosine triphosphate. In this case adenosine triphosphate increased both the maximum velocity as well as the affinity of the enzyme for its substrate, inosine diphosphate. From these and other differences noted it is concluded that the microsomal and supernatant nucleoside diphosphatase activities are due to two different enzymes. The possible metabolic significance of opposite modulatory effects of adenosine triphosphate on these rat liver isoenzymes is discussed.

During recent studies with rat liver microsomal glucose 6-phosphatase and other activities associated with it, a very active nucleoside diphosphatase was observed. Later studies showed that this nucleoside diphosphatase was different from the one reported by Nordlie and Arion (1965) as it could be separated from glucose 6-phosphatase activity by deoxycholate treatment and ammonium sulfate fractionation. Furthermore this preparation (after glucose 6-phosphatase removal) was not able to catalyze phosphoryl transfers from nucleoside di- or triphosphates to glucose.

Nucleoside diphosphatase which catalyzes the hydrolysis of IDP, GDP, and UDP, but not of ADP or CDP, to the corresponding nucleoside monophosphate and P_i has been studied earlier by Plaut (1955), by Gibson *et al.* (1955), by Novikoff and Heus (1963), and by Ernster and Jones (1962). An allosteric activation by ATP of a nucleoside diphosphatase preparation

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purified from rat liver acetone powder has been reported by Yamazaki and Hayaishi (1965). We wish to report here that nucleoside diphosphatase obtained from the rat liver microsomal fraction is inhibited by physiological concentrations of ATP while the nucleoside diphosphatase present in microsome free rat liver supernatant fraction was activated by ATP. From these observations as well as other differences noted in their kinetic properties it is concluded that the microsomal and supernatant nucleoside diphosphatases are two different enzymes. The possible significance of the opposite modulatory effect of ATP on the microsomal enzymes is also considered.

Materials and Methods

ATP, UDP, UTP, IDP, ADP, and CTP were obtained from P-L Biochemicals; AMP, GDP, and CDP from Calbiochem; and glucose 6-phosphate dehydrogenase and Tris (Sigma-121) from Sigma Chemical Co. $MgCl_2$ and sucrose were Baker Analyzed product.

Rat liver homogenate (10% w/v) was prepared in 0.25 M sucrose. The supernatant obtained after sedimenting mitochondria at 10,000*g* for 10 min was centrifuged at 105,000*g* for 1 hr to obtain microsomes and microsome-free supernatant.

Enzyme activity was assayed by measuring the P_i formed by the method of Marsh (1959). The usual assay system contained 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM nucleoside diphosphate, and enzyme in a final volume of 0.5 ml. The reaction was allowed to proceed at 30° for 10 min and was stopped by the addition of 0.1 ml of 12% trichloroacetic acid. P_i was estimated on an aliquot after removal of protein by centrifugation. A zero-time control was always included to account for the presence of P_i in the reactants. When effects of ATP on nucleoside diphosphatase were being studied, adequate controls were concurrently run to allow correction for a small ATPase activity of the enzyme preparation. One unit of enzyme activity was defined as the amount of enzyme catalyzing the liberation of 1 μmole of P_i/mg of protein per min under the assay condition.

Results

The microsomal enzyme was partially purified to remove other interfering enzymes. Rat liver microsomes were treated with 0.3% deoxycholate in 0.25 M sucrose. The supernatant obtained from the deoxycholate-treated microsomes on centrifugation for 2 hr at at 105,000*g*, was fractionated with ammonium sulfate. The fraction, precipitated between 60 and 80% saturation of ammonium sulfate, was tenfold purified over the microsomal preparation. The specific activity of this preparation was 0.81, 1.07, and 0.63 units per mg of protein with UDP, IDP, and GDP, respectively, while ADP and CDP were not hydrolyzed. The following enzymes were not detectable in the purified preparation: adenosine triphosphatase, nucleoside triphosphate AMP kinase (as judged by incubating UTP and AMP with nucleoside diphosphatase), ATP monophosphate kinase (by incubating ATP and UMP with enzyme), nucleoside diphosphate kinase (Bessman, 1963, and by estimating ATP concentration after incubating with enzyme and UDP), myokinase, and glucose-6-phosphatase.

The following procedure was used for the partial purification of the supernatant enzyme. The microsome free supernatant fraction was subjected to ammonium sulfate fractionation. The fraction obtained between 40 and 70% ammonium sulfate saturation was taken up in 0.05 M Tris-HCl (pH 7.5) containing 1 mM mercaptoethanol and applied on a Sephadex G-200 column (3.5 × 38 cm) equilibrated with buffer of the same composition. Elution was carried out using the same Tris–mercaptoethanol solution and fractions of 10 ml each were collected. The peak enzyme activity was usually found around the 24th fraction but this enzyme was very unstable under these conditions and because of loss of activity the extent of over-all purification was only about fourfold. However, this fractionation sufficed to remove most of the ATPase activity found in the original supernatant fraction. The enzyme could not easily be stabilized even by the inclusion in the buffer of mercaptoethanol and EDTA. It lost activity on storage at 4 or −15°.

The effect of some nucleoside phosphates on the microsomal nucleoside diphosphatase activity is given in Table I. Various nucleoside phosphates tested inhibited the enzyme activity; however, ATP was most effective in this regard. The inhibition pattern remained the same when UDP was used in place of IDP as substrate. It was ascertained by the enzymatic estimation of ATP (Kornberg, 1950) at different time intervals that ATP itself did not undergo transformation while altering the nucleoside diphosphatase activity. The effect of varying IDP concentration on the enzyme activity with or without ATP is given in Figure 1A. As is evident, the ATP inhibition increased with ATP concentration. A double-reciprocal plot of the data (Figure 1B) shows that ATP inhibition is competitive with respect to IDP. Thus the apparent Michaelis constant of the microsomal enzyme for IDP increased 2.5- and

TABLE I: Effect of Nucleoside Phosphates on the Partially Purified Microsomal Nucleoside Diphosphatase Activity[a]

Addition (final concn 5 mM)	P_i Formed (μmoles)	Inhibn (%)
None	204	
ATP	64	68.5
UTP	125	38.6
ADP	130	36.2
CTP	166	18.6
AMP	180	11.8

[a] Assay system in 0.5 ml contained: 50 μmoles of Tris-HCl (pH 7.5), 2.5 μmoles of $MgCl_2$, 0.25 μmole of IDP, 100 μg of the purified enzyme, and 2.5 μmoles of indicated nucleoside phosphate were present.

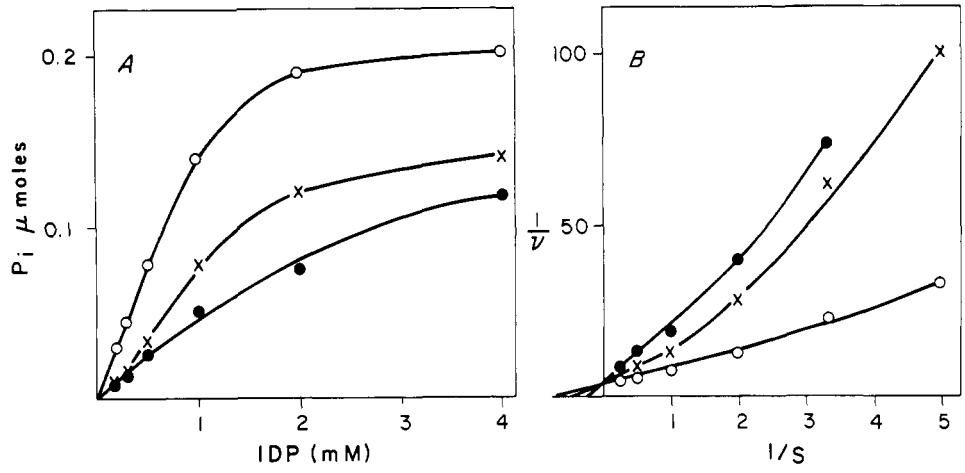

FIGURE 1: Effect of ATP on the rate of the microsomal nucleoside diphosphatase reaction as a function of substrate concentration. (A) The assay system contained 0.1 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 25 μg of partially purified enzyme, and IDP as indicated. Final volume 0.5 ml. (●—●) With 5 mM ATP, (x—x) with 3 mM ATP, and (○—○) no ATP. (B) The reciprocal of data of part A.

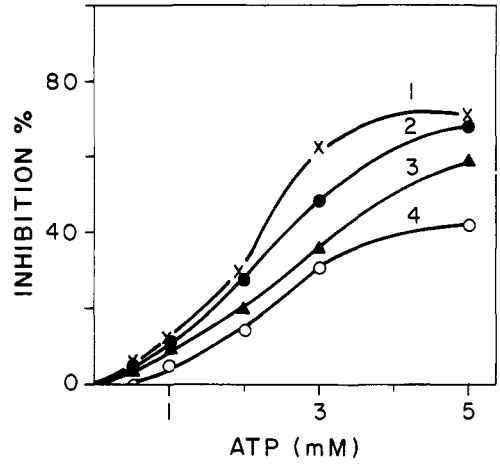

FIGURE 2: Inhibition of microsomal nucleoside diphosphatase by ATP. The assay system was the same as in Figure 1, except that the IDP concentration for curve 1, 2, 3 and 4 was, respectively, 0.3, 0.5, 2.0, and 4.0 mM. Concentration of ATP was as indicated.

TABLE II: The Effect of Nucleoside Polyphosphates on the Microsome-Free Supernatant Nucleoside Diphosphatase.[a]

Substrate		Activator	P_i Liberated (mμmoles)	% Act.
IDP			96	100
None		ATP	4	
None		UTP	12	
None		CTP	45	
None		ADP	9	
None		AMP	48	
IDP	+	ATP	259	159
IDP	+	UTP	175	62
IDP	+	CTP	182	24
IDP	+	ADP	118	12
IDP	+	AMP	140	00

[a] Assay system in 0.5 ml contained: 50 μmoles of Tris-HCl (pH 7.5), 2.5 μmoles of $MgCl_2$, 1 μmole of IDP, and 0.5 mg of supernatant protein from peak Sephadex fraction; 0.3 μmole of other nucleoside phosphates was present.

4-fold by the presence of 3 and 5 mM ATP, respectively, showing that ATP markedly decreased the affinity of the enzyme for IDP. As expected, it was found that inhibition due to any given concentration of ATP was decreased by increasing IDP concentration. Similar results were obtained when UDP was used as substrate for the enzyme. It is evident that this enzyme showed near-normal kinetics in the absence of ATP (Figure 1B), but in the presence of ATP the reciprocal plots were clearly concave upward.

The inhibitory effect of ATP on the microsomal nucleoside diphosphatase catalyzed reaction, observed at four different substrate levels, when plotted as percentage inhibition against ATP concentration (Figure 2) was sigmoidal, indicating possible cooperative effects.

An allosteric effect of ATP on nucleoside diphosphatase was reported by Yamazaki and Hayaishi (1965), but in this case ATP acted as a positive effector on an enzyme obtained by extraction of a rat liver acetone powder. We confirmed their results and demonstrated that an aqueous extract of rat liver acetone powder contained a nucleoside diphosphatase which was activated by ATP. Examination of our rat liver fractions revealed that the nucleoside diphosphatase of the microsome-free supernatant fraction was similar to the enzyme studied by Yamazaki and Hayaishi (1965). Presumably, the ATP-activated nucleoside diphosphatase selectively survived acetone treatment or was more easily extracted from acetone powders.

The effect of ATP concentration on the activation of nucleoside diphosphatase from the supernatant fraction shows (Figure 3) that the enzyme activity increased with increasing ATP concentration up to 0.3 mM. A maximum

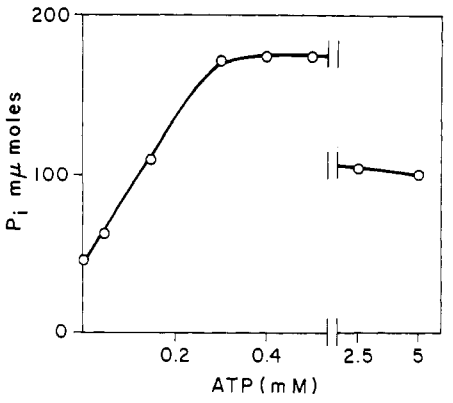

FIGURE 3: Activation of the supernatant nucleoside diphosphatase by ATP. Assay system contained 0.1 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM IDP, and 0.84 mg of supernatant protein. Volume of assay mixture was 0.5 ml.

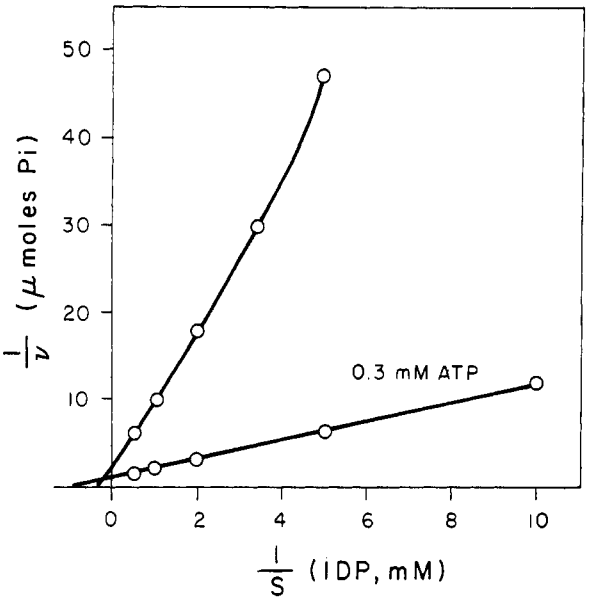

FIGURE 4: Effect of ATP on the affinity of the supernatant nucleoside diphosphatase for IDP. Assay system contained 0.1 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1.3 mg of supernatant protein separated between 40 and 70% saturation of ammonium sulfate, and IDP.

of about fourfold stimulation of enzyme activity was seen in the presence of ATP. As the concentration of ATP was further increased (2.5–5 mM), the activation effect was relatively decreased, although even at 5 mM ATP catalytic activity was twice as high as in the absence of ATP. The order of effectiveness of different nucleoside polyphosphates in stimulating nucleoside diphosphatase activity (Table II) was ATP > UTP > CTP > ADP. AMP was without any effect.

A reciprocal plot of the effect on the supernatant enzyme of increasing IDP concentration in the absence and in the presence of 0.3 mM ATP is presented in Figure 4. ATP not only increased the maximum velocity but it also reduced the apparent Michaelis constant of the supernatant nucleoside diphosphatase for IDP from 3.3 to 0.9 mM. Thus ATP markedly increased the affinity of the enzyme for IDP. This nucleoside diphosphatase, in contrast to the microsomal enzyme, showed sigmoidal kinetics in the absence of ATP and normal kinetics in the presence of ATP. The kinetic behavior of the supernatant enzyme is similar to the behavior of the ezyme[1] studied by Yamazaki and Hayaishi (1965).

With repeated freezing and thawing or standing at 4°, solutions of the supernatant nucleoside diphosphatase slowly lost the stimulatory response to ATP. However, as shown in Table III, precincubation of the enzyme with thiol compounds prior to assay restored the response to ATP. The suggestion based on these results, that reduced sulfhydryl groups were involved in the ATP stimulation, was strengthened by the observation that *p*-mercuribenzoate prevented ATP activation unless a thiol compound was also added. It further seems probable that only the allosteric site involves an SH group since the enzyme activity in the absence of ATP is not affected by thiol reagents. In contrast, the microsomal nucleoside diphosphatase was not affected by the presence of SH group inhibitors or thiol reagents either in the presence or absence of ATP.

TABLE III: Effect of SH Reagents on the Supernatant Nucleoside Diphosphatase in the Presence or Absence of ATP.[a]

Additions	mμmoles of P_i Liberated	
	IDPase	IDPase in presence of ATP
No addition[b]	45.0	67.5
Mercaptoethanol (4 mM)	45.7	206.0
Dithiothreitol (4 mM)	43.5	228
Oxidized glutathione (4 mM)	45.0	54.0
p-Mercuribenzoate (0.2 mM)	40.0	40.2
p-Mercuribenzoate (0.4 mM)	38.0	40.7
p-Mercuribenzoate (0.2 mM) + dithiothreitol (4 mM)	43.5	215.0

[a] Assay system in 0.5 ml contained: 50 μmoles of Tris-HCl (pH 7.5), 2.5 μmoles of $MgCl_2$, 1 μmole of IDP, and 1.5 mg of supernatant protein separated between 40 and 70% saturation of ammonium sulfate and stores at −15° for about 1 month. ATP when present was 0.6 mM. [b] This enzyme loses its response to ATP on repeated freezing and thawing or upon storage at −15°.

Both the supernatant and the microsomal nucleoside diphosphatases, assayed in the presence or absence of ATP, showed an absolute requirement for a bivalant cation for activity since in the absence of added Mg^{2+}

[1] During revision of this manuscript another paper (Schramm and Morrison, 1968) has appeared on this enzyme.

TABLE IV: Effect of Mg^{2+} ion Concentration on the Rate of Microsomal Nucleoside Diphosphatase Activity with or without ATP.[a]

Mg (mM)	mμmoles of P_i Liberated		
	Without ATP	With 2.5 mM ATP	With 5 mM ATP
0	35		
1	103	34 (69)[b]	31 (70)[b]
3	112	57 (49)	33 (70)
5	111	78 (29)	43 (61)
10	112	88 (20)	61 (45)
16	111	96 (15)	66 (40)

[a] Assay system in 0.5 ml contained: 50 μmoles of Tris-HCl (pH 7.5), 0.25 μmole of IDP, and 50 μg of the partially purified microsomal enzyme preparation. [b] Values in parentheses give per cent inhibition.

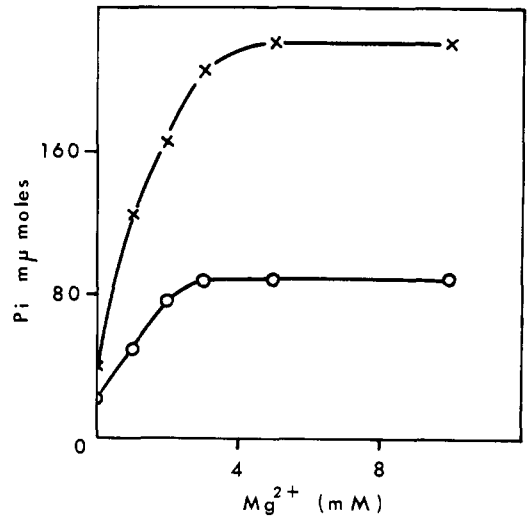

FIGURE 5: Effect of Mg^{2+} ion concentration on supernatant nucleoside diphosphatase activity. Assay system contained: 0.1 M Tris-HCl (pH 7.5), 2 mM IDP, and 0.5 mg of supernatant protein from the peak Sephadex fraction in the presence of (X) and absence (○) of 0.6 mM ATP.

and the presence of 1 mM EDTA in the assay system no activity was observed. However, the effects of ATP on these nucleoside diphosphatases could not be explained by Mg^{2+} chelation with the nucleotide alone. The optimum level of Mg^{2+} ion for the microsomal enzyme was about 1 mM. To check that Mg^{2+} was not being limited by chelation with ATP, Mg^{2+} was increased up to 8 times its saturation level. While the results in Table IV show that the inhibition of enzyme activity by ATP was partially relieved by increasing Mg^{2+}, even at this high ratio of Mg^{2+} to ATP as much as 40% inhibition at 5 mM ATP was observed. ATP inhibition was much greater at lower Mg^{2+} concentrations, possibly due to a Mg^{2+} binding effect or more probably because free ATP is the inhibitory species. It should be emphasized (Table I) that at equimolar levels, ATP was a much more effective inhibitor than other nucleoside triphosphates tested.

The stimulatory effect of ATP on the supernatant nucleoside diphosphatase was evident at the levels of Mg^{2+} tested (Figure 5). The K_m value for Mg^{2+} was 1.9 mM, and the presence of 0.6 mM ATP did not change that value. Thus, in these experiments ATP did not affect the Mg^{2+} ion requirement of the supernatant enzyme.

Discussion

As seen from these results, the rat liver supernatant and microsomal nucleoside diphosphatase differ widely in their kinetic behavior and in their response to ATP. ATP acts as a positive modifier for the supernatant enzyme by increasing the maximal velocity as well as the affinity of the enzyme for nucleoside diphosphate. Contrariwise for the microsomal enzyme, ATP acts as a negative modifier and further behaves as an apparent competitive inhibitor, reducing the affinity of the enzyme for its substrate.

Whereas the stimulatory effect of ATP on hepatic nucleoside diphosphatase (rat and bovine) has been examined thoroughly (Yamazaki and Hayaishi, 1965, 1968), the inhibitory effect of ATP on rat liver microsomal enzyme has not been investigated before in any detail. That a nucleoside diphosphatase of rate liver is subject to inhibition by ATP was noted earlier (Plaut, 1955).

Since these opposite modulatory effects of ATP on the two enzymes are exerted at levels of ATP that are well within the physiological range (Hohorst *et al.*, 1959; Mandel, 1964), it appears that this phenomenon may have metabolic significance *in vivo*. It has been suggested that the allosteric activation of nucleoside diphosphatase by ATP might function in shifting the equilibrium in synthetic reactions where IDP, GDP, or UDP are products of the reaction (Yamazaki and Hayaishi, 1965). Such an effect was considered important for the reversal of glycolysis by displacing the equilibrium of the phosphoenolpyruvate–carboxykinase reaction in the direction of phosphoenolpyruvate formation.

Although rat liver microsomal nucleoside diphosphatase is one of the most active microsomal enzymes, its physiological function is unknown. In view of the finding that ATP acts as a negative effector for this enzyme we suggest that this microsomal nucleoside diphosphatase may function differently from the ATP-stimulated nucleoside diphosphatase.

It is known that the level of adenine nucleotides in liver and other tissues is greater than that of other nucleotides (Marchetti *et al.*, 1962) and that most of the adenine nucleotide exists as ATP (Mandel *et al.*, 1963, 1964). Since the tissue concentration of the substrates of nucleoside diphosphatase (IDP, GDP, and UDP) is thus much lower than that of ATP (Mandel *et al.*, 1963, 1964), it is evident that effective competitive inhibition of microsomal nucleoside diphosphatase by ATP may be all the more pronounced *in vivo*. Evidently the activity of this nucleoside diphosphatase *in vivo*

would not depend upon its substrate level alone but upon the prevailing ATP level as well. Under conditions of excess energy with high ATP levels, the microsomal nucleoside diphosphatase will be inhibited by ATP resulting in an accumulation of nucleoside diphosphates. These conditions would be favorable for the formation of nucleoside triphosphates and in turn for biosynthesis of polysaccharide, nucleic acid, and proteins. On the other hand, under conditions of energy deprivation with decreased ATP concentration an unimpeded nucleoside diphosphatase may favor hydrolysis of UDP, IDP, and GDP. This depletion of nucleoside diphosphate level coupled to the decreased ATP concentration will prevent the formation of nucleoside triphosphates by nucleoside diphosphate kinase, thus halting various synthetic reactions dependent upon these specific nucleoside triphosphates. This explanation is in line with the suggestion of Atkinson (1966) that modulation of enzymatic activities by adenine nucleotides is related to the control of energy metabolism.

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Preparation of Glycopeptides from Bovine Submaxillary Mucin by Chemical Degradation*

Fred Downs and Ward Pigman

ABSTRACT: The disaccharide side chains (85% of them) of bovine submaxillary mucin were cleaved by alkali through a β-elimination reaction. The resulting peptide contained unsaturated amino acids at most of the original serine and threonine positions. Hydrolytic scission of the 2-aminopropenoic acid residues was carried out by heating at 100° for 1 hr at pH 2.2, but cleavage did not occur at 2-amino-3-butenoic acid residues. Gel filtration of this material resulted in the separation on Sephadex G-25 of a glycopeptide fraction which was shown to have a molecular weight of 3000. This glycopeptide accounted for 40% by weight of the original bovine submaxillary mucin and had a relative amino acid composition equivalent to bovine submaxillary mucin and alkali-treated bovine submaxillary mucin; it still contained one disaccharide side chain. This suggests the existence of repeating sequences of about 28 amino acids in bovine submaxillary mucin.

Bovine submaxillary mucin is a glycoprotein with a molecular weight of about 4×10^5. It contains about 70% carbohydrate mainly in the form of disaccharides consisting of a sialic acid and *N*-acetylgalactosamine unit (Gottschalk, 1960; Tsuiki *et al.*, 1961; Tettamanti and Pigman, 1968). The disaccharides are attached by *O*-glycosidic linkages to most of the threonine and serine residues (Tanaka and Pigman, 1965; Bertolini and Pigman, 1967). The disaccharide side chains undergo a β-elimination reaction on treatment with alkali, and the serine and threonine units are converted into 2-aminopropenoic acid and 2-amino-3-butenoic acid units (Tanaka and Pigman, 1965).

Using model serine peptides, Patchornik and Sokolovsky (1964) have shown that 2-aminopropenoic acid residues are cleaved by mild acids at the amino group, and the "NH_2-terminal group" is pyruvic acid. Harbon *et al.* (1968) found that the β-elimination product from

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