Isolation and Characterization of Multiple Forms of Ovine Pancreatic Deoxyribonuclease. Chromatographic Behavior of the Enzyme on Concanavalin A–Agarose and Carboxymethylcellulose Columns†

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ABSTRACT: A new procedure has been devised for the purification of ovine DNase, including $(NH_4)_2SO_4$ fractionation, two steps of CM-cellulose chromatography, concanavalin A–agarose chromatography, and gel filtration on Sephadex G-100. The enzyme, like bovine DNase, exhibits multiplicity due to changes in the primary structure and the sugar structure of the carbohydrate moiety. Unlike bovine DNase, ovine DNase does not have sialic acid in any of its multiple forms. Concanavalin A–agarose is useful in the purification of not only ovine but also bovine DNase. For ovine DNase, it is a necessary and key step of purification; for bovine DNase, it can be used to purify commercial preparations of DNase free from proteases in a single step as judged by its stability in Ca^{2+}-free media at pH 8.0. The purified enzyme has a specific activity equal to that of a highly purified DNase and presumably contains predominantly DNases A and C. Two of the four forms of ovine DNase have been purified to apparent homogeneity and subjected to chemical analysis. The present results show that bovine and ovine DNases have indistinguishable molecular weights and identical end groups, suggesting that they may have the same number of amino acid residues. The amino acid composition indicates that two enzymes may have six residues of amino acids subject to substitution which can be explained by single base changes in their genetic code words. Amino acid analyses also indicate that the most likely difference between two forms of ovine DNase is the substitution of Leu for Arg.

Deoxyribonuclease (EC 3.1.4.5) from bovine pancreas, readily available in relatively pure form (Kunitz, 1950), has received a great deal of study not only as an enzyme but also as a protein (Laskowski, 1971). Bovine DNase consists of four forms which separate on phosphocellulose (Salnikow et al., 1970). Multiple forms of DNase are due to a histidine residue replacing a proline in the primary sequence and the presence or absence of a sialic acid residue in the carbohydrate side chain. The primary structure of one of the four forms is known (Salnikow et al., 1973; Liao et al., 1973a). As shown by peptide mapping, the histidine–proline replacement is at residue 118 (Salnikow & Murphy, 1973), and the two variant proteins have either a neutral or a sialic acid containing carbohydrate side chain (Liao, 1974), thus making four forms of DNase that differ in charge.

Multiple forms of DNase occur not only in the bovine species but also in other mammals as well (Antonoglou & Gerogatsos, 1971; Ball & Rutter, 1971; Ball, 1974). It has recently been reported that malt DNase also shows multiple forms (Liao, 1977). The chemical basis of multiplicity for species other than bovine has not yet been established; however, it probably resembles bovine multiplicity since very similar amino acid composition is observed among different forms within the same species. Nevertheless, the biological significance of multiple forms of DNase is not understood. In the present communication we show that multiple forms of DNase also exist in ovine pancreas. Further investigation of ovine DNase is therefore necessary in order to understand the multiplicity aspect of the enzyme.

Specific adsorption of carbohydrates by Con A[1] has been well documented (Sumner & Howell, 1936; Goldstein et al., 1965). Examples of the use of this binding property to purify glycoproteins by Con A–agarose are numerous (Aspberg & Porath, 1970; Dufau et al., 1972; Bessler & Goldstein, 1973; Steinemann & Stryer, 1973; Rush et al., 1974; Norden & O'Brien, 1974; Davey et al., 1976; Neary et al., 1977; Lotan et al., 1977). Since bovine pancreatic DNase is a glycoprotein (Catley et al., 1969; Price et al., 1969) and it is possible that ovine pancreatic DNase is also a glycoprotein, we decided to study the potential use of Con A–agarose for the purification of pancreatic DNases. Moreoever, there is a need for a convenient preparation of a stable DNase free from proteases for use in investigations of protein biosynthesis and related fields. Indeed, this concern has led Hugli (1973) to use a combination of DEAE-Sephadex and phosphocellulose chromatography, Liao (1974) to use affinity elution with Ca^{2+} on DEAE-cellulose, Otsuka & Price (1974) to try lima bean protease inhibitor affinity chromatography, and Wang & Moore (1978) to design a simple, one-step procedure using affinity chromatography and salting-out adsorption on lima bean protease inhibitor bound to Sepharose. Therefore, we also report here a simple, one-step procedure for obtaining the stable DNase using Con A–agarose, which completely removes proteases and their zymogens from DNase since these contaminants are not glycoproteins.

Materials and Methods

Materials. Frozen ovine and bovine pancreas was purchased from Pel-Freez. Fresh ovine pancreas was obtained from the local slaughter house at Oklahoma State University. Bovine

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[1] Abbreviations used: Con A, concanavalin A; $NaDodSO_4$, sodium dodecyl sulfate; NANA, *N*-acetylneuraminic acid; Pu, purines; Py, pyrimidines; X, all four bases of nucleotides.

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Table I: Summary of the Purification Steps of Ovine DNase

purification step	total act. (units × 10^{-3})	act. recovery (%)	sp act. (units/A_{280})	purification (x-fold)
(1) crude extract	46.0	100	1.8	1
(2) $(NH_4)_2SO_4$ precipitate	34.0	74	5.6	3
(3) first CM-cellulose chromatography	14.4	31	101	57
(4) Con A-agarose				
fraction I	4.2	9.1	142	80
fraction II	6.3	13.7	373	216
total	10.5	22.8		
(5) second CM-cellulose chromatography of each fraction from step 4				
fraction IA	1.6	3.5	276	156
fraction IB	1.9	4.1	351	198
fraction IIA	1.9	4.0	703	397
fraction IIB	3.0	6.6	517	292
total	8.4	18.2		
(6) Sephadex G-100 chromatography[a]				
fraction IA			657[b]	365
fraction IB			353	196
fraction IIA			778	432
fraction IIB			1000	556

[a] The fractions in this step (step 6) were obtained from the combination of many preparations; therefore, the data on the total activity as well as its recovery are not available. [b] Due to low concentrations of the protein, accurate A_{280} could not be obtained; it was calculated from the elution profile in which the base line was subtracted from the A_{280} curve.

pancreatic DNase (DP grade) was obtained from Worthington; four forms of bovine DNase (A, B, C, and D) were prepared from it according to Salnikow et al. (1970). Calf thymus DNA, Con A, Con A–agarose, and phenylmethanesulfonyl fluoride were from Sigma Chemical Co. CM-cellulose (Whatman; CM-52) was from Reeve Angel. Ampholines were from LKB.

Preparation of Con A–Agarose. Con A–agarose was either purchased from Sigma or prepared as described by Steinemann & Stryer (1973). Agarose was first activated by CNBr, followed by the addition of Con A; remaining active groups were blocked by treatment with 1 M glycine for 2 h at pH 9.0 (Sica et al., 1973). The capacity of Con A–agarose to bind glycoproteins was measured with purified bovine DNase A. Usually, 1 mL of bed volume of Con A–agarose containing 2 mg of Con A is capable of binding 0.2–0.3 mg of DNase A.

Amino Acid Analysis. Protein was hydrolyzed in 200 μL of 6 N HCl in evacuated, sealed tubes at 110 °C (Salnikow et al., 1973). For the determination of half-cystine and methionine, protein was first oxidized with performic acid and then hydrolyzed with 6 N HCl in separate tubes (Liao et al., 1973a). Amino acid analyses were performed on the nanomole scale with an analyzer (Spackman et al., 1958), modified for use with a 2.8-mm bore column (Liao et al., 1973b). For the determination of tryptophan, protein was hydrolyzed in 6 N HCl containing 0.25% phenol, 0.5% thioglycolic acid, and 0.1% 3-(2-aminoethyl)indole hydrochloride. The indole derivative protected tryptophan (Liu & Chang, 1971); under the present conditions the method gives 2.3 residues of tryptophan per molecule of bovine DNase A which is known to contain three residues of the amino acid (Liao et al., 1973a).

DNase Assay. DNase activity was determined by a modification of the hyperchromicity assay of Kunitz (1950), as described by Liao (1974). One unit of enzyme is defined as the activity that causes the increase of 1 absorbance unit at 260 nm per min per mL of assay medium at 25 °C. The specific activity is designated as units of 1 mL of enzyme solution per absorbance unit of that solution at 280 nm (units/A_{280}).

Electrophoresis. Isoelectrofocusing electrophoresis was performed according to the procedure of Vesterberg & Svensson (1966). $NaDodSO_4$ gel electrophoresis was carried out according to that of Weber & Osborn (1969).

End-Group Analysis. Qualitatively, the NH_2-terminal amino acid was determined by the dansylation procedure (Gray, 1967), and the identification of dansyl amino acids was performed by thin-layer chromatography on polyamide sheets as described by Woods & Wang (1969). Quantitatively, it was determined by the Edman degradation procedure (Dopheide et al., 1967); the anilinothiazolinone derivatives were back-hydrolyzed and determined on the amino acid analyzer (Bailey et al., 1977). The COOH-terminal amino acids were analyzed on the amino acid analyzer after hydrolysis with carboxypeptidase Y in the presence of $NaDodSO_4$ as described by Liao (1975).

Sialic Acid Analysis. Sialic acid was released by hydrolysis of glycoproteins in 0.1 N H_2SO_4 at 80 °C for 1 h and determined by the procedure of Warren (1959) in a reduced scale (Liao, 1974).

Results

Purification of Ovine DNase. The purification steps are summarized in Table I. The procedure of extraction and $(NH_4)_2SO_4$ fractionation was adapted and modified from that of Kunitz (1950). Frozen or fresh ovine or bovine pancreas (213 g) was minced and stirred in 213 mL of cold water. The crude extract of frozen bovine pancreas had 3–4 times more DNase activity than that of ovine, and there was no significant difference in activity between fresh and frozen pancreas. The pH of the homogenates was adjusted slowly to 3.0 with 0.5 N H_2SO_4. After centrifugation, the precipitate was washed with another volume of cold water and centrifuged. Both supernatants were combined and designated as the "crude extracts". This extract was first subjected to $(NH_4)_2SO_4$ fractionation; the fraction of 0.1–0.4 $(NH_4)_2SO_4$ saturation which contained most of the activity was recovered as precipitate after centrifugation. This fraction was then dissolved and dialyzed against 5 mM calcium acetate, pH 4.7, containing 0.2 mM phenylmethanesulfonyl fluoride, an inhibitor of serine protease (Fahrney & Gold, 1963). We find that this reagent

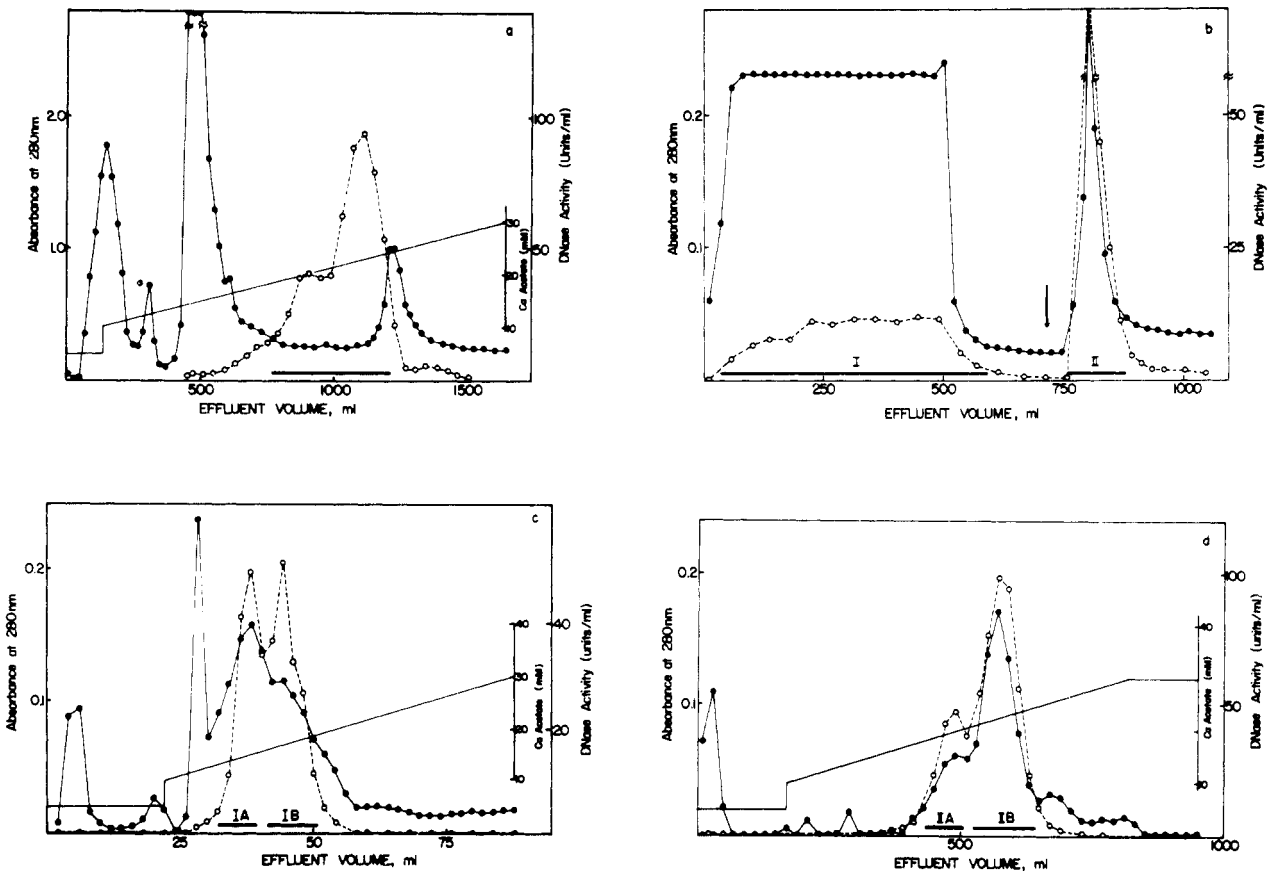

FIGURE 1: Purification of ovine DNase. Bars indicate pooled active fractions; solid lines indicate the absorbance at 280 nm; broken lines indicate the DNase activity. (a) Chromatography of the $(NH_4)_2SO_4$ fraction on a CM-cellulose column, 1.5 × 42 cm. Elution was performed with 500 mL each of 0.01 and 0.03 M calcium acetate buffer, pH 4.7. (b) Chromatography of the active fraction from (a) on a Con A–agarose column, 2.5 × 20 cm. The column was equilibrated and eluted with 0.1 M Tris-HCl, pH 7.0, and 10 mM in $CaCl_2$. The arrow indicates the change of elution to 5% glucose in the same buffer. (c) Rechromatography of fraction I from (b) on a CM-cellulose column, 0.9 × 18 cm. Elution was performed with 200 mL each of 0.01 and 0.03 M calcium acetate buffer, pH 4.7. (d) Rechromatography of fraction II from (b) on a CM-cellulose column, 0.9 × 18 cm. Elution was performed with 200 mL each of 0.01 and 0.03 M calcium acetate, pH 4.7.

is more convenient to use than diisopropyl phosphofluoridate (Price et al., 1969), because it does not form insoluble products in the presence of $CaCl_2$ and it requires less precaution in handling. All of the above centrifugations were performed on a Sorvall centrifuge (Model RC-2B) at 27000*g*. The dialysate was loaded on the first CM-cellulose column previously equilibrated with 5 mM calcium acetate, pH 4.7 (Figure 1a); the enzyme was eluted in a gradient from 10 to 30 mM calcium acetate, pH 4.7. The active fraction was adjusted to pH 7.0 with 1 M Tris–hydroxide and was loaded on a Con A–agarose column, equilibrated with 0.1 M Tris-HCl buffer, pH 7.0, containing 10 mM $CaCl_2$ (Figure 1b). The enzymatic activity appeared in two fractions. Each fraction was chromatographed separately, as before, on a second CM-cellulose column which separated it into another two fractions (Figure 1c,d). Therefore, four fractions were obtained, designated as fractions IA, IB, IIA, and IIB. A further purification was obtained when each fraction was chromatographed on a Sephadex G-100 column (1.5 × 95 cm), eluted with 0.1 M Tris-HCl, pH 7.0, containing 10 mM $CaCl_2$. Unless otherwise indicated, all the above operations were performed at 4 °C.

Figure 1a shows that the first CM-cellulose chromatography (step 3) partially separated into two active fractions. Since at this point the aim was to remove a large amount of other proteins which were eluted early during chromatography, no attempts were made to improve the separation, and these two fractions were combined as one. The elution profile of Con A–agarose chromatography in step 4 is shown in Figure 1b. Two fractions were obtained: the unbound fraction (fraction I) and the bound fraction (fraction II). When each of the two fractions was rechromatographed on a second CM-cellulose column (step 5), each separated into two fractions. These two fractions very likely represented the two fractions that were partially separated on the first CM-cellulose column, since the conditions used were similar. The elution profile of the absorbance at 280 nm and of the activity as shown in Figure 1c indicates that fractions IA and IB were not homogeneous. In fact this is reflected in their specific activities shown in Table I. In contrast, fractions IIA and IIB were nearer homogeneity, suggesting that glycoproteins in ovine pancreas contain mainly DNase and the purification of fractions IIA and IIB is simpler than that of fractions IA and IB because of their glycoprotein nature.

Step 6, gel filtration on a Sephadex column, is a useful step for fraction IA in which a substantial amount of large molecular weight proteins was removed in addition to some UV-absorbing materials which were eluted in the small molecular weight region. Fraction IB exhibited a similar purification, but denaturation must have occurred during gel filtration, since it did not show much increase in specific activity. The great increase of specific activity from step 5 to step 6 in fraction IIB was due primarily to the removal of some UV-absorbing materials which were eluted in the small molecular weight region.

Heterogeneity at the Carbohydrate Moiety and Sialic Acid Contents. In one of the purification steps (step 4, Table I), Con A–agarose was used, originally with the intent of puri-

Table II: Capability of Various Forms of Both Ovine and Bovine DNases to Bind Con A-Agarose in Relation to Their Sialic Acid Contents

	bovine[a]			ovine[b]	
	sialic acid[c]	Con A-agarose bound (%)		sialic acid[c]	Con A-agarose bound (%)
DNase A	no	100	fraction IA	no	0
DNase B	yes	70	fraction IB	no	0
DNase C	no	100	fraction IIA	no	100
DNase D	yes	70	fraction IIB	no	100

[a] For the separation and nomenclature of bovine DNases, see Salnikow et al. (1970). [b] Fractions of ovine DNase were obtained from step 5 in Table I. [c] The sialic acid assays are shown in Table III.

Table III: Sialic Acid Assay of Four Fractions of Ovine DNase and Bovine DNases B and D[a]

		absorbance at 549 nm[d]	total amount of protein (μg)	residues/mol of DNase
ovine	fraction IA	0.03	300	0.1
	fraction IB	0.03	310	0.1
	fraction IIA	0.02	302	0.1
	fraction IIB	0.05[c]	159	0.2
bovine	DNase B unbound[b]	0.13	113	0.9
	DNase B bound[b]	0.20	262	0.4
	DNase D unbound[b]	0.06	56	0.8
	DNase D bound[b]	0.07	125	0.4

[a] It has previously been shown (Salnikow et al., 1970) that bovine DNases A and C do not contain any sialic acid and therefore they are not assayed. [b] The unbound and bound fractions are referring to those in Table II for their ability to bind Con A-agarose. [c] The slight absorbance (0.05) at 549 nm which is reflected as 0.2 residue/mol of sialic acid is probably not due to sialic acid because the color of this solution (cyclohexanone layer) after reactions is yellowish and is not the same pink color as that of 1.3 nmol of NANA which also shows the same amount of absorbance. [d] The absorbances for 10 and 1.3 nmol of standard NANA are 0.39 and 0.05, respectively.

fication of DNase owing to its glycoprotein nature. The presence of DNase activity in the unbound fraction (fraction I) posed a problem that only fraction II was purified by the Con A–agarose chromatography. To further investigate this aspect, we rechromatographed each of the four fractions from step 5 of Table I on a similar Con A–agarose column. The results, shown in Table II, again indicate no binding for fractions IA and IB and complete binding for fractions IIA and IIB and provide strong evidence that fraction I in step 4 is not due to overloading of the Con A–agarose column.

From previous studies (Salnikow & Murphy, 1973; Liao, 1974) it is known that bovine DNase exists as four forms which differ in either the primary amino acid sequence or the carbohydrate structure. Bovine DNases A and C are devoid of sialic acid, whereas bovine DNases B and D contain one residue each of sialic acid. For the purpose of comparison and better understanding of the carbohydrate moiety of ovine DNase, bovine DNases were also tested for their binding to Con A–agarose (Table II).

Table III shows the sialic acid assays of various fractions obtained from bovine and ovine DNases after Con A–agarose chromatography. It is evident that ovine DNase contains no sialic acid in any of its multiple forms. On the other hand, bovine DNases B and D, bound or unbound, contain a significant amount of sialic acid, and perhaps all possess one residue of sialic acid per molecule; the low values (0.4 residue/molecule) in the bound fractions are probably due to the presence of DNases A and C in the original preparations.

In a recent report, Baenziger & Fiete (1979) measured association constants between Con A and various glycopeptides and studied the binding of these glycopeptides to Con A–Sepharose. Our results on the binding of bovine DNase to Con A–agarose (Table II) are consistent with their findings. DNases A and C, which have the same carbohydrate composition (Liao, 1974) and probably the same saccharide structure as their ovalbumin glycopeptide, GP IV, are similarly bound to Con A–agarose strongly. On the other hand, DNases B and D, which contain sialic acid, are partially bound (Table III). Taking the present knowledge of the carbohydrate compositions of DNases B and D (Liao, 1974) into consideration, we found that the bound fractions may have a glycopeptide similar to their glycopeptide G-A_4 which is not bound to Con A–Sepharose and the bound fraction may have a glycopeptide similar to their glycopeptide G-A_3 which is bound to Con A–Sepharose. The same type of analogy cannot be drawn for the multiple forms of ovine DNase, due to the lack of information on the carbohydrate composition other than the fact that they do not contain sialic acid. However, it is probably correct that forms IIA and IIB which are bound to Con A–agarose may have a similar carbohydrate structure as that of bovine DNases A and C, whereas forms IA and IB which are not bound to Con A–agarose may have a carbohydrate side chain not bound by Con A–agarose as did those studied by Baenziger & Fiete (1979) or may be completely devoid of carbohydrates.

Con A–Agarose Chromatography of DNases. Table I shows that Con A–agarose is very useful for the purification of bound ovine DNase (fraction II) in which approximately fourfold purification over the previous step was obtained. In addition, fraction II after this step was very stable even in the absence of Ca^{2+} or phenylmethanesulfonyl fluoride, indicating a complete removal of proteases, because it was shown by McCarty (1946) that the major cause of instability is digestion by proteases. The fact that proteases and their zymogens in pancreas are not glycoproteins accounts for their complete separation from DNase by Con A–agarose chromatography.

The stabilization effect by Con A–agarose chromatography is also observed with bovine DNase. When a commercial preparation of bovine DNase (Worthington; DP grade) was chromatographed on a Con A–agarose column, under conditions similar to those used in ovine DNase purification, 7.3% of the DNase activity along with inert proteins appeared in the first buffer washing fraction, and most of the remaining activity (73.4%) was recovered in the 5% glucose washing fraction (Figure 2). This latter fraction very likely contained only DNase because it had a specific activity of approximately 800 units/mg, equivalent to that of a highly purified DNase (Liao, 1974). It was free from proteases as judged by its stability after exhaustive dialysis to remove Ca^{2+} and the retention of activity during 8 h at pH 8.0 and 37 °C (Hugli, 1973).

Isoelectrofocusing and NaDodSO$_4$ Gel Electrophoresis. When either fraction I or II from step 4 in Table I was isoelectrofocused, two activity peaks, very close to each other, with an isoelectric point near 4.7, were observed. These results and those obtained by CM-cellulose chromatography (Table I, step 5) strongly suggest that the multiple fractions of DNase activity in ovine pancreas are due, in part, to charge differences.

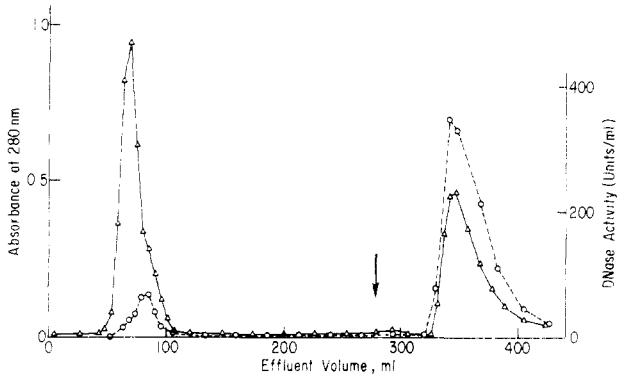

FIGURE 2: Con A–agarose chromatography of bovine DNase. Fifty milligrams of DNase (Worthington; DP grade) was dissolved in 10 mL of 0.1 M Tris-HCl, pH 7.0, containing 10 mM $CaCl_2$ and placed on a Con A–agarose column (2 × 16 cm) previously equilibrated in the same buffer. The arrow indicates the position of elution by 5% glucose in the same buffer. Solid line, absorbance at 280 nm; broken line, DNase activity.

$NaDodSO_4$ gel electrophoresis revealed that fractions IA and IB were contaminated by other proteins, whereas fractions IIA and IIB contained a single band representing a molecular weight of 31 000. The accuracy of this molecular weight was verified by coelectrophoresis of bovine and ovine DNases, which showed comigration of the two; bovine DNase has a molecular weight of 30 072 as calculated from its structure (Liao et al., 1973a). A similar molecular weight was obtained from calculation of the elution volume by gel filtration on Sephadex G-100.

End-Group Analysis. The NH_2-terminal residue was determined by the Edman degradation method in which the anilinothiazolinone amino acids were back-hydrolyzed in 6 N HCl at 150 °C to parent amino acids and analyzed on the amino acid analyzer. One residue of leucine was obtained from fraction IIB of ovine DNase. The same residue was also identified qualitatively by using the dansyl procedure. Thus, ovine and bovine DNases have the same NH_2-terminal residue. The COOH terminal was determined from amino acids released by carboxypeptidase Y. In 10 min, 0.4 residue each of Thr and Leu was detected, and in 60 min, 1.7 of Thr, 0.7 of Glu, 0.6 of Pro, 1.5 of Val, and 1.0 residue of Leu were found. This pattern of releasing amino acids from the COOH terminal in ovine Dnase is very similar to that obtained with bovine DNase. It is therefore very likely that ovine and bovine DNases have a homologous COOH-terminal sequence in which both contain a COOH-terminal Thr (Liao, 1975).

Amino Acid Analysis. Results of amino acid analysis of fractions IIA and IIB are expressed as residues per molecule and shown in Table IV. Because of impurities present in fractions IA and IB, their amino acid compositions are not meaningful and therefore not reported. For amino acids at 15 or less residues per molecule, the integral differences are probably significant. Thus, in comparison with bovine DNase A, the extra residues of proline, glycine, and isoleucine and the fewer residues of threonine, tyrosine, and histidine are very likely. The numbers for aspartic acid, serine, glutamic acid, and alanine are less reliable. However, they are probably nearly correct because these amino acid compositions are the average of six (for fraction IIB) and three (for fraction IIA) analyses. For the calculations in Table IV, the proteins are assumed to have the same number of residues as in bovine DNase, because of the identical end groups and indistinguishable molecular weights of bovine and ovine DNases.

Other Properties of Ovine DNase. Catalytical properties of ovine DNase are identical with those of bovine DNase

Table IV: Amino Acid Compositions of Ovine DNase[a]

	ovine		bovine
	fraction IIA	fraction IIB	DNase A
aspartic acid	32.9 (33)	33.3 (33)	32
threonine[b]	14.1 (14)	13.8 (14)	15
serine	30.9 (31)	31.3 (31)	30
glutamic acid	18.0 (18)	18.4 (18)	19
proline	11.3 (11)	10.7 (11)	9
glycine	10.2 (10)	10.0 (10)	9
alanine	20.9 (21)	21.1 (21)	22
half-cystine	nd (4)[e]	3.8 (4)[c]	4
valine[d]	23.9 (24)	23.9 (24)	24
methionine	4.2 (4)	3.9 (4)[c]	4
isoleucine[d]	12.0 (12)	12.0 (12)	11
leucine	22.8 (23)	22.3 (22)	23
tyrosine	13.7 (14)	13.5 (14)	15
phenylalanine	10.5 (11)	10.7 (11)	11
histidine	4.1 (4)	3.9 (4)	6
lysine	9.3 (9)	9.2 (9)	9
tryptophan	nd (3)[e]	2.3 (3)	3
arginine	11.3 (11)	11.7 (12)	11

[a] Results are expressed as the calculated number of residues per enzyme molecules based on the average of six (for fraction IIB) and three (for fraction IIA) analyses. The possible number of residues is in parentheses. The values for bovine DNase A are established in its sequence study (Liao, 1973a) which showed a total of 257 amino acid residues in the protein. The total number of residues in ovine DNase is also assumed to be 257. [b] Extrapolated to zero-time hydrolysis. [c] Determined as cysteic acid or methionine sulfone after performic acid oxidation. [d] Values obtained from 96-h hydrolysis. [e] Not determined; residues are assumed from homology between fraction IIA and fraction IIB.

(Laskowski, 1971); these include the metal requirements, protections against proteases by Ca^{2+}, and the pH optimum. However, judging from their behavior on CM-cellulose columns and isoelectrofocusing, ovine DNase is slightly more basic than bovine DNase (the isoelectric point of the bovine enzyme, by isoelectrofocusing, is near 4.5).

Discussion

In the present study, a revised procedure was developed to obtain a sufficient amount of homogeneous materials for analyses. The conventional procedure for the purification of bovine DNase cannot be employed for two reasons. First, ovine pancreas contains much less DNase than does bovine pancreas. This low activity poses a problem in achieving the first stage of purification of ovine DNase, that is, to obtain so-called "crystalline" DNase as in bovine (Kunitz, 1950). Second, the preparation of crystalline DNase requires drastic treatment with acid and heat, resulting in very low recoveries (1–3% yield from the crude extract). Also, the crystalline DNase still contains other proteins. A further purification by either phosphocellulose (Salnikow et al., 1973) or DEAE-cellulose (Liao, 1974) chromatography gives a preparation containing only DNase but with a 40–60% further loss in activity. Thus, if one follows the same procedure for the purification of ovine DNase, one will at most recover only 0.3–1.0% of the DNase originally present in pancreas. With our new procedure, final yields of ovine DNase are 10–20%. The essence of the successful purification of two of the four forms of ovine DNase is Con A–agarose chromatography. This finding led us to a simple, one step purification of a stable bovine DNase from commercial preparations. This DNase probably contains very little other proteins as judged from its high specific activity; however, it may contain a mixture of predominantly forms A and C, since they are bound strongly to Con A–agarose (Table II).

Due to the unstable nature of ovine DNase in Ca^{2+}-free media, chromatography on a phosphocellulose column, which

was used successfully in the separation of four forms of bovine DNase, is not applicable here because of the absence of Ca^{2+} in the eluting buffer. Thus, a direct comparison of the two is not available, and conclusions concerning structural analogies must rely on more detailed analyses of ovine DNase. CM-cellulose chromatography and isoelectrofocusing as well as Con A–agarose chromatography clearly demonstrate that ovine DNase contains four forms which differ in the charge on the primary sequence or the structure of the carbohydrate side chain, as in the case with bovine DNase. However, ovine DNase is devoid of sialic acid.

Results from amino acid analyses (Table IV) indicate there are possibly 12 amino acid residue differences between bovine DNase A and ovine DNase fraction IIA. If these differences are assumed to be due to amino acid substitution rather than addition or deletion, one can pair these amino acids to predict a substitution that may occur at a particular position in the primary sequence. Assuming conservation of amino acids and single base changes, the best pairings of the residue changes from bovine to ovine are derived: Glu (GAPu) → Asp (GAPy), Thr (ACPy, ACA) → Ile (AUPy, AUA), Tyr (UAPy) → Ser (UCPy), Ala (GCX) → Gly (GGX), 2His (CAPy) → 2Pro (CCPy). It has been shown (Salnikow et al., 1970) that bovine DNase C has five His and ten Pro instead of six His and nine Pro as found in bovine DNase A, making only five residue substitutions between bovine DNase C and ovine DNase fraction IIA. Also, since there are only four His in ovine DNases, one of the five His in bovine DNase C must not be essential for activity. Since fraction IIA was eluted ahead of fraction IIB in CM-cellulose chromatography, fraction IIB must be slightly more basic. The amino acid composition data (Table IV) also show this, as fraction IIB may have one more arginine and one less leucine as compared with fraction IIA, a variation which can also be accounted for by single base mutation: Leu (CUX) → Arg (CGX). It is not clear why fraction IIA has only 78% of the specific activity of fraction IIB (Table I) and yet they differ only in one amino acid residue. A similar discrepancy was also observed in multiple forms of bovine DNase (Liao, 1974).

According to Marchalonis & Weltman (1971), the relativeness ($S\Delta Q$) of two proteins can be calculated from their amino acid compositions. The $S\Delta Q$ of bovine and ovine DNases is below 3. This means that the similarity between the two is much higher than that of hemoglobin β chains of human and rhesus monkey ($S\Delta Q$ = 7.5), whose sequences differ by nine amino acid residues (Braunitzer et al., 1961; Matsuda et al., 1968).

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S-Adenosylhomocysteinase from Mouse Liver. Effect of Adenine and Adenine Nucleotides on the Enzyme Catalysis†

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ABSTRACT: The kinetics of the synthesis and hydrolysis of *S*-adenosylhomocysteine catalyzed by the enzyme *S*-adenosylhomocysteinase from mouse liver was determined in the presence of adenine and adenine nucleotides. At a concentration of 1 μM adenosine, the time course of the synthesis of *S*-adenosylhomocysteine in the presence of adenine acquired a smaller slope after about 5 min of incubation. The synthesis in the presence of AMP was linear for about the same time as that in the absence of inhibitor whereas the curve obtained with ADP and ATP gradually leveled off until a plateau was reached after about 10 min of incubation. The hydrolysis of *S*-adenosylhomocysteine in the presence of AMP, ADP, and ATP was linear with respect to time for about the same time as that in the absence of these nucleotides. The time course in the presence of adenine acquired a smaller slope after about 5 min of incubation. The enzyme was preincubated in the presence of adenine (25 μM), AMP (2 mM), ADP (2 mM), and ATP (2 mM), diluted to minimize the effect of purines during incubation, and assayed for *S*-adenosylhomocysteine synthase activity. After a lag phase lasting for 2–5 min, the activity decreased in a time-dependent manner in the presence of ADP and ATP, and about 50% of the initial activity remained after 30 min of preincubation. Only a slight decrease was observed with AMP. Adenine effected a rapid initial fall in synthase activity after which no further decrease was observed. Kinetic analysis of initial velocity data indicates linear competitive inhibition of *S*-adenosylhomocysteine hydrolase activity in the presence of adenine, AMP, and ADP, and the inhibitor constants (K_i) increased in the order mentioned. The inhibitor constants for these purines seem to be the same during synthesis and hydrolysis of *S*-adenosylhomocysteine. Cyclic AMP and ATP were not or only slightly inhibitory. The hydrolysis of *S*-adenosylhomocysteine (50 μM) was allowed to proceed to equilibrium. Adenine, AMP, and ADP increased the time needed for equilibrium to be obtained.

A protein not associated with cyclic AMP dependent protein kinase was identified in mouse liver because of its ability to bind cyclic AMP (Døskeland & Ueland, 1975). Further binding kinetic studies revealed the following binding properties of this protein. The protein was isolated in a form possessing low binding capacity for cyclic AMP (Ueland & Døskeland, 1977). The cyclic AMP binding was increased by preincubating the protein in the presence of ATP. Thus, the ATP-treated protein had two distinct binding sites, called the adenosine binding site(s) and the cyclic AMP binding site, respectively (Ueland & Døskeland, 1977). The adenosine binding site(s) showed rather high specificity toward this nucleoside whereas the structural requirements for the binding of ligands to the cyclic AMP site was low. The following adenine derivatives were shown to interact with this site with decreasing affinity in the order mentioned: cyclic AMP, adenosine, AMP, ADP, and ATP (Ueland, 1978).

The binding protein which interacts with various adenine analogues was eventually identified as *S*-adenosylhomocysteinase (EC 3.3.1.1) (Saebø & Ueland, 1978; Hershfield & Kredich, 1978). This enzyme catalyzes the reversible thioether bond formation between adenosine and L-homocysteine according to the equation

adenosine + L-homocysteine ⇌ *S*-adenosylhomocysteine

(De la Haba & Cantoni, 1959). This enzyme has been implicated in the regulation of the cellular level of *S*-adenosylhomocysteine (SAH[1]) which is a product formed from *S*-adenosylmethionine (SAM) upon transmethylation from SAM to cellular acceptors (Cantoni & Scarano, 1954; De la Haba & Cantoni, 1959). SAH is a potent inhibitor of most transmethylases using SAM as a methyl donor (Hurwitz et al., 1964; Zappia et al., 1969; Deguchi & Barchas, 1971; Kerr, 1972; Coward et al., 1974; Pugh et al., 1977). The tissue level of SAH equals that of SAM (Salvatore et al., 1971; Hoffman, 1975). On this basis SAH has been suggested to be a regulator of biological methylation (Hurwitz et al., 1964; Zappia et al., 1969; Deguchi & Barchas, 1971; Salvatore et al., 1971; Kerr, 1972; Coward et al., 1974; Hoffman, 1975; Pugh et al., 1977).

In this paper the effect of adenine and adenine nucleotides on the metabolism of SAH in the presence of *S*-adenosylhomocysteinase from mouse liver was investigated for the following reasons. (a) The binding of adenine and adenine nucleotides to the enzyme (Ueland & Døskeland, 1977; Ueland, 1978) suggests that the metabolism of SAH may be regulated by the concentration of these purines. To our knowledge, a detailed study on the effect of the adenine and adenine nucleotides on *S*-adenosylhomocysteinase has not been carried out before. (b) By measurement of the synthesis and hydrolysis of SAH in the presence of adenine and adenine nucleotides, data on the binding properties of the catalytic site(s) may be obtained which may suggest the relation between the adenosine binding site(s), the cyclic AMP site, and the catalytic site(s).

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[1] Abbreviations used: SAH, *S*-adenosylhomocysteine; SAM, *S*-adenosylmethionine; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Hcy, homocysteine; Ado, adenosine.

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