Biosynthesis of Chondroitin Sulfate. Sulfation of the Polysaccharide Chain†

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ABSTRACT: A microsomal preparation from chick embryo epiphyseal cartilage has previously been shown to be capable of synthesizing chondroitin on endogenous protein linked primers. Sulfation (exclusively in the 6 position) of as much as 85% of the newly formed chondroitin has now been accomplished by inclusion of 3′-phosphoadenosine 5′-phosphosulfate in incubation mixtures. Sulfation during chondroitin polymerization occurred essentially in an "all or nothing" rather than a random fashion so that maximally 75% of the final chondroitin chains was 96% sulfated, while approximately 5% of the chondroitin chains was essentially free of sulfate. Only 20% of the newly formed chondroitin (representing less than 11% of the total sulfate incorporated) might have contained intermediate amounts of sulfate. Timed sulfation studies of newly formed chondroitin confirmed the "all or nothing" pattern of sulfation. The data suggested that the sulfating enzymes were located together with polymerizing enzymes in an enzyme complex so that sulfation of the heteropolysaccharide chain proceeded during polymerization or immediately following polymerization.

Sulfation of chondroitin sulfate by 3′-phosphoadenosine 5′-phosphosulfate was first described by D'Abramo and Lipmann (1957). Subsequently, 3′-phosphoadenosine 5′-phospho[^{35}S]sulfate has been utilized with soluble enzyme systems from several different sources to demonstrate the incorporation of sulfate into various exogenous or endogenous glycosaminoglycan acceptors (Adams, 1960; Suzuki and Strominger, 1960; Perlman *et al.*, 1964; Meezan and Davidson, 1967; Robinson, 1969). Incorporation of [^{35}S]sulfate could be demonstrated readily, but in all cases the amount of sulfate incorporated was extremely small in comparison to the sites available on the acceptors. In no case was the amount of sulfate incorporated sufficient to produce a demonstrable change in the chemical nature of an acceptor polysaccharide.

Recently it has become clear that the bulk of the chondroitin sulfating activity is particulate, occurring as microsomal enzymes rather than as soluble enzymes (DeLuca and Silbert, 1968; Silbert and DeLuca, 1969), although solubilization can be achieved readily. The sulfation of newly formed polysaccharide has been sufficient to modify the glycosaminoglycan products in a measurable fashion (Silbert and DeLuca, 1969). In the case of chondroitin sulfate, 25% of the hexosamine units in the newly formed glycosaminoglycan became sulfated. The distribution of sulfate within the newly synthesized polysaccharide chain was not determined, nor was it known whether the product was chondroitin 4-sulfate, chondroitin 6-sulfate, or a mixture. It also could not be determined from the data whether sulfation occurred during or after partial or full chondroitin polymerization. It was not known whether the sulfate was added to newly formed chondroitin in a random fashion or in a more ordered manner. Experiments were therefore undertaken with the microsomal system in order to examine these aspects of chondroitin sulfate synthesis in greater detail.

Experimental Procedures

UDP-[^{14}C]glucuronic acid, UDP-*N*-[^{3}H]acetylgalactosamine, and 3′-phosphoadenosine 5′-phosphosulfate were prepared as previously described (Silbert, 1962, 1964, 1967). Glycosaminoglycans, glycosaminoglycan-degrading enzymes, and disaccharide standards were obtained as described in the first paper of this series (Richmond *et al.*, 1973a). Microsomal preparations of chick embryo epiphyseal cartilage were prepared as previously described (Silbert, 1964, 1966). Separation of the radioactively labeled products was carried out as described (Silbert, 1964, 1966; Richmond *et al.*, 1973a) by treating the origins of paper chromatograms with pancreatin or alkali followed by dialysis.

Microsomal preparations were incubated at 37° in mixtures containing 0.05 M Mes (2-(*N*-morpholino)ethanesulfonic acid)[1] (pH 6.5), 0.01 M $MnCl_2$, and radioactive sugar nucleotides with or without pAdo-5′-phosphosulfate. In one series of experiments the sugar nucleotides and pAdo-5′-phosphosulfate were added at the same time; in another series, pAdo-5′-phosphosulfate was added subsequent to incubation of the microsomal preparation with the sugar nucleotides. In this latter series of experiments, aliquots of the reaction mixtures were removed at varying times after the addition of pAdo-5′-phosphosulfate.

Aliquots of reaction mixtures together with standards of hyaluronic acid and chondroitin 4-sulfate were chromatographed on a DEAE-cellulose column and eluted with a logarithmic gradient of LiCl as described in the first paper of this series (Richmond *et al.*, 1973a). Pooled fractions from the DEAE-cellulose column were dialyzed to remove LiCl and

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[1] Abbreviations used are: Mes, 2-(*N*-morpholino)ethanesulfonic acid; ΔDi-0S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-D-galactose; ΔDi-4S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-4-*O*-sulfo-D-galactose; ΔDi-6S, 2-acetamido-2-deoxy-3-*O*-(β-D-gluc-4-enepyranosyluronic acid)-6-*O*-sulfo-D-galactose; pAdo, 3′-phosphoadenosine.

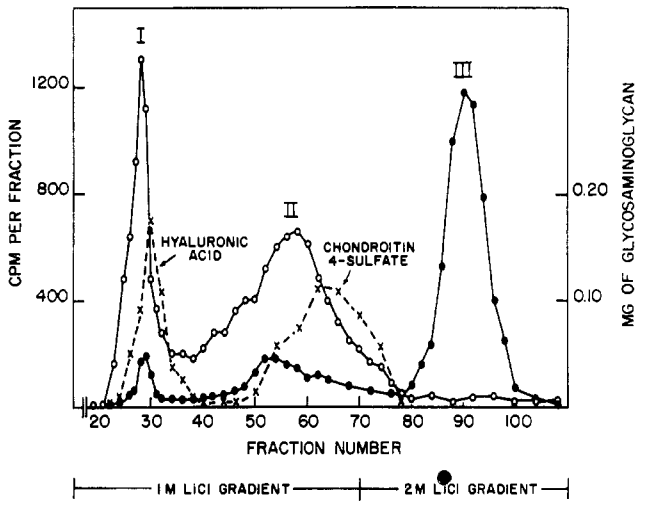

FIGURE 1: DEAE-cellulose chromatography of [^{14}C,^{3}H]glycosaminoglycan formed in the absence and presence of 3′-phosphoadenosine 5′-phosphosulfate. Incubations were carried out at 37° for 2.5 hr as follows: (1) 0.05 M Mes (pH 6.5), 0.01 M $MnCl_2$, UDP-[^{14}C]glucuronic acid (12.5 nmol (3 × 10^5 cpm)), UDP-*N*-[^{3}H]-acetylgalactosamine (9 nmol (2.5 × 10^5 cpm)), pAdo-5′-phosphosulfate (20 nmol), and 10 μl of microsomal preparation in a total volume of 25 μl. An additional 20 nmol of pAdo-5′-phosphosulfate was added after 15 min, 30 min, 1 hr, 1.5 hr, and 2 hr of incubation. (2) The same as (1) with the substitution of equivalent amounts of ATP instead of pAdo-5′-phosphosulfate. [^{14}C,^{3}H]Glycosaminoglycan was isolated as described under Experimental Procedures. Aliquots of each mixture were chromatographed together with hyaluronic acid (1 mg) and chondroitin 4-sulfate (5 mg) on a DEAE-cellulose column (1 × 5 cm) with a logarithmic gradient of LiCl. The mixing flask contained 125 ml of water and the reservoir initially contained 1 M LiCl. This was changed to 2 M LiCl at fraction 70. Fractions of 2.5 ml were collected and assayed for radioactivity and glycosaminoglycan standards. [^{14}C,^{3}H]Glycosaminoglycan formed in the absence of pAdo-5′-phosphosulfate (○—○) and formed in the presence of pAdo-5′-phosphosulfate (●—●) are shown superimposed on the results of elution of standard glycosaminoglycans (×- - -×).

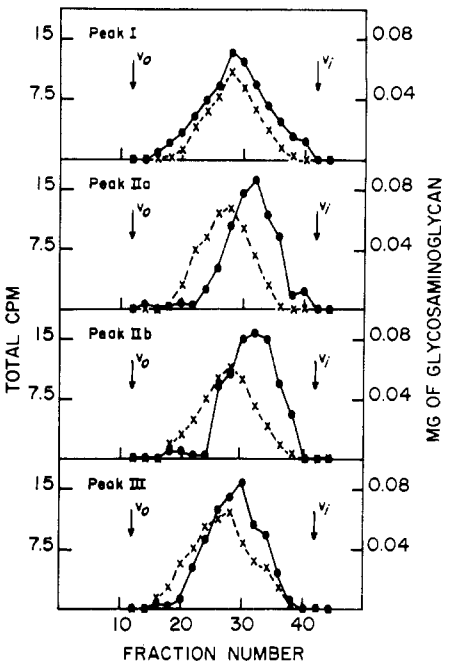

FIGURE 2: Sepharose 4B chromatography of [^{14}C,^{3}H]glycosaminoglycan formed in the presence of pAdo-5′-phosphosulfate. Aliquots of pooled fractions from the DEAE-cellulose column of [^{14}C,^{3}H]-glycosaminoglycan formed in the presence of pAdo-phosphosulfate (as shown in Figure 1 and Table I) were chromatographed on a Sepharose 4B column (1 × 60 cm) together with Blue Dextran (V_0), Phenol Red (V_i), and a standard of chondroitin 6-sulfate (1 mg). The eluent utilized was 0.1 M LiCl, with a flow rate of 4 ml/hr. Fractions of 1 ml were collected and assayed for radioactivity (●—●) and the chondroitin 6-sulfate standard (×- - -×).

lyophilized. Aliquots of the pooled fractions were then analyzed for molecular size by Sepharose 4B column chromatography as previously described (Richmond *et al.*, 1973a). Other aliquots were degraded with chondroitinase ABC, chondro-6-sulfatase, and chondro-4-sulfatase (Saito *et al.*, 1968) to determine the degree and nature of sulfation. The resulting disaccharides were identified as previously described (Richmond *et al.*, 1973a). Uronic acid containing material was assayed by the Bitter and Muir (1962) carbazole method. Radioactivity was determined with a low background (0.5 cpm) gas flow counter or with a liquid scintillation counter.

Results

Characterization of Products by Charge and Charge Density. The gradient elution of radioactive glycosaminoglycan from a DEAE-cellulose column is shown in Figure 1. All fractions contained equimolar amounts of ^{14}C and ^{3}H, although only the ^{14}C is shown. As has been described in the preceding paper (Richmond *et al.*, 1973b), material formed in the absence of pAdo-5′-phosphosulfate was found in two peaks. The first (peak I) appeared in an area near standard hyaluronic acid and the second (peak II) before, but overlapping, standard chondroitin 4-sulfate (mol wt 26,000). However, when pAdo-5′-phosphosulfate was present in incubation mixtures, the glycosaminoglycan product had a markedly different pattern when chromatographed on DEAE-cellulose. Material chromatographing in the area of peaks I and II was greatly diminished, while a new peak (peak III), appearing later, accounted for the majority of the material.

Characterization of Products by Size. Glycosaminoglycans were chromatographed on Sepharose 4B to determine approximate molecular size. The results of Sepharose chromatography of glycosaminoglycan formed in the absence of pAdo-5′-phosphosulfate have been shown earlier (Richmond *et al.*, 1973b). The results of chromatography on Sepharose 4B of glycosaminoglycan formed in the presence of pAdo-5′-phosphosulfate are shown in Figure 2. The small amount of peak I material was found to be approximately the same size as standard chondroitin 6-sulfate (mol wt 41,000) and thus similar in size to peak I formed in the absence of pAdo-5′-phosphosulfate. The average size of the peak II material (divided into subpeaks IIA and IIB) appeared to be somewhat smaller than the average size of the material found in peak I or in peak II of the glycosaminoglycan formed in the absence of pAdo-5′-phosphosulfate. Glycosaminoglycan that was found in peak III had approximately the same average size as the chondroitin 6-sulfate standard (mol wt 41,000) and thus was similar in size to the peak I glycosaminoglycan and to glycosaminoglycan formed in the absence of pAdo-5′-phosphosulfate.

Position of Sulfate. Aliquots from each peak of the glycosaminoglycan formed in the presence of pAdo-5′-phosphosulfate were degraded with chondroitinase to identify the sulfated glycosaminoglycan and to measure the degree of sulfation. Figure 3 shows the chromatogram of the degradation products of glycosaminoglycan from peak III. The radioactive areas contained equimolar amounts of ^{14}C and ^{3}H. Approximately 90% of the products obtained after degradation of peak III was found to co-chromatograph with a

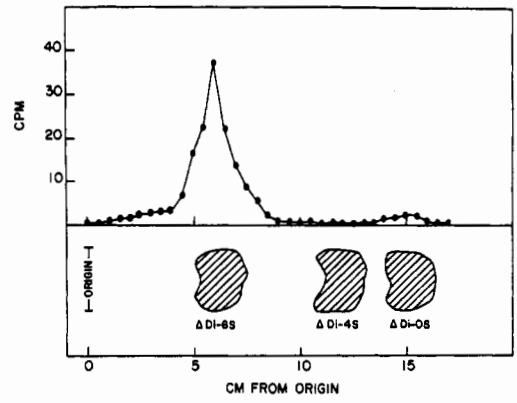

FIGURE 3: Products of chondroitinase treatment of [^{14}C,^{3}H]glycosaminoglycan formed in the presence of pAdo-5′-phosphosulfate. An aliquot of peak III (as described in Figure 1 and Table I) was incubated for 2 hr at 37° in a reaction mixture containing 20 μl of "enriched Tris buffer" (pH 8.0) (Saito *et al.*, 1968), 0.12 mg of chondroitin 4-sulfate, 0.12 mg of chondroitin 6-sulfate, and 0.5 unit of chondroitinase ABC in a total volume of 75 μl. The total reaction mixture was chromatographed and the products were identified as described previously (Richmond *et al.*, 1973a).

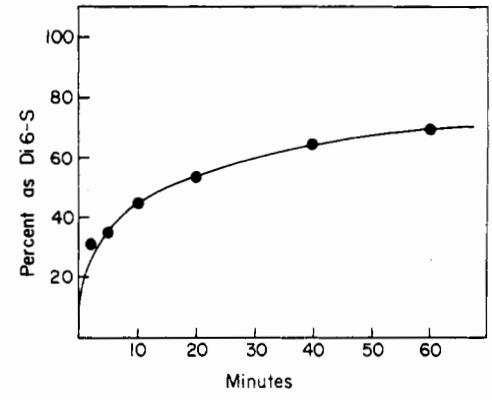

FIGURE 4: Sulfation of pre-formed [^{3}H]glycosaminoglycan with time. [^{3}H]Glycosaminoglycan was synthesized in a reaction mixture containing 0.05 M Mes (pH 6.5), 0.01 M $MnCl_2$, UDP-glucuronic acid (125 nmol), UDP-*N*-[^{3}H]acetylgalactosamine (45 nmol (12.5 × 10^5 cpm)), and 50 μl of microsomal preparation in a total volume of 75 μl. After 1 hr of incubation at 37°, 65 nmol of pAdo-5′-phosphosulfate was added, and incubation continued. At the indicated times following the addition of pAdo-5′-phosphosulfate, 15 μl was removed and the radioactive glycosaminoglycan isolated. An extra 10 nmol of pAdo-5′-phosphosulfate was added after 20 and 40 min of incubation. Chondroitinase digestions were performed directly on chromatographic origins, and the products were identified as described previously (Richmond *et al.* 1973a). The per cent of the total degradation products identified as ΔDi-6S is shown.

standard of ΔDi-6S,[1] while only 4% co-chromatographed with a standard of ΔDi-0S. There was no material appearing in the area of a standard of ΔDi-4S, although there was some material chromatographing earlier, in the area where a standard of acetylchondrosine 6-sulfate (the saturated disaccharide 6-sulfate) was found. (A saturated disaccharide would be produced from the terminal nonreducing end of a chondroitin sulfate chain.) The material chromatographing as ΔDi-6S could be degraded quantitatively to ΔDi-0S by chondro-6-sulfatase. Chondro-4-sulfatase was not effective on any of the degradation products.

Results of the chondroitinase digestions of all the peaks are shown in Table I. As well as ΔDi-0S and ΔDi-6S, there was in each case "other" incompletely identified radioactive material that chromatographed in the areas of standard saturated disaccharides. A larger percentage of this material was noted after chondroitinase digestions of peaks IIA and IIB than after digestions of peaks I and III. This might suggest that the newly formed glycosaminoglycans of peaks IIA and IIB were of shorter chain length, which is consistent with the observed smaller size of peaks IIA and IIB on Sepharose 4B. Peak IIB was found to contain a greater percentage of sulfated hexosamine units than peak IIA. All of the newly formed sulfated heteropolysaccharide chains were thus identified exclusively as chondroitin 6-sulfate, with no sulfate added to the 4 position.

More than 75% of the newly synthesized chondrosine repeating units were sulfated in these experiments. If the sulfate had been randomly distributed throughout all of the newly synthesized glycosaminoglycan chains, individual newly synthesized chains of all degrees of sulfation would have been seen and discrete peaks would not be expected to appear on the DEAE-cellulose chromatogram. Instead, the product chromatographed in three discrete peaks. Peak III, representing 75% of the newly formed heteropolysaccharide chains, was essentially completely (96%) sulfated (the appearance of peak III after the standard chondroitin 4-sulfate is consistent with a size larger than and a sulfate content greater than that of the standard); peak I, representing 5% of the newly formed chains, was essentially completely free of sulfate. Only 20% of the total material (peak II) containing, at most, 11% of the total sulfate incorporated might have consisted of individual chondroitin chains with intermediate amounts of sulfate. It should be noted, however, that peak II material contained smaller chains than peak III material and may in part have represented smaller fully sulfated chondroitin sulfate instead of chondroitin sulfate with intermediate amounts of sulfation. This could not be determined with the present studies.

TABLE I: Chondroitinase Digestion of Radioactive Glycosaminoglycans from a DEAE-Cellulose Chromatogram.

			% of Product Chromatographing as			
Peak	Fractions	% of Total	ΔDi-0S	ΔDi-4S	ΔDi-6S	Other
I	25–32	5	93	0	3	4
IIa	46–56	11	60	0	23	17
IIb	57–76	9	37	0	41	22
III	80–100	75	4	0	90	6

Time Course of Sulfation. In order to examine the nature of this "all or nothing" pattern, sulfation of preformed glycosaminoglycan was investigated. Sulfation with time is shown in Figure 4. Within 2 min of the addition of pAdo-5′-phosphosulfate to the microsomal preparation containing preformed radioactive chondroitin there was sulfation (exclusively in the 6 position) of approximately 30% of the galactosamine units. In this experiment, sulfation (all at the 6 position) reached approximately 70% at 1.5 hr. Thus, it could be seen that sulfation proceeded to a similar extent subsequent to polymerization as it did during polymerization.

Degree of Sulfation Relative to Time Course. Aliquots of radioactive glycosaminoglycan obtained from the incubation mixture 2, 10, and 40 min after the addition of pAdo-5′-phosphosulfate were chromatographed on a DEAE-cellulose column. Results are shown in Figure 5. A discrete peak III was clearly present at the end of 2 min and became increasingly

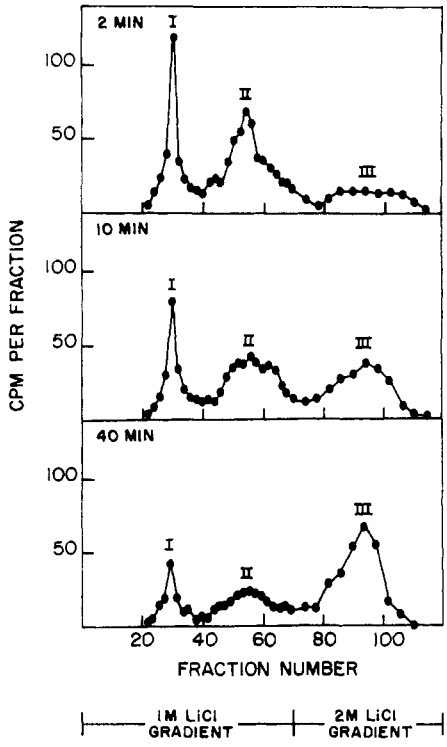

FIGURE 5: DEAE-cellulose chromatography of pre-formed [^{3}H]-glycosaminoglycan after varying periods of incubation with pAdo-5′-phosphosulfate. Aliquots of [^{3}H]glycosaminoglycan incubated with pAdo-5′-phosphosulfate for varying times as described in Figure 4 were chromatographed on a DEAE-cellulose column (1 × 5 cm) as described in Figure 1.

larger with time. There was a concomitant decrease in both peaks I and II. At no time were there significant amounts of radioactivity intermediate between any of the peaks. As before, chondroitinase digestion of the peak III materials yielded 90–95% ΔDi-6S with only a trace of ΔDi-0S. In each case, ~85–95% of the total sulfate incorporated was found in the peak III glycosaminoglycan with only 5–15% found in the peak II material.

Thus, it was concluded that, in general, once sulfation was initiated on a nonsulfated chain, it rapidly continued until the chain was nearly completely sulfated. An occasional missed galactosamine was indicated by the presence of some ΔDi-0S (4%) after chondroitinase digestions of peak III. This is consistent with the results shown in the first paper of the series (Richmond *et al.*, 1973a) indicating the presence of only an occasional nonsulfated galactosamine in endogenous chondroitin sulfate of high sulfate content and no detectable chondroitin sulfate of low sulfate content.

Discussion

Sulfation of ~25% of newly formed chondrosine units was described in a previous paper (Silbert and DeLuca, 1969). This has now been increased to more than 75% by changing the incubation mixtures from 0.05 M Tris (pH 7.8) to 0.05 M Mes (pH 6.5). It is likely that the increased sulfation in the pH 6.5 system was due to the greater stability of pAdo-5′-phosphosulfate, since it is rapidly degraded in reactions carried out in Tris (pH 7.8) but much less so in the pH 6.5 system. Sulfation at given time intervals occurred in an "all or nothing" pattern so that some chains were essentially completely sulfated, while others were essentially nonsulfated. Longer incubation with pAdo-5′-phosphosulfate resulted in more chains being sulfated rather than an increase in the sulfate content of individual chains.

The newly formed sulfated heteropolysaccharide chains were identified exclusively as chondroitin 6-sulfate. This is in contrast to the results obtained with endogenous material where 30–40% of the sulfate incorporated was into the 4 position (Richmond *et al.*, 1973a). Therefore, enzymatic activity capable of forming the chondroitin 4-sulfate was present, even though none of the newly formed heteropolysaccharide was sulfated in the 4 position. It could be suggested that the primer molecule on which the chondroitin chain was built plays a role in determining the site of sulfation. In this fashion, it is possible that in these experiments chondroitin was only formed on primer "programmed" to lead to chondroitin 6-sulfate.

No definitive conclusion can be made for the precise time of sulfation relative to polymerization. The results of these experiments are consistent with a process of sulfation concurrent with polymerization or a process of sulfation immediately following polymerization. The data presented in this series of papers would tend to indicate that sulfation occurs primarily during polysaccharide polymerization, since no nonsulfated chondroitin could be found in the microsomal preparation and smaller sulfated chains were found to be present in a small amount. However, it would also be possible that some sulfation occurs *in vivo* immediately after completion of the chain. In either case, it would be necessary for sulfation to take place at the same site in the cell as polymerization. Thus, it does not seem likely, as has been suggested (Horwitz and Dorfman, 1968), that polymerization occurs as the growing polysaccharide moves along the reticuloendothelial system, with sulfation occurring later after the completed polysaccharide has moved to a separate site. The present data are also inconsistent with the suggestion that *in vivo* sulfation is a necessary prerequisite for further polysaccharide formation (Derge and Davidson, 1972), since it is clearly shown that, at least *in vitro*, extensive polymerization can precede extensive sulfation.

The mechanism of rapid sulfation together with the previously described rapid polymerization (Richmond *et al.*, 1973b) suggest that a synthesizing "complex" of enzymes might be localized together in the cell to complete the total assembly and sulfation of chondroitin sulfate. The completed product would then be moved for export to the extracellular matrix.

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Functional Arginyl Residues in Carboxypeptidase A. Modification with Butanedione†

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ABSTRACT: Treatment of carboxypeptidase A with butanedione in borate buffer markedly decreases peptidase activity correlating with the reversible modification of a single arginyl residue. This correlation supports the hypothesis that the specificity of carboxypeptidase is due to interaction of the terminal carboxyl group of peptide substrates with an arginyl residue of the enzyme. However, this modification simultaneously increases apparent esterase activity threefold indicating that the mode of binding of esters differs from that of peptides. If modification is carried out in the absence of borate, peptidase activity still decreases but esterase activity is unchanged. Binding of esters, like that of inhibitors, may involve a metal ion–carboxyl group interaction whereas peptide binding requires an arginyl residue as the positively charged substrate recognition site.

Carboxypeptidases catalyze the hydrolysis of carboxyl-terminal peptide and ester bonds of peptides or their depsipeptide analogs. The presence of a free terminal α-carboxyl group of the substrate is a strict specificity requirement of these enzymes. A positively charged residue in the active center of bovine carboxypeptidase A has long been thought to account for this specificity (Waldschmidt-Leitz, 1931; Smith, 1949; Vallee *et al.*, 1963). Since the zinc atom does not appear to be essential for peptide binding (Coleman and Vallee, 1964), we have considered a positively charged residue such as lysine or arginine to be the most likely alternative. Acylation experiments have excluded lysyl residues from binding functions (Riordan and Vallee, 1963). Hence, it seemed probable that arginine might be important for the specificity of this enzyme, and therefore attempts were made to identify a functional arginyl residue by means of chemical modifications (Vallee and Riordan, 1968; Riordan, 1970).

Reagents suitable for the modification of arginyl residues in proteins under relatively mild conditions have been described only recently (Yankeelov *et al.*, 1968). One of the first of these, butanedione, had been used successfully in the identification of antibody-combining sites and seemed particularly promising (Grossberg and Pressman, 1968).

Indeed, under the conditions to be described this reagent and a number of other α-dicarbonyl compounds block arginyl residues of carboxypeptidase A, and one of these appears to be involved in the productive binding of peptide but not of ester substrates (Vallee and Riordan, 1968). Since then similar effects on activity have been found for carboxypeptidase B suggesting a common basis for their analogous substrate specificities (Werber and Sokolovsky, 1972).

Materials and Methods

Bovine carboxypeptidase A, prepared according to the procedure of Anson (1937), and porcine carboxypeptidase B were obtained from the Worthington Biochemical Corp. Butanedione was a product of Eastman and was distilled immediately prior to use. Crystalline trimeric butanedione was kindly provided by Dr. John A. Yankeelov, Jr. All other chemicals were of the highest grade available. All buffer solutions were extracted with 0.1% dithizone in CCl_4 and other precautions, previously described, were taken to prevent contamination by adventitious metal ions (Thiers, 1957).

In preliminary experiments, a 15% solution of butanedione was incubated overnight at room temperature according to the method of Yankeelov *et al.* (1966) as modified by Grossberg and Pressman (1968) prior to addition to carboxypeptidase. Borate, Tris, Veronal, bicarbonate, and *N*-hydroxyethylpiperazine-*N*-2-ethanesulfonate (Hepes),[1] all at 0.05 M, pH 8.6, were examined for their suitability as buffers. Both borate and Tris were found to interact with butanedione as evidenced by an immediate fall in pH on addition of the reagent to the buffer solution. The remaining three buffers did not appear to react with butanedione nor to have any other specific effects. Subsequently, it was found that preincubation of butanedione was unnecessary for modifying carboxypep-

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[1] Abbreviations used are: Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulfonate; Mes, 2-(*N*-morpholino)ethanesulfonate.