Butyl Isocyanate, an Active-Site-Specific Reagent for Yeast Alcohol Dehydrogenase†

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ABSTRACT: Butyl isocyanate has been found to inactivate yeast alcohol dehydrogenase in a reaction involving 3 mol of sulfhydryl groups/mol of enzyme. Based on the specificity of the reaction and on the effect of the substrate, 1-butanol and of the coenzymes as protective agents in the inactivation, it is concluded that butyl isocyanate acts as a true substrate analog and thus can be considered to be an active-site specific reagent for yeast alcohol dehydrogenase. The identification of sulfhydryl groups as the sites of the chemical modification is based on the disappearance of free sulfhydryl groups in the reaction, on the similarity in chemical properties of the butylcarbamoyl-enzyme derivative and authentic *S*-(butylcarbamoyl)cysteine, and on the isolation and identification of *S*-(butylcarbamoyl)cysteine from an enzymatic digest of the inactive yeast alcohol dehydrogenase derivative. Using [^{14}C]-butyl isocyanate in the inactivation, a single radioactive peptide was obtained after pepsin digestion. The peptide was purified (16% yield) and preliminary sequence studies suggest that its structure is Cys-Ala-Gly-Ile-Thr-Ala.

Active-site-specific reagents, substrate analogs which contain chemically reactive groups suitable for covalent bond formation with the enzyme once the Michaelis–Menten (the enzyme–analog) complex has formed, have become very useful tools in the exploration of enzyme structure and function. It was recently reported that simple alkyl isocyanates can be used as active-site-specific reagents for serine esterases (Brown and Wold, 1971). For these compounds, the alkyl chain provides the active-site recognition component and the isocyanate the chemically reactive group which can form covalent derivatives of several functional groups in proteins. In the case of the esterases chymotrypsin and elastase the reaction is between the isocyanate and the active-site serine to form the substituted carbamate (W. E. Brown, personal communication) but reaction with the SH group of cysteine to yield substituted thiocarbamates, or with amino groups to form substituted ureas are also very likely to occur with these reagents. In connection with the studies on the esterases, it was also found that papain was inactivated by reaction with stoichiometric amounts of butyl isocyanate at low pH, and in this case it was demonstrated that the active-site SH group was derivatized (L. E. Wyborny, personal communication). The reason for the low pH optimum for the reaction between butyl isocyanate and papain is due to the lability of the thiocarbamate product, which readily breaks down at a pH at or above neutrality.

Given the rather nonselective reactivity of the isocyanates and the fact that a large number of enzymes act on substrates containing simple aliphatic chains (all the enzymes catalyzing reactions involving fatty acids, for example) it occurred to us that the alkyl isocyanates may be useful as simple and quite general active-site-specific reagents, applicable to the exploration of the active-site structure of a large number of enzymes. As a first test of this hypothesis we have reacted yeast alcohol dehydrogenase with butyl isocyanate. Since butanol is a substrate for this enzyme, we predicted that the reagent should satisfy the requirements as a substrate analog; furthermore, since the enzyme is known to contain reactive sulfhydryl groups in the active site, we also predicted that covalent bond formation between the reagent and the active site should be likely to occur. These predictions have been tested and found to be correct. The characteristics of the enzyme–butyl isocyanate reaction, the properties of the reaction product, and the structure of the isolated butylcarbamoyl-peptide are reported in this paper.

The results of these tests of butyl isocyanate as an active-site-specific reagent for the dehydrogenase have also given some new information about the enzyme's active-site structure. It is well established that the dehydrogenase is a tetramer made up of four identical or very nearly identical subunits (Pfleiderer and Auricchio, 1964; Harris, 1964a). It has also been concluded that the enzyme has four active sites based on coenzyme binding (Hayes and Velick, 1954), on Zn^{2+} binding (Kagi and Vallee, 1960), and on the specific inactivation of the enzyme associated with the reaction of four sulfhydryl groups with iodoacetamide (Whitehead and Rabin, 1964). However, Dickinson (1970) has recently questioned this exact stoichiometry of four, finding only three NADH binding sites upon fluorescence titration of the dehydrogenase with NADH in the presence of acetamide, and he has suggested that a negative cooperativity may exist between the four sites. The work presented here leads to the same conclusions in showing that the inactivation of the dehydrogenase by butyl isocyanate is associated with the incorporation of only 3 mol of reagent. Furthermore, since the sulfhydryl groups involved in the reaction with butyl isocyanate will be shown to be different from those reacting with iodoacetamide (Harris, 1964b), the work also suggests that there must be at least two "critical" sulfhydryl groups in or near each active site of the dehydrogenase.

Experimental Section

Materials. Yeast alcohol dehydrogenase (twice crystallized), NAD, and cellulose phosphate were purchased from Sigma Chemical Co. 5,5′-Dithiobis(2-nitrobenzoic acid) and NADH were obtained from Calbiochem. [^{12}C]- and [^{14}C]butyl iso-

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cyanate (specific radioactivity of 3.25 Ci/mol) were the products of Aldrich Chemical Co. and New England Nuclear, respectively. Pepsin was obtained from Pentex, Inc.; aminopeptidase M from Henley and Co. Inc.; *Streptococcus griseus* protease (Pronase) from Sigma Chemical Co.; and agarose-bound Pronase was prepared in this laboratory by Mr. C. C. Q. Chin.

The purity of the yeast alcohol dehydrogenase samples used in this work was checked by acrylamide gel electrophoresis, and based on both protein and activity stains, the enzyme was estimated to be at least 90% pure.

Assays. Yeast alcohol dehydrogenase activity was measured in the following reaction mixture (final concentration): 4.8×10^{-4} M NAD^+, 1×10^{-2} M semicarbazide, 0.27 M ethanol, 0.1 M potassium phosphate (pH 7.0), and a suitable amount of diluted enzyme in a final volume of 3 ml. The formation of NADH was followed at 340 nm with a Cary 15 recording spectrophotometer at room temperature. The dehydrogenase concentrations were determined either by the method of Lowry *et al.* (1951) or by the absorbance at 280 nm using an absorbancy value of 12.6 for a 1% solution (Hayes and Velick, 1954). Radioactivity was assayed with a Beckman LS-133 liquid scintillation spectrometer using glass vials containing 10 ml of Beckman toluene–fluorally TLA cocktail containing 10% (v/v) Bio Solv formula BBS-3 solubilizer. The counting efficiency for ^{14}C was 97% in this system. Sulfhydryl content of yeast alcohol dehydrogenase and its derivatives was determined by titration with 5,5′-dithiobis(2-nitrobenzoate) (Ellman, 1959) according to the method described by Vanaman and Stark (1970) and using the molar absorbancy of 13,600 at 412 nm for the 5-thio-2-nitrobenzoate anion. Quantitative amino acid analyses were performed with a Beckman Model 120C automatic amino acid analyzer according to the method of Spackman *et al.* (1958). The [^{14}C]butyl isocyanate labeled peptide was oxidized with performic acid (Moore, 1963) before it was hydrolyzed with 6 N HCl at 110° for 24 hr.

Quantitative organic microanalyses were carried out by Galbraith Laboratories, Inc., Knoxville, Tenn.

Synthesis of S-(Butylcarbamoyl)-L-cysteine. A solution containing 1 g (6.4 mmol) of cysteine hydrochloride in 200 ml of 0.1 mM EDTA was adjusted to pH 5.7 with 0.1 M sodium hydroxide. The solution was deaerated and stirred with a constant stream of nitrogen gas while 2 ml (20 mmol) of butyl isocyanate was slowly added to the reaction mixture at room temperature over a period of 20 min. After lyophilization, one-third of the dried material was dissolved in 50 ml of 0.17 M pyridine acetate (pH 4.7) (molarity based on acetate) and chromatographed on a 1.5×58 cm column of Dowex 50W-X2 resin equilibrated and eluted with the same buffer (250 ml). Fractions containing ninhydrin-positive material were located by spotting small aliquots on paper and spraying with ninhydrin reagent after removing the solvent by heating at 100° for 20 min. Two ninhydrin-positive peaks, one at 40–60 ml and one at 70–100 ml, were obtained. The fractions making up each peak were pooled and lyophilized. Ion-exchange chromatography on the long column of the amino acid analyzer showed that the first peak contained cysteic acid, cysteine and cystine, while the second peak contained a single ninhydrin-positive compound which eluted at the position of the buffer change (close to methionine). The lyophilized material from the second peak was dissolved in a minimal amount of distilled water and the solution was filtered. The filtrate was then treated with 3.4 volumes of cold acetone and after several hours at −20°, crystallization occurred. The fine crystals were collected by filtration, washed with cold acetone, and stored in a desiccator. The yield of crystalline material was approximately 25%. The crystals melt at 189–190° (uncor). The yield can undoubtedly be improved by taking proper note of the lability of the product (see Results sections), and carrying out the reaction and the subsequent purification steps at low temperature, for example. *Anal.* Calcd for $C_8H_{16}N_2O_3S$ (220.3): C, 43.63; H, 7.32; N, 12.72; S, 14.56. Found: C, 43.50; H, 7.26; N, 12.63; S, 14.48 (average of two separate analyses).

Reaction of Enzyme with Butyl Isocyanate. In a typical experiment 25 mg (0.17 μmol) of enzyme in 5 ml of 0.1 M potassium phosphate buffer (pH 5.7) and at 0° was treated with an acetone solution of [^{14}C]butyl isocyanate (91.2 μmol/ml). The reagent was added in increments of 4 μmol of reagent/μmol of enzyme, and after each addition the reaction was allowed to proceed for 15 min. At this time aliquots were removed for the determination of activity and sulfhydryl content in comparison to reagent-free controls, and a 0.5-ml aliquot was also removed for the determination of reagent incorporation. The 0.5-ml sample was subjected to gel filtration on a 1×45 cm column of Sephadex G-25 (coarse) with 0.1 M potassium phosphate buffer (pH 5.7) as eluent. The excluded peak was collected and protein and radioactivity were determined as described above. For larger scale preparations of the butyl isocyanate inactivated enzyme, most of the individual analyses were omitted, and the addition of reagent was monitored only by activity measurements. The reaction was stopped when less than 5% activity remained (usually at the addition of 8 mol of reagent/mol of enzyme) and the product was collected and characterized. It should be noted here that the half-life of alkyl isocyanate in aqueous solutions is of the order of 2 min, so little or no intact reagent remains when the above protocol is followed. Since the ^{14}C-labeled reagent used was labeled in the isocyanate group, the radioactivity of decomposed reagent was released as CO_2.

Results

Some Properties of S-(Butylcarbamoyl)-L-cysteine. As a prerequisite to the characterization of the butyl isocyanate yeast alcohol dehydrogenase reaction product, some properties of the model compound were established. The elemental analysis together with the positive ninhydrin color reaction (with a color yield identical with that of methionine) established the identity of the synthetic product as *S*-(butylcarbamoyl)-L-cysteine. A summary of the stability of this compound is given in Table I and shows that at acid pH (from about pH 2 to 6) the compound is quite stable. At pH values above neutrality, however, the rate of decomposition becomes significant and at pH 10.5 and 30° complete decomposition was observed in 1 hr. In all cases cystine appeared to be the product of the decomposition. As expected strong acid and oxidation also destroyed the derivative; after 21-hr hydrolysis with 6 N HCl at 110° a mixture of cystine and cysteine, accounting for a normal yield of half-cystine from protein hydrolysates was obtained, and upon oxidation with performic acid a quantitative conversion to cysteic acid was observed.

Inactivation of Yeast Alcohol Dehydrogenase by Butyl Isocyanate. Stoichiometry and Specificity of the Reaction. When the dehydrogenase was treated with increasing levels of butyl isocyanate, the activity decreased, and a concomitant decrease in SH groups was also observed. A typical plot of loss of activity and SH groups as a function of reagent incorporation is given in Figure 1. Several conclusions can be drawn from these results. First of all, since the highest level of butyl

TABLE I: Effect of pH and Temperature on the Stability of *S*-(*N*-Butylcarbamoyl)-L-cysteine (BCC).

	Incubation for 1 hr at 30°[a]		Incubation for 19 hr at 4°[b]	
pH	Recov of BCC[c]	$^1/_2$-Cys Produced[d]	Recov of BCC	$^1/_2$-Cys Produced
2.5	100	0	100	0
5.5	99	0	100	0
7.0	91	4		
7.5	76	11	80	13
8.5	13	89	60	41
9.0			44	52
10.0			18	90
10.5	0	100		

[a] Samples (1 ml) containing 0.07 μmol of BCC were used. [b] Samples (3 ml) containing 0.14 μmol of BCC was used. In both cases the solvent was 0.1 M ammonium acetate buffer adjusted to the indicated pH. The samples were incubated as specified, lyophilized and analyzed on the amino acid analyzer for BCC and Cys content.

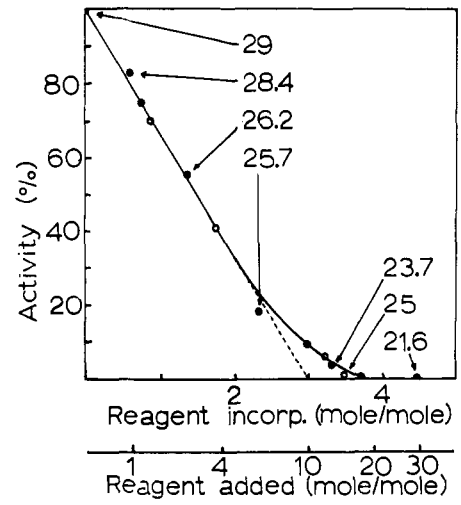

FIGURE 1: Inactivation of yeast alcohol dehydrogenase by [^{14}C]butyl isocyanate at 0°. The enzyme (3×10^{-5} M) in 0.1 M potassium phosphate buffer (pH 5.7) was treated with successive additions of butyl isocyanate. After each addition the reaction mixture was incubated for 15 min and aliquots were then removed for the determination of activity, sulfhydryl content, and reagent incorporation as described in the text. Reagent incorporation as well as the amount of reagent added to the reaction mixture (both in moles of reagent per mole of enzyme) are given on the abscissa. The sulfhydryl content is indicated directly on the graph in moles of sulfhydryl groups per mole of enzyme. The open and closed circles represents results from two separate experiments.

isocyanate added was 10 mol of reagent/mol of enzyme, the reaction is quite specific, especially when one considers the fact that the reagent is rapidly hydrolyzed. It is also clear from the data in Figure 1 that 3 mol of reagent must react before all activity is lost, and that within experimental errors the 3 groups reacting are sulfhydryl groups. All these observations are consistent with the thesis that butyl isocyanate is an active-site-specific reagent for yeast alcohol dehydrogenase. In an attempt to obtain further evidence that the active site is indeed directly involved in the inactivation, we investigated the effect of coenzymes and substrates on the reaction. The results are presented in Table II and are in good general agreement with the observations by Whitehead and Rabin (1964) on the effect of the same compounds as protective agents against inactivation of the dehydrogenase by iodoacetate and iodoacetamide. The relative protection afforded by NADH and NAD^+ is consistent with the respective K_m values for these two coenzyme forms; and complete protection was observed with the combination of NAD^+ and pyrazole, which is known to form a very tight ternary complex with alcohol dehydrogenase (Theorell and Yonetani, 1963). It is most significant, however, that butanol itself gave substantial protection, since this finding represents the most direct argument for competition between substrate and reagent for a single site. Another more indirect argument in favor of the thesis that butyl isocyanate is a substrate analog for the dehydrogenase is based on the fact that another similar enzyme, lactate dehydrogenase, which is also inhibited by sulfhydryl reagents, was not inactivated by butyl isocyanate at concentrations 100× those used to inactivate yeast alcohol dehydrogenase.

It has been implied above that the inactivation is caused by the specific formation of the *S*-(butylcarbamoyl) derivative of the active-site sulfhydryls in yeast alcohol dehydrogenase. With some knowledge of the properties of such derivatives based on the studies on the model compound, it should be possible to establish whether the proposed derivative is a reality. To this end, the inactive butylcarbamoyl-enzyme derivative was incubated under different conditions, and the reactivation of the enzyme was followed in parallel with loss of ^{14}C and reappearance of SH groups. The results are given in Table III, and together with the fact that performic acid oxidation caused complete loss of radioactivity from the butylcarbamoyl-enzyme, they demonstrate the close similarity between the authentic *S*-(butylcarbamoyl)cysteine and the enzyme derivative. An attempt was also made to isolate butyl-

TABLE II: Effect of Substrates and Coenzymes on the Inactivation of Yeast Alcohol Dehydrogenase by Butyl Isocyanate.

Additions (Concn)	Act. Remaining after Treatment with Butyl Isocyanate (% of Native Enzyme)[a]
None	0
NADH (2.9×10^{-5} M)	55
NAD^+ (3.6×10^{-5} M)	15
1-Butanol (0.14 M)	21
Ethanol (0.4 M)	5
Acetaldehyde (4×10^{-4} M)	0
NAD^+ (5.8×10^{-3} M)	37
pyrazole (1.2×10^{-2} M)	2
NAD^+ + pyrazole	110

[a] Aliquots of a 7.3×10^{-6} M enzyme solution in 0.1 M potassium phosphate buffer (pH 5.7), and containing the additions indicated, were treated with 10 mol of butyl isocyanate/mol of enzyme. After 15 min at 0°, the enzyme activity was determined and compared to the butyl isocyanate free control.

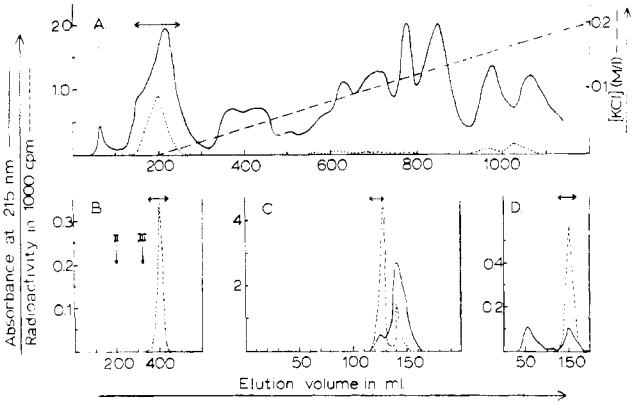

FIGURE 2: Isolation of the derivatized peptic peptide from butyl isocyanate inactivated yeast alcohol dehydrogenase. The experimental details are given in the text. (A) Fractionation of the initial peptic digest on phosphocellulose. (B) Fractionation of the main radioactive peak from part A on Dowex 50W-X2. The vertical arrows indicate the changes in elution buffer. (C) Gel filtration of the radioactive peak from B on Sephadex G-25. (D) Fractionation of the main radioactive peak from C on phosphocellulose. The horizontal arrows indicate the fractions pooled in each step. The final pooled fraction from D was subjected to amino acid analysis and sequence determination.

carbamoylamino acids from enzymatic digests of inactive yeast alcohol dehydrogenase. The modified enzyme was subjected to proteolytic digestion with pepsin (3 hr at pH 2) and Pronase plus aminopeptidase M (5 hr at pH 7). When the resulting digest was analyzed on the amino acid analyzer, 15% of the total radioactivity in the starting material was recovered. Of this 15%, only one-half could be identified as *S*-(butylcarbamoyl)cysteine. Perhaps the most significant part of this experiment was the total absence of other more stable butylcarbamoylamino acid derivatives in the digestion mixture (both the *O*-serine, and the N^{ϵ}-lysine and the *O*-tyrosine derivatives have been prepared and characterized by Mr. C. C. Q. Chin in this laboratory).

Isolation and Characterization of the S-(Butylcarbamoyl)-cysteine-Containing Peptide. The experimental plan for the peptide isolation was standard for this type of work, with only one unusual stipulation, that all the steps be carried out below pH 6.5–7. The starting material was 185 mg of inactivated yeast alcohol dehydrogenase, containing 4.25 μmol of ^{14}C/μmol of enzyme (total radioactivity 3.63×10^7 cpm). The lyophilized protein was dissolved in 50 ml of 0.02 N H_3PO_4, the pH was adjusted to 2.0 with concentrated H_3PO_4 and the protein was digested with 4 mg of pepsin for 4 hr at room temperature. The total digest was then applied to a 1.5×40 cm column of phosphocellulose, equilibrated and eluted with 200 ml of the original 0.025 N H_3PO_4 solution (Chin and Wold, 1972). It was further eluted with a three-chamber gradient of 350 ml each of 0.025 N H_3PO_4–0.01 M KCl in 0.025 N H_3PO_4 and 0.2 M KCl in 0.025 N H_3PO_4 (Figure 2a). The major radioactive material emerging before the start of the gradient contained 1.8×10^7 cpm (50%) while the other minor radioactive components eluting later accounted for a total of 2.5×10^6 cpm (15%). Thus, 65% of the starting material was accounted for at this stage. The fractions containing the main radioactivity peak were pooled and lyophilized, and the dried residue, dissolved in 5 ml of 0.05 M pyridine acetate buffer (pH 3.3) (molarity based on pyridine) was subjected to ion-exchange chromatography on a 1.5×45 cm column of Dowex 50W-X2, equilibrated and eluted with

TABLE III: Effect of pH on Butyl Isocyanate Modified, Inactive Yeast Alcohol Dehydrogenase.[a]

		Changes Observed		
pH	Incubn Time at 4° (hr)	Act. Increase[b] (%)	SH Increase[c] (mol/mol of Enzyme)	^{14}C Decrease[d] (%)
5.7	19	12		15
6.7	14	35	1.5 ± 1.5	
	19	47		64
	60	80	2.7 ± 1.5	
7.5	14	40	2.2 ± 1.5	
	19	60		68
	60	90	3.8 ± 1.5	
10.5	1			84

[a] Aliquots containing 0.017 μmol of inactive *N*-butylcarbamoyl-enzyme in 0.1 M potassium phosphate buffer adjusted to the indicated pH and incubated at 4°. [b] Activity is reported as per cent of control samples of native enzyme incubated with the experimental samples. The actual activity loss in the 100% control was 12% in 60 hr. The activity of the inactivated enzyme was 2%. [c] SH increase is reported as the difference between the incubated sample and the zero-time value for the butylcarbamoyl-enzyme. Our values for SH content in the native enzyme (27–30 mol/mol) ran consistently lower than the literature value (36 mol/mol, and we had an observed error of about ±2% in our measurements. The accumulated error for the SH content in incubated samples, relative to the zero-time control is therefore ±1.5 mol/mol. The inactive starting material had lost 5.2 ± 1.5 SH groups relative to the native enzyme. [d] Determined after gel filtration on a 1×50 cm column of Sephadex G-25, eluted with 0.1 M potassium phosphate buffer (pH 5.7). The results are given as per cent decrease to facilitate the direct comparison to activity recovery. The radioactive starting material at zero time thus represents 0, and a sample completely freed of radioactivity 100% on the scale. [e] Incubated in 0.1 M ammonium acetate buffer at 30° to parallel the conditions of the experiments reported in Table I.

200 ml of the same 0.05 M pyridine acetate buffer. The column was further eluted with 150 ml of 0.2 M pyridine acetate (pH 4.0) (molarity based on pyridine) and finally with 200 ml of 0.17 M pyridine acetate (pH 4.7) (molarity based on acetate). As shown in Figure 2b, only one homogeneous radioactive peak appeared, containing 1.3×10^7 cpm (36%). The pooled radioactive material was lyophilized and dissolved in 2.0 ml of 0.003 N HCl and subjected to gel filtration on a 1.5×120 cm column of Sephadex G-25 (fine) equilibrated and eluted with 0.003 N HCl (Figure 2c). The main radioactive fraction, 9.7×10^6 cpm (25%) was lyophilized. The residue was dissolved in 5 ml of 0.025 N H_3PO_4 and rechromatographed on the phosphocellulose column (1.5×40 cm) equilibrated and eluted with the same buffer. As seen in Figure 2d, a single homogeneous radioactive peak coincident with the peptide absorption peak at 215 nm was obtained. This radioactive peak accounted for 5.7×10^6 cpm, or 16% of the starting material. An aliquot containing about 2.5×10^5 cpm was oxidized with performic acid and then hydrolyzed. Amino acid analyses showed that the isolated peptide consisted of

Cys, Thr, Gly, Ala, Val, and Ile (Table IV). The quantity of peptide subjected to amino acid analysis was 18 nmol (based on the specific radioactivity), which agreed well with the amount of amino acids released (18–22 nmol).

Attempts to identify cysteine as the residue modified by butyl isocyanate by digestion of the isolated radioactive peptide with either carboxypeptidse A or leucine aminopeptidase were unsuccessful in that neither enzyme gave any release of a radioactive amino acid derivative. Therefore, the isolated radioactive peptide (8×10^4 cpm) was incubated with 1.5 ml of agarose-bound Pronase in 0.05 M sodium borate buffer (pH 7.0) containing 0.001 M $CaCl_2$, for 1 hr at 37°. After the incubation, half of the incubation mixture (about 4×10^4 cpm) was placed on the long column of the amino acid analyzer and the eluted material was collected at 2-min intervals directly from the column. Only one radioactive peak was detected; it eluted at the position of the buffer change, coincident with a sample of authentic *S*-(butylcarbamoyl)-L-cysteine (Figure 3).

The amino acid sequence of the hexapeptide was determined by subtractive Edman degradation (Konigsberg, 1967) after performic acid oxidation and by carboxypeptidase digestion. Some difficulties were encountered with the differential solubility of the peptides and the phenylthiohydantoins in the Edman degradation using either benzene or ethyl acetate as the organic solvent, and the yields were both variable and low at each step. The most reasonable interpretation of the data in Table V is that the sequence of the peptide is Cys-Ala-Gly-Ile-Thr-Val.[1] Even if this sequence can only be considered as a tentative one, the data clearly establish that the Cys residues modified by butyl isocyanate are not the same as those modified by iodoacetamide (Harris, 1964b).

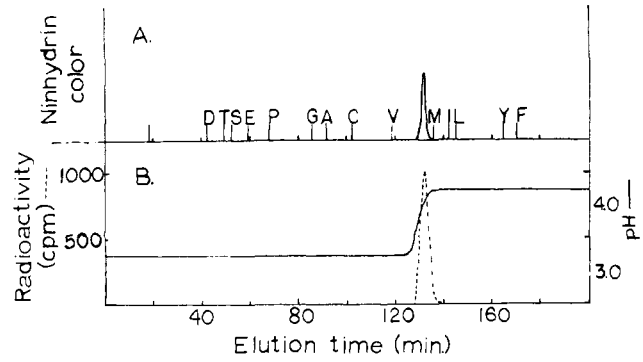

FIGURE 3: Ion-exchange chromatography of *S*-(butylcarbamoyl)-cysteine on the amino acid analyzer. (A) Ninhydrin peak positions observed with a standard amino acid mixture (coded with one-letter symbols) containing cysteic acid (first unlabeled line) and butylcarbamoylcysteine (drawn as peak in front of methionine). (B) Elution position of the radioactive product obtained either from Pronase digestion of butylcarbamoyl-hexapeptide or from pepsin–Pronase–aminopeptidase M digestion of butylcarbamoyl-enzyme. The pH change, signifying the buffer change position serves as a convenient marker for the elution position of butylcarbamoyl-cysteine.

Discussion

The work reported in this paper was initiated to test the proposition that butyl isocyanate is an active-site specific reagent of yeast alcohol dehydrogenase. On the basis of the criteria of selectivity and specificity of the inactivation reaction, we feel that the results support the proposition. Butyl isocyanate has a half-life of about 2 min under the reaction conditions used in this work (Brown and Wold, 1971), and a rapid and efficient interaction with the enzyme is therefore required to compete with the hydrolytic decomposition of the reagent. At an enzyme concentration of 3×10^{-5} M nearly 50% of the added reagent was incorporated into the enzyme in the initial (linear) part of the titration curve shown in Figure 1, and even at the later stages of the titration only a 20-fold molar excess of reagent was required to give an incorporation of 4 mol of reagent/mol of enzyme. The curvature in the titration curve clearly identifies three uniquely reactive sulfhydryl groups in the enzyme, and since these groups are protected by coenzymes and by butanol, we conclude that their unique reactivity is due to the formation of a Michaelis–Menten complex between the enzyme and the reagent, thus bringing the sulfhydryl group and the isocyanate group in the proper juxtaposition for the facilitated reaction to occur. The absence of similarly reactive sulfhydryl groups in lactate dehydrogenase is consistent with this conclusion.

The conclusion that the reaction between the enzyme and butyl isocyanate is specific is derived at two levels, by establishing first that only sulfhydryl groups are involved in the reaction and next that only a certain sulfhydryl group (an active-site SH) is involved. The first of these is well established in spite of the analytical uncertainties encountered in

TABLE IV: Amino Acid Composition of the Isolated *N*-Butylcarbamoyl-peptide.[a]

Amino Acid	nmol Found	No. of Residues
Tryptophan	nd	
Lysine	0	
Histidine	0	
Arginine	0	
Aspartic acid	0	
Threonine	18.6	1.0
Serine	0	
Glutamic acid	0	
Proline	0	
Glycine	21.7	1.2
Alanine	21.8	1.2
Cysteine (as cysteic acid)	17.8	1.0
Valine	17.7	1.0
Methionine	0	
Isoleucine	21.2	1.2
Leucine	Trace	
Tyrosine	0	
Phenylalanine	0	

[a] A sample containing 18 nmol of performic acid oxidized and hydrolyzed peptide (based on radioactivity measurements on the isolated peptides) was applied to the analyzer column. The number of residues is thus calculated relative to one butylcarbamoyl residue.

[1] It has been called to our attention that the small decrease in glycine in the third degradation step with return to a high value after the subsequent step could be expected if an oxidized tryptophan residue were present in the third position. Although we have no data to eliminate this possibility, it appears very unlikely that an intact x-x-Trp-x-x- peptide should be recovered in such a high yield after pepsin digestion of the enzyme.

TABLE V: Sequence Determination of the Butylcarbamoyl-hexapeptide by Subtractive Edman Degradation and Carboxypeptidase A Digestion.

Subtractive Edman Degradation of Oxidized Peptide	Amino Acid Ratio in Peptide[a]					
	$CySO_3$	Thr	Gly	Ala	Val	Ile
Initial	0.8	1.0	1.2	1.0	1.0	1.1
First degradation	*0.3*	1.0	1.2	1.0	1.0	1.0
Second degradation	0.4	1.1	1.2	*0.5*	1.0	1.0
Third degradation	0.6	1.2	*0.9*	0.6	1.0	1.0
Fourth degradation	0.2	1.0	1.1	0.8	1.0	*0.4*

Carboxypeptidase A Digestion	Amino Acids Released[b]					
	Cys[c]	Thr	Gly	Ala	Val	Ile
Of oxidized peptide at pH 7						
2 hr	0	0.3	0	0	1.0	0.1
3 hr	0	0.3	0	0	1.0	0.2
Of intact butylcarbamoyl-peptide at pH 8						
1 hr	0	0.8	0	0	0.8	0
22 hr	0	1.0	0	0	1.0	0.1

[a] Calculated relative to Val in all experiments. [b] Calculated relative to Val content in starting material. [c] Analyzed as cysteic acid in the oxidized peptide, as Cys and *S*-(butylcarbamoyl)-Cys in the unoxidized peptide.

the sulfhydryl titrations. Primarily because of the errors involved in establishing the protein concentration in each sample, the titrations have a 2% error which means an uncertainty of about 0.7 of a residue, or an accumulated error of 1.5 SH residues in comparing sulfhydryl content before and after reaction with butyl isocyanate. For this reason it cannot be quantitatively established that the number of sulfhydryl groups reacted corresponds exactly to the number of moles of reagent incorporated. However, the stability properties of the derivative formed between reagent and enzyme can only be explained on the basis that all the reagent formed the *S*-butylcarbamoylcysteine derivative. The butylcarbamoyl derivatives of lysine, tyrosine, and serine have been prepared in this laboratory and shown to be completely stable to performic acid oxidation and incubation at pH 10.5 and 37° (C. C. Q. Chin, personal communication). These treatments led to complete decomposition of both the enzyme derivative and authentic *S*-(butylcarbamoyl)cysteine. The second part of the argument, that a single, unique type of SH group was involved in the reaction, requires the isolation of a single derivatized peptide sequence in a 100% yield. The overall yield of pure peptide in this work was only 16%. However, considering the lability of the thiocarbamate derivative, the relatively low specificity of pepsin, and the fact that only insignificant amounts of radioactive side fractions were observed in the peptide isolation, we feel justified in concluding that a single polypeptide sequence was derivatized. Based on these arguments we consequently also conclude that butyl isocyanate inactivates yeast alcohol dehydrogenase through reaction with a specific sulfhydryl group in three of the enzyme's four active sites. This finding of only three "essential" sites is in conflict with the data of Whitehead and Rabin (1964) who found that four sulfhydryl groups had to react with iodoacetamide for complete inactivation of the dehydrogenase. However, since the sequence determined for the iodoacetamide-derivatized sulfhydryl groups (Harris, 1964b) is clearly different from that found in this work, it is evident that a different set of "active-site sulfhydryl groups" react with the two reagents. It is in fact tempting to use the observation of only three reactive groups in this work as an argument in favor of butyl isocyanate being a true substrate analog. Since Dickinson (1970) has found only three coenzyme binding sites in the dehydrogenase and proposed a negative cooperativity between the four sites lead to a very low affinity for the fourth site, a similar situation for the substrate binding would be a most reasonable proposition. Work is in progress to attempt to resolve this question.

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