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Heavy-Chain Variable-Region Sequence from an Inulin-Binding Myeloma Protein†

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ABSTRACT: The entire variable-region sequence of the heavy chain from ABE-47N, a BALB/c inulin-binding myeloma protein, has been determined. This protein is unusual in that the third complementarity region (H3) is extremely short, consisting of at the most three and probably only one amino acid. A comparison of the heavy-chain hypervariable regions from mouse, human, and rabbit proteins shows that the variability in length of H3 is greater than that seen in the first or second hypervariable regions. This variability in H3 length suggests a specialized function for this region.

Hapten-binding mouse myeloma proteins have been used for several years as models to study the structural relationships among proteins with similar binding specificities and the three-dimensional structure of the antibody combining site. Extensive primary structural data have been obtained on the heavy chains from seven phosphorylcholine-binding proteins (Rudikoff and Potter, 1974; Hood et al., 1975; Rudikoff and Potter, 1976; Rudikoff et al., 1977 (submitted for publication)).

We are currently analyzing a second group of heavy chains from myeloma proteins with specificity for inulin[1] (Vrana et al., 1976; Potter et al., 1976b,c) to determine if the pattern of variation observed is similar to that of the heavy chains of phosphorylcholine-binding proteins. The eleven anti-inulin proteins characterized to date have been shown to possess individual, as well as cross-reacting, variable-region antigenic determinants (idiotypes) (Lieberman et al., 1975) and are thus a potential model for attempting to analyze the structural basis of idiotypy. We are currently performing structural determinations on five of these inulin-binding proteins (Lieberman et al., 1975; Potter et al., 1976c), and report here the complete heavy-chain variable-region sequence from the ABE-47N protein, which we intend to use as a prototype for this group.

Materials and Methods

Protein Purification. ABE-47N was purified by affinity chromatography on Sepharose–inulin columns as previously described (Vrana et al., 1976).

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[1] Inulin is a short linear polyfructan of β2-1 linked fructofuranose terminating with a nonreducing D-glucopyranose residue. Eleven proteins have been isolated that bind inulin, which thus provide a large group of functionally related homogeneous immunoglobins.

TABLE I: Sequence of ABE-47N Heavy-Chain Variable Region.[a,b]

Positions	10 · 20 · 30 · 40 (H1: 31–35)
Sequence	E V K L E E S G G G L V Q P G G S M K L S C V A S G F T F S N Y (W)M N W V R Q S P
Method	NH₂-Term (—)
Positions	50 · a b · 60 · 70 (H2: 50–65)
Sequence	E K G L E W V A E I R L K S H N Y A T H Y A E S V K G R F T I S R D D S K S S V
Method	CN-2 sed (—); →→ from F onward; ↔ from K onward
Positions	80 · 90 · H3 ↓ (at G) · 100[b]
Sequence	Y L Q M N N L R A E D T A I Y Y C S T G F A Y W G Q G T L V T V
Method	CN-1C (—, from N to V); ↔ Y L Q M

[a] Sequence determined by automated degradation in liquid sequencer (—). Sequence determined by solid phase sequencing of CN-2 sed following citraconylation and tryptic digestion (→→). Sequence determined by solid-phase sequencing of CN-2 sed following tryptic digestion (↔). Repetitive yield of the NH_2 terminus, CN-2 sed, and CN-1C was 92.6, 92.4, and 91.3%, respectively. [b] Numbering is according to Rudikoff and Potter (1974) and requires a deletion between Cys-96 and Gly-109 to maintain homology. See Discussion. Single letter amino acid code of Dayhoff (1972) is used.

TABLE II: Mouse Heavy-Chain Variable Region Sequences.[a]

	10 · 20 · 30
A-47N (α, VH_{III})	E V K L E E S G G G L V Q P G G S M K L S C V A S G F T F S
T15 (α, VH_{III})	E V K L V E S G G G L V Q P G G S L R L S C A T S G F T F S
M315 (α, VH_I)	D V Q L Q E S G P G L V K P S Q S L S L T C S V T G Y S I T
M173 (γ_{2a} VH_{III})	E V K L L E S G G P L V Q L G G S L K L S C A A S G F D F S
M-21 (γ_1, VH_{III})	D V Q L V E S G G G L V Q P G G S R K L S C A A S G F T F S
	40 · 50 · ←H1→ · ←H2→ · a b
A-47N	N Y (W) M N - W V R Q S P E K G L E W V A E I R L K S H N Y A T
T15	D F Y M E - W V R Q P P G K R L E W I A A S R N K A N D Y T T
M315	S G Y F W N W I R Q F P G N K L E W L G F I K Y D G S B - - -
M173	R Y W M S - W V R Q A P G K G L E W I G E I D P N S S T I - -
M-21	S F G M H - W V R Q A P E K G L E W V A Y I S S G S S T L - -
	60 · 70 · 80
A-47N	H Y – A E S V K G R F T I S R D D S K S S V Y L Q M N N L R A
T15	E Y – S A S V K G R F I V S R D T S Q S I L Y L Q M N A L R A
M315	– (Y G) B P S L K N R V S I T R D T S E N Q F F L K L D S V T (T
M173	N Y – T P S L K D K F I I S R N D A K N T L Y L Q M S K V R S
M-21	D Y – A H T V K G R F T I S R N D P K N T L F L Q M T S L R S
	90 · 100 · 110 · ←H3→
A-47N	E D T A I Y Y C S T G – – – – – – – – – F A Y W G Q G T L V T V
T15	E D T A I Y Y C A R D Y Y G S S Y W Y F D Y W G A G T T V T V
M315	Z B) T A T Y Y C A G D N D H L Y – – – F D Y W G Q G T T L T V
M173	E D T A L Y Y C A R S P Y Y A – – – – M D Y W G Q G T S V T V
M-21	E D T A M Y Y C A R H G N Y P W Y A – M D Y W G Q G T S V T V

[a] Mouse sequences are as follows: T15, Rudikoff and Potter (1976); M315, Francis et al. (1974); M173, Bourgois et al. (1972) and Rocca-Serra et al. (1975); M21, Milstein et al. (1974).

Heavy-Chain Preparation. ABE-47N protein (30–40 mg/mL) was dialyzed against 0.15 M Tris[2]-HCl-0.15 M NaCl-2 mM Na_2EDTA. The protein was reduced with 10 mM dithiothreitol for 2 h at room temperature in the dark, followed by alkylation for 15 min with 20 mM iodoacetamide (Bridges and Little, 1971), and then dialyzed overnight against 6 M urea–1 M acetic acid. Heavy and light chains were separated by chromatography on a Sephadex G-100 column equilibrated in 6 M urea–1 M acetic acid.

[2] Abbreviations used are: Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA (ethylenedinitrilo)tetraacetic acid; Pth, phenylthiohydantoin; TETA, triethylenetetraamine.

Cyanogen Bromide Cleavage. Protein was dissolved in 70% formic acid and CnBr was added at a 4:1 weight ratio (CnBr:protein). The mixture was allowed to stand 24 h at 4 °C and then diluted with water and lyophilized.

Sequence Determination. Intact heavy chains and appropriate CnBr fragments were subjected to automated degradation on a Beckman 890C sequencer using the standard dimethylallylamine (DMAA) program with two modifications. (1) An additional 2-s delivery of coupling buffer was performed following the initial coupling reaction. (2) Benzene and ethyl acetate were delivered simultaneously for 200 s following coupling. The addition of ethyl acetate to the benzene wash removed many of the non-amino acid spots previously seen on

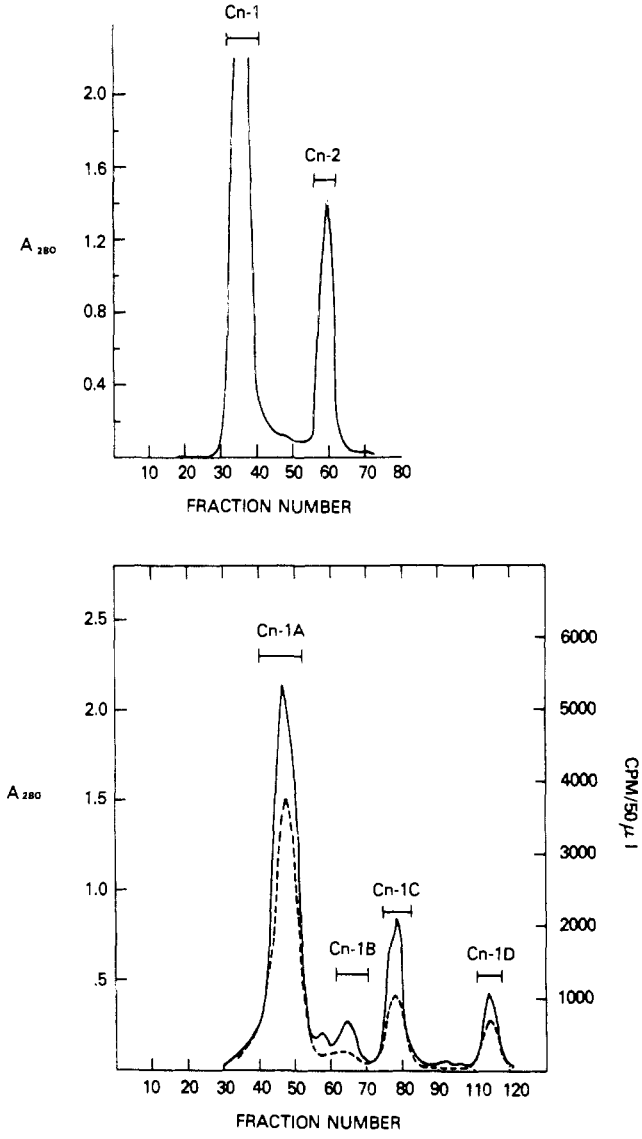

FIGURE 1: (A, top) Sephadex G-100 gel filtration of CnBr cleaved ABE-47N. Columns were equilibrated in 5 M quanidine hydrochloride–0.2 M NH_4HCO_3. (B, bottom) Gel filtration as in A of CN-1 following full reduction and alkylation with [^{14}C]iodoacetamide. A_{280} (—); cmp (- - -).

thin-layer chromatography with the DMAA program and thus permitted the use of this technique throughout the sequencer runs. Pth derivatives were identified by gas chromatography (Pisano et al., 1972) and two-dimensional thin-layer chromatography (Summers et al., 1973). Acid hydrolysis products of the Pth derivatives (Smithies et al., 1971) were identified on a Beckman 119 amino acid analyzer. All positions in a sequencer run were initially analyzed by gas chromatography. Residues not conclusively identified by this technique were subjected to thin-layer chromatography and HI hydrolysis. In addition, all residues in the latter portion of most sequencer runs were also hydrolyzed. When necessary, certain CnBr fragments were digested with trypsin (1:50) at 37 °C for 5 h in their native state or following citraconylation. The peptide containing homoserine at its C terminus was then coupled to triethylenetetraamine (TETA) resin (Horn and Laursen, 1973) and sequenced on a Sequemat, Model 12, solid-phase sequencer.

Citraconylation. Protein was dissolved in 0.5 M borate buffer, pH 8.2, and a 25 molar excess of citraconic anhydride was added. The mixture was incubated at room temperature for 45 min and then dialyzed against 0.02 M ammonium bicarbonate.

TABLE III: Comparison of Lengths of Heavy-Chain Hypervariable Regions.[a]

	H1	H2	H3
Mouse			
47N	5	14	1
S107	5	14	9
M511	5	14	10
H8	5	14	9
M603	5	14	8
T15	5	14	9
M167	5	14	11
M315	6	11	6
M173	5	12	5
M21	5	12	8
Human			
Zap	5	12	7
Ou	7	11	13
Nie	5	12	7
Pom	5	12	10
Lay	5	12	10
Gal	5	12	4
Eu	5	12	5
Daw	7	11	6
He	7	12	7
Cor	7	11	10
Tei	5	12	10
Was	5	12	8
Jon	5	12	6
Tur	5	12	7
Rabbit			
BS-5	5	11	10
K-25	5	11	11

[a] Mouse and human sequences not referenced in other tables were obtained from Hood et al. (1975) and Capra and Kehoe (1975). H1, H2, and H3 correspond to residues 31–35, 51–62, and 99–104, respectively, according to the numbering of M603 (Rudikoff and Potter, 1974).

Results

Heavy-Chain NH_2-Terminal Sequence. Purified heavy chain was subjected to 36 cycles of degradation. The sequence obtained is given in Table I.

Heavy-Chain Cyanogen Bromide Fragments. The partially reduced and alkylated heavy chain following cleavage with cyanogen bromide was dissolved in 5 M guanidine–0.2 M NH_4HCO_3 and chromatographed on a Sephadex G-100 column (2.5 × 125 cm) equilibrated in the same buffer. Two peaks were obtained (Figure 1A). CN-1 and CN-2 were dialyzed against 0.2 M NH_4HCO_3 and 5% acetic acid, respectively, and lyophilized.

CN-1 was then dissolved in 5 M guanidine–0.05 M Tris (pH 8.2) and fully reduced with 10 mM dithiothreitol for 2 h at 37 °C followed by alkylation with 20 mM [^{14}C]iodoacetamide for 30 min. CN-1 was then applied to the same Sephadex G-100 column as above and the results are shown in Figure 1B. The peaks were pooled as indicated, dialyzed against 5% acetic acid, and lyophilized.

CN-2 by sequence analysis was a mixture of four peptides, one of which was the variable-region peptide from position 35 through 83. This peptide alone was found to be insoluble in 0.02

TABLE IV: Comparison of Representative H3 Sequences[a] from Mouse, Human, and Rabbit

		Binding[b] Specificity	Framework	H3	Framework	Length of H3
Mouse						
A47N	(α, κ, VH_{III})	Inulin	C S T	G	F A Y W	1
M603	(α, κ, VH_{III})	PC	C A R	N Y Y G S T W Y	F D V W	8
T15	(α, κ, VH_{III})	PC	C A R	D Y Y G S S Y W Y	F D V W	9
M167	(α, κ, VH_{III})	PC	C T R	D A D Y G D S Y F G Y	F D V W	11
M511	(α, κ, VH_{III})	PC	C A R	D G D Y G S S Y W Y	F D V W	10
M315	(α, λ2, VH_I)	DNP	C A G	D N D H L Y	F D V W	6
M173	(γ2a, κ, VH_{III})	unknown	C A R	S P Y Y A	M D Y W	5
M21	(γ1, κ, VH_{III})	unknown	C A R	H G N Y P W Y A	M D Y W	8
Human						
Ou	(μ,κ, VH_{II})	unknown	C A R	V V N S V M A G Y Y Y Y Y	M D V W	13
Nie	(γ1, κ, VH_{III})	unknown	C A R	I R D T A M F	F A H W	7
Lay	(μ, κ, VH_{III})	IgC	C A R	D A G P Y V S P T F	F A H W	10
Gal	(μ, κ, VH_{III})	unknown	C A R	G W G G	G D Y W	4
Eu	(γ1, κ, VH_I)	unknown	C A G	G Y G I Y	S P E E	5
Rabbit						
BS-5	(γ, κ)	pneumococcus	C A R	Q G T G L V H L A F	V D V W	10
K-25	(γ, κ)	pneumococcus	C A R	G H T G L S Y L K S S	V D V W	11

[a] H3 begins three residues after the invariant Cys at position 96 and ends at the fourth residue before the relatively constant Trp at position 108 (Rudikoff and Potter, 1974; Capra and Kehoe, 1974a). Mouse sequences are as follows: M603, Rudikoff and Potter (1974); T15 and M167, Rudikoff and Potter (1976); M511, cited as personal communication by E. Appella in Hood et al. (1975); M315, Francis et al. (1974); M173, Bourgois et al. (1972) and Rocca-Serra et al. (1975); M21, Milstein et al. (1974); Lay, Capra and Kehoe (1974b); Nie, Ponstingl et al. (1970); Ou, Shimizu et al. (1971); Eu, Edelmar, et al. (1969); Gal, Watanabe et al. (1973); BS-5 and K-25, Jaton (1975). [b] PC = phosphorylcholine; DNP = dinitrophenol.

M NH_4HCO_3. The insoluble peptide (CN-2 sed) was obtained by centrifugation after standing overnight at room temperature and dissolved in 1% acetic acid. The same procedure was previously used by Rudikoff and Potter (1974) to purify the corresponding peptide from McPC 603 heavy chain. Four hundred nanomoles of this fragment was added to the spinning cup sequencer, resulting in 43 cycles of degradation in which positive amino acid identification could be made (Table I).

The C-terminal nine residues of this peptide were analyzed by use of solid-phase sequencing techniques. Two hundred nanomoles of peptide was citraconylated and digested with trypsin, and the homoserine-containing peptide was coupled to TETA resin. The sequence of this peptide began at residue 68 (Phe) and was extended through residue 79 (Val) (Table I). Significant trypsin cleavage did not occur at Arg-72, presumably due to the two adjacent negatively charged side groups (Haschemeyer and Haschemeyer, 1973). A second aliquot of the CnBr fragment was then trypsin digested without citraconylation, coupled to TETA resin, and sequenced as above. This peptide began at Ser-77 and was sequenced through to the C terminus, thus completing the sequence of Cn-2.

Cn 1-A was previously been reported as a large fragment from the constant region (Francis et al., 1974; Rudikoff and Potter, 1974) and was not examined further. Cn 1-C was found to be a single fragment and subsequently subjected to 28 cycles of degradation. The sequence of this fragment is given in Table I and its position in the chain can be assigned by homology with other heavy-chain sequences from both mice (Table II) and humans as extending from residue 84 in the variable region to residue 116 in the constant region.

Cn 1-D was identified as the small peptide extending from residue 19 to 34. As position 33 in the N-terminal sequencer run was only tentatively identified as Trp, this peptide was examined for the presence of this amino acid. Spectrophotometric analysis (Edelhoch, 1967) and *p*-toluenesulfonic acid hydrolysis (Liu and Chang, 1971) both confirmed the presence of Trp in this peptide. Accordingly, position 33 was assigned as Trp.

Discussion

The ABE-47N heavy chain is typical of the mouse VH_{III} proteins, showing 76% homology to T15, another VH_{III} protein, in the framework[3] portion of the variable region. A comparison of five of the available mouse heavy-chain sequences (Table II) clearly demonstrates the presence of three regions of hypervariability.[4] Analysis of this group of proteins, admittedly a small sample, by the method of Kabat and Wu (1971) has failed to provide evidence for a fourth hypervariable region in the area of residues 84–91 as is seen in human heavy chains (Capra and Kehoe, 1974a). This analysis may, of course, change as more sequences accumulate.

The most striking feature of the ABE-47N heavy chain is the very short third complementarity region (H3) consisting of one amino acid, glycine, at position 99. Positions 97 and 98, the two residues following the Cys at position 96, are conventionally considered framework residues (Kabat and Wu, 1971). In ABE-47N, the two amino acids at these positions are uncommon ones, suggesting the possibility that they are part of the H3 region, and that the two framework residues, which are usually Ala and Arg (see Table II), have been deleted. A three-dimensional model of ABE-47N, based on the x-ray crystallographic data of M603 (E. Padlan, personal communication) shows that positions 97 and 98 are not exposed to solvent, and could not participate in antigen binding. We therefore prefer to think of them as part of the framework and not hypervariable or complementarity determining.

[3] "Framework" refers to the portions of the variable region which have been shown by x-ray crystallographic analysis not to participate in antigen binding and appear to function as a backbone for hypervariable or complementarity determining regions. The sequence in these areas appears to be highly conserved compared to the hypervariable regions.

[4] Hypervariable regions refer to the three areas of the heavy-chain variable region (H1, H2 and H3) shown by Kabat and Wu (1971) to exhibit the greatest degree of primary sequence variation. These regions have subsequently been shown by x-ray crystallographic analysis to be the antigen contacting or complementarity regions.

In contrast to H3, the H1 and H2 regions of ABE-47N are quite similar in length to those of other heavy chains. The H1 segment from available mouse sequences, including ABE-47N, is found to be five amino acids in length with the exception of M315 which contains 6 residues (Table III). A representative sample of human heavy chains also shows only two different lengths in H1 (5 or 7 residues). The two rabbit chains sequenced to date are the same size in H1. H2 in the mouse chains may contain 11, 12, or 14 amino acids and similar limited variability can be seen among the human heavy chains.

The very short size of H3 in ABE-47N is an example of the marked variability in length H3 may assume. If one compares heavy-chain variable regions among proteins of the same and different species, it is obvious that size variation in H3 is more marked than in H1 or H2 (Tables III and IV). Among the 10 mouse heavy-chain sequences available, 7 of the H3 regions vary in size, being from 1 to 11 residues in length (Table III). This observation is even more striking in that four of the proteins with H3 size differences, T15, M603, M167 (Rudikoff and Potter, 1976), and M511 (Hood et al., 1975), bind the same haptenic determinant, phosphorylcholine. If one includes representative human heavy-chain sequences, the variability in size of H3 is maintained, the shortest being 4 and the longest being 13 amino acids. The two rabbit VH_{III} regions sequenced also vary in length, even though they are from proteins raised against the same antigenic determinant. The extreme size variability observed in H3 suggests that this segment may possess some special functions. In fact, in the three-dimensional model of M603 (Segal et al., 1974), H3 is found to be in intimate contact with the first light-chain complementarity region (L1) where it forms a very stable structure. L1 has been found in mouse proteins to be the most variable complementarity region in size in the light chain (Potter et al., 1976a) in a similar manner as H3 in the heavy chain. Thus, size variations within H3, in addition to the localized alteration of the topography of the antigen binding site, may produce further changes in binding-site conformation by altered interactions with the light chain. An example of this altered interaction is seen in the three-dimensional model of ABE-47N. In ABE-47N, the combined lengths of L1 and H3 are 12 residues shorter than in M603, making it impossible for L1 and H3 to interact, although the lengths of all other complementarity regions appear to be the same in both proteins (M. Vrana, unpublished data). The three-dimensional effect of this size difference is that, instead of formation of a cavity as is seen in M603, a shallow groove is formed in ABE-47N. Thus, modulation in size of H3 (and/or L1) may markedly alter the structure of the binding surface.

It is interesting that a protein with an essentially deleted third complementarity region not only is a functional molecule in that it binds moieties containing 2,1 linked fructosans such as inulin (Vrana et al., 1976; Potter et al., 1976b), but it also shares antigenic determinants (idiotypes) with other myeloma proteins reacting with inulin and antibodies induced to inulin (Lieberman et al., 1975, 1976), suggesting that all of these proteins are structurally related. Thus, the large size variations observed in H3 rather than being genetic "accidents" may be an inherent mechanism of extreme importance in the generation of functional antibody diversity.

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Acid-Catalyzed Hydration of Reduced Nicotinamide Adenine Dinucleotide and Its Analogues†

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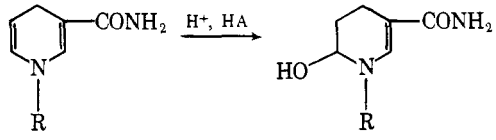
ABSTRACT: The rate of the primary acid modification reaction of 1,4-dihydronicotinamide adenine dinucleotide (NADH) and 1,4-dihydro-3-acetylpyridine adenine dinucleotide (APADH) and their analogues has been studied over a wide pH range (pH 1–7) with a variety of general acid catalysts. The rate depends on $[H^+]$ at moderate pH and becomes independent of $[H^+]$ at low pH. This behavior is attributed to substrate protonation at the carbonyl group (pK of NADH = 0.6). The reaction is general acid catalyzed; large solvent deuterium isotope effects are observed for the general acid and lyonium ion terms. Most buffers cause a linear rate increase with increasing buffer concentration, but certain buffers cause a hyperbolic rate increase. The nonlinear buffer effects are due to complexation of the buffer with the substrate, rather than to a change in rate-limiting step. The rate-limiting step is a proton transfer from the general acid species to the C_5 position of the substrate. Anomerization is not a necessary first step in the case of the primary acid modification reaction of β-NADH, in which β to α anomerization takes place.

NADH[1] and its analogues are unstable in acid solutions or even in buffered neutral solutions because they undergo a general acid catalyzed hydration reaction, according to eq 1,

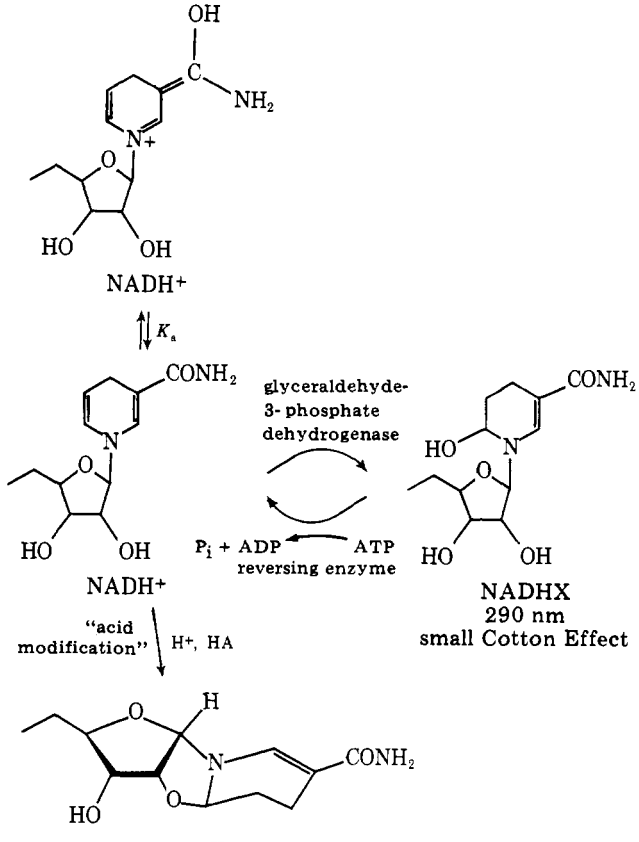


(1)

I

to form the primary acid product, **I**, which is characterized by an absorption band at 290–300 nm. Secondary reactions destroy **I** (Kim and Chaykin, 1968; Stock et al., 1961; Johnston et al., 1963; Alivisatos et al., 1965; Burton and Kaplan, 1963). In the case of the ribose-containing NADH, the primary acid product results from a hydration step, an anomerization at the ribosyl–dihydronicotinamide bond (Miles et al., 1968), and a cyclization reaction to form **II** as shown in Scheme I (Oppenheimer and Kaplan, 1974a). Both α- and β-NADH form **II**, which is characterized by a large positive Cotton effect at 290 nm. NADH also undergoes a hydration reaction catalyzed by glyceraldehyde-phosphate dehydrogenase to form NADHX (Rafter et al., 1954; Chaykin et al., 1956, Hilvers et al., 1966), which is the hydrated form of NADH which has not undergone anomerization at the ribosyl–dihydronicotinamide bond (Oppenheimer and Kaplan, 1974b; Oppenheimer, 1973). NADHX and **II** have very similar spectroscopic properties and

Scheme I



are associated with various enzyme systems as products, inhibitors, or by-products (Huennekens et al., 1955; Pfleiderer and Stock, 1962; Weiland et al., 1960; Gelderman and Pea-

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[1] Abbreviations used are: NADH, dihydronicotinamide adenine dinucleotide; NMNH, dihydronicotinamide mononucleotide; BzNH, 1-benzyl-1,4-dihydronicotinamide; PrNH, 1-propyl-1,4-dihydronicotinamide; APADH, 1,4-dihydro-3-acetylpyridine adenine dinucleotide; BzAPH, 1-benzyl-1,4-dihydro-3-acetylpyridine; MeAPH, 1-methyl-1,4-dihydro-3-acetylpyridine; Pic, 4-picoline; Py, pyridine; Im, imidazole; P_i, inorganic phosphate; PP_i, pyrophosphate; AcOH, acetic acid; ClAcOH, chloroacetic acid; PhP_i, phenyl phosphate.