p-Methoxybenzyloxycarbonyl	Z(OMe)–
p-Methoxyphenylazobenzyloxycarbonyl	Mz–
p-Phenylazobenzyloxycarbonyl	Pz–
t-Butyloxycarbonyl	Boc–
Cyclopentyloxycarbonyl	Poc–

6.2 *Other N-Protecting Groups.*

Acetyl	Ac–
Benzoyl	Bz–
Tosyl	Tos–
Trifluoroacetyl	Tfa–
Phthalyl	Pht–
Benzyl	Bzl–
Trityl	Trt–
Tetrahydropyranyl	Thp–
Dinitrophenyl	Dnp–
Benzylthiomethyl	Btm–
o-Nitrophenylsulfenyl	Nps–

6.3 *Carboxyl-Protecting Groups.*

Methoxy (methyl ester)	–OMe
Ethoxy (ethyl ester)	–OEt
Tertiary butoxy (*trty*-butyl ester)	–OBu^t
Benzyloxy (benzyl ester)	–OBzl
Diphenylmethoxy (benzhydryl ester)	–OBzh
p-Nitrophenoxy (*p*-nitrophenyl ester)	–ONp
Phenylthio (phenyl thiolester)	–SPh
p-Nitrophenylthio	–SNp
Cyanomethoxy	–OCH$_2$CN

Note: Contrary to the symbols for amino acid residues, the position of the dashes in the symbols for substituents carries no significant information.

A New N-Terminal Blocking Group Involving a Schiff Base in Hemoglobin A_{Ic}*

W. R. Holmquist and W. A. Schroeder

ABSTRACT: Hemoglobin A_{Ic} is a minor hemoglobin component in the erythrocytes of normal adult humans. Chemically, it is the condensation product, a Schiff base, between one molecule of hemoglobin A and one molecule of a ketone or aldehyde R=O. The pK_a of this Schiff base is 6.64. The point of linkage of R=O to hemoglobin A to form hemoglobin A_{Ic} is at the N terminus of one of the two β chains. Other than this, no difference has been found between the primary amino acid sequence of the α and β chains of hemoglobins A_{Ic} and A.

The ketone or aldehyde R=O has a molecular weight of approximately 280, is not an aromatic aldehyde, is not a steroid, and does not contain phosphorus, carbohydrates, or amino acids. Esterified nonketo acyl groups of less than five carbon atoms are absent. It is probable that R=O contains neither nitrogen nor free carboxyl groups. There is as yet no basis for excluding the possibility that R=O is a long-chain aliphatic aldehyde or ketone. Methods are described for isolating A_{Ic} as well as smaller peptides which contain the blocking group R, such as R-Val-His. The lability of the Schiff base linkage at alkaline pH has limited the experimental procedures. This lability can be circumvented by reduction with $NaBH_4$. A practical method for calculating ionization constants from paper electrophoretic data is described and used to obtain the pK_a of the Schiff base nitrogen in A_{Ic}.

Hemoglobin A_{II}, which has the subunit structure $\alpha_2\beta_2$, is the major hemoglobin protein of most adult humans. The major component has been variously designated as A, A_{II}, A_0, and A_1. Partial correlations between different systems of nomenclature are given by Schnek and Schroeder (1961), Huehns and Shooter (1965), and Schroeder and Jones (1965). The only sure guide to the identity of a hemoglobin is an understanding of the methods by which it was isolated and characterized. This paper will designate the main component as hemoglobin A_{II} to be consistent with earlier publications from this laboratory. The amino acid sequences of both the α and β polypeptide chains and much of their secondary, tertiary, and quaternary structures are known.[1] Whereas there is only a single major component, A_{II}, several minor components exist,

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† Contribution No. 3355.

[1] References to the original literature can be found in the reviews by Huehns and Shooter (1965) and Schroeder and Jones (1965).

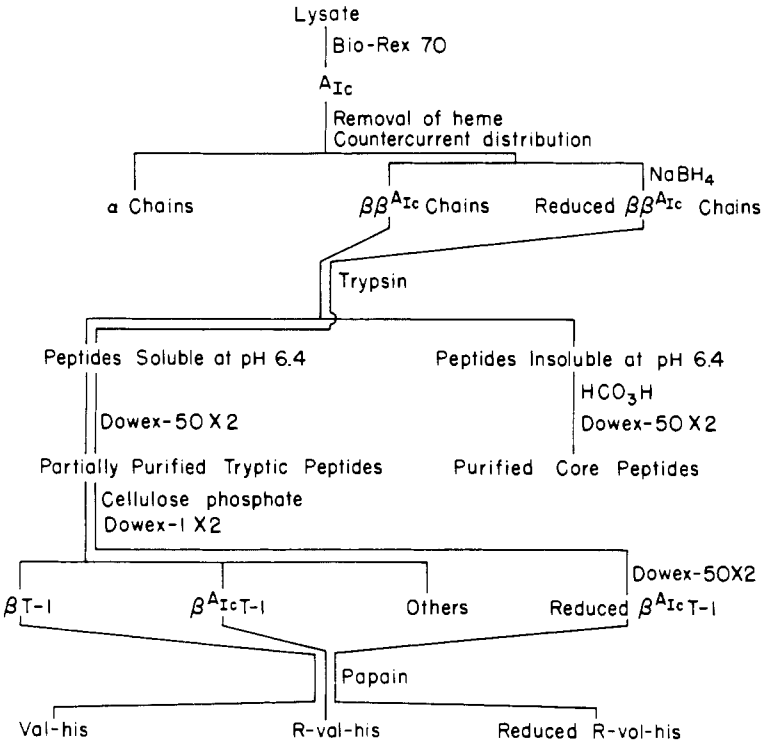


FIGURE 1: Flow sheet for isolation procedures. See text for explanation.

and of these, hemoglobin A_{Ic} is present in the greatest relative amount.

Hemoglobin A_{Ic} was first isolated and purified by chromatographing normal adult hemoglobin on IRC-50 (Allen *et al.*, 1958). Hemoglobin A_{Ic} comprises 5–7% of the total hemoglobin (Clegg and Schroeder, 1959; Schnek and Schroeder, 1961) and is eluted more rapidly than hemoglobin A_{II} from the chromatographic column at neutral values of pH. Because IRC-50 is a carboxylic type cation exchange resin, it follows that A_{Ic} is more negatively charged at these pH values than is A_{II}.

Further advances toward an understanding of the structure of A_{Ic} were made by Jones (1961). He found that the ultraviolet spectra, molecular weights, and amino acid compositions of A_{Ic} and A_{II} were the same. Using Sanger's technique, he was able to localize the chemical difference(s) in the A_{Ic} molecule to the β chains: from the quantity of DNP-Val-Leu (Rhinesmith *et al.*, 1957a,b), it was concluded that two α chains were present, whereas the amount of di-DNP-Val-His and di-DNP-Val-His-Leu together (Rhinesmith *et al.*, 1957b, 1958) suggested some abnormality in the N terminals of the β chains. He obtained additional support for this interpretation by showing that the α chains of radioactive hemoglobin S_{II}, which is the major component of persons with sickle cell anemia, would exchange with the α chains of A_{Ic} under conditions suitable for subunit hybridization.

At the time the experiments which are reported here were begun, the structure that was most consistent with the above data was $\alpha_2\beta\beta^{A_{Ic}}$, where $\beta^{A_{Ic}}$ differed from β in that its N terminus was blocked by some chemical group R whose nature was not known. Jones (1961) himself proposed the structure α_2X_2 for A_{Ic}, where the polypeptide chain X was assumed to be similar to, but not identical with, the β chain of A_{II}. The presence of some N-terminal peptides from the β chains was attributed to partial loss of the blocking group from two X chains during Sanger's procedure. In view of the known presence of an N-terminal acetyl group in other proteins, the cytochromes *c*, the protein of the tobacco mosaic virus (see the reviews by Hirs, 1964; and Keil, 1965), and especially hemoglobin F_I (Schroeder *et al.*, 1962a), we first searched for that group. It is absent from A_{Ic}. The experiments reported below are the outgrowth of that fact.

It is the purpose of this paper to present additional data on the subunit structure of A_{Ic} and to give a partial chemical characterization of the blocking group R and its mode of linkage to the β chain. In a companion paper (Holmquist and Schroeder, 1966a), the biosynthesis and function of hemoglobin A_{Ic} are considered.

Experimental Section

Isolation Procedures. The general plan of the isolation procedures is presented in the flow sheet of Figure 1. In Figure 1, βT-1, $\beta^{A_{Ic}}$T-1, and reduced $\beta^{A_{Ic}}$T-1 are the N-terminal tryptic peptides from the β, $\beta^{A_{Ic}}$, and

reduced $\beta^{A_{Ic}}$ chains, respectively.[2] Similarly, Val-His, R-Val-His, and reduced R-Val-His are the N-terminal dipeptides from these chains. This flow sheet shows 2 schemes of isolation: the first, which uses $\beta\beta^{A_{Ic}}$-globin as the source of R-Val-His, was briefly described earlier (Holmquist and Schroeder, 1964), whereas the second uses $\beta\beta^{A_{Ic}}$-globin that has been reduced with $NaBH_4$ as a source of reduced R-Val-His. For reasons that will become clear, reduced $\beta\beta^{A_{Ic}}$-globin is the preferred starting material.

SOURCE AND ISOLATION OF HEMOGLOBIN A_{Ic}. For small-scale isolations, approximately 50 ml of venous blood from one of the authors was collected in Alsever's solution (Delta Laboratories, Inglewood, Calif.). For large-scale operations, packed erythrocytes (in anticoagulant ACD solution, U.S.P., formula A) from 2 pints of whole blood were obtained from Hyland Laboratories (Los Angeles, Calif.). In each case the blood was worked up within a few hours of its drawing. The hemolysate was prepared as described by Clegg and Schroeder (1959). Purified CO-hemoglobin A_{Ic} was isolated by chromatography on Bio-Rex 70 (Holmquist and Schroeder, 1964; Schroeder and Holmquist, 1966). By means of the 10 × 100 cm chromatographic column that is described in the latter reference, approximately 2 g of carbonmonoxy-A_{Ic} could be isolated from the hemolysate from 1 pint of blood.

ISOLATION OF α AND $\beta\beta^{A_{Ic}}$ POLYPEPTIDE CHAINS. The heme was removed from A_{Ic} by the method of Schroeder *et al.* (1963), and the α and $\beta\beta^{A_{Ic}}$ subunits were separated from one another by countercurrent distribution (Hill *et al.*, 1962; Babin *et al.*, 1964, 1966). The initial hope that β and $\beta^{A_{Ic}}$ subunits also could be separated from each other in this manner was not realized in the small number (18) of transfers that were used.

ENZYMATIC HYDROLYSES. Tryptic hydrolysis of the α- and mixed $\beta\beta^{A_{Ic}}$-globins followed Babin *et al.* (1964). Papain hydrolyses of peptides were done according to Hill *et al.* (1960).

COLUMN CHROMATOGRAPHIC ISOLATION OF PEPTIDES. A preliminary separation of the peptides from a tryptic hydrolysate was achieved on Dowex 50-X2 (Schroeder *et al.*, 1962b, and Schroeder, 1966b). The relatively few peptides in any given zone from the Dowex 50 chromatography were separated from each other either on cellulose phosphate (Holmquist and Schroeder, 1966b) or on Dowex 1-X2 (Schroeder *et al.*, 1962b; Schroeder and Robberson, 1965; Schroeder, 1966c). However, the peptide $\beta^{A_{Ic}}$T-1 (but not reduced $\beta^{A_{Ic}}$T-1) *must* be purified under acidic conditions, for example, on cellulose phosphate (Holmquist and Schroeder, 1964). Those peptides from the tryptic hydrolysis which were insoluble at pH 6.4 (the tryptic "core") were oxidized with performic acid (Hirs, 1956), and the oxidized peptides were isolated on Dowex 50-X2 as described by Goldstein *et al.* (1961).

Smaller peptides, and in particular the dipeptides Val-His and R-Val-His, from papain hydrolysates of βT-1 and $\beta^{A_{Ic}}$T-1 were isolated on a single chromatogram by modifying the method of Goldstein *et al.* (1963) to use Dowex 50-X8 and buffers of lower ionic strength. A 0.6 × 45 cm Dowex 50-X8 column (particle size 25–30 μ) was equilibrated at 38° with 200 ml of buffer B of Table I. The mixture of peptides from the papain hydrolysate of $\beta^{A_{Ic}}$T-1 (or βT-1) was applied to the column in 0.4 ml of buffer A and followed by three 0.2-ml rinses of buffer B. The column was developed stepwise with 100 ml each of buffers B to F in succession. A flow rate of 10 ml/hr was maintained, and 1-ml fractions were collected.

TABLE I: Buffer Compositions for Dowex 50-X8 Chromatography.

Buffer	pH	Ionic Strength[a]	ml/l. Pyridine	ml/l. Acetic Acid
A	2.41	0.004	0.00	62.50
B	3.28	0.05	4.03	61.80
C	4.21	0.05	4.59	10.40
D	5.28	0.05	9.42	4.20
E	5.28	0.10	18.85	7.80
F	5.64	0.30	686	181

[a] Calculated from pK values.

Paper Electrophoretic Procedures. Electrophoresis was carried out on a 70-cm length of Whatman 3MM paper for 45 min at 3 kv with 0.62 F pyridine–0.035 F acetic acid, pH 6.4, buffer (Ingram, 1958; Schroeder *et al.*, 1963). The dried papers were sprayed with 0.5% ninhydrin in acetone to locate the peptides. Histidyl peptides were then located with Karler's (1952) modification of Pauly's reagent (see Block *et al.*, 1955).

Amino Acid Analyses and Edman Degradations. The determination of the amino acid composition of purified peptides was carried out as described by Schroeder *et al.* (1963). The sequence of the amino acids in a given peptide was determined by the paper-strip modification of the Edman degradation (Fraenkel-Conrat *et al.*, 1955; Schroeder *et al.*, 1963; Schroeder, 1966a).

Determination of the N-Terminal Amino Acids in A_{Ic} and in $\beta\beta^{A_{Ic}}$-Globin by Sanger's Procedure. The experimental procedures for dinitrophenylation and the isolation of N-terminal DNP peptides and DNP-amino acids followed Rhinesmith *et al.* (1957a,b, 1958).

Starch Gel Electrophoresis of A_{Ic}-Globin. A_{Ic}-Globin and the globin subunits α_2 and $\beta\beta^{A_{Ic}}$ were electrophoresed on starch gel at pH 1.8 by the method of Muller (1961).

Results

The Subunit Structure of Hemoglobin A_{Ic}. Four ex-

[2] Throughout this paper we shall designate hemoglobin peptides according to the nomenclature of Baglioni (1961).

periments were done to confirm the subunit structure $\alpha_2\beta\beta^{A_{Ic}}$ which was proposed above for hemoglobin A_{Ic}. These experiments were a reexamination of the N termini, self-hybridization (see below), a detailed study of the polypeptide chains, and electrophoresis of the A_{Ic}-globin subunits.

The results of the reexamination of the N terminals (Table II) confirm the earlier work of Jones (1961) and support the subunit structure $\alpha_2\beta\beta^{A_{Ic}}$ in which the N terminus of the $\beta^{A_{Ic}}$ chain is unreactive toward 2,4-dinitrofluorobenzene because this N terminus is blocked by some group R.

TABLE II: N Terminals Found in Hemoglobin A_{Ic} by Sanger's Method.

		Amt. of DNP-peptide Isolated[a]	
		From α Subunits	From β and $\beta^{A_{Ic}}$ Subunits
Starting Compd	Sample No.	Val-Leu + Val	Val-His-Leu + Val-His + Val
DNP-A_{II}	1	1.75	1.24
	2	1.76	1.43
	3[b]	1.84	1.31
	Av	1.78	1.33
DNP-A_{Ic}	1	1.70	0.97
	2	1.54	0.76
	3	1.80	0.96
	4[b]	1.57	0.53
	5[b]	2.10	0.79
	Av	1.74	0.80
DNP-$\beta\beta^{A_{Ic}}$	1	0.06	0.86

Number of α chains in A_{Ic} = $1.74/1.78 \times 2 = 1.96$
Number of β chains in A_{Ic} = $0.81/1.33 \times 2 = 1.22$

[a] In micromoles per mole of starting compound.
[b] From Jones (1961).

The results of the self-hybridization experiments have been reported earlier (Holmquist and Schroeder, 1964): when carbonmonoxy-A_{Ic} is dissociated into its subunits at pH 3.66, 4.66, or 11.24 and reassociated at neutral pH, in each case approximately 12% of the A_{Ic} is converted to a hemoglobin that chromatographs like A_{II}.[3] This is consistent with the presence of the normal, *i.e.*, unblocked β chain in A_{Ic}. The small amount of A_{II} that is formed suggests that A_{Ic} is more stable, either kinetically or thermodynamically, toward acid–base dissociation than A_{II}. This could explain why A_{Ic} is chromatographically isolable, a fact that is contrary to its expected behavior according to the "dynamic equilibrium" hypothesis of Guidotti *et al.* (1963).

If $\alpha_2\beta\beta^{A_{Ic}}$ is the correct subunit structure of hemoglobin A_{Ic}, then the sequences of the α, β, and $\beta^{A_{Ic}}$ chains of A_{Ic} should be identical with those of the α and β chains, respectively, of A_{II}, with the exception of a blocking group R at the N terminus of the $\beta^{A_{Ic}}$ polypeptide chain. Because our primary interest has been in the blocking group R, only the amino acid composition of the tryptic peptides of the α chain has been investigated, and the primary sequence of the $\beta\beta^{A_{Ic}}$ chains has been carried only as far as could be conveniently done through the application of the phenylthienylhydantoin procedure to individual tryptic peptides. The amino acid compositions of the tryptic peptides of the α chain as they are summarized in Table III are in accord with the known composition of the α chain from A_{II} (Braunitzer *et al.*, 1961). The β chains were somewhat more extensively studied with the results that are presented in Figure 2. From Figure 2, the experimentally found amino acid composition of the peptides can be calculated as described in the legend. Thus for βT-10,11 there were 1.76 residues of threonine, 3.15 residues of leucine, etc. The tryptic peptides have been arranged by analogy to the β^A chain. Methionine and tryptophan are partially destroyed during the hydrolysis; consequently, the number of residues that were found for these two amino acids was often considerably less than 1. βT-4 and βT-5 provide examples. Again the results are as anticipated (Braunitzer *et al.*, 1961; Konigsberg *et al.*, 1963).

Starch gel electrophoresis at pH 1.8 revealed no differences between the α and β chains of A_{II} and the α and $\beta\beta^{A_{Ic}}$ chains of A_{Ic}. The $\beta^{A_{Ic}}$ chain appeared to have a mobility identical with that of the β chain. Huisman and Horton (1965) have shown that A_{Ic} moves toward the anode barely perceptibly faster than A_{II} on starch gel. Thus at both pH 1.8 and 8.1 the blocked N-terminal nitrogen of the $\beta^{A_{Ic}}$ chain appears to have approximately the same charge as the unblocked N-terminal nitrogen of the β chain.[4] This lack of a charge difference between β and $\beta^{A_{Ic}}$ chains at pH 1.8 and 8.1 can reasonably be explained by assuming that the bond

[3] Through a calculational error, it was stated (Holmquist and Schroeder, 1964) that "approximately 25% of the A_{Ic} is converted to A_{II}... the equilibrium constant (K_e) is therefore 0.1." These numbers should read 12% and 0.03.

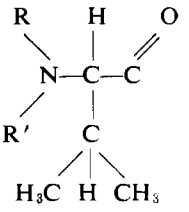
[4] It is doubtful that starch gel electrophoresis of proteins, especially those of unknown structure, is sufficiently well understood to state with certainty that if charge differences *did* exist between β and $\beta^{A_{Ic}}$, separate bands would have been observed in the gel. For example, at pH 1.8 the α and β chains of A_{II} have positive charges of 25 and 24, respectively; yet, the β chain has the higher cathodic mobility, exactly the opposite of the expected behavior. The starch gel experiments are thus only corroborative of more definite experiments.

TABLE III: Amino Acid Composition[a] of the Tryptic Peptides of α Chain of Hemoglobin A_{Ic}.

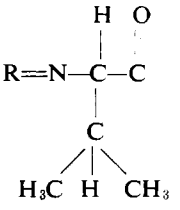
	αT-1	αT-2	αT-3	αT-4	αT-5	αT-6	αT-7	αT-8	αT-9	αT-10	αT-11	αT-12 + αT-13[b]	αT-14
Lys	0.97	1.00	1.00		1.14	0.95	1.10	1.00	0.96		1.01	2	
His				0.98		1.78	0.93		2.82			3	
Arg				0.98						1.00			1.04
Asp	1.06	1.00				1.01			6.25		2.04	1	
Thr		0.92			1.76	0.88			1.02			4	
Ser	0.73				0.92	1.90			1.93		0.11	5	
Glu	0.16			2.96		1.07						1	
Pro	1.01				1.21	1.22			1.05		0.94	2	
Gly			1.06	3.08	0.29	1.18	1.94	0.12					
Ala	1.01		1.94	4.34		1.06	0.23	0.10	7.09			6	
Cys/2												1	
Val	1.12	1.00		1.16		1.09			2.99		2.02	4	
Met					0.24				0.31				
Ile													
Leu	0.83			1.19	1.06	1.01			4.06	1.00		9	
Tyr				0.93		0.78							0.94
Phe	0.15				1.89	1.85					0.96	2	
Try			0.14										

[a] The data are the number of amino acid residues relative to each other. Amounts <0.10 residue are not reported. [b] The number of residues in this column is only accurate to about ±0.6 residue. They were calculated by subtracting the sum of the (integral) number of amino acid residues for the other $\alpha^{A_{Ic}}$ tryptic peptides from the known amino acid composition of the $\alpha^{A_{II}}$ chain. To within the error of the analysis (3–5%), the amino acid compositions of A_{II} and A_{Ic} were identical.

between R and the N-terminal valine is one of the following two types: (a) R is bonded to the N-terminal amino group to form a secondary or tertiary amine



(b) R is bonded to the N-terminal amino group as a Schiff base[5]



[5] The pK_a for the protonated imine nitrogen lies in the range 6–7 at 25° (Coryell and Pauling, 1940; Greenstein and Winitz, 1961; Cordes and Jencks, 1963).

We shall return to this matter in a subsequent section.

At this point in the investigation, it became clear that the identification of the N-terminal blocking group R would require either the isolation of R itself or of a small compound to which it was covalently bonded and through which it could be unambiguously characterized. Since there was no very rational way of directly detecting R itself (because it was both colorless and of unknown structure), the second approach was necessary. This was accomplished (Figure 1) by first isolating the blocked N-terminal octapeptide $\beta^{A_{Ic}}$T-1, whose expected structure was R-Val-His-Leu-Thr-Pro-Glu-Glu-Lys, from a tryptic hydrolysate of $\beta\beta^{A_{Ic}}$-globin and then by degrading this octapeptide with papain to obtain R-Val-His.

Preliminary Separation of Tryptic Peptides on Dowex 50-X2. ISOLATION OF $\beta^{A_{Ic}}$T-1. A typical chromatogram of the hydrolysate from the tryptic digestion of $\beta\beta^{A_{Ic}}$-globin is shown in Figure 3. Figure 4 shows the paper electrophoretic behavior of the peptide mixture in each zone of Figure 3. The only difference between the pattern in Figure 4 and that obtained from the tryptic peptides of the β chains from A_{II} is the presence in zone 5 of Figure 4 of a histidine-positive peptide of low anodic mobility. This peptide is indicated by an arrow in Figure 4 and is designated as $\beta^{A_{Ic}}$T-1. The following

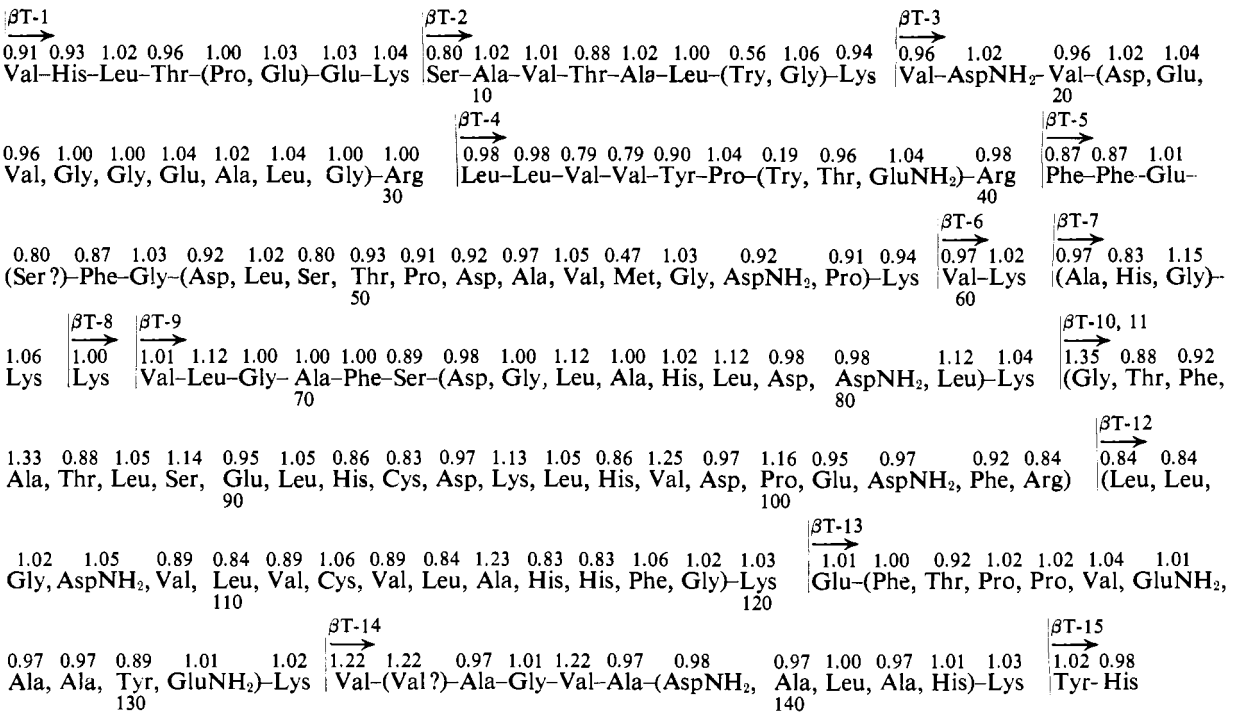


FIGURE 2: Amino acid composition and sequence of the tryptic peptides from the mixed $\beta\beta^{A_{Ic}}$ chains of A_{Ic}. The symbols βT-1, etc., represent the tryptic peptides as designated by Baglioni (1961). A question mark indicates an uncertain identification. For a given amino acid within a peptide, the sum of the numbers above that amino acid is the number of residues of that amino acid that was found in the peptide. See text for further explanation.

points are relevant. From previous chromatographic experience,[6] the histidine-positive peptide in zone 7 of Figure 4 is βT-1, which has the structure Val-His-Leu-Thr-Pro-Glu-Glu-Lys. Because $\beta^{A_{Ic}}$T-1 is eluted from the Dowex 50 column before the peptides in zone 7, it must have a relatively more negative net ionic charge than βT-1. This fact is in agreement with both the chromatographic behavior of A_{Ic} on Bio-Rex 70 and with the slight anodic mobility of $\beta^{A_{Ic}}$T-1. However, the new peptide is only slightly more negative than leucine at pH 6.4. Thus, whatever property of the blocking group R is responsible for the more negative charge of $\beta^{A_{Ic}}$T-1, that property does not seem to correspond to a fully ionized carboxyl group.

An attempt to separate $\beta^{A_{Ic}}$T-1 from the other peptides in zone 5 on Dowex 1 failed because $\beta^{A_{Ic}}$T-1 was quantitatively converted to βT-1 through the removal of the blocking group R during the chromatography (Holmquist and Schroeder, 1964). The βT-1 so isolated had N-terminal valine as detected by the Edman degradation. This was the first indication of the lability at neutral and basic pH values of the bond between R and the N-terminal nitrogen. However, $\beta^{A_{Ic}}$T-1 could be purified at acidic pH without the loss of R by chromatographing the peptides in zone 5 on cellulose phosphate (Figure 5). The βT-1 in Figure 5 arose from the loss of R from $\beta^{A_{Ic}}$T-1 *prior* to the chromatography. On acid hydrolysis, $\beta^{A_{Ic}}$T-1 had an amino acid composition identical with βT-1. Consequently, the blocking group R cannot be bonded to the N-terminal α-amino group of valine to form a secondary or tertiary amine,[7] nor can R contain common amino acids. $\beta^{A_{Ic}}$T-1 could thus be formulated at this point as R-Val-(His,Leu,-Thr,Pro,Glu,Glu)-Lys.

ISOLATION OF R-VAL-HIS FROM A PAPAIN HYDROLYSATE OF $\beta^{A_{Ic}}$T-1. An electrophoretic comparison at pH 6.4 of the papain hydrolysates from $\beta^{A_{Ic}}$T-1 and βT-1 is shown in the top two lines of Figure 6. From Figure 6 it can be calculated (see Appendix) that the pK_a for the proton of the blocked α-amino nitrogen of valine in R-Val-His is 6.64 ± 0.14. This fact is consistent with a Schiff base type bond between R and the α-amino group of the valine that has been suggested by the starch gel experiments, for it has been shown by Cordes and Jencks (1963) that the pK_a of the Schiff base between *t*-butylamine and benzaldehyde is 6.70 at 25°. When electron-withdrawing groups are substituted on the benzene ring, the pK_a is less, and when electron-donating

[6] See Schroeder *et al.* (1962b). Zone 10 of Figure 4 in that reference corresponds to zone 7 of Figure 4 in this paper.

[7] Although compounds other than α-amino acids, among these the secondary amines, give positive reactions with ninhydrin, their color yields are only 2–25% of that of the free α-amino acids (Neuzil *et al.*, 1959; Josselin and Breton, 1960; McCaldin, 1960; Zacharius and Talley, 1962; Schilling *et al.*, 1963; Hu *et al.*, 1965).

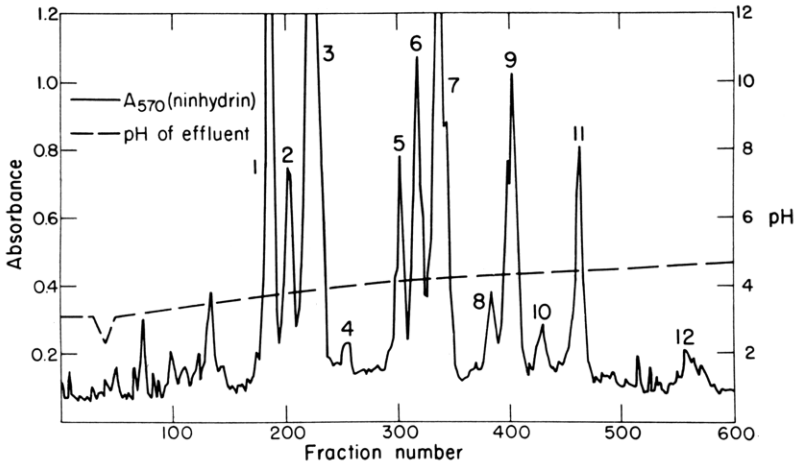


FIGURE 3: Dowex 50-X2 chromatogram of the soluble tryptic peptides from 1.7 g of $\beta\beta^{A_{Ic}}$-globin. Column size 3.5 × 100 cm. Fraction size 15.5 ml. The zones have been numbered consecutively from left to right. See text for discussion.

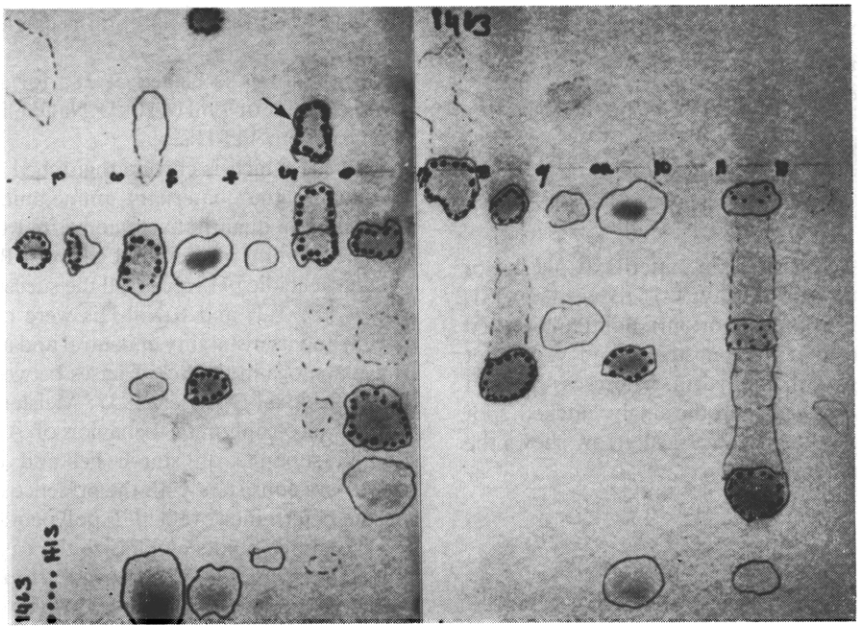


FIGURE 4: Paper electrophoresis at pH 6.4 of the zones of the chromatogram illustrated in Figure 3.

groups are present in the benzene ring, the pK_a is larger.

The chromatographic separation on Dowex 50-X8 of the peptides from the papain hydrolysate of $\beta^{A_{Ic}}$T-1 is shown in Figure 7. The base line in this chromatogram is somewhat more erratic than usual. In part this is due to the expanded scale for the ordinate, and in part to the fact that alkaline hydrolysates of zones from a Dowex 50-X8 chromatogram sometimes give small spurious peaks. Only those zones that are labeled were found to contain amino acids or peptides. Val-His emerges from the column after lysine (Goldstein *et al.*, 1963), so that if a mixture of R-Val-His and Val-His had been present (due to a partial loss of the blocking group—in practice this loss did not occur), it would have readily been resolved by chromatography. The separation between Glu-Lys and R-Val-His was approximately five fractions; amino acid analysis of these two peptides showed that neither was contaminated by the other.

The Schiff Base Nature of Hemoglobin A_{Ic}

After describing a few preliminary experimental observations which support the Schiff base nature of A_{Ic}, definitive chemical evidence will be presented. These preliminary observations are given because, taken individually, they were at first puzzling. The behaviors herein described may be of use to other in-

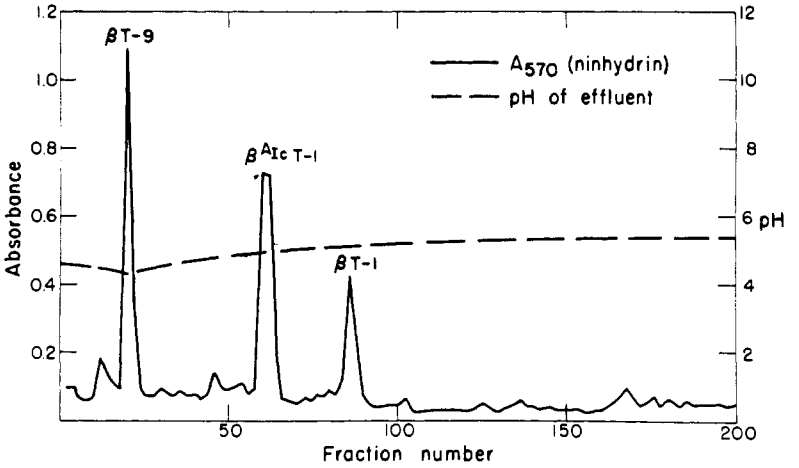


FIGURE 5: Separation of the tryptic peptides from zone 5 of Figure 3 on cellulose phosphate. Column size 0.9 × 100 cm. Fraction size 4.4 ml.

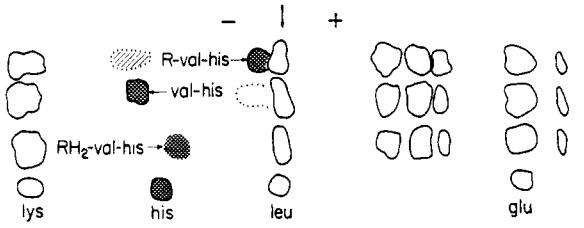


FIGURE 6: Paper electrophoretic patterns at pH 6.4 of papain hydrolysates of $\beta^{A_{Ic}}$T-1, βT-1, reduced $\beta^{A_{Ic}}$T-1, and a standard (from top to bottom). Solid and dotted lines outline spots that gave a strong and very faint color, respectively, with ninhydrin. The cross-hatched spots reacted very strongly and the singly hatched spot very weakly for histidine. The vertical arrow shows the line of application.

vestigators who may encounter similar situations with other proteins.

Preliminary Observations. On paper, $\beta^{A_{Ic}}$T-1 or R-Val-His react only very weakly with ninhydrin at room temperature. However, at 80°, a deep purple, comparable in intensity to that given by an equivalent amount of βT-1 or Val-His, develops. These facts suggest that $\beta^{A_{Ic}}$T-1 and R-Val-His have a potentially free primary amino group. For example, N=R by hydrolysis would furnish an amino group and the ketone or aldehyde R=O.

When $B^{A_{Ic}}$T-1 is concentrated from large volumes of pyridine–acetic acid buffers by evaporation at room temperature, sometimes only βT-1 is found in the residue; *i.e.*, the blocking group R is cleaved from $\beta^{A_{Ic}}$T-1.

Each time that purified $\beta^{A_{Ic}}$T-1 or R-Val-His is dissolved in water and the water is then evaporated, the amount of $\beta^{A_{Ic}}$T-1 or R-Val-His decreases and a corresponding amount of βT-1 or Val-His, respectively, is formed. More of the latter two compounds are formed when the evaporation is carried out at 38 than at 25°.

If R-Val-His is heated at 38° for 16 hr with either 1 ml of water or 1 ml of 10^{-4} F NaOH, it is quantitatively converted to Val-His.

$\beta^{A_{Ic}}$T-1 which is chromatographed under basic conditions on the quaternary amine anion-exchange resin Dowex 1 is quantitatively converted to βT-1.

The stability of the bond between R and N-terminal valine at acidic pH values (all the successful isolations of $\beta\beta^{A_{Ic}}$, $\beta^{A_{Ic}}$T-1, and R-Val-His were carried out below pH 5) and its instability at neutral and alkaline pH values have analogs in the Schiff bases between some proteins and pyridoxal (Metzler, 1957; Meister, 1965).

The electrophoretic behavior of A_{Ic}-globin and the globin subunits on starch gel and of R-Val-His on paper are consistent with the presence of an imine bond at the N terminus of the $\beta^{A_{Ic}}$ polypeptide chain.

The above points emphasize the ease with which the blocking group R is removed from the N-terminal nitrogen under very mild hydrolytic conditions. This lability has been the yield-limiting factor in obtaining sufficient R-Val-His for chemical characterization.

Chemical Evidence for the Schiff Base Linkage. If, indeed, the bond between R and valine is of the Schiff base type, R=NC<, then it should be possible to reduce this bond to a secondary amine by the following reaction

$$R{=}N{-} + 2[H] \longrightarrow \overset{H}{HRN}{-}$$

If reduced $\beta^{A_{Ic}}$T-1 or R-Val-His were then isolated, acidic hydrolysis should not cleave R from valine. Consequently, *valine would be missing from the amino acid analysis of either reduced $\beta^{A_{Ic}}$T-1 or reduced R-Val-His* because reduced R-valine should have a different chromatographic behavior and should give little or no color with ninhydrin. The absence of valine

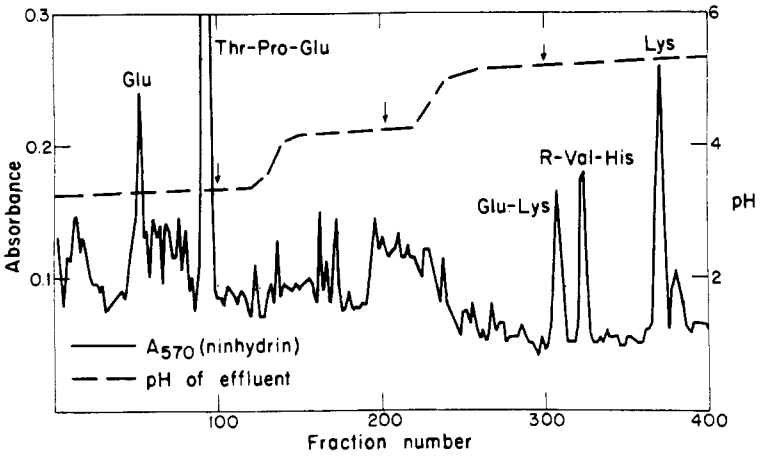


FIGURE 7: Separation of peptides from a papain hydrolysate of $\beta^{A_{1c}}$T-1 on Dowex 50-X8. Column size 0.6 × 45 cm. Fraction size 1 ml. Arrows indicate fraction at which buffer changes were made.

provides an experimental criterion for the presence of the Schiff base.

REDUCTION OF $\beta\beta^{A_{1c}}$-GLOBIN WITH $NaBH_4$. Reductions with $NaBH_4$ are usually carried out at alkaline pH values because of the instability of the hydride anion under acidic conditions. However, Meyers and Libano (1961) and Crestfield *et al.* (1963) have shown that even at acidic pH values the hydride anion has a sufficiently long half-life to accomplish those reductions that are normally done in alkaline media. Because of the lability of the blocking group R under neutral or alkaline conditions, we carried out the reduction in acidic solution.

A 249-mg sample of $\beta\beta^{A_{1c}}$-globin was suspended in 20 ml of water, and the pH was adjusted to 3.5 with approximately 0.02 ml of 6 F HCl. After 1 hr of stirring, the solution was slightly turbid and a very pale yellow. A solution of 37 mg of $NaBH_4$ in 1 ml of water which contained 4 μl of 4 F NaOH was added dropwise to the solution of globin, and the pH was maintained at 3.5 by the manual addition of 2 F HCl. Two more 37-mg portions of $NaBH_4$ were added at 1-hr intervals. Three hours after the first addition of $NaBH_4$, dialysis of the reaction mixture was begun at 2° for three 8-hr periods *vs.* 25 times its volume of water. The dialysate was lyophilized to dryness. The reduced $\beta\beta^{A_{1c}}$-globin had the same amino acid composition as the unreduced globin so that no extensive degradation of the protein appeared to have occurred during the reduction. Crestfield *et al.* (1963) have shown that under some conditions reduction of a protein with $NaBH_4$ does lead to smaller peptide fragments, so that the above statement must be verified for each individual case.

ISOLATION OF REDUCED $\beta^{A_{1c}}$T-1 AND REDUCED R-VAL-HIS. Reduced $\beta\beta^{A_{1c}}$-globin from the preceding procedure was hydrolyzed with trypsin, and the tryptic peptides were isolated on Dowex 50-X2. The Dowex 50 chromatogram was similar in detail to that of Figure 3 except that zone 5 had moved into the region of zone 6, although a shoulder was still visible on the left-hand side of the latter zone. If $\beta^{A_{1c}}$T-1 is a Schiff base, the reduced octapeptide (being a secondary amine) would be more basic and would move more slowly than $\beta^{A_{1c}}$T-1 on Dowex-50. $\beta^{A_{1c}}$T-1 is normally found in zone 5 of Figure 3. The observed movement of zone 5 into the region of zone 6 was thus as anticipated. Indeed, when the zones from the Dowex 50 chromatogram were analyzed by paper electrophoresis and paper chromatography, no peptide having the electrophoretic mobility (*cf.* Figure 4) of $\beta^{A_{1c}}$T-1 was found. Instead, a histidine-positive peptide with the paper electrophoretic and paper chromatographic mobility of βT-1 was found in zone 6; one would expect the mobilities of reduced $\beta^{A_{1c}}$T-1 and (normal) βT-1 to be very similar, because the basicities of a secondary amine (reduced $\beta^{A_{1c}}$T-1) and a primary amine (βT-1) differ little. Because zone 6 is adjacent to zone 7, in which βT-1 is normally found, it was possible (but unlikely from previous experience) that the βT-1-like peptide in zone 6 was not reduced $\beta^{A_{1c}}$T-1, but rather was βT-1 from zone 7 which had overlapped into zone 6. This possibility was ruled out by rechromatographing the mixture of peptides in zone 6 on Dowex 1 by the method of Schroeder and Robberson (1965). The chromatogram is shown in Figure 8. Three zones were isolated from the Dowex 1 chromatography. Two of these contained βT-14 and βT-6, which were expected, because these peptides are usually found in zone 6 from Dowex 50 chromatography. The third contained a peptide which was contaminated with βT-9, and which from its amino acid analysis could have been either reduced $\beta^{A_{1c}}$T-1 or βT-1. [An amino acid analysis of βT-1 would give the amino acid composition (Val,His,Leu,Thr,Pro,Glu_2,Lys), whereas an analysis of reduced $\beta^{A_{1c}}$T-1 would give the composition (His,Leu,Thr,Pro,Glu_2,Lys).] The ambiguous analysis was due to the presence of about 0.3 residue of valine; this suggested that the peptide was still contaminated with some βT-9. To free the peptide from the contaminating βT-9, it was again chromatographed on Dowex 50-X2, but this time buffers of lower ionic

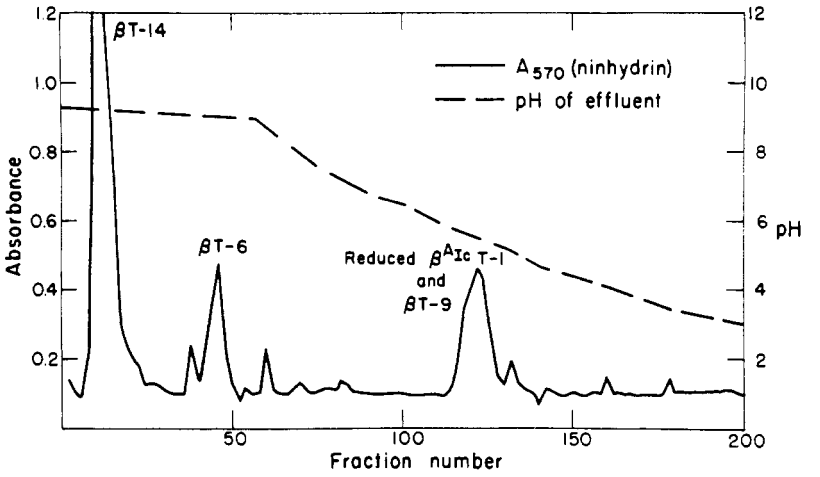


FIGURE 8: Isolation of reduced $\beta^{A_{Ic}}$T-1 on Dowex 1-X2. Column size 0.6 × 60 cm. Fraction size 1.0 ml.

strength were used (see Table II in Schroeder, 1966b). The results of this chromatogram are shown in Figure 9.

The amino acid composition of the peptide designated "Reduced $\beta^{A_{Ic}}$T-1" in Figure 9 is compared in Table IV with that of βT-1 from reduced $\beta\beta^{A_{Ic}}$-globin.

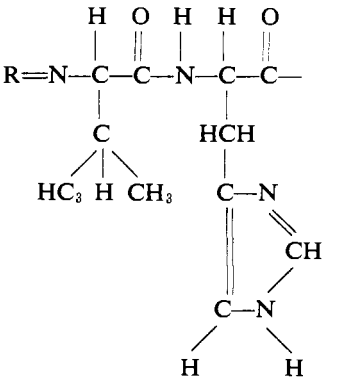
TABLE IV: Amino Acid Composition[a] of Reduced $\beta^{A_{Ic}}$T-1 and βT-1.

Amino Acid	Reduced $\beta^{A_{Ic}}$T-1	βT-1
Lys	0.95	0.99
His	0.87	0.86
Thr	0.95	0.95
Glu	2.00	1.97
Pro	1.01	1.01
Val	0.04	0.99
Leu	0.82	1.00

[a] The numbers in the table are the number of amino acid residues in the peptide.

The important point in Table IV is the absence of valine in the new peptide. The absence of valine could conceivably be the result of hydrolysis of the amide bond between valine and histidine during the reduction. This is unlikely both because the normal yield of βT-1 was found and because $NaBH_4$ does not usually attack amide bonds. Evidence that the bond between valine and histidine was intact was obtained by hydrolyzing reduced $\beta^{A_{Ic}}$T-1 with papain with the results shown in the third line of Figure 6. Had the amide bond between valine and histidine been split during the reduction, the papain hydrolysate of reduced $\beta^{A_{Ic}}$T-1 would be expected to contain free histidine, which would give positive ninhydrin and Pauly tests. However, as can be seen from Figure 6, the papain hydrolysate contained only a *single* peptide which contained histidine, and this peptide gave a *negative* ninhydrin test and had an electrophoretic behavior different from that of Val-His and R-Val-His. Thus, the α-amino group of the histidyl residue in this fragment is not free. But as Table IV shows, this α-amino group can be released, and free histidine produced, by hydrolysis with HCl. Amino acid analysis of reduced R-Val-His showed the presence of histidine alone.

Conclusion. The isolation of reduced $\beta^{A_{Ic}}$T-1 proves the hypothesis of a Schiff base type bond between the blocking group R and the N terminus of the $\beta^{A_{Ic}}$ polypeptide chain



Accordingly, R itself derives from either an aldehyde or a ketone R=O, the characterization of which is the subject of the next few sections.

Chemical Characterization of the Blocking Group R

The Molecular Weight of R. METHODOLOGY. The molecular weight was obtained by weighing a known number of moles of R-Val-His. In practice, the experimental procedure was complicated by the presence in R-Val-His of contaminants from the chromatographic procedures (resin material and impurities from the

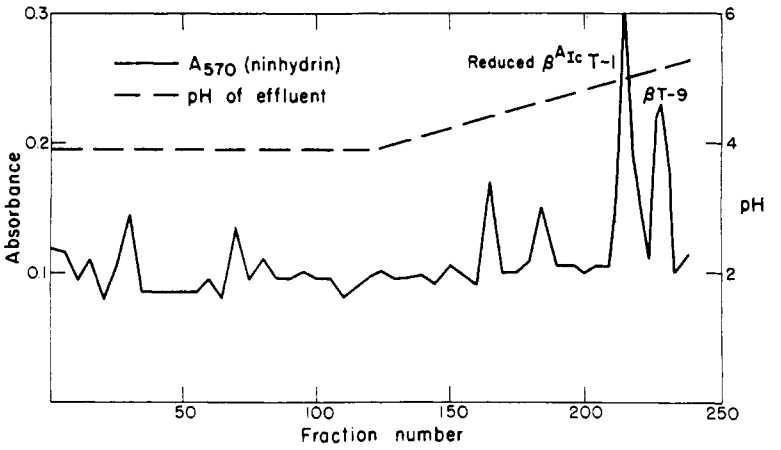


FIGURE 9: Purification of reduced $\beta^{A_{Ic}}$T-1 on Dowex 50-X2. Column size 0.6 × 60 cm. Fraction size 1.0 ml.

evaporation of large volumes of volatile buffers). It was therefore necessary to determine the percentage of these contaminants in the weighed sample. This was accomplished by taking as controls several chromatographic zones on either side of the zone which contained R-Val-His. In this way, the possibility that the amount of contaminant was a function of the effluent volume was allowed for. Some of these control zones contained known amounts (by analysis) of peptides whose molecular weights were about the same as that of Val-His, and so they were closely equivalent to the zone which contained R-Val-His. As a check on the procedure, the molecular weight of L-Ala-L-Ala-L-Ala was determined.

EXPERIMENTAL. After a Pyrex test tube had been dried to constant weight over P_2O_5, an aliquot portion from fractions 316–330 (Figure 7) which contained approximately 600 μg of R-Val-His was added and dried *in vacuo* at room temperature over P_2O_5 (heating would have removed the blocking group). The sample was hydrolyzed in an evacuated sealed tube for 22 hr at 110° in 2 ml of doubly glass-distilled 6 F HCl which was added in such a way as to rinse the sample from the sides of the tube. Analysis for valine and histidine determined the number of moles of R-Val-His which were present in the weighed sample. Exactly the same procedure was followed with the controls.

RESULTS. Calculation of the molecular weight of the blocking group R is shown in Table V. From the molecular weight of 265 ± 27 (probable error) for R, the molecular weight of the ketone or aldehyde R=O must be 281 ± 27. Because of the magnitude of the probable error, it is unlikely that the true molecular weight of R=O lies outside the range 281 ± 80.

Tests for Specific Chemical Groupings in the Blocking Group R. General considerations, arising from the known structures of other proteins, from certain facts about hemoglobins in general, from the principles of biochemistry, and from the available chemical evidence, led us to test for the following groups: nonketo acyl groups that produce volatile acids, R = —COR′, and in particular acetyl; carboxyl, R = COO^-; carbamyl, R = —$CONH_2$; aromatic aldehydes, and pyridoxal in particular; phosphorus, both organic and inorganic; carbohydrate, R = a sugar, or derivative thereof; and steroids. At the time that most of these characterizations were made, the Schiff base nature and the molecular weight of R were unknown. Therefore, some of the above groupings lie without the molecular weight range found in the preceding section. The tests do, however, exclude many possible groups.

METHODOLOGY. Tests for the above chemical groupings were carried out on either A_{Ic} itself, $\beta^{A_{Ic}}$T-1,

TABLE V: Calculation of the Molecular Weight of R.

Molecular weight of L-Ala_3[a]	243 ± 2
Weight of R-Val-His + contaminants (μg)	774 ± 17
Weight of column contaminants[b]	136 ± 28
Weight of R-Val-His	638 ± 33
R-Val-His (μmoles)	1.23 ± 0.04
Molecular weight of R-Val-His	518 ± 27
Molecular weight of Val-His	254
Molecular weight of R	265 ± 27[c]

[a] Determined in triplicate. Calculated molecular weight is 231.34. The small error must be considered accidental, for the sum of the weighing and amino acid analysis errors was ±7 awu. [b] Determined from fractions 130–137, 302–315, and 367–376 of Figure 7. The weight of the residue from each zone was reduced by the weight of any amino acids or peptides which it contained (none, Glu-Lys, and Lys, respectively), and was then adjusted proportionally for differences in the number of chromatographic fractions in that zone and the zone from which R-Val-His was taken. The average of these adjusted values for the three zones was 136 ± 28 μg. [c] This error is divided as follows: weighing 9, amino acid analysis 9, and contaminants 9.

R-Val-His, or reduced R-Val-His. Wherever feasible, a given chemical group was assayed for by two independent methods. Standard curves covering the appropriate nano- to micromole range were prepared from substances of known composition.

Because of the presence of nonprotein impurities (from the chromatography), the assays were carried out quantitatively in such a way that any contribution from these impurities could be allowed for. A test was considered negative if <0.10 μmole of group/μmole of compound was found, since ratios >0.80 μmole of group/μmole of compound were always obtained for standards that were known to contain the group in question.

Some of the assays were modified to make them applicable, in a quantitative manner, to the submicromole range. Because all the groups in question were shown to be absent, detailed descriptions have been omitted here. A complete description can be found elsewhere (Holmquist, 1966).

EXPERIMENTAL. Nonketo acyl groups that produce volatile acids (less than five carbon atoms) were assayed by titration with $Ba(OH)_2$ (Bartley, 1953); the acetyl group was also tested for by the method of Ludowieg and Dorfman (1960) as modified for gas chromatography by Schroeder *et al.* (1962a).

The possibility that the blocking resulted from the addition of CO_2 to the α-amino group of valine was tested for indirectly by incubating synthetic L-Val-L-His with CO_2 in a sealed tube and comparing the resulting compound electrophoretically (pH 6.4) with R-Val-His and Val-His. The incubation was carried out at 25° for 108 hr. The reacting mixture contained the following species at the indicated concentrations when under 8 atm of CO_2 pressure: L-Val-L-His, 0.0015 F; Na^+, 0.26 F; $H_2PO_4^-$, 0.14 F; HCO_3^-, 0.12 F; H_2CO_3, 0.27 F; pH 5.6. For a discussion of the formation of CO_2 compounds with amino acids and proteins, the reader is referred to Faurholt (1925), Ferguson and Roughton (1934), Stadie and O'Brien (1937), Margari (1952), Giustina *et al.* (1952a,b), and Milla *et al.* (1953a,b).

Stark and Smyth (1963) carbamylated proteins to determine the N-terminal amino acid present therein. If the treatment with potassium cyanate is omitted, and the procedure is otherwise left unchanged, the Stark and Smyth method can be used to determine whether an N-terminal carbamyl group is initially present in a peptide or protein.

The possible presence of pyridoxal was investigated by synthesizing the Schiff base between pyridoxal and L-valine and comparing the color of this base with that of an equal concentration of R-Val-His. The synthesis followed Heyl *et al.* (1948). The Schiff bases of aromatic aldehydes are a characteristic deep yellow (Metzler, 1957).

Phosphorus was assayed by the method of Nakamura (1952). Carbohydrates were tested for by both the anthrone test (Dreywood, 1946; Scott and Melvin, 1953; Whistler and Wolfrom, 1962) and the phenol test (Dubois *et al.*, 1962). The microdetermination of steroids was made with antimony trichloride (Neher, 1964; Hais and Macek, 1963).

RESULTS. All of the above groups were found to be quantitatively absent. R is not itself nor does it contain esterified nonketo acyl groups of less than five carbon atoms, is not carboxyl, carbamyl, pyridoxal, or other aromatic aldehyde, and does not contain phosphorus, carbohydrate, or steroids. The electrophoretic data make it unlikely that R contains any basic nitrogen atoms or any acidic groups.

General Discussion

Difficulties in the Isolation of Schiff Base Compounds. As has already been mentioned, the isolation of the octapeptide $\beta^{A_{Ic}}$T-1 and the dipeptide R-Val-His was difficult because of the susceptibility to hydrolysis of the imine double bond at the N terminus of these compounds. The fact that these difficulties were not encountered with compounds of larger molecular weights such as $\beta\beta^{A_{Ic}}$-globin or hemoglobin A_{Ic} itself may be due to stabilizing interactions of the N terminus of the $\beta^{A_{Ic}}$ chain with the tertiary or quaternary structures that are possible for these larger molecules but impossible in the smaller peptides (see below). This would be consistent with the increased stability toward acid–base dissociation of A_{Ic} relative to A_{II} that has been observed in the self-hydridization experiments.

As an illustration of the problems that can actually arise, we have observed the complete conversion of 18 μmoles of $\beta^{A_{Ic}}$T-1 to βT-1 during the evaporation at room temperature of 300 ml of pyridine–acetic acid effluent from a 3.5 × 100 cm Dowex 50 column. Successful isolations on a smaller scale had not led us to expect such a loss. It seems reasonable to attribute the loss to the increased concentrations of buffer and resin impurities during the evaporation.

It is now clear that the above difficulties can be obviated by reduction with $NaBH_4$ at an early stage in the isolation procedures. The comments of Meister (1965, pp 392–393) on the isolation of glutamate–aspartate transaminase may also be relevant with respect to difficulties in isolating free Schiff bases. Currently, a more highly purified sample of reduced R-Val-His is being isolated in order to examine the structure of R by mass spectrometry.

The Biological Uniqueness of Hemoglobin A_{Ic}. Although several proteins, all enzymes, are known in which pyridoxal is bonded to the ε-amino group of non-N-terminal lysine in a Schiff base linkage, hemoglobin A_{Ic} is the first example of a protein in which an N-terminal Schiff base involving neither pyridoxal nor lysine has been found. For example, the presence of a Schiff base in pig heart glutamate–aspartate transaminase (Hughes *et al.*, 1962), rabbit skeletal muscle phosphorylase (Fischer *et al.*, 1958), and acetoacetic acid decarboxylase from *Clostridium acetobutylicum* (Westheimer, 1963) has been proven; and the evidence is strong for a similar linkage of pyridoxal with an ε-amino group of lysine for cystathionase, aspartic-β-decarboxylase, and glutamic-α-decarboxylase (Meister, 1965, pp 386–398).

Stability of the Schiff Base Bond in Hemoglobin A_{Ic}.

Cordes and Jencks (1963) have shown that at pH 2 the half-life at 25° for the hydrolysis of aromatic Schiff bases increases from 6 sec for *p*-nitrobenzene to 2.5 hr for *p*-methoxybenzene. These differences gradually become less as the pH increases, until at pH 8 the half-life is of the order of 1 min for all monosubstituted benzenes. One would expect Schiff bases formed from nonaromatic aldehydes to be less stable than those formed from aromatic aldehydes because the former have no resonance stabilization. The blocking group R is known not to be an aromatic aldehyde or ketone. Yet hemoglobin A_{Ic} is stable *in vitro* for periods of over a month at 4° and for at least 7 days at 25° (Huisman and Horton, 1965). Their data are fully in accord with our experience. This relative stability of A_{Ic} compared to the Schiff bases that were discussed in the preceding paragraph suggests that a rather large stabilizing force is present in this protein, and it may be related to the tertiary or quaternary structure of hemoglobin A_{Ic}.

From the chemical tests, individuals from many classes of compounds must be excluded as identical with or as components of R=O. There is yet no basis for excluding long-chain aliphatic lipidlike, possibly hydroxylated, aldehydes such as stearaldehyde (mol wt 268). A chain of 18 carbon atoms would be capable of interacting at distances of at least 20 A from the N terminus, and therefore, could interact with other parts of the β chain to which it is attached as well as with the second β chain or one of the α chains of the molecule.

Matters of Nomenclature. Because of the multiplicity of nomenclatures for hemoglobins, an attempt is made here to clarify several possible ambiguities which may exist with respect to A_{Ic}. First, A_{Ic} is not (Schnek and Schroeder, 1961) to be identified with Kunkel's (1958) and Kunkel and Wallenius' (1955) "fast minor component" hemoglobin A_3. Normally, A_{Ic} is a constituent of Kunkel's and Wallenius' A_1, though it is possible that some A_{Ic} may be present in the A_3 fraction if the electrophoretic separation of A_1 and A_3 is incomplete. Thus A_{Ic} is not the hemoglobin A_{II}–glutathione complex found by Muller (1961): the latter complex may be an artifact of the isolation procedures (Huisman and Dozy, 1962; Huisman and Horton, 1965). A_{Ic} is probably one of the components of Huisman and Meyering's (1960) A_1^c that was isolated on carboxymethylcellulose, inasmuch as the latter amounts to 11.0% (Meyering *et al.*, 1960) of the total hemoglobin while the former comprises only 5.3%.

The paper by Atassi (1964) has unintentionally further confused the matter of nomenclature. Approximately 33% of Huisman and Meyering's A_1 can be equated to Schnek and Schroeder's (1961) A_I, and the former's A_0 is identical with the latter's A_{II}. But Huisman and Meyering's A_1 and A_0 cannot be equated to Schnek and Schroeder's "A_3" and "A_1" because the latter use only Roman subscripts. Atassi reports the interconversion of Huisman and Meyering's A_1 and A_0 by "simply allowing what was originally an electrophoretically and chromatographically homogeneous fraction to stand in a buffered solution at 0–2°." Because no blocking group is present in A_0 (= A_{II}), the latter's conversion to A_{Ic} (which comprises 33% of A_1) is impossible. Atassi was therefore probably studying the interconversion of part of a heterogeneous mixture.

General Comments. There has been a tendency to dismiss the minor hemoglobin components as artifacts of the isolation procedures or as the biochemical products of erythrocyte aging. Yet the human minor components that have been fairly well characterized, namely Kunkel's A_2 (Kunkel, 1958), hemoglobin F_I (Schroeder *et al.*, 1962a), Hb-α^A (Chernoff, 1964; Fessas and Loukopoulos, 1964), and A_{Ic}, have been shown to be neither artifacts nor products of aging; and further, from their diverse structures, it is clear that both their biogenesis and function in the erythrocyte differ significantly.

It is hoped that in addition to elucidating the structure of A_{Ic} partially, this paper may give impetus to detailed chemical studies of other minor hemoglobin components (of humans and other species). One of the problems in such studies is to obtain enough material to characterize. Some of the methods described in this paper may be useful toward achieving that goal.

Appendix

Calculation of Ionization Constants from Paper Electrophoretic Data

The pK_a of the protonated imine nitrogen of R-Val-His was calculated from the Henderson–Hasselbach equation

$$pK_a = pH - \log\left(\frac{\alpha}{1-\alpha}\right)$$

The degree of ionization α (at pH 6.4) was found from

$$\alpha = Q_{\text{R-Val-His}} + 1 - \left(\frac{Q_{\text{Val-His}} + Q_{\text{reduced R-Val-His}}}{2}\right)$$

where Q_i is the charge on the species i and is given by

$$Q_i = \frac{D_i}{\frac{D_{lys} - D_{glu}}{2}}$$

The distances D_i were measured from the neutral marker Leu ($Q_{Leu} = D_{Leu} \equiv 0$) and were taken as positive or negative if the species i migrated toward the cathode or anode, respectively. In the formula for α, $Q_{\text{R-Val-His}}$ includes the negative charge of the carboxyl group, the positive charge of the imidazolium ion, and the positive charge on the imine nitrogen to which the blocking group R is attached. The third term, in parentheses, corrects for the charges on the carboxyl and imidazolium ions, but it also subtracts out a full positive charge because the N-terminal amino group of Val-His and reduced R-Val-His bears a unit positive charge at pH 6.4. The second term, +1, adds this positive charge back in, so that α is simply that fraction

of the molecules of R-Val-His which have a unit positive charge on the imine nitrogen to which R is attached.

Waldron-Edward (1966) has given both theoretical and experimental justification for the validity of this type of calculation. From our data (Figure 6 and other results) it was found that $Q_{Lys} = -Q_{Glu} = 1.00 \pm 0.03$ as anticipated. Further the pK_a of the imidazolium ion in free histidine, in Val-His, and in reduced R-Val-His were calculated by this method (for these compounds $\alpha = Q_i$) to be 6.35, 6.62, and 6.34, respectively. These values are in satisfactory agreement with the literature values (Coryell and Pauling, 1940; Greenstein and Winitz, 1961), which range from 6.00 for free histidine to 6.83 for carnosine in dilute aqueous solutions at room temperature. Some allowance should be made for the fact that the electrophoretic determinations of Figure 6 were made on paper in pyridine–acetate buffer at about 10°. In view of these agreements, the pK_a of 6.64 ± 0.14 that has been calculated for R-Val-His should be accurate to within the limits stated.

For some compounds, such as *N*-acetyl-Val-His and the octapeptide βT-1, the above method of obtaining pK_a values fails completely. This is probably due to the dissimilarity between these compounds and the reference compounds Leu, Lys, and Glu, especially with regard to their ability to bind to the paper.

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References

Allen, D. W., Schroeder, W. A., and Balog, J. (1958), *J. Am. Chem. Soc. 80*, 1628.

Atassi, M. Z. (1964), *Biochem. J. 93*, 189.

Babin, D. R., Jones, R. T., and Schroeder, W. A. (1964), *Biochim. Biophys. Acta 86*, 136.

Babin, D. R., Schroeder, W. A., Shelton, J. R., Shelton, J. B., and Robberson, B. (1966), *Biochemistry 5*, 1297.

Baglioni, C. (1961), *Biochim. Biophys. Acta 48*, 392.

Bartley, W. (1953), *Biochem. J. 53*, 305.

Block, R., Durrum, E. L., and Zwieg, G. (1955), A Manual of Paper Chromatography and Paper Electrophoresis, New York, N. Y., Academic, p 95.

Braunitzer, G., Gehring-Müller, R., Hilschmann, N., Hilse, K., Hobom, G., Rudloff, V., and Wittmann-Liebold, B. (1961), *Z. Physiol. Chem. 325*, 283.

Chernoff, A. I. (1964), *Abstr. 10th Congr. Intern. Soc. Hematol. Stockholm*, L:3.

Clegg, M. D., and Schroeder, W. A. (1959), *J. Am. Chem. Soc. 81*, 6065.

Cordes, E. H., and Jencks, W. P. (1963), *J. Am. Chem. Soc. 85*, 2843.

Coryell, C. D., and Pauling, L. (1940), *J. Biol. Chem. 132*, 769.

Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.

Dreywood, R. (1946), *Ind. Eng. Chem., Anal. Ed. 18*, 499.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., and Smith, F. (1956), *Anal. Chem. 28*, 350.

Faurholt, C. (1925), *J. Chim. Phys. 22*, 1.

Ferguson, J. K. W., and Roughton, F. J. W. (1934), *J. Physiol. 83*, 68.

Fessas, P., and Loukopoulos, D. (1964), *Science 143*, 590.

Fischer, E. H., Kent, A. B., Snyder, E. R., and Krebs, E. G. (1958), *J. Am. Chem. Soc. 80*, 2906.

Fraenkel-Conrat, H., Harris, J. I., and Levy, A. L. (1955), *Methods Biochem. Analy. 2*, 393.

Giustina, G., Milla, E., and Margaria, R. (1952a), *Giorn. Biochem. 1*, 357.

Giustina, G., Milla, E., and Margaria, R. (1952b), *Giorn. Biochim. 1*, 475.

Goldstein, J., Guidotti, G., Konigsberg, W., and Hill, R. J. (1961), *J. Biol. Chem. 236*, PC77.

Goldstein, J., Konigsberg, W., and Hill, R. J. (1963), *J. Biol. Chem., 238*, 2016.

Greenstein, J. P., and Winitz, M. (1961), Chemistry of the Amino Acids, New York, N. Y., Wiley, p 498.

Guidotti, G., Konigsberg, W., and Craig, L. C. (1963), *Proc. Natl. Acad. Sci. U. S. 50*, 774.

Hais, I. M., and Macek, K., Ed. (1963), Paper Chromatography, New York, N. Y., Academic, pp 368, 799.

Heyl, D., Harris, S. A., and Folkers, K. (1948), *J. Am. Chem. Soc. 70*, 3429.

Hill, R. J., Konigsberg, W., Guidotti, G., and Craig, L. C. (1962), *J. Biol. Chem. 237*, 1549.

Hill, R. L., Swenson, R. T., and Schwartz, H. C. (1960), *J. Biol. Chem. 235*, 3182.

Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.

Hirs, C. H. W. (1964), *Ann. Rev. Biochem. 33*, 627.

Holmquist, W. R., (1966), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.

Holmquist, W. R., and Schroeder, W. A. (1964), *Biochim. Biophys. Acta 82*, 639.

Holmquist, W. R., and Schroeder, W. A. (1966a), *Biochemistry 5*, 2504 (this issue; following paper).

Holmquist, W. R., and Schroeder, W. A. (1966b), *J. Chromatog.* (in press).

Hu, C. L., Rendig, V. V., and McComb, E. A. (1965), *J. Chromatog. 19*, 622.

Huehns, E. R., and Shooter, E. M. (1965), *J. Med. Genet. 2*, 48.

Hughes, R. C., Jenkins, W. T., and Fischer, E. H. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1615.

Huisman, T. H. J., and Dozy, A. M. (1962), *J. Lab. Clin. Med. 60*, 302.

Huisman, T. H. J., and Horton, B. F. (1965), *J. Chromatog. 18*, 116.

Huisman, T. H. J., and Meyering, C. A. (1960), *Clin. Chim. Acta 5*, 103.

Ingram, V. M. (1958), *Biochim. Biophys. Acta 28*, 539.
Jones, R. T. (1961), Ph.D. Thesis, California Institute of Technology, Pasadena, Calif.
Josselin, J., and Breton, J. C. (1960), *Bull. Mem. Ecole Natl. Med. Pharm. Dakar 8*, 149.
Karler, A. (1952), U. S. Atomic Energy Pamphlet UCRL-1838, May 29.
Keil, B. (1965), *Ann. Rev. Biochem. 34*, 185.
Konigsberg, W., Goldstein, J., and Hill, R. J. (1963), *J. Biol. Chem. 238*, 2028.
Kunkel, H. G. (1958), *in* Conference on Hemoglobin, National Academy of Science–National Research Council, Publication No. 557, p 157.
Kunkel, H., and Wallenius, G. (1955), *Science 122*, 288.
Ludowieg, J., and Dorfman, A. (1960), *Biochim. Biophys. Acta 38*, 212.
Margari, R. (1952), *Schweiz. Med. Wochschr. 82*, 990.
McCaldin, D. J. (1960), *Chem. Rev. 60*, 39.
Meister, A. (1965), Biochemistry of the Amino Acids, 2nd ed, New York, N. Y., Academic, pp 375–437.
Metzler, D. E. (1957), *J. Am. Chem. Soc. 79*, 485.
Meyering, C. A., Israels, A. L. M., Sebens, T., and Huisman, T. H. J. (1960), *Clin. Chim. Acta 5*, 208.
Meyers, A. I., and Libano, W. Y. (1961), *J. Org. Chem. 26*, 4399.
Milla, E., Giustina, G., and Margaria, R. (1953a), *Giorn. Biochim. 2*, 80.
Milla, E., Giustina, G., and Margaria, R. (1953b), *Giorn. Biochim. 2*, 153.
Muller, C. J. (1961), Molecular Evolution, Assen, The Netherlands, Van Gorcum and Co.
Nakamura, G. R. (1952), *Anal. Chem. 24*, 1372.
Neher, R., Ed. (1964), Steroid Chromatography, 2nd ed, New York, N. Y., Elsevier, pp 129, 135.
Neuzil, E., Breton, J. C., and Plagnol, H. (1959), *Bull. Mem. Ecole Natl. Med. Pharm. Dakar 7*, 157, 195.
Rhinesmith, H. S., Schroeder, W. A., and Martin, N. (1958), *J. Am. Chem. Soc. 80*, 3358.
Rhinesmith, H. S., Schroeder, W. A., and Pauling, L. (1957a), *J. Am. Chem. Soc. 79*, 609.
Rhinesmith, H. S., Schroeder, W. A., and Pauling, L. (1957b), *J. Am. Chem. Soc. 79*, 4682.
Schilling, E. D., Burchill, P. I., and Clayton, R. A. (1963), *Anal. Biochem. 5*, 1.
Schnek, A. G., and Schroeder, W. A. (1961), *J. Am. Chem. Soc. 83*, 1472.
Schroeder, W. A. (1966a), *Methods Enzymol.* (in press).
Schroeder, W. A. (1966b), *Methods Enzymol.* (in press).
Schroeder, W. A. (1966c), *Methods Enzymol.* (in press).
Schroeder, W. A., Cua, J. T., Matsuda, G., and Fenninger, W. (1962a), *Biochim. Biophys. Acta 63*, 532.
Schroeder, W. A., and Holmquist, W. R. (1966), *J. Chromatog.* (in press).
Schroeder, W. A., and Jones, R. T. (1965), *Fortschr. Chem. Org. Naturstoffe 23*, 113.
Schroeder, W. A., Jones, R. T., Cormick, J., and McCalla, K. (1962b), *Anal. Chem. 34*, 1570.
Schroeder, W. A., and Robberson, B. (1965), *Anal. Chem. 37*, 1583.
Schroeder, W. A., Shelton, J. R., Shelton, J. B., Cormick, J., and Jones, R. T. (1963), *Biochemistry 2*, 992.
Scott, T. A., and Melvin, E. H. (1953), *Anal. Chem. 25*, 1656.
Stadie, W. C., and O'Brien, H. (1937), *J. Biol. Chem. 117*, 439.
Stark, G. R., and Smyth, D. G. (1963), *J. Biol. Chem. 238*, 214.
Waldron-Edward, D. (1966), *J. Chromatog. 20*, 556.
Westheimer, F. H. (1963), *Proc. Chem. Soc.*, 253.
Whistler, R. L., and Wolfrom, M. L. (1962), *Methods Carbohydrate Chem. 1*, 390, 479.
Zacharius, R. M., and Talley, E. A. (1962), *Anal. Chem. 34*, 1551.