lyzed with 0.25*N* sodium hydroxide (0.2 ml) for 18 hours at 37°C. The mixture was then neutralized with carbon dioxide and applied to a DEAE-Sephadex column (HCO_3^- form, *p*H 9.0; 0.4 by 100 cm). Elution with water gave uridine (16 nmole) as the only nucleoside, and subsequent elution with 0.2*M* ammonium bicarbonate (*p*H 9.0) gave cytidine 2′(3′)-phosphate (49 nmole) as the only nucleotide. The ratio of Cp to U was 3.02 to 1.0, indicating that the structure of the oligonucleotide was C-C-C-U. These results and the results of the ten degradation cycles taken together with the known specificity of ribonuclease T_1 and the results of the terminal base determination of the intact Qβ RNA (*2*) demonstrate that the 3′-terminal hexadecanucleotide sequence of the RNA is as shown above.

Recently, Dahlberg has also studied the 3′ terminus of Qβ RNA and has proposed (*7*) a nucleotide sequence which, like the present work, was based on the compositional formula -G(C_9U_4)C-A reported earlier (*2*). However, this sequence differs from that reported here in four positions (residues 7, 8, 9, and 10 from the 3′ terminus). A revised sequence has also been communicated by the same author (*8*), and this sequence differs in two positions (residues 5 and 6) from that derived here. At the moment the reasons for these conflicting results are not clear because the methods of sequence analysis used by Dahlberg differ quite markedly from ours and the experimental details of that analysis are not yet available for comparison with those reported here.

While the periodate-beta-elimination method yields results which appear to be unambiguous, it can be seen that the experimental operations suffer from the fact that they are rather time-consuming. However, because the method employs a cyclic process of degradation it is open to improvement by automation. Initial experiments have shown that it is possible to carry out all of the steps required in each degradative cycle while the polynucleotide is bound to a solid support. We are presently using this principle to develop a sequencing machine which will carry out the degradative cycles automatically, and this approach should reduce the time of analysis to about 2 hours per base.

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1. Abbreviations: RNA, ribonucleic acid; DEAE, diethylaminoethyl; O.D.U., optical density unit; Cp, cytidine 2′(3′)-phosphate; U, uridine; C, cytidine; A, adenosine; and G, guanosine. Sequences are presented in the standard way by the linear array of nucleosides, with the 3′-hydroxyl terminus to the right. Nucleosides in parentheses are of still-undetermined sequence.
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Single Chain Alkali Resistance in Hemoglobin Rainier: β 145 Tyrosine → Histidine

Abstract. *The mechanism of alkali resistance in hemoglobin Rainier, an adult human hemoglobin variant (β 145 tyrosine → histidine), has been investigated. Alkali denaturation kinetics, electrophoretic and hybridization study, and ultracentrifuge analysis provided evidence for a monomeric β Rainier chain resisting denaturation at alkaline pH. These data provide evidence for a previously unrecognized effect of a single amino acid substitution, that is, the change of the alkali denaturation properties of monomeric chains.*

Increased resistance to denaturation by alkali is a characteristic of human fetal hemoglobin ($\alpha_2\gamma_2$) and several adult animal hemoglobins ($\alpha_2\beta_2$). The structural basis of this property is not known. Dissociation studies and the first-order alkali denaturation kinetics in human Hb $\alpha_2\gamma_2$ and in bovine Hb $\alpha_2\beta_2$ support the postulate that the increased alkali resistance is confined to symmetrical dimers ($\alpha\beta$) rather than asymmetrical subunits (α_2 and β_2) (*1*). Among many variant hemoglobins caused by single amino acid substitutions, only Hb Rainier (β 145 Tyrosine → Histidine) exhibits resistance to alkali denaturation (*2*). Thus, the structural alteration causing alkali resistance is known. The mechanism, however, by which an amino acid substitution near the carboxyl terminal of the β chain alters the alkali denaturation properties of the hemoglobin is not apparent. Therefore, we tested whether in Hb Rainier alkali resistance is a property of the $\alpha\beta$ Rainier dimer, the β_2 Rainier dimer, or the monomeric β Rainier chain.

Alkali denaturation of Hb Rainier differs strikingly from that of Hb $\alpha_2\gamma_2$ (Fig. 1). In contrast to the first-order kinetics of fetal hemoglobin, approximately 50 percent of Hb Rainier is denatured within the first minutes of the reaction, while denaturation of the remaining hemoglobin fraction proceeds significantly slower than the denaturation of Hb F (Fig. 1). These findings are inconsistent with alkali resistance of symmetrical $\alpha\beta$ Rainier dimers, because first-order kinetics would be expected in that case. The observed biphasic alkali denaturation is more consistent with alkali resistance of only one type of hemoglobin chain. To test this possibility, hemoglobin Rainier was treated with 0.06*N* NaOH from 2 to 30 minutes. The hemoglobin solution was then neutralized, concentrated by vacuum dialysis, and studied by starch-gel electrophoresis in a buffer system of tris(hydroxymethyl)aminomethane(tris), ethylenediaminetetraacetate (EDTA), and borate at *p*H 8.6 (Fig. 2). After 2 minutes in 0.06*N* NaOH, only traces of hemoglobin with the electrophoretic migration of Hb Rainier were observed. However, a new fast-moving hemoglobin fraction with a mobility expected for the β Rainier chain appeared on electrophoresis (Fig. 2). The electrophoretic migration of this fraction was identical to that of the β Rainier chain prepared by treatment of Hb Rainier with *p*-chloromercuribenzoate (PCMB) (*3*), an indication that it consists of β Rainier chains. The β Rainier chains recovered after treatment of Hb Rainier with 0.06*N* NaOH were mixed with equal concentrations of αA chains prepared by treatment with PCMB; the mixture was incubated for 2 hours at 4°C and examined by starch-gel electrophoresis in the tris-EDTA-borate buffer system at *p*H 8.6. The β Rainier chain recombined with the αA chain to form hemoglobin Rainier (Fig. 2). These findings indicate that the hemoglobin recovered after alkali treatment is free β Rainier chain; therefore, the biphasic alkali denaturation kinetics of Hb Rainier are due to the original rapid denaturation of the αA chain and the subsequent resistance to denaturation of the β Rainier chain.

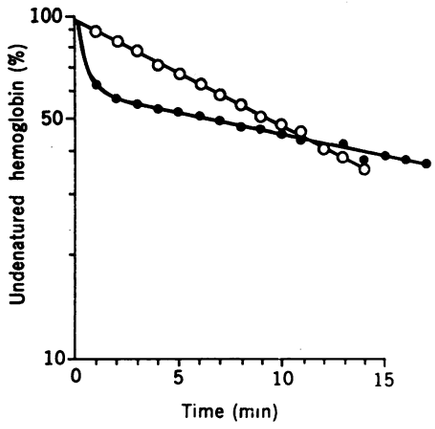

Fig. 1. The rate of alkali denaturation of hemoglobin Rainier purified chromatographically (●) and Hb F (○) in 0.06*N* NaOH, *p*H 12.2. The decrease in absorbance at 415 nm at 20°C was measured in a Beckman Model DU spectrophotometer. The reaction was assumed to be complete when the samples were warmed at 37°C for 30 minutes.

Dissociation of hemoglobin tetramers in high *p*H involves initial dissociation into symmetrical dimers, followed by further dissociation into monomers (*4*, *5*): $\alpha_2\beta_2 \rightleftarrows 2\alpha\beta \rightleftarrows 2\alpha + 2\beta$. It has been suggested that the rate of denaturation of hemoglobin at alkaline *p*H is related to the rate of monomer formation (*1*, *5*). Thus, in human fetal and in animal hemoglobins, alkali resistance

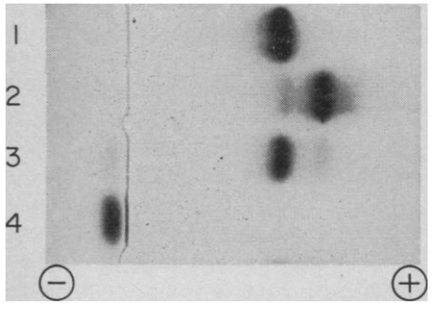

Fig. 2. Electrophoresis of hemoglobin on starch gel in tris-EDTA-borate buffer at *p*H 8.6. (1) Chromatographically purified Hb $\alpha_2\beta_2$ Rainier. (2) $\alpha_2\beta_2$ Rainier after treatment with 0.06*N* NaOH for 5 minutes at 24°C. Note that only traces of $\alpha_2\beta_2$ Rainier remain. The alkali resistant hemoglobin consists mainly of fast-migrating hemoglobin β Rainier. (3) Mixture of β Rainier [as prepared in (2)] and αA chains. Only traces of αA and β Rainier are found. αA and β Rainier recombine to form hemoglobin $\alpha_2\beta_2$ Rainier. (4) αA Chains prepared after treatment of Hb $\alpha_2\beta_2$ with excess of PCMB (*3*). The chains were separated by starch-block electrophoresis in tris-EDTA-borate buffer at *p*H 8.6.

implies a slower rate of dissociation of symmetrical dimers into monomers at high *p*H. For the explanation of alkali resistance in Hb Rainier, two different possibilities were encountered. The first was that Hb Rainier dissociates into monomers ($\alpha_2\beta_2R \rightarrow 2\alpha\beta R \rightarrow 2\alpha + 2\beta R$) exactly as the normal hemoglobin does at high *p*H, but the soformed β Rainier monomers are resistant to denaturation. The second possibility was that asymmetrical β_2 Rainier dimers are formed and these dimers are not denatured even at high *p*H. The latter possibility was excluded because the sedimentation coefficient ($s_{20,w}$) measurements in alkaline solvents (*p*H 12.05 and 12.7) were 2.07*S* and 2.0*S*, respectively, an indication that, at that *p*H, hemoglobin Rainier, like other alkali-labile and alkali-resistant hemoglobins (*1*, *6*), exists in a monomeric form.

This β Rainier preparation recovered after the treatment with alkali had a sedimentation constant of 2.89*S* at neutral *p*H (Table 1). Determination of the molecular weight shows that the low sedimentation constant is not due to a high frictional coefficient of a tetrameric form of β Rainier but that it is due to a formation of β Rainier dimers [weight average molecular weight = 31,000, determined by sedimentation equilibrium (Table 1)]. In view of the fact that the naturally occurring βA chains in Hb H exist in a tetrameric form (*7*) and also that the βA chains prepared by treatment with PCMB (*3*) or *p*-hydroxymercuribenzoate (*8*) form tetramers at neutral *p*H, the finding of β Rainier dimers at neutral *p*H indicates that the β Rainier has altered association properties. Because the β Rainier treated with alkali recombines with αA to form $\alpha_2\beta_2$ Rainier and because the naturally occurring Hb Rainier is found on tetrameric configuration, the abnormality in association properties appears to involve at least the lack of association of β_2 Rainier dimers into β_4 Rainier tetramers.

Thus in hemoglobin Rainier a single amino acid substitution is responsible for the alkali resistance of the monomeric chain. A possible mechanism relating alkali resistance to the alteration in structure in Hb Rainier has been suggested by Perutz and Lehmann (*9*). They postulate that a hydrogen bond between the carbonyl group of leucine at β H 19 and the imidazole group of

Table 1. Sedimentation constants of Hb Rainier and β chain of Hb Rainier, measured in a Spinco Model E centrifuge at 56,100 rev/min at 20°C. The protein was dissolved in the solvents at a concentration of 0.6 to 0.8 percent. The solvents were: A, 0.05*M* Na_2HPO_4–KH_2PO_4 (*p*H 6.8) containing 0.005*M* KCN and 0.2*M* NaCl; B, same as A, plus 0.1*M* NaOH; C, same as A, plus 0.2*M* NaOH; D, same as A, except containing 0.217*M* NaCl. Single and symmetrical sedimentation boundaries were observed in all cases. The weight average molecular weight was determined by the sedimentation equilibrium. Protein concentrations were 0.65 and 0.32 mg/ml. Partial specific volume was taken as 0.749 ml/g.

Solvent	Final *p*H	$s_{20,w}$ (Svedberg units)*	Molecular weight
		Hb Rainier	
A	6.8	4.0 (3.97–4.03)	
B	12.05	2.07	
C	12.7	2.0 (1.9–2.1)	
		Rainier β chain	
D	6.8	2.89 (2.80–2.98)	31,000

* Mean value and range of duplicate analysis.

substituted histidine at H 23 of β Rainier might be more stable at alkaline *p*H than that between the tyrosine residue at H 23 and the carbonyl group of valine at FG 5 of the normal β chain. This theoretical consideration fits with the observed alkali resistance of the monomer, but does not elucidate the reason for the abnormal association properties of β Rainier. The latter may indicate a more drastic change in the three-dimensional structure which may also be responsible for the abnormal alkali denaturation kinetics of the β Rainier chain.

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