

## SYMPOSIUM

### Protein Methodology

Recently developed techniques for the investigation of the structure of proteins have made it possible to obtain detailed information on the chemical structure (the amino acid sequence) in a number of cases [see, for example, (3)], and, in at least one case (myoglobin), the detailed three-dimensional structure of a protein has been established by the application of special techniques in X-ray crystallography (10). One of the primary tasks facing protein chemists today is to relate the chemical structure of proteins to their physical and biological behavior. A very fruitful approach to this problem consists in the comparison of the behavior of two proteins almost identical chemically. By cancelling out their identical features, it should be possible to identify their chemical differences with the resulting differences in physical or biological behavior. Ideally suited for such investigations are families of proteins differing in chemical composition by a very few amino acids, undoubtedly as a result of a mutation in the gene which controls their synthesis. Two such systems have undergone extensive investigation. These are: human hemoglobin and bovine  $\beta$ -lactoglobulin.

Being the most abundant protein of milk whey,  $\beta$ -lactoglobulin has attracted the attention of scientists over a number of years. Its physical properties at the isoelectric point are summarized in Table 1. In 1955, Aschaff-

$\beta A$  has two more free carboxyl groups per 35,500 molecular weight unit than  $\beta B$ , thus accounting for the electrophoretic mobility difference (19) between the two.

*Physical differences between  $\beta A$  and  $\beta B$ .* Systematic studies (17-21, 24-29) carried out by us have resulted in a detailed characterization of the molecular behavior of the two  $\beta$ -lactoglobulins at pH's below their isoelectric points. The principal techniques used were ultracentrifugation, Tiselius electrophoresis, light scattering, small-angle X-ray scattering, and radioactive tagging. Results of these investigations of the solution behavior of  $\beta$ -lactoglobulin below pH 6 are summarized in Figure 2. Here, the weight average molecular weights of the two  $\beta$ -lactoglobulins are plotted as a function of pH. Three zones of pH can be distinguished: I. Between pH 6.0 and 5.2. II. Between pH 5.2 and 3.5. III. Below pH 3.5.

I. In the zone between pH 6.0 and 5.2, no molecular association or dissociation phenomena are observed.

II. Between pH 5.2 and 3.5, a highly specific aggregation of  $\beta A$  (18, 20) to a tetramer (27, 29) is observed at low temperatures. This is a reaction in rapidly reversible equilibrium, with the maximum degree of association occurring at pH 4.65 (25). A complete thermody-

TABLE 1  
Molecular parameters of  $\beta$ -lactoglobulin

Isoelectric point (iep)	pH 5.1-5.3
Molecular weight (at iep)	35,500
Hydration	35-40%
$s_{20,w}^{\circ}$ (at iep)	3.05 Svedberg units
Axial ratio (at iep)	2:1 (prolate ellipsoid)

burg and Drewry (1) discovered that  $\beta$ -lactoglobulin is actually a mixture of two genetically linked proteins which can be distinguished by paper electrophoresis at pH 8.5. Typical patterns are shown in Figure 1. It was found that some cows produce only  $\beta$ -lactoglobulin A ( $\beta A$ ) (rapid-moving band), some only  $\beta$ -lactoglobulin B ( $\beta B$ ) (slow-moving band), and some a mixture of the two in approximately equal quantities (two bands). In no case was an intermediate mixed species protein ( $\beta AB$ ) found. In 1959, Tanford (16) reported that

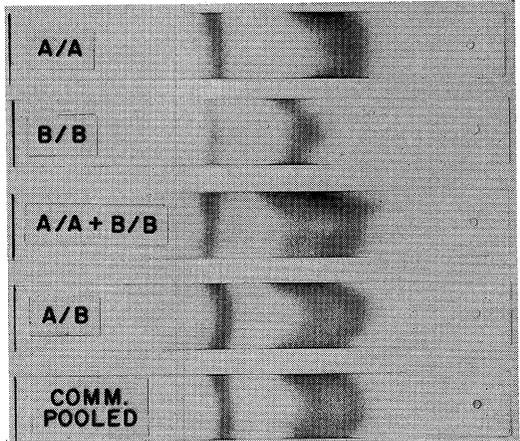


FIG. 1. Paper electrophoresis patterns, obtained in pH 8.5 veronal buffer ( $\Gamma/2 = 0.05$ ). From top to bottom: homozygous A cow; homozygous B cow; synthetic mixture of the two; heterozygous cow; commercial milk.

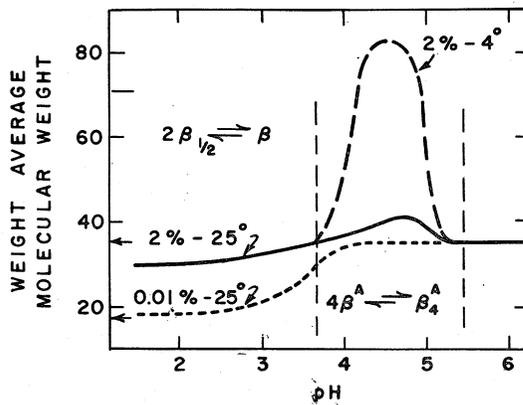
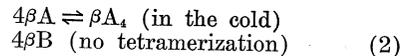


FIG. 2. Aggregation state of the  $\beta$ -lactoglobulins as a function of pH.

dynamic characterization of this aggregation has been carried out. The possible mechanisms involved in the tetramer formation will be described elsewhere. In Figure 2, the values of the molecular weights observed for a protein concentration of 20 g/liter and a temperature of 4 C are shown by the dashed line. This being a system in equilibrium, only average molecular weights can be obtained at any given set of conditions. The values plotted in Figure 2 are weight average molecular weights, obtained in light-scattering experiments. The weight average molecular weight,  $\overline{M}_w$ , is defined as:

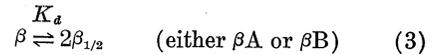
$$\overline{M}_w = \frac{\sum n_i M_i^2}{\sum n_i M_i} \quad (1)$$

where  $n_i$  is the molar concentration of particles in a given state of aggregation (monomer and tetramer in the case of  $\beta$ A aggregation),  $M_i$  is their molecular weight. At 25 C (shown by the solid line on Figure 2) no comparable aggregation is observed. In the case of  $\beta$ B no such tetramerization takes place at any temperature. Thus, between pH 5.2 and 3.5, the molecular behavior of the  $\beta$ -lactoglobulins can be described as:



III. In the zone below pH 3.5 both  $\beta$ A and  $\beta$ B have been found to dissociate into subunits of 17,750 molecular weight, i.e., both proteins split in half (26). This dissociation is strongest at pH's below two and the driving force has been found to be that of nonspecific electrostatic repulsion (28). The values of  $\overline{M}_w$  plotted in Figure 2 for two concentrations of protein can be used as a guide to the degree of dissociation as a function of pH. Thermodynamic analysis of the dissociation data on the two  $\beta$ -lactoglobulins (21) have shown that the two behave essentially identically. At 25 C and

pH 2.7 ( $\Gamma/2 = 0.1$ ), the dissociation constant,  $K_d$ , of the reaction



was found to be  $1.3 \times 10^{-4}$  mole/liter for  $\beta$ A,  $5.08 \times 10^{-5}$  for  $\beta$ B, and  $7.11 \times 10^{-5}$  for the normal mixture of  $\beta$ A and  $\beta$ B prepared from pooled milk (referred to as  $\beta$ N). These three values are very close to each other and their difference does not exceed experimental error.

The apparent equilibrium constant observed for the protein mixture could represent one of two cases:

- (1) Both  $\beta$ A and  $\beta$ B dissociate identically and in reassociation can form mixed molecules:  $K_d^{(N)}$  would then be a real equilibrium constant;
- (2) Both  $\beta$ A and  $\beta$ B dissociate, but can reassociate only specifically, each with an identical subunit ( $A_{1/2}$  only with  $A_{1/2}$ ,  $B_{1/2}$  only with  $B_{1/2}$ ). In this case  $K_d^{(N)}$  is only an apparent equilibrium constant which reflects the simultaneous occurrence of two independent dissociation reactions.

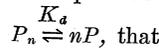
An attempt was made to determine which situation prevailed by calculating  $K_d^{(N)}$ , apparent, from the experimental values of  $K_d^{(A)}$  and  $K_d^{(B)}$  and to compare this with the value of  $K_d^{(N)}$  obtained from dissociation experiments on the  $\beta$ -lactoglobulin mixture. Such a calculation can be done in the following way:

If  $\alpha$  is the fraction of protein (in grams/liter) that dissociates,  $(1 - \alpha)$  remains undissociated, then, for the reaction of Equation 3,

$$K_d = \frac{[\beta_{1/2}]^2}{[\beta]} = \frac{2\alpha^2 C_2}{(1-\alpha)M_{1/2}} \quad (4)$$

where  $[\beta]$  is the molar concentration of the undissociated protein,  $[\beta_{1/2}]$  is that of the dissociated protein,  $C_2$  is the total  $\beta$ -lactoglobulin concentration in grams/liter, and  $M_{1/2}$  is the molecular weight of the subunit (taken as 17,500 in the present calculations).

From the definition of the weight average molecular weight,  $\overline{M}_w$  (Equation 1), it follows for a protein system in dissociative equilibrium,



$$\overline{M}_w = M_m [n + \alpha(1-n)] \quad (5)$$

and

$$K_d = \frac{n(nM_m - \overline{M}_w)^n C_2^{n-1}}{(n-1)^{n-1} (\overline{M}_w - M_m) M_m^{2(n-1)}} \quad (6)$$

where  $M_m$  is the molecular weight of the monomer, i.e., the dissociated species ( $P$ ). (In the present case  $n = 2$ ).

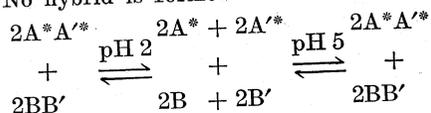
The dependence of the weight average molecular weight on concentration for  $\beta$ A and  $\beta$ B at pH 2.7 is shown in Figure 3a. Values of  $\alpha$ , calculated from this, using Equation 5, are shown in Figure 3b. Using the values of  $\alpha$

and the knowledge that the particular lot of mixed  $\beta$ -lactoglobulin ( $\beta N$ ) used consisted of 59%  $\beta A$  and 41%  $\beta B$  (19), an apparent equilibrium constant ( $K_d^{(N)},_{app}$ ) could be calculated for this composition. This calculation is illustrated in Table 2. First, the amount of  $\beta A$  and  $\beta B$  present in the form of monomer at each given total protein concentration is calculated. From the sum of the two, the apparent total degree of dissociation ( $\alpha_N,_{app}$ ) is obtained. From these values of  $\alpha_N,_{app}$ ,  $\bar{M}_w$  is calculated as a function of concentration, using Equation 5, and  $K_d^{(N)},_{app}$  is calculated, using Equation 6. Results of these calculations are shown in Figure 3a and Table 2, respectively.  $K_d^{(N)},_{app}$  turns out to have a value of  $1.8 \times 10^{-4}$  mole/liter, resulting in an apparent standard free energy of dissociation ( $\Delta F^\circ$ ) of 5.1 Kcal/mole ( $\Delta F^\circ = -2.303 RT \log K$ ).

The values of  $\Delta F^\circ$  for  $\beta A$ ,  $\beta B$ , and  $\beta N$ , found experimentally, are 5.3, 5.9, and 5.7 Kcal/mole, respectively. The difference between the calculated and experimental values of 5.1 and 5.7 Kcal/mole would correspond to an experimental error of only 4% in light scattering. This is quite reasonable and, thus, the two values are not significantly different and it is not possible to conclude from equilibrium data alone whether the observed dissociation occurs independently and specifically in  $\beta A$  and  $\beta B$ , or whether the two species are able to reassociate randomly in mixed form.

The answer to this particular problem was suggested by the fact that any cow produces only  $\beta$ -lactoglobulin A or B, or a separable mixture of the two. In no case has a mixed (intermediate) AB form of the protein been observed, i.e., no intermediate band is ever found in the paper electrophoresis experiments (see Figure 1). This fact, however, could be only a reflection of the mechanism of in vivo synthesis of this protein. Therefore, experiments were designed to see if mixed, hybrid molecules could be formed from  $\beta A$  and  $\beta B$ . The essence of such experiments is as follows: one of the proteins is marked in some manner (7, 14), and after a cycle of dissociation and reassociation of the mixture, the two are separated and examined for a transfer of the marking; thus:

I. No hybrid is formed:



II. A hybrid is formed:

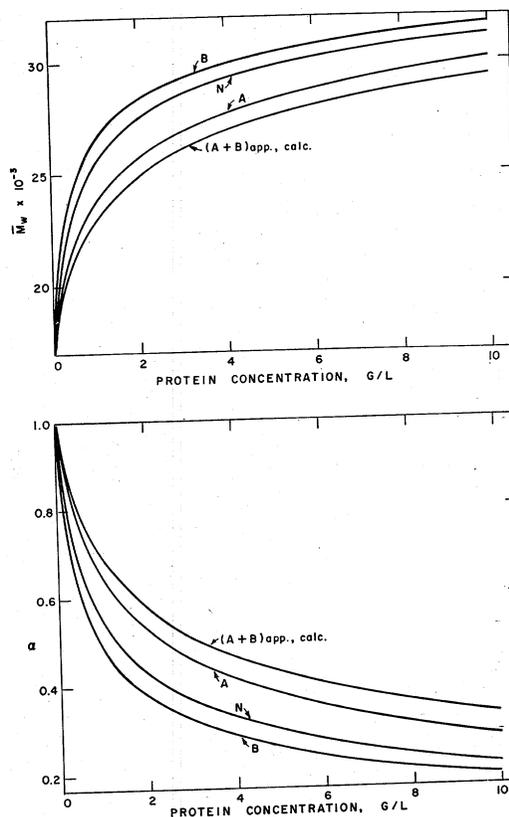
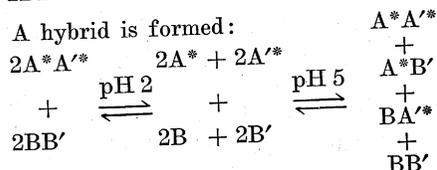


FIG. 3. Degrees of dissociation and weight average molecular weights of  $\beta A$ ,  $\beta B$ , and  $\beta N$  (experimental), and  $\beta(A + B)$  calculated from  $\beta A$  and  $\beta B$ . pH 2.7,  $\Gamma/2 = 0.1$  HCl-NaCl, 25 C.

In the case under consideration (24), a radioactively tagged  $\beta$ -lactoglobulin A was prepared by injecting  $C^{14}$ -valine into a homozygous A/A cow and isolating the protein from the milk produced by this cow during the first 48 hr after injection. The tagged  $\beta A$  was mixed with untagged  $\beta B$ , the mixture was adjusted to pH 2 (dissociation), then neutralized to pH 5.3 (reassociation), the two proteins were separated in a Tiselius electrophoresis cell at pH 5.3, and the amount of radioactivity present in each was measured. Had subunit exchange occurred (Scheme II), 25% of the radioactivity (one  $A'^*$  unit) would have been transferred from  $\beta A$  to  $\beta B$  (to form  $BA'^*$ ). In repeated experiments, it was found that no transfer of radioactivity took place from  $\beta A$  to  $\beta B$ , indicating that no mixed reassociation can occur, i.e., there is no interchange of subunits between  $\beta A$  and  $\beta B$ . Therefore,  $\beta A$ - $\beta B$  hybrids cannot be formed. The equilibrium constant deduced from the dissociation experiments on  $\beta N$  is only an apparent value and reflects the simultaneous occurrence of two similar independent reactions which are indistinguishable from each

TABLE 2  
 Calculation of  $K_d^{(N)},_{app}$ .

$C_d$ grams/ liter	Dissociated, grams/liter					$a_N,_{app}$	$K_d^{(N)},_{app}$ mole/liter
	$a_A$	$a_B$	$\beta A$	$\beta B$	Total		
10	0.36	0.28	2.12	1.15	3.27	0.33	$1.88 \times 10^{-4}$
4	0.50	0.40	1.18	0.66	1.84	0.46	1.79
1	0.73	0.64	0.43	0.26	0.69	0.69	1.77
0.5	0.83	0.74	0.25	0.14	0.39	0.78	1.59
0.3	0.88	0.81	0.16	0.10	0.26	0.86	1.81

Ave =  $1.8 \times 10^{-4}$ 

other by the criterion of molecular weight measurements.

The physical-chemical examination of the state of aggregation of the  $\beta$ -lactoglobulins below pH 5.2 reveals, then, two differences:

1. Between pH 5.2 and 3.5,  $\beta A$  can tetramerize specifically,  $\beta B$  can not;
2. Below pH 3.5 both proteins can dissociate into halves, the driving force being generalized electrostatic repulsion between the subunits; their reassociation is specific, however, pointing to a structural difference in the area of subunit contact.

*Chemical differences between  $\beta A$  and  $\beta B$ .* The physical-chemical differences described above are a result of the genetic difference between  $\beta A$  and  $\beta B$ . This must be reflected also in structural differences. Such differences can be of three types: (1) amino acid composition; (2) amino acid sequence (primary structure); (3) chain configuration (secondary and tertiary structures).

No chemical differences between  $\beta A$  and  $\beta B$  were known until Tanford (16) showed that  $\beta A$  contains two more ionizable carboxyls per 35,500 molecular weight unit than does  $\beta B$ . It was further known that all analytical data gave even-numbered results per 35,500 molecular weight [see, for example, (15, 28)], suggesting that  $\beta$ -lactoglobulin is composed of two identical chains, a fact demonstrated in the hybridization experiments just described. Furthermore, Kalan and Greenberg (9) have shown  $\beta A$  and  $\beta B$  to have identical sequences at the C-terminal ends of the peptide chains.

The chemical nature of the difference in carboxyls found by Tanford has been investigated by Townend and Ingram (23) and Kalan et al. (8). Townend and Ingram carried out tryptic digestions of derivatives of the two  $\beta$ -lactoglobulins, followed by high-voltage electrophoresis. They found one pair of difference peptides between  $\beta A$  and  $\beta B$ . Amino acid compositions of these peptides revealed that the peptide from  $\beta A$  contained one more residue of aspartic acid (Asp) than that from  $\beta B$  and one less residue of glycine (Gly). From the number of peptides obtained, it could be concluded that the two chains in  $\beta A$  and the two

in  $\beta B$  are, in each case, identical. In this way, the difference in free carboxyls reported by Tanford was accounted for:  $\beta A$  contains one more Asp per chain than  $\beta B$  and the isoelectric protein is a dimer of two identical chains, held together probably by hydrophobic forces (28). Independent studies by Kalan et al. (8) on chymotryptic digests have revealed at least one pair of difference peptides essentially the same in amino acid composition except for an excess of one residue of aspartic acid in the  $\beta A$  peptide, which appears to be compensated for by an excess of one residue of glycine in the  $\beta B$  peptide.

The total amino acid composition of  $\beta A$  and  $\beta B$  has been determined by Gordon, Basch, and Kalan (4, 5), and by Piez et al. (11, 12). These authors find the amino acid compositions of the two to be identical, with the exception of the four amino acids listed in Table 3. Re-

 TABLE 3  
 Differences in amino acid composition between  $\beta A$  and  $\beta B$  (residues per 17,750 molecular weight unit)

	Asp	Gly	Val	Ala
$\beta A$	16	3	10	14
$\beta B$	15	4	9	15

sults of these studies indicate a second difference to exist in the amino acid composition between  $\beta A$  and  $\beta B$ , namely:  $\beta A$  has one more valine (Val) and one less alanine (Ala) per chain than  $\beta B$ , in addition to the Asp-Gly difference. Although this second chemical difference has not been unequivocally found yet in experiments on peptides, it would seem reasonable to assume that this represents a second replacement in the chains between  $\beta$ -lactoglobulins A and B.

*Structural implications.* The various studies, summarized above, show that there are two known differences in the physical behavior of the  $\beta$ -lactoglobulins and two differences in their chemical structure. The question arises: Can these be mutually related in any reasonable manner?

Although no definite assertions can be made, it is possible to attempt such a correlation. As shown in Figure 2, the tetramerization of  $\beta$ A occurs in the pH region of carboxyl ionization, the reaction being maximal at pH 4.65. This is close to the pK of carboxyl ionization and suggests the participation of the aspartic acid residues in the tetramerization. The stoichiometry (27) and geometry (17) of the aggregation require two identical sites per 35,500 molecular weight entity. The presence of one extra aspartic acid residue in each chain of  $\beta$ A would satisfy this requirement.

The second structural difference is reflected in the lack of hybrid formation after dissociation and reassociation in a mixture of  $\beta$ A and  $\beta$ B. Since there is no difference between the two in the pH dependence of this phenomenon, one might wish to assign this difference as a consequence of the Val-Ala replacement.

The resulting model of the protein would be one in which the neutral amino acid residue replacement site would be present in the area of subunit contact, while the one involving charge and leading to tetramerization would be removed from this area, as shown in Figure 4a. The fact that the difference amino acids,

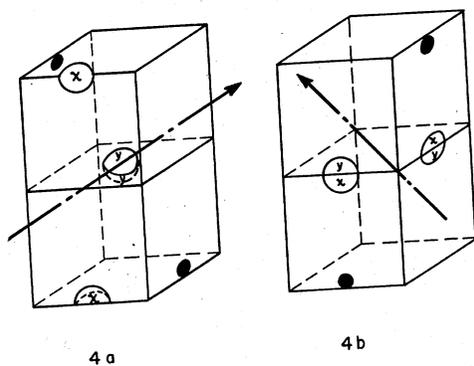


FIG. 4. Models of  $\beta$ -lactoglobulins A and B at the isoelectric point. X = Asp in  $\beta$ A; Gly in  $\beta$ B; Y = Val in  $\beta$ A; Ala in  $\beta$ B. Arrow indicates dyad axis of symmetry.

represented by Y, are located in such manner that they face each other in the three-dimensional schematic model, reflects the requirement that the surface of contact facing the difference amino acid also be different between  $\beta$ A and  $\beta$ B as, otherwise, hybrid formation would be possible. The simplest way to accomplish this is to let the chemical differences from each chain face each other. Another possibility is that the difference in the surface of contact is due to a conformational difference induced over a long range by the change in primary structure (22). This could impede hybrid formation without the difference amino acid being actually located in the area of subunit contact. This possibility is certainly not excluded. It

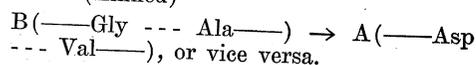
is sufficient to realize that the bulky side chain of valine could easily interfere with the formation of certain secondary structures (e.g., the  $\alpha$ -helix), whereas the smaller alanine residue might display no similar steric effect.

Another attractive model for the structure of the  $\beta$ -lactoglobulin two-chain dimer is depicted in Figure 4b. In this case, both chemical differences are found in the area of contact, the Asp residue facing a Val in  $\beta$ A and the Gly facing the Ala in  $\beta$ B. In this model the two chemical differences become genetically linked. Formation of the tetramerization sites in  $\beta$ A is contingent on the integrity of the two-chain (35,500 molecular weight) structure. This is consistent with the observation that tetramerization occurs only at pH's above the pH of the two-chain separation. In this model the two subunits are arranged in space in such a way that they face each other and are turned by  $90^\circ$  with respect to each other. This is shown schematically in the figure, where the two black dots represent identical spots in the three-dimensional structure of the two chains. The result of this arrangement (b) permits the tetramerization sites to be located at  $90^\circ$  angles to each other in such position that the tetramer formed must be a compact structure, as is required by the geometric dimensions of the tetramer (17). Furthermore, the dyad axis of symmetry found in the X-ray crystallographic studies of Green and Aschaffenburg (6) is maintained. The model of Figure 4a also respects the dyad axis of symmetry.

In both models the sites of amino acid replacement are far removed from each other along the polypeptide chain. This is consistent with the evidence deduced from the composition of the tryptic (23) and chymotryptic (8) difference peptides. This indicates that the Val:Ala and Asp:Gly positions must be removed from each other along the chain by at least several amino acid residues. In the model of Figure 4b the replacement positions are adjacent to each other in spatial configuration, but the two positions are chemically parts of different polypeptide chains.

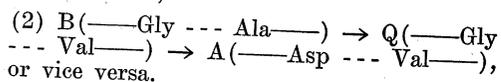
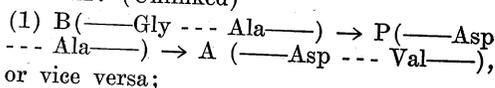
*Genetic implications.* From studies of family groups (2, 13) it is known that the synthesis of  $\beta$ A and  $\beta$ B is under the control of a single pair of genes. Since the operational distinction between  $\beta$ A and  $\beta$ B is electrophoretic, i.e., it is based on a charge difference, strictly speaking, the genetic studies reflect only the Asp-Gly replacement. Now, two possibilities exist for the second (Val-Ala) replacement: (1) it is linked genetically to the first one; (2) it is the result of an independent mutation. If it is linked, then the immediate consequence is that the single mutation has produced two changes in genetic information, as shown in the scheme:

Case I: (Linked)



If the two replacements are not linked, then two mutations must have occurred by either of two paths:

Case II: (Unlinked)



Both structures of Figure 4 could be the result of either case.

A single mutation resulting in two changes (Case I) poses certain difficulties, since the amino acid positions in question are removed from each other along the chain. For a single mutagenic event to produce these two mutations and no others would require the information on the two genetic sites to be adjacent to each other in space, while remote along the linear sequence of genetic information. Such a requirement could be met by the presence of a loop in the genetic information, as pictured schematically in Figure 5b. In Figure 5a, the genetic information on the amino acid sequences in  $\beta A$  and  $\beta B$  is shown as it might exist in linear arrangement: the two replacement sites ( $X$  and  $Y$ ) are far removed from each other. In Figure 5b, the chain of genetic

information is looped, bringing  $X$  and  $Y$  in juxtaposition in three-dimensional space; an event (shown by  $*$ ) could easily result in a mutation at both sites  $X$  and  $Y$ . Since the association of the two identical chains (in  $\beta A$  and  $\beta B$ ) seems to require a definite configuration in the two surfaces of contact and the geometry of the two-chain structure (Figures 4a and b) requires the amino acid chains containing the Asp and Val residues in each contact site to run opposite to each other, a suitable spatial arrangement of the genetic information would be that depicted in Figure 5c. Here the chain of genetic information loops back on itself and two straight sections run parallel to each other in opposite directions, giving rise to the necessity of complementarity between the amino acid chain sections involved in the surface of contact. An event occurring in the proper position ( $*$ ) would result in mutation both in  $X$  and  $Y$ . Since the bond holding the two subunits together at the isoelectric point probably involves more than just the two amino acids specified by loci  $X$  and  $Y$ , the rest of the complementarity would be modulated by the genetic information contained on either side of  $X$  and  $Y$ . It must be emphasized that in the synthesized protein this complementarity is realized not between sections of the same chain but between sections of two chains. This is shown schematically in Figure 6.

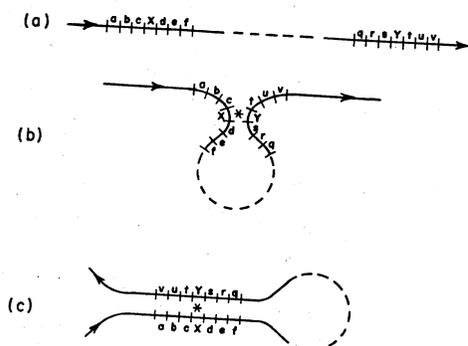


FIG. 5. Relative location of genetic information for the two sites of amino acid replacement. (a) Linear arrangement of information leading to amino acid sequence. (b) Chain of genetic information looped, bringing into spatial juxtaposition two loci remote from each other linearly. (c) Chain of genetic information looped, and running back parallel to another part of the chain. This brings together in spatial juxtaposition two sequences of loci remote from each other linearly. In each case,  $X$  and  $Y$  indicate the genetic loci corresponding to the two replacement sites in amino acid sequence; other letters indicate other neighboring loci;  $*$  represents mutagenic event.

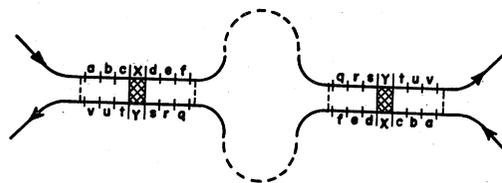


FIG. 6. Schematic representation of two identical complementarities between two polypeptide chains. Vertical dashed lines between chains indicate limits of subunit contact areas; shaded area indicates region in which side chains are different for  $\beta A$  and  $\beta B$ .

If the true situation is that represented by Case II, then intermediate mutants ( $P$  or  $Q$ ) may well be present. These would not be detected electrophoretically, as they would migrate with  $\beta A$  and  $\beta B$ , respectively. It is known that  $\beta A$  contains ca. 10% of material which does not tetramerize in the cold between pH 5.2 and 3.5 (20). It is not known whether this is related in any respect with a possible chemical difference. Studies aimed at the chemical detection of the presence of mutants  $P$  or  $Q$  are presently in progress in our laboratory. In conclusion, it should be emphasized again that neither the correlation between the chemical and physical differences between  $\beta A$  and

$\beta$ B, nor the discussion of possible genetic implications is to be considered as an established fact. These should be regarded as working hypotheses that future work should either confirm or disprove.

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