

(3) Actinomycin D suppresses estrogen-induced synthesis of RNA to levels below those of controls, but protein synthesis in this case is restricted to control values. Since actinomycin D in hormone-free controls limits protein synthesis to lower levels, and since combined estrogen-puromycin-actinomycin D treatments give maximum inhibition of RNA and protein synthesis, evidence exists for a component of uterine hormone response which is not sensitive to actinomycin D, but is sensitive to puromycin.

(4) The findings (1-3) hint that an early action of estrogen on the uterus involves induction or activation of protein synthesis which underwrites DNA-dependent RNA synthesis, and thereby controls the following more extensive phase of protein synthesis. The mechanism for this awaits elucidation.

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The following abbreviations are used: RNA for ribonucleic acid; DNA for deoxyribonucleic acid; RNase for ribonuclease; DNase for deoxyribonuclease; TCA for trichloroacetic acid; PCA for perchloric acid; PVS for polyvinyl sulfate; SDS for sodium dodecyl sulfate; and cpm for counts per minute.

Concentrations of compounds used in this study for isolation of protein, RNA, and ribonucleotides are as follows: RNase, 20  $\mu$ g per ml; Dnase, 20  $\mu$ g per ml; PVS, 2%; SDS, 0.2%.

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### DEVELOPMENTAL HEMOGLOBIN ANOMALIES IN A CHROMOSOMAL TRIPPLICATION: D<sub>1</sub> TRISOMY SYNDROME\*

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Recent work on the structure and genetic control of human hemoglobin suggests that there are four loci determining the structure of each of the four different  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -globin chains.<sup>1</sup> It has also been suggested that other genetic loci control their rates of synthesis.<sup>2</sup> Family studies indicate that the structural genes

determining the  $\beta$  and  $\delta$  chains are closely linked,<sup>3</sup> while the gene for the  $\alpha$  chain segregates independently.<sup>4</sup> No information is available to indicate on which chromosome the four structural loci and their related controlling elements are situated. In order to localize genes controlling hemoglobin synthesis to specific chromosomes, hemoglobins of individuals with autosomal trisomies were studied. It was postulated that if a given triplicated chromosome carries genes directly or indirectly affecting the synthesis of one of the chains, the additional genetic material might lead to the formation of increased amounts of one of the normal hemoglobins or of one of the hemoglobin species consisting of a single type of polypeptide chain.

*Methods.—Purification of the hemoglobins:* The hemoglobins were purified by column chromatography on carboxymethyl Sephadex C-50 using a gradient from pH 6.8 to pH 8.9. When almost all the Hb-A and Hb-F had been eluted from the column, Hb-Gower 2 (cf. ref. 5) was eluted by a solution of 0.02 M  $\text{Na}_2\text{HPO}_4$ . Hb-Gower 2 was further purified by starch block electrophoresis in barbiturate buffer pH 8.6. Hb- $\gamma_4$  was purified from the first protein fraction eluted from the chromatogram as previously described.<sup>6</sup>

*Chromosome examinations* were made on cultures of peripheral blood leukocytes.<sup>7</sup> Hb-F in whole hemolysates was measured as described by Betke *et al.*<sup>8</sup>

The methods used for the preparation of hemolysates, electrophoresis on starch block and starch gel, estimation of the alkali denaturation rate, dissociation and recombination studies, peptide mapping, and measurement of ultraviolet spectra have been previously described.<sup>6</sup>

*Patients studied:* Table 1 lists the cases of  $D_1$  trisomy syndrome. Cases I–VII

TABLE 1

Case	Chromosomal findings	Age	Hb-Gower 2 %	Hb-A <sub>2</sub> Starch gel	Hb-F %	Hb-A %	Hb- $\gamma_4$ %
I	D trisomy	4 days	0.7	trace	76	23	1.5
II	D trisomy	5 days	≈0.5	trace	75	24	≈1.5
III	D trisomy	5 days	trace	trace	77	22	≈1.5
IV	D/D translocation	17 days	trace	trace	62	37	≈1.5
V	Not examined	5 days	≈0.5	trace	77	22	≈1.5
VI	D trisomy	2 <sup>2</sup> / <sub>12</sub> years	nil	normal	6.4	91	nil
VII	D trisomy	4 <sup>9</sup> / <sub>12</sub> years	nil	normal	2.6	95	nil
VIII	D trisomy mosaic	2 <sup>6</sup> / <sub>12</sub> years	nil	normal	5.9	92	nil

showed the characteristic clinical picture of this syndrome.<sup>9</sup> Case VIII, with D trisomy mosaicism, clinically only presented with cleft palate and mental retardation; however, dermal print analysis revealed characteristic hallucal patterns hitherto seen only in the  $D_1$  trisomy syndrome.<sup>10</sup>

Case IV had 46 chromosomes with 1 chromosome missing from the D group and an extra large metacentric chromosome interpreted as a D/D translocation. The other cases showed 47 chromosomes, the extra chromosome being a member of the D group.

Cases I and III were studied hematologically and had no anemia. The red cell indices, as well as the red cell appearances on the stained smear, were normal.

Table 2 lists the other cases. Case IX was a 7-year-old boy with D trisomy mosaicism.<sup>11</sup> He had none of the major features of the  $D_1$  trisomy syndrome and his hallucal patterns were normal. It is therefore possible that he had trisomy for

TABLE 2

Case	Clinical Picture	Chromosomal Findings	Age	Hb-F %
IX	Multiple anomalies <sup>11</sup>	D trisomy mosaic	7 years	0.2
X	Trisomy 17-18 syndrome (Case 2, ref. 25)	Trisomy 17-18	14 days	32.4
XI	Trisomy 17-18 syndrome (Case 3, ref. 25)	Trisomy 17-18	4 days	66
XII	Mongolism (newborn)	Trisomy 21	3 days	48
XIII-XVII	Mongolism (older children)	—	7-13 years	0.3-0.6
XVIII-XIX	Normal premature infants (1,520 gm, 760 gm)	—	1 day	74, 69
XX	Multiple congenital malformations	Normal	1 day	64
XXI			16 days	71
XXII			8 weeks	39
XXIII			4 months	2.5
XXIV			3 days	70
	Neonatal jaundice, cause unknown	—		

a chromosome in the 13-15 group other than that triplicated in the D<sub>1</sub> trisomy syndrome.

*Results.—D<sub>1</sub> trisomy syndrome in the newborn (cases I-V):* Starch gel electrophoresis in a sodium phosphate buffer at pH 7.4 of the hemolysates from cases I and II showed two abnormal hemoglobins, one migrating more rapidly than Hb-A toward the anode, and the other migrating toward the cathode. These were shown to be Hb- $\gamma_4$  and Hb-Gower 2, respectively (see below). Measurement of the proportions of these abnormal hemoglobins by starch block electrophoresis in Case I showed that Hb- $\gamma_4$  constituted approximately 1.5 per cent of the total hemoglobin, and Hb-Gower 2, 0.7 per cent. In case II, similar amounts were indicated by visual comparison of the starch gel analyses.

In cases III-V, Hb- $\gamma_4$  was present in similar concentrations as in cases I and II. Comparison with normal cord bloods by starch gel electrophoresis showed that Hb- $\gamma_4$  was present in 3 to 4 times the normal amount. In cases III and IV, Hb-Gower 2 could only just be detected in the whole hemolysate by starch gel electrophoresis at pH 8.6 after staining the gel for hemoglobin. (Insertion into the starch gel of two, rather than one, filter paper wicks containing the test hemolysate facilitated the detection of the rather small quantities of Hb-Gower 2.) The presence of Hb-Gower 2 was confirmed by starch gel electrophoresis of the residual hemoglobin eluted with a 0.02 M Na<sub>2</sub>HPO<sub>4</sub> solution from the CM Sephadex column (Fig. 1). The amount of Hb-Gower 2 present in cases III and IV was estimated to be approximately 0.1 per cent of total hemoglobin. The hemolysate from case V was examined by starch gel electrophoresis only. Hb-Gower 2 was present in amounts similar to those found in case I. In cases III and IV, Hb-Gower 2 and Hb- $\gamma_4$  had markedly decreased in amount and were barely detectable when the patients were 30 and 62 days old, respectively.

*Identification of the two abnormal hemoglobins: (a) Hemoglobin  $\gamma_4$ :* The "fast" hemoglobin from all five D<sub>1</sub> trisomy cases was similar in electrophoretic mobility to that of known Hb- $\gamma_4$ . The ultraviolet spectrum in the region 285-295 m $\mu$  of the isolated hemoglobin from cases I, II, and IV showed a tryptophan fine-structure band with approximately three times the resolution of that of Hb-F (Fig. 2). The alkali denaturation rate of the presumed Hb- $\gamma_4$  isolated from case I was similar to that of proved Hb- $\gamma_4$  and, unlike that of Hb-A and Hb-F, did not follow first-order kinetics. The degree of resolution of the tryptophan fine structure band

and the unusual alkali denaturation rate are specific properties of Hb- $\gamma_4$  (cf. ref. 6).

(b) *Hemoglobin Gower 2*: On electrophoresis in starch gel at pH 8.6, the second abnormal hemoglobin migrated slightly more slowly toward the anode than Hb-G/C (Hb- $\alpha_2^G\beta_2^C$ ) (cf. ref. 12) and was identical to the Hb-Gower 2 found in a small human embryo<sup>13</sup> (Fig. 1). In phosphate buffer at pH 7.4, this pigment migrated toward the cathode more rapidly than Hb-G/C but more slowly than Hb- $\alpha^A$ .

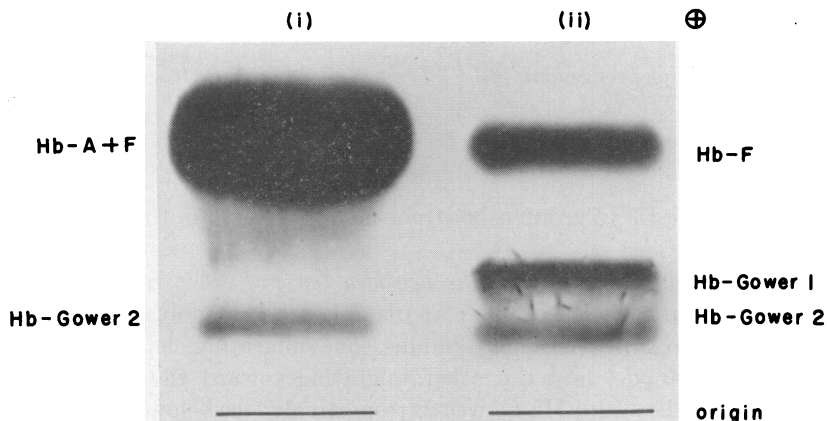


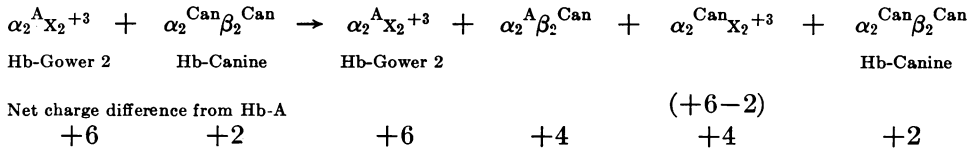
FIG. 1.—Starch gel analysis in the *tris*-citrate borate system: (i) chromatographic fraction from the hemolysate of case IV eluted from the CM Sephadex column with 0.02 M  $\text{Na}_2\text{HPO}_4$ ; (ii) hemolysate from a 34-mm (crown-rump length) human embryo.

On electrophoresis in agar gel (sodium citrate buffer pH 6.2) (cf. ref. 14), which clearly separates Hb-A from Hb-F, Hb-Gower 2 migrated with Hb-A. The spectrum of the cyanmet derivative of this hemoglobin was similar to that of Hb-F. The tryptophan fine-structure band was partially resolved at a wavelength just below 2900 Å (Fig. 2), as had previously been found when Hb-Gower 2 from a human embryo was examined.<sup>5</sup> Alkali denaturation of Hb-Gower 2 in dilute solution, isolated from case I, gave a biphasic reaction. Thirty per cent of the pigment was alkali-labile and denatured within the first two minutes. The remaining 70 per cent was denatured at a rate intermediate between that of Hb-F and Hb-A, and its denaturation apparently followed first-order kinetics. The time taken for 50 per cent denaturation was *ca.* 6 min for the resistant fraction of Hb-Gower 2, while it was *ca.* 0.2 min for Hb-A and *ca.* 17 min for Hb-F.

*Sedimentation studies:* The sedimentation coefficient of Hb-Gower 2 was 4.5 *S*. This value agrees closely with that reported for Hb-A (cf. ref. 15) and strongly suggests that Hb-Gower 2 consists of four polypeptide chains.

*Dissociation and recombination experiments* of Hb-Gower 2 with canine hemoglobin (Fig. 3iii) indicated that, in addition to the parent species, a single new zone was formed. The formation of only one new zone rather than the two usually seen in human/canine recombination experiments suggests that the second expected hybrid was not resolved in the analysis. This is explained by considering the charge differences between the various hemoglobin species. On electrophoresis at pH 8.6, Hb-Gower 2 moves slightly more slowly than Hb-G/C. Since Hb-G/C is known to differ from Hb-A by six positive charges,<sup>12</sup> Hb-Gower 2 also differs from

Hb-A by at least six positive charges. The formation of a new zone of identical electrophoretic mobility to that of Hb- $\alpha_2^A\beta_2^{Can}$  in the recombination experiment (Fig. 3*iii*) suggests that the  $\alpha$  chains of Hb-Gower 2 are normal. This difference of six positive charges must then reside in the non- $\alpha$ -chains. The structure of Hb-Gower 2 can therefore be provisionally written Hb- $\alpha_2^AX_2^{+3}$ . (The three extra positive charges apply to each of the non- $\alpha$ -chains at pH 8.6.) The recombination reaction would then proceed as follows:



On this hypothesis, both the expected hybrids should have the same net charge or electrophoretic mobility for the following reasons. Hb- $\alpha_2^{Can}X_2^{+3}$  should differ in charge from Hb- $\alpha_2^{Can}\beta_2^A$  by the same amount that Hb-Gower 2 differs from Hb-A (+6, see above). Hb- $\alpha_2^{Can}\beta_2^A$  carries two negative charges more than Hb-A (cf. ref. 16); therefore, the net charge difference of Hb- $\alpha_2^{Can}X_2^{+3}$  from Hb-A would be (-2+6) or 4 positive charges. Since Hb- $\alpha_2^A\beta_2^{Can}$  also has a net charge difference from Hb-A of +4 (cf. ref. 16), the two hybrid species would have the same electrophoretic mobility. Starch gel analysis (Fig. 3*iii*) shows a single hemoglobin zone containing a relatively large amount of pigment migrating in this position.

These experiments indicate that Hb-Gower 2 is a hemoglobin consisting of four polypeptide chains, two  $\alpha^A$  chains joined to two other chains, each of which differs from the  $\beta$  or  $\gamma$  chains by three positive charges. The maximum difference in net charge due to a single amino acid substitution in one chain is two (e.g., the substitution of a basic by an acidic amino acid residue, as in Hb-C; glutamyl  $\rightarrow$  lysyl). The localization of the six extra positive charges of Hb-Gower 2 to the two non- $\alpha$  chains therefore suggests that these chains differ by more than one amino acid substitution from the  $\beta$  or  $\gamma$  chains. The possibility that the extra positive charges in Hb-Gower 2 when compared with Hb-A or Hb-F are due to secondary changes in the polypeptide chains rather than amino acid differences cannot be ruled out, but appears unlikely. The *in vivo* formation

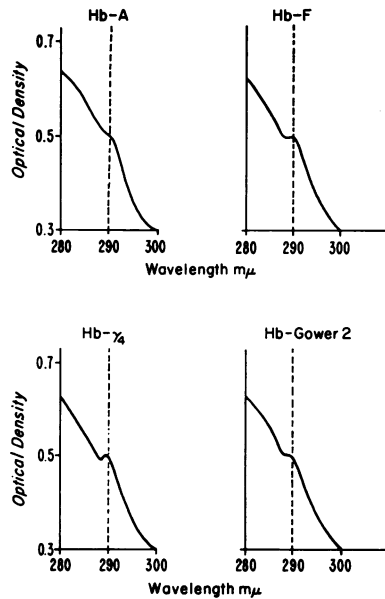


FIG. 2.—Absorption spectra of various cyanmethemoglobins in the 280-300  $m\mu$  region. The fractional resolution ( $R = \frac{E_{max} - E_{min}}{E_{max}}$ ) of the tryptophan fine structure band was  $0.4 \times 10^{-2}$  for Hb-F, and  $1.6 \times 10^{-2}$ - $2.0 \times 10^{-2}$  for Hb- $\gamma_4$  (from cases I, II, and IV). The latter is within the reported range for Hb- $\gamma_4$  (cf. ref. 5). The resolution for Hb-Gower 2 could not be measured.

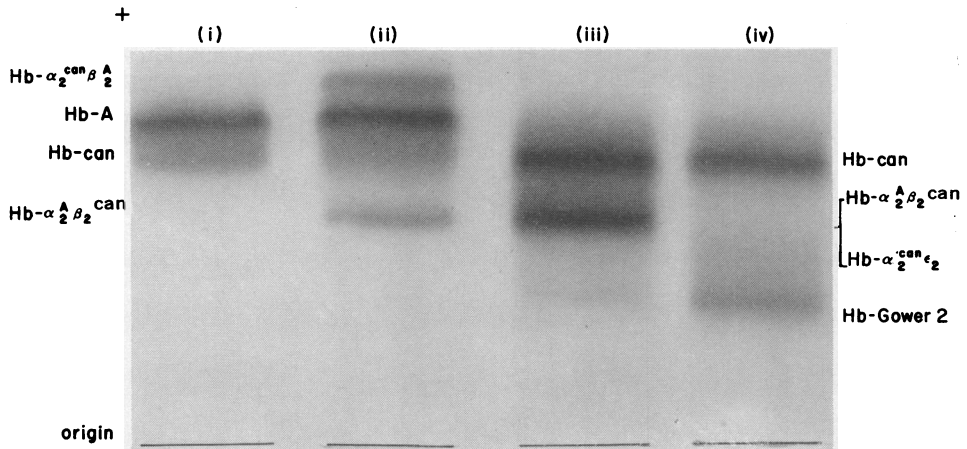


FIG. 3.—Recombination of Hb-Gower 2 with Hb-canine using starch gel electrophoresis in the tris-citrate borate system: (i) mixture of Hb-A with Hb-canine, untreated; (ii) same mixture as (i) dissociated and recombined; (iii) same mixture as (iv) dissociated and recombined; (iv) mixture of Hb-Gower 2 with Hb-canine, untreated.

of N-acetyl<sup>17</sup> and glutathione<sup>18</sup> derivatives of hemoglobin is known to occur, but these lead to the formation of extra negative charges. The *in vivo* formation of more positively charged derivatives has not, to our knowledge, been described.

*Peptide mapping:* A small amount of purified Hb-Gower 2 from case I was digested with trypsin and one peptide analysis carried out. On this "fingerprint," all the peptides derived from the  $\alpha^A$  chains could be clearly identified. The pattern of the remaining peptides differed from that of the  $\beta$ ,  $\gamma$ , or  $\delta$  chains by more than one peptide. With the small amount of material available, it was impossible to delineate these differences further. However, these data lend further support to the postulate that the non- $\alpha$  chains of Hb-Gower 2 differ from the  $\beta$ ,  $\gamma$ , and  $\delta$  chains by more than one amino acid substitution.

*Family studies:* The parents of cases I, III, IV, and VIII were examined. No abnormal hemoglobin was detected by starch gel electrophoresis at either pH 8.6 or pH 7.4. Hb-A<sub>2</sub> and Hb-F were within normal limits. The father of case IV is a carrier of the translocation (D/D) chromosome. He showed no clinical or hemoglobin abnormality.

*D<sub>1</sub> trisomy in older children (cases VI–VIII):* Case VI (aged 2 years 2 months) carried 6.4 per cent Hb-F; case VII (aged 4<sup>3</sup>/<sub>4</sub> years) carried 2.6 per cent Hb-F; case VIII (aged 2 years 6 months) carried 5.9 per cent Hb-F. These values are higher than those found in normal children of these ages.<sup>19</sup> The increased amounts of fetal hemoglobin were confirmed by examining the blood films (cases VI and VII) for Hb-F by the acid elution technique.<sup>20</sup> Hb-A<sub>2</sub> appeared within normal limits on starch gel analysis in the three cases.

*Other cases (Table 2):* Case IX, aged 7 years, with mosaicism involving trisomy of a chromosome in the 13–15 group possibly other than the chromosome triplicated in the D<sub>1</sub> trisomy syndrome, showed no hemoglobin abnormality on starch gel electrophoresis. Hb-F was within normal limits.

Cases X–XII were newborns with other autosomal trisomies. In these cases, no abnormal hemoglobins were detected by starch gel electrophoresis. The absence

of Hb-Gower 2 was confirmed by starch gel electrophoresis of the final hemoglobin fraction eluted from CM-Sephadex columns.

In the other control cases (XIII-XXIV), including 2 normal premature infants, no abnormal hemoglobins were detected by starch gel analyses and Hb-F was within normal limits.

*Discussion.*—D<sub>1</sub> trisomy affects hemoglobin synthesis in several ways. In the neonatal period both increased Hb- $\gamma_4$  and Hb-Gower 2 could be demonstrated in five consecutively examined newborns. Older children with D<sub>1</sub> trisomy syndrome showed increased amounts of fetal hemoglobin but no Hb-Gower 2 or Hb- $\gamma_4$ . Examination of the hemoglobin from infants with other trisomies or congenital malformations failed to show any hemoglobin abnormalities.

Hb-Gower 2 has been demonstrated on five occasions when very young, apparently normal human embryos were examined.<sup>5, 13</sup> It was found in only one out of 300 cord blood samples.<sup>21</sup> It is therefore likely that Hb-Gower 2 is a normal embryonic hemoglobin which usually disappears early in development.<sup>13</sup> The persistence of Hb-Gower 2 in D<sub>1</sub> trisomy suggests that this pigment is a hemoglobin synthesized as a result of genetic information normally present, for it is difficult to visualize how a chromosomal triplication could lead to the formation of an abnormal hemoglobin with an altered primary structure.

Hb-Gower 2 consists of four polypeptide chains with two normal  $\alpha$  chains joined to two non- $\alpha$  chains with properties which suggest that they differ from the  $\beta$  and  $\gamma$  chains by more than one amino acid substitution (see *Results*). These findings and the suggestion<sup>13</sup> that Hb-Gower 2 is probably a normal embryonic hemoglobin indicate that the non- $\alpha$  chains of Hb-Gower 2 are biochemically and functionally distinct from the other human hemoglobin polypeptide chains. It is, therefore, suggested that these be called  $\epsilon$  chains and the structure of Hb-Gower 2 would then be written  $\alpha_2^A \epsilon_2$ .

It is of interest to note that Hb-Gower 1, which was found together with Hb-Gower 2 in the five embryos mentioned above,<sup>5, 13</sup> was not found in the cases of D<sub>1</sub> trisomy reported here or in the one cord blood sample found to contain Hb-Gower 2 (cf. ref. 21). As, at present, the relationship between these two embryonic hemoglobins is unknown, the reasons for this are not apparent.

The hemoglobin anomalies in the D<sub>1</sub> trisomy syndrome can be interpreted in various ways. It seems possible, but unlikely, that generalized developmental immaturity is responsible. The absence of Hb-Gower 2 from the hemolysates of two premature infants (cases XVIII and XIX) argues against this interpretation. A more specific effect is suggested by the recent demonstration<sup>22</sup> of clones of Hb-F containing red cells in an adult patient with erythroleukemia and acquired D trisomy mosaicism.

The hemoglobin anomalies described in the D<sub>1</sub> trisomy syndrome may then be due to triplication of some of the genetic loci (structural or regulatory) controlling the synthesis of  $\gamma$  or  $\epsilon$  chains.

Variable increases of  $\gamma$  and  $\epsilon$  chain synthesis might occur if there were an *imbalance* between the amount of structural genetic material and the amount of regulatory substance (repressor) formed. Two possible mechanisms are illustrated in Figure 4. In the first, the structural loci would be triplicated and the normal amount of repressor would not be sufficient to inactivate three genes (Fig. 4A).

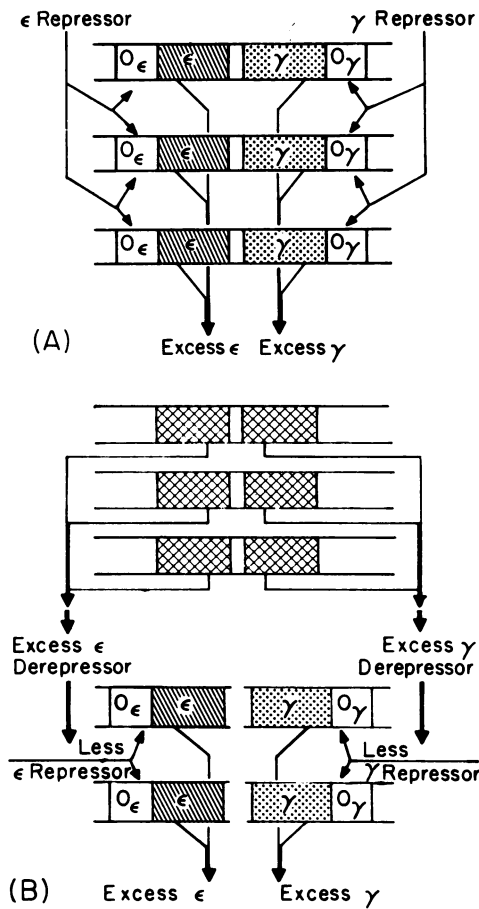


FIG. 4.—Models to explain hemoglobin anomalies in D<sub>1</sub> trisomy. O<sub>ε</sub> and O<sub>γ</sub> represent operator genes regulating structural genes ε and γ. Model A—structural loci for ε and γ chains are linked and located on the triplicated chromosome. The normal amount of repressor is not sufficient for complete inactivation of three loci, and this leads to excess formation of ε and γ chains. Model B—genes affecting production of ε and γ derepressor (inducer) are located on the triplicated chromosome. Excess derepressor leads to decreased repression of the ε and γ genes with consequent excess formation of the corresponding chains. ε and γ structural loci need not be linked in this model.

Alternatively, genes which indirectly regulate the synthesis of the γ and ε chains may be located on the triplicated chromosome and these indirectly lead to deficient repression of the normal loci controlling γ and ε chain formation (Fig. 4B).

At present it is clearly impossible to state whether structural genes or genetic elements which affect hemoglobin synthesis more indirectly have been triplicated. The problem raised is similar to that of the increased leukocyte alkaline phosphatase<sup>23</sup> and erythrocyte galactose-1-phosphate uridyl-transferase<sup>24</sup> levels in trisomy 21. Genes affecting synthesis of these enzymes are undoubtedly located on the triplicated chromosome, but the nature of these genes is not known.

*Summary.*—Hb-Gower 2 appears to be a normal embryonic hemoglobin detectable in very young human embryos. This hemoglobin was found to persist in small amounts in five newborns with D<sub>1</sub> trisomy. These infants also carried increased amounts of Hb-γ<sub>4</sub>. Hb-Gower 2 is a tetramer with two normal α chains. It is suggested that the non-α-chains differ from the other hemoglobin chains by more than one amino acid substitution and that they represent a hitherto undescribed, biochemically and functionally distinct chain, the ε chain. Three older children with D<sub>1</sub> trisomy had slightly elevated amounts of Hb-F. Presumably, genetic elements which, directly or indirectly, affect the synthesis of γ and the postulated ε chains are located on the chromosome triplicated in D<sub>1</sub> trisomy.

*Note added in proof:* Since completion of this manuscript, we have examined two more newborns with D<sub>1</sub> trisomy. In both cases Hb-Gower 2 and Hb-γ<sub>4</sub> were detected in amounts similar to those observed in our original cases.

Cases I and III were seen in Seattle, cases II and IV by Dr. J. R. Miller in Vancouver, B. C., case V by Drs. S. Breibart, W. L. Mellman, and R. B. Young in Philadelphia, cases VI and VII by Dr. D. W. Smith in Madison, Wisconsin (Cases 286 and 20, cf. ref. 9).



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## STRAIN ELECTROMETRY AND PASSIVATION OF ALUMINUM ELECTRODES

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The relationship between the chemistry at an electrode-solution interphase and the potential difference,  $V$ , across the interphase may be expressed as

$$i_{\text{ext}} - \frac{d(C_{dl}V)}{dt} = \sum_i C_i^{n_i} Z_i F k_i e^{\frac{\alpha_i F Z_i V}{RT}} - \sum_j C_j^{n_j} Z_j F k_j e^{-\frac{\alpha_j F Z_j V}{RT}} \quad (1)$$

where  $i_{\text{ext}}$  is the external current,  $C_{dl}$  is the differential capacitance of the electrical