EVIDENCE FOR MULTIPLE STRUCTURAL GENES FOR THE γ CHAIN OF HUMAN FETAL HEMOGLOBIN

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A sequence with a specific residue at each position was proposed for the γ chain of human fetal hemoglobin by Schroeder *et al.*[1] after a study in which hemoglobin from a number of individual infants was used. We have now examined in part the fetal hemoglobin components of 17 additional infants and have observed that position 136 of the γ chain may be occupied not only by a glycyl residue, as previously reported, but also by an alanyl residue.

Experimental.—Source of hemoglobin: Samples were taken from the umbilical cord or by venipuncture. Four specimens contained an abnormal hemoglobin F (or $\alpha_2\gamma_2^x$) and one an elevated percentage of hemoglobin Bart's or γ_4 (Table 1).

Nomenclature: The dual system of nomenclature for hemoglobin components as used in this paper distinguishes between the methods of isolation to be described below. F_I, F_{II}, etc., apply to hemoglobins isolated by IRC-50 chromatography, and F_1, F_0, etc., to those isolated by *O*-(diethylaminoethyl)-Sephadex (DEAE-Sephadex) chromatography. They may be equated as follows: $F_{II} = F_0$, major fetal hemoglobn; $F_I = F_1$, minor fetal hemoglobin; $A_{II} = A_0$, major adult hemolgobin; A_2, the minor adult component $\alpha_2\delta_2$; and F_x, an abnormal fetal hemoglobin $\alpha_2\gamma_2^x$.

Isolation of hemoglobin: Solutions of hemoglobin were prepared from the cells as described by Clegg and Schroeder.[2] In a few instances, cord hemoglobin was used without separation of the fetal and adult hemoglobins. Individual hemoglobins were isolated by chromatography on Amberlite IRC-50 (ref. 1) or on DEAE-Sephadex.[3] Hemoglobins F_x from subjects D. P., W. B., C. W., and J. B. are aberrant in the γ chain. F_x from D. P. has an electrophoretic mobility similar to hemoglobin F_{Texas} (ref. 4), those from W. B. and C. W. much like hemoglobin F_{Warren} (ref. 5), and that of J. B. near to hemoglobin Bart's or γ_4; further characterization will be published elsewhere. Heme was removed from all preparations as previously described,[1] but the globin was dried and not dialyzed. Some samples were aminoethylated.[6] γ Chains were prepared by chromatography.[7]

Reaction with cyanogen bromide: The sample was dissolved in 70% formic acid at a concentration of 5 mg/ml, and cyanogen bromide (Matheson, Coleman, and Bell, or Eastman) equivalent to 30 times the methionine content was added. After 16 hr at room temperature in the dark, the reaction mixture was diluted with 10 times its volume of water and lyophilized.

Isolation of peptides: Initial separation of peptides was made by passage through Bio-

TABLE 1. *Data for subjects with abnormal fetal hemoglobins.*

			Percentages of Hemoglobin Components						$F_x/(F_0 + F_1 + F_x) \times 100$
Subject	Race	Sex	F_0	F_1	F_x	γ_4	A_0	A_2	
D. P.	Cauc.	M	68.2	7.5	10.5	...	13.8	<0.1	12.2
W. B.	Negro	M	64.6	9.6	11.6	...	14.1	0.1	13.5
C. W.	Negro	M	59.9	10.3	10.3	...	19.4	0.15	12.8
J. B.	Negro-Cauc.	F	60.3	9.4	8.9	...	21.3	0.1	11.5
W. S.	Negro	M	68.8	7.9	0	6.9	16.3	<0.1	—

Gel P-100 (Bio-Rad Laboratories, Richmond, Calif.). During the equilibration of Bio-Gel P-100 in 50% acetic acid, a small amount of fine material was removed. Columns were then poured in tubes that had been treated with Desicote (Beckman Instruments, Inc., Fullerton, Calif.). For most separations, two columns in series were used: effluent from the first was passed through small-bore polyethylene tubing into the second at a point several millimeters below the upper surface of the Bio-Gel. Each column of the series was 1 × 160 cm when the sample was 50–100 mg and 2.2 × 160 cm when it was 200–300 mg. The sample was placed on the column in 50% acetic acid, which was also used as developer at a flow rate of 6 ml per square centimeter of cross-sectional area per hour. All separations were made at room temperature.

The desired peptide was purified by chromatography on a 0.6 × 60-cm or a 1 × 100-cm column of Dowex-1 (ref. 8). In later experiments, this system was modified to use 10 ml of pH 9.4 buffer, 30 ml of pH 9.0 buffer, 40 ml of pH 8.4 buffer, 60 ml of pH 6.5 buffer, and 100 ml each of 0.5 *N* and 2 *N* acetic acid for the smaller column. For special examination, the peptide was also chromatographed on a 0.6 × 60-cm column of Dowex-50-X8 (equivalent to fraction C of Moore *et al.*[9]). The gradient system was like that of Schroeder *et al.*[10] except that 83 ml of 0.1 *M* buffer at pH 3.1 and 166 ml of *M* buffer at pH 5.0 were used. The flow rate of developer was 10 ml per hour.

Amino acid analyses: The hydrolytic procedure has been described.[1] The analyses were made on spherical resin in a model 120 amino acid analyzer (Beckman Instruments, Inc., Palo Alto, Calif.), according to Benson and Patterson,[11] with high-sensitivity cuvettes.[12]

Results.—Human hemoglobin F has methionyl residues at positions 32 and 76 of the α chain and at positions 55 and 133 of the γ chain. If we label the prospective peptides from cyanogen bromide cleavage[13] analogously to tryptic peptides,[14] the number of residues in the several peptides is as follows: αCB-1, 32; αCB-2, 44; αCB-3, 65; γCB-1, 55; γCB-2, 78; and γCB-3, 13. The separation of peptides from the cyanogen bromide cleavage of globin F is depicted in Figure 1*a*. The identity of the peptides in the various zones is given.

The primary concern of this investigation was peptide γCB-3. The sequence of the portion of the γ chain that comprises this peptide is Val-Thr-Gly-Val-Ala-Ser-Ala-Leu-Ser-Ser-Arg-Tyr-His.[1] When peptide γCB-3 was isolated from globin F_{II} of subject J. H. (Table 2), the amino acid composition in terms of residues was His, 0.97; Arg, 0.98; Thr, 0.90; Ser, 2.70; Gly, 0.71; Ala, 2.31; Val, 2.00; Leu, 0.99; and Tyr, 0.88. If we take into account the usual hydrolytic destruction of threonine, serine, and tyrosine, only glycine and alanine do not give essentially integral values. These results suggest that two peptides are present and that they are identical in sequence except for glycine or alanine in the third position. When the peptide was degraded for ten steps by the phenylthiohydantoin (PTH) method on paper strips,[1] the expected results were found except in the third position where, indeed, both glycine and alanine were detected (Fig. 2). On the other hand, alanine alone and no glycine was detected in the fifth and seventh positions. Further evidence of two peptides was obtained when the zone was pooled in several portions and each was analyzed; the analyses showed an increasing ratio of glycine to alanine from the front to the tail of the zone.

Data for additional samples are summarized in Table 2.

Discussion.—Except for nos. 18, 19, 21, and 23, which are the abnormal fetal hemoglobins, all samples in Table 2 show more than one half and less than one

residue of glycine in peptide γCB-3 and, therefore, force the conclusion that a mixture of peptides is normally present. Consequently, hemoglobin $F_{II}(=F_0)$ as isolated must have contained at least two types of γ chains, which will be designated as γ^{136Gly} and γ^{136Ala} chains in the subsequent discussion. When the context requires, the position at which the chains differ will also be referred to synonymously as "residue 3 of the γCB-3 peptide" or "residue 136."

It is pertinent to ask, "If almost all samples have a mixture of γ^{136Gly} and γ^{136Ala} chains, why was the sequence originally reported as γ^{136Gly} only[1]?" In the original investigation, the data were derived from tryptic peptide γT-15, which includes residues 133–144. To answer the above question, γT-15 was isolated from globin F_{II} of J. H. (subject 1, Table 2) by chromatography on Dowex 50-X2 and Dowex-1-X2 as was originally done. Just as the mixed peptides of γCB-3 may be partially separated on Dowex-50-X8 and Dowex-1, so the mixed peptides of γT-15 separate partially on Dowex-50-X2. Depending upon the way in which the fractions were pooled, most of the alanine-containing γT-15 could be excluded in the initial isolation. We believe that the fortuitous pooling of fractions eliminated the alanine-containing γT-15 in our original investigation. It is not probable that the present results are distorted in this way. In the first place, Bio-Gel chromatography is unlikely to bring about even a partial separation; and secondly, care was taken to exclude no portion of the zone either at this point or on purification by Dowex-1. This point will be further discussed in another context below.

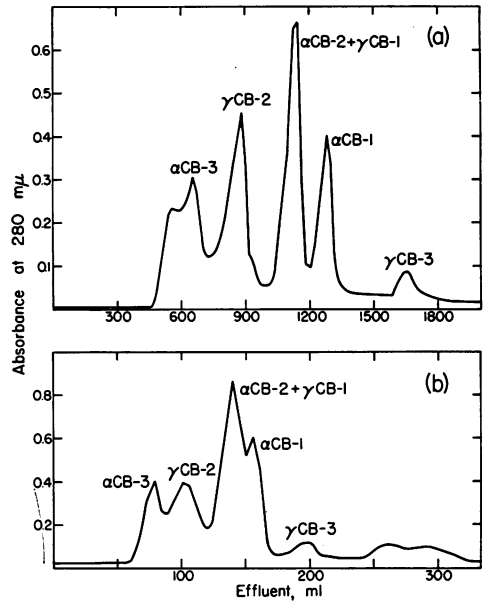


FIG. 1.—(*a*) Peptides from cyanogen bromide treatment of 220 mg of globin F_0 from D. P. Four 2.2 × 160-cm columns of Bio-Gel P-100 in series were developed with 50% acetic acid. (*b*) Peptides from cyanogen bromide treatment of 50 mg of aminoethylated globin F_0 from D. P. Two 1 × 160-cm columns of Bio-Gel P-100 in series were developed with 50% acetic acid.

The data of Table 2 resemble results that have been reported by Bargetzi *et al.*[15] and Walsh *et al.*[16] for bovine carboxypeptidase A, by von Ehrenstein[17] for the α chain of rabbit hemoglobin, by Rifkin *et al.*[18] and Popp[19] for the α chain of several strains of mouse hemoglobin, by Kilmartin and Clegg[20] for the α chain of horse hemoglobin, and by Huisman *et al.*[21] for the α chain of goat hemoglobin. In general, the data have been explained by assuming either a genetic cause or an ambiguous translation of genetic material. Walsh *et al.*[16] concluded that two allelomorphs occurred in the general bovine population, and Huisman *et al.*[21] presented strong evidence that nonallelic genes were respon-

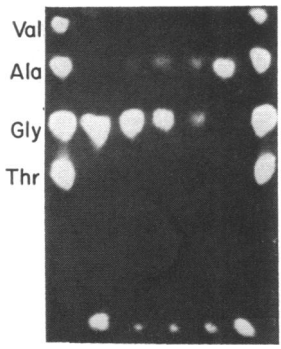


FIG. 2.—Results of PTH-degradations at residue 3 of several γCB-3 peptides. Standards are at left and right. Samples from left to right derive from W. B.-F_x (no. 19), D. P.-F_0 (no. 16), J. H.-F_0 (no. 1), J. L.-F_0 (persistent high fetal hemoglobin carrier), and D. P.-F_x (no. 18).

sible, but no conclusion could be reached in the other cases.

The hemoglobins F_x of D. P., W. B., C. W., and J. B. (nos. 18, 19, 21, and 23, Table 2) have either glycine or alanine alone in position 136 of the γ chain. The results of PTH-degradations at residue 3 of γCB-3 from various sources (Fig. 2) show that F_x of D. P. has only alanine and no trace of glycine, whereas F_x of W. B. has only glycine and no trace of alanine. The small amount of glycine reported for D. P.'s F_x (no. 18, Table 2) is therefore probably due to impurities. Although J. B.'s F_x, like D. P.'s, is believed to have only alanine at residue 136, the data are less definitive because J. B.'s F_x, which emerges last from the DEAE-Sephadex column, contained some F_0 and F_1.

These results from the hemoglobins F_x are interpreted to mean that the observed effects in normal infants come from a genetic factor and not from ambiguous translation. If ambiguous translation is involved, the different F_x's should each show ambiguity at residue 136. If, on the other hand, multiple structural genes are involved, some F_x's should have glycine in residue 136 and some should have alanine. The latter state is observed: the mutated genes which produce the F_x's yield distinct products that are related *either* to γ^{136Gly} *or* to γ^{136Ala} chains. Consequently, on the basis of this evidence, it may be concluded that the two types of γ chains in normal samples of hemoglobin $F_{II}(=F_0)$ (as represented by nos. 1–12 in Table 2) stem from a genetic factor and not from ambiguous translation.

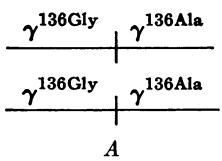
TABLE 2. *Analytical data on the γCB-3 peptide from several sources.*

Subject	Residues: Glycine	Residues: Alanine	Material	Subject	Residues: Glycine	Residues: Alanine	Material
1. J. H.	0.71	2.31	Globin F_{II}	13. W. S.	0.72	2.43	Globin F_0
2. P. L.	0.77	2.23	Globin F_{II}	14. W. S.	0.83	2.33	Globin F_1
3. B. F.	0.88	2.14	Globin F_{II}	15. W. S.	0.65	2.41	Globin γ_4
4. N. N.	0.68	2.35	Globin F_{II}	16. D. P.	0.97	2.18	Globin F_0†
5. B. G.	0.74	2.30	Globin F_{II}	D. P.	0.87‡	2.19	Globin F_0†
6. J. M.	0.85	2.19	Globin F_{II}	D. P.	0.87	2.22	Globin F_0†
7. C. O.*	0.76	2.28	γ Chain	17. D. P.	0.87	2.22	Globin F_1
8. C. C.*	0.73	2.32	γ Chain	18. D. P.	0.16‡	2.97	Globin F_x
9. G. W.	0.77	2.26	Cord globin	19. W. B.	1.01‡	2.01	Globin F_x
10. B. W.	0.81	2.26	Cord globin	20. C. W.	0.83	2.21	Globin F_0
11. B. K.	0.71	2.29	Cord globin	21. C. W.	1.01‡	2.03	Globin F_x
12. G. S.	0.79	2.31	Cord globin	22. J. B.	0.83	2.23	Globin F_0
				23. J. B.	0.38‡	2.73	Globin F_x

* Samples 7 and 8 are mixtures of blood from two individuals.
† Three analyses of the same sample.
‡ Aminoethylated sample.

It is significant that both γ^{136Gly} and γ^{136Ala} are present in hemoglobin $F_{II}(=F_0)$ of samples 1–13, 16, 20, and 22 (Table 2). None of the individuals is "homozygous" at position 136. If a pair of allelic genes at a single locus was involved in producing γ^{136Gly} and γ^{136Ala} chains, homozygotes for both γ^{136Gly} and γ^{136Ala} should have been detected even in this relatively small number. For example, Walsh *et al.*[16] observed an almost exact Mendelian distribution of heterozygotes and homozygotes in a similarly sized population. As would be expected, γ^{136Ala} chains are present in the F_0's of W. B. and C. W. whose F_x's are derived solely from γ^{136Gly} chains. However, it is important to realize that γ^{136Ala} chains are also present in the F_0's of D. P. and J. B. (samples 16 and 22 which were not contaminated with hemoglobins F_x), where F_x is derived solely from γ^{136Ala} chains. The analysis (Table 2) and PTH-degradation of F_0 from D. P. show this. Ambiguity of translation cannot be invoked to explain the presence of γ^{136Ala} chains in samples 16 and 22 because the abnormal hemoglobins give no evidence of ambiguity.

The data, therefore, cannot be explained by ambiguity of translation or by heterozygosity at a single locus. The situation may be analogous to that of the goat α chains.[21] More than one pair of allelic genes that code for the γ chain is assumed. In the simplest case, this may be represented by diagram *A* on homologous chromosomes; the relative arrangement is, of course, arbitrary and the genes may or may not be closely linked or adjacent. Although the normal individual would be homozygous at each locus, both products would appear, the abnormal fetal hemoglobins would result from a mutation of one of the alleles of the appropriate gene, and hemoglobin F_0 from an individual with an F_x would have the two types of γ chains.

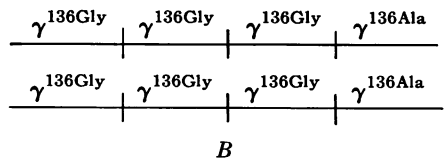


A

The quantitative aspects of the data, on the other hand, cannot easily be explained by this simple model. If equal gene products resulted, the ratio of γ^{136Gly} to γ^{136Ala} chains would be 1 and the analysis of the mixed γCB-3 peptide should have 0.5 residue of glycine and 2.5 residues of alanine. Actually, the average of samples 1–12 is 0.77 residue of glycine and 2.27 residues of alanine. It is pertinent to consider the meaning of the average because of the range of values in samples 1–12 and because sample 18, which by PTH-degradation has no glycine in residue 3 of γCB-3 (Fig. 2), shows 0.16 residue by analysis (Table 2). The anticipated sum of glycine and alanine is 3.00 residues. Of the analyses in Table 2, all but five fall between 3.00 and 3.10 residues or within a range of 3 per cent, which is the expected precision of the analyses. The fact that the sum always equals 3.00 or more and averages 3.06 suggests that a small and somewhat variable amount of extraneous glycine may be present. This problem has been further investigated by sectioning the peak from Dowex-1 chromatography into parts and analyzing each: sections at front and tail of the peak each constituted about 5 per cent of total material and two center sections about 45 per cent. The presence of extraneous glycine was evident in both the front and tail sections because the sum of glycine and alanine was sometimes as much as 3.5; in the center sections values of 3.0–3.1 were observed. If all the data are used to cal-

culate the analytical results for the unsectioned peak, the results differ by only a few per cent from those of the center sections. When the sum of glycine and alanine is 3.0–3.1 residues, a difference of more than 0.1 residue of glycine between two samples may be real. Consequently, all samples depart from the value of 0.5 residue of glycine in residue 136 that would be expected from the simple model.

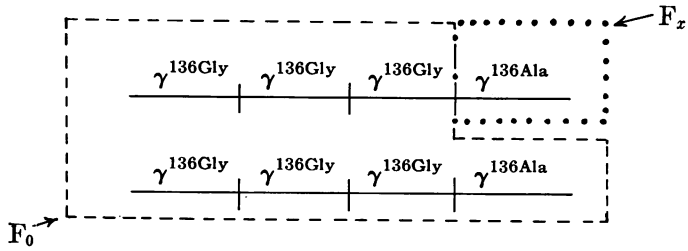
If the variation around the average is disregarded for the moment, it is evident that the observed average of 0.77 residue of glycine in residue 136 could result from diagram *B*, if each cistron yielded equal product.



B

This concept is especially attractive when we examine the observed percentages of hemoglobins F_x. Table 1 lists the amount of abnormal hemoglobins in per cent of the fetal hemoglobin components ($F_0 + F_1 + F_x$). These values are near 12.5 per cent or $^1/_8$ as would be expected if a mutation of any of the eight alleles produced a hemoglobin F_x. This fraction remains essentially constant as the fetal hemoglobin decreases with the age of the child. Other fetal hemoglobins abnormal in the γ chain are present in similar percentage: F_{Roma}, 17% (ref. 22); F_{Texas}, 12% (ref. 4); $F_{Houston}$, 15% (ref. 23); and F_{Warren}, 14% (ref. 5).

On the basis of this model, the ratio of γ^{136Gly} to γ^{136Ala} chains in the hemoglobin F_0 of an individual with a hemoglobin F1 would depend upon whether F_x derived from a γ^{136Gly} or a γ^{136Ala} allele. Thus, when F_x has alanine at residue 136, F_0 and F_x would be derived from the eight alleles as follows:



The ratio of γ^{136Gly} to γ^{136Ala} chains in the F_0 should be 6:1, and the calculated residues of glycine in residue 136 of F_0 should be 0.86: the data from F_0 of D. P. (sample 16) and J. B. (sample 22) are in excellent agreement. In like manner, when F_x derives from a γ^{136Gly} allele, the ratio should be 5:2, which corresponds to 0.71 residue of glycine at residue 136 of F_0. W. B. (sample 19) and C. W. (sample 20) are cases in point. The value of 0.83 residue of glycine in F_0 from C. W. does not agree well if the accuracy of the determination is as good as was suggested above; F_0 of W. B. was not available. There is, in fact, no appreciable difference in the glycine content at residue 136 for the F_0's of D. P., C. W., and J. B., despite the basic difference in the F_x's. Because the number of examples is small, the conflicting results do not entirely rule out the possibility of four structural genes for the γ chain, but the idea is less tenable.

On the other hand, an explanation of these results is not more easily elicited from current concepts of the control of protein synthesis especially as they relate to the hemoglobins. It is well known that the heterozygote for hemoglobin A and an abnormal hemoglobin does not usually produce the two in equal amount. It has been suggested that this inequality of production by allelic genes results either because the structure of the polypeptide chain itself determines the rate of production[24, 25] or because regulator genes determine the rate of synthesis according to the model of Jacob and Monod for bacterial systems.[26]

The structure-rate hypothesis is easily applicable to the first model of two structural genes for the γ chain. One simply assumes that the γ^{136Gly} gene synthesizes at three times the rate of the γ^{136Ala} gene so that the product as isolated has 0.75 residue of glycine in residue 136. In the same way, when an individual has an F_x that derives from a γ^{136Ala} allele, the structure-rate hypothesis can explain the percentage of F_x and the ratio of γ^{136Gly} to γ^{136Ala} chains in the F_0. When the F_x derives from a γ^{136Gly} allele, the hypothesis is unsatisfactory: one would expect the percentage of F_x to be 37.5 per cent, and F_0 should have 0.6 residue of glycine in residue 136.

Many authors have pointed out that evidence does not exist for regulator genes in mammalian systems, and have also noted that a sufficiently complex arrangement of inducers, repressors, and controlling elements can be made to play any kind of trick.[27] Although it may be possible to explain the present results in these terms, this exercise has not been carried out.

That some rate-controlling factor, whatever it may be, is responsible for the observed relationships in the product of the γ chain genes is suggested not only by the range of values from subjects 1–12, but also by data from the goat α chains.[28] In this instance, a mutant of one of the two probable structural genes has been observed in heterozygous and homozygous state. The data indicate a difference in the rates of chain production by the two related structural genes and by the mutant form of one of them. Unavailability of some of the data in the case of the human γ chain prevents strict comparison. It is also relevant that Winslow and Ingram[29] have observed distinctly different rates of synthesis for the human β and δ chains, the genes for which are believed to be closely linked.

Although it is not possible to interpret all aspects of the present data, there is persuasive evidence that more than one structural gene codes for the synthesis of the human γ chain. It is hoped that pertinent information will result from the examination of fetal hemoglobin that persists into adult life as is observed, for instance, in thalassemia, sickle cell anemia, and the hereditary persistence of fetal hemoglobin. We are currently examining hemoglobin F_0 from individuals with such conditions. Data about J. L., who has hereditary persistence of fetal hemoglobin combined with hemoglobin C, are shown in Figure 2. The presence of approximately equal amounts of glycine and alanine observed in residue 3 of J. L.'s γCB-3 peptide is substantiated by amino acid analysis. On the other hand, the fetal hemoglobins from a mother and two daughters in an unrelated family have only glycine in residue 136. The data will be discussed in detail elsewhere.

Summary.—Human fetal hemoglobin in the normal infant at birth is composed

of at least two components that are not separable by chromatography or electrophoresis. At a minimum, they differ in the exchange of glycine and alanine in position 136 of the γ chain. On the basis of evidence from abnormal human fetal hemoglobins, it is concluded that these types of γ chains are the products of more than one structural gene and are not the results of ambiguity of translation of the genetic material. The quantitative relationships that have been observed are not easily explained on the basis of current concepts of the control of protein synthesis.

We appreciate the helpful discussions on genetics with Drs. E. B. Lewis and Ray D Owen. Dr. Audrey Brown (Department of Pediatrics, Medical College of Georgia) assisted us in obtaining additional samples from babies D. P., W. B., and C. W. We are also indebted to Dr. Edgar Ahern (Department of Pathology, University College Hospital, Jamaica, W.I.) who made samples from baby J. B. available to us. Mr. Gerald Apell assisted in some of the experimental work. This investigation was supported in part by grants (HE-02558 and HE-05168) from the National Institutes of Health, U.S. Public Health Service. E. F. K. was supported by USPHS grant 1 FOS-TW-1127-01 as an International Research Fellow on leave from the Department of Pediatrics, University of Munich, Germany.

* Contribution no. 3668.

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