A PARTIAL SEQUENCE OF THE AMINO ACID RESIDUES IN THE γ CHAIN OF HUMAN HEMOGLOBIN F

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That human fetal hemoglobin differs from human adult hemoglobin in its rate of denaturation by alkali was detected almost a century ago by Körber.[1] In the intervening years, other differences (for example, in spectrum, in amino acid composition, etc.) have become apparent. When it was reported[2-4] that the number of N-terminal residues of fetal hemoglobin was only about half that of adult hemoglobin, it was to be expected that the structures of the two molecules would be very different. However, Schroeder and Matsuda[5] showed that fetal hemoglobin has four N-terminal residues of which two are valine and two are glycine; hence, the number of N-terminal residues is the same as in adult hemoglobin, which has four N-terminal valyl residues,[6] and the likelihood of vastly different structures is lessened. Indeed, both fetal[5] and adult[6] hemoglobin have a pair of polypeptide chains that possess the N-terminal sequence val-leu-; available evidence[7-9] suggests that these chains (the α chains) are identical. The other chains (the β chains) of adult hemoglobin are N-terminal in the sequence val-his-leu-,[10] and thus differ from the second kind of chains (the γ chains) of fetal hemoglobin, which are N-terminal in the sequence gly-his-phe-.[9] It is the purpose of this communication to present preliminary data on the sequence of amino acid residues in the γ chain of fetal hemoglobin and to compare these results with sequences in the α and β chains as they have been determined in these laboratories and elsewhere.

General Experimental Procedure.—Umbilical cord hemoglobin from which fetal hemoglobin is ordinarily obtained has been shown by chromatographic procedures[11] to contain not only fetal and adult hemoglobin as has long been known but also other components. In order to have a definite molecular species to study, the main component, termed F_{II}[11], has been isolated chromatographically and used in the present investigation.

Hemoglobin F_{II} was readily isolated in quantity by chromatographing 3 gm of cord hemoglobin on a 3.5 × 35-cm column of ion-exchange resin Amberlite IRC-50 at 6°C with Developer No. 4.[11] When the front of F_{II} reached the bottom of the column, the upper portion that contained the hemoglobin A was removed. By warming the remaining column to 28°C, the F_{II} was eluted from the column in a few hundred ml of developer. It was concentrated for further experiments by high speed centrifugation[12] and then dialyzed free of salt.

The α and γ chains were separated chromatographically by the method of Wilson and Smith[13] as described by them and, in one instance, with the modification of an interrupted gradient as described by Chernoff and Liu.[14]

Enzymatic hydrolyses of individual chains or of heat-denatured hemoglobin F_{II} with trypsin or chymotrypsin were maintained at constant pH by using a Radiometer automatic titrator as a pH-stat. The conditions of hydrolysis were: pH 8.0, 40°C., 0.01 *M* calcium ion, an enzyme: substrate ratio of 1 to 2 per cent by weight, and 90 minutes of hydrolysis with trypsin or 180 minutes with chymotrypsin. After hydrolysis the pH was brought to 6.5 and any insoluble material was removed by centrifuging.

The initial separation of peptides was carried out by column chromatography. The peptides from about 0.5 gm of individual chains or one gm of hemoglobin were chromatographed on a 3.5 × 100-cm column of Dowex 50-X2 ion-exchange resin with a gradient in pH and pyridine concentration The gradient was produced after the principle of the device described by Bock and Ling[15]

in their Figure 6. Three containers of equal diameter were connected in series; in the mixer (the container from which developer went directly to the chromatogram) were placed 4.3 liters of pH 3.1 pyridine-acetic acid buffer that was 0.2 *N* pyridine and in each of the other containers were placed 4.3 liters of pH 5.0 pyridine-acetic acid buffer that was 2 *N* pyridine. A flow-rate of 60 ml per hour was maintained with a Milton Roy Minipump (Milton Roy Co., Philadelphia, Pa.). The ninhydrin procedure[16] was applied to 0.5-ml portions of alternate fractions in order to evaluate the progress of the chromatogram. Fractions which were thus shown to contain peptides were pooled and the solvent was removed largely in a rotary evaporator and finally in a stream of air at 40°C. The homogeneity of the zones thus isolated was investigated by paper chromatography and paper electrophoresis. As necessary, further separation was made by paper chromatography, paper electrophoresis, or column chromatography on Dowex 1.[17]

The amino acid composition of hemoglobin preparations and of purified peptides was determined chromatographically with a Spinco Automatic Amino Acid Analyzer, Model 120. Hydrolysates were prepared by heating in doubly glass-distilled 6 *N* hydrochloric acid at 110°C. for 22 or 24 hours (or for 70 hours also in the case of hydrolysates of hemoglobin) in evacuated sealed tubes. When applicable, hydrolyses were also made with leucine aminopeptidase[18] (LAP) at 40° and pH 8.5 for 16 hours.

The sequence of amino acids in a peptide was determined by the application of a variety of procedures. These procedures included the determination of the N-terminal amino acid by the DNP-method, stepwise degradation by the Edman procedure in a modified form[19] of the paper-strip method, and partial hydrolysis by chemicals or enzymes followed by separation and identification of the fragments.

Results.—Amino acid composition of hemoglobin F_{II} and of the γ chains: The amino acid composition of hemoglobin F_{II} and of the γ chains is presented in Table 1. These data were obtained from two different preparations of hemoglobin F_{II}. A sample from each preparation was hydrolyzed for 22 and 70 hours. The first quantity stated under each time of hydrolysis is from the first sample. The values for serine and threonine after correction for destruction during hydrolysis disagree appreciably. The behavior of cystine is unexpected. If the weight of the heme

TABLE 1

AMINO ACID COMPOSITION OF HEMOGLOBIN F_{II} AND OF THE γ CHAINS

	Gm Amino Acid per 100 gm Protein				Residues	Residues				
Amino acid	22-hr. hydrolysis		70-hr. hydrolysis		Residues[a] 66,000 M.W.	$1\alpha + 1\gamma$[b]	αA chain[c]	γ chain by difference	γ chain[d] from γ_4	γ chain from lit.[e]
Ala	9.09,	8.31	8.78,	8.48	64.2	32	21	11	11.6	13
Arg	3.25,	3.02	3.12,	3.09	11.8	6	3	3	3.3	3
Asp	10.48,	9.73	10.10,	10.01	50.0	25	12	13	12.9	14
Cys/2	0.70,	0.50	0.53,	0.72	3.9	2	1	1	1.1	
Glu	7.99,	7.37	7.68,	7.58	34.3	17	5	12	12.0	12
Gly	4.59,	4.27	4.46,	4.34	38.8	19	7	12	12.2	13
His	8.13,	7.83	7.99,	7.99	34.0	17	10	7	6.7	6
Ileu	1.54,	1.44	1.52,	1.51	7.6	4	0	4	3.7	4
Leu	14.36,	13.35	13.94,	13.79	69.8	35	17–18	17–18	16.3	19–20
Lys	10.15,	10.10	10.04,	10.28	45.8	23	11	12	11.0	12
Met	1.84,	1.73	1.87,	1.74	7.9	4	2	2	1.9	2
Phe	7.74,	7.18	7.46,	7.18	29.6	15	7	8	7.8	8
Pro	4.06,	3.92	4.02,	3.91	22.8	11–12	8	3–4	4.5	4
Ser	6.19,	5.85	4.57,	4.53	43.6 40.6	20–22	11	9–11	9.9	9
Thr	6.71,	6.19	5.77,	5.69	39.6 35.6	18–20	9	9–11	9.5	10
Try							1	4[f]		
Tyr	2.57,	2.75	2.28,	2.41	10.2	5	3	2	1.9	3
Val	9.45,	8.63	9.31,	9.02	51.3	26	13	13	12.6	14

[a] Average of all values except threonine, serine, and tyrosine which are corrected for hydrolytic destruction, and cystine/2 for which only the two highest values are used.
[b] Preceding column divided by 2.
[c] From data of ref. 20.
[d] Values for γ_4 molecule divided by 4 (ref. 21).
[e] Based on ref. 22 by difference.
[f] Tentative estimate from spectrophotometric data

groups and the destruction of serine, threonine, tyrosine, and tryptophan during hydrolysis are taken into account, essentially all of the weight that was taken for analyses is accounted for. Previous analyses of "fetal" hemoglobin[22] have all been performed on material that must have been heterogeneous to some degree. The analyses here presented have been made on preparations from which the other obvious components of cord blood hemoglobin have been removed. The results of the present analyses in general are in good agreement with the average of the sometimes disparate quantities previously reported.[22] If it is assumed that the α^A and α^F chains are identical, the amino acid composition of the γ chain can be calculated. An amino acid analysis of the separated γ chains has not been made because N-terminal analyses suggest that our best preparations contain 15 to 25 per cent of α chains.

Initial chromatographic separation of tryptic and chymotryptic peptides: The chromatographic separation of the soluble tryptic peptides in a hydrolysate of hemoglobin F_{II} is depicted in Figure 1, in which the optical density after reaction

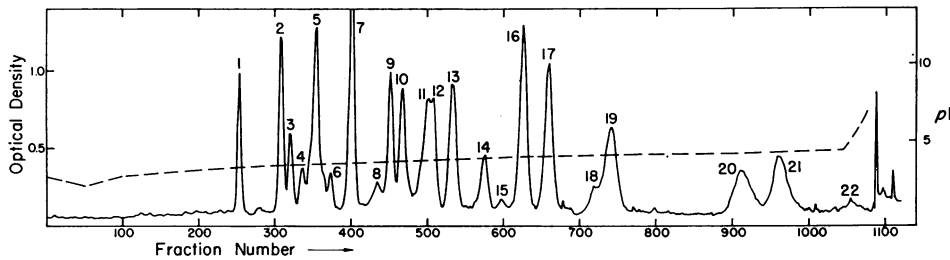

FIG. 1.—Separation of peptides from a tryptic hydrolysate of hemoglobin F_{II} by chromatography on a column of Dowex 50-X2 with pyridine-acetic acid developers. At Fraction No. 985, elution was begun with 2 *N* sodium hydroxide.

with ninhydrin is plotted against the fraction number of the 10-ml fractions. The pH gradient is also shown. The peptides in the peaks of this chromatogram are derived from both the α and the γ chains, but the chain of origin is known from similar chromatograms of hydrolysates of the individual chains. In this chromatogram, the peaks that are numbered 1, 2, 3, 5, 9, 10, 11, 12, 13, 16, 18, 19, and 21 contain peptides that derive from the γ chain, and those numbered 2, 3, 5, 6, 7, 11, 13, 14, 16, 17, 19, 20, and 21 contain peptides from the α chain.

The chromatographic isolation of the peptides in a chymotryptic hydrolysate of γ chains is presented in Figure 2. The chromatographic conditions were identical in the chromatograms that are shown in Figures 1 and 2, but the amount of sample was different. The ninhydrin reaction was used only roughly quantitatively to detect the zones; comparison of the sizes of the peaks is indicative of the relative amount only in a given chromatogram.

The peptides from both hydrolysates were separated and purified by paper chromatography, paper electrophoresis, or column chromatography as necessary.

Sequence of the tryptic peptides of the γ chain: After N-terminal determination and amino acid analysis had demonstrated the purity of a given peptide, the sequence was determined by the application of such methods as were suggested by the amino acid composition. The sequence of amino acid residues in the soluble tryptic peptides of the γ chain is presented in Table 2.

It is known that the γ chain is N-terminal in the sequence gly-his-phe-.[9] The

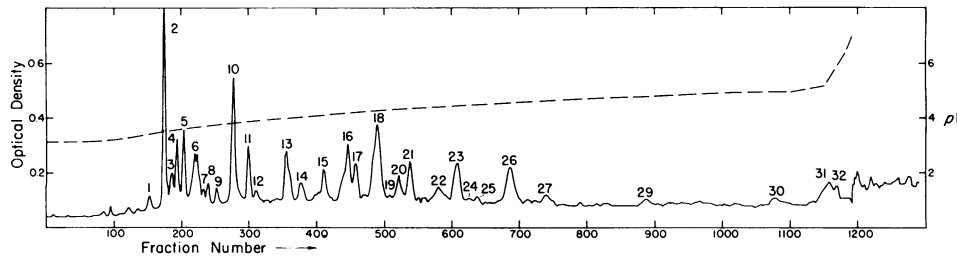

FIG. 2.—Separation of peptides from a chymotryptic hydrolysate of γ chains by chromatography on a column of Dowex 50-X2 with pyridine-acetic acid developers. At Fraction No. 1098, elution was begun with 2 *N* sodium hydroxide.

sole peptide that contains this sequence is that from Zone 19. In this peptide, which contains two lysyl residues, the bond between lysine and alanine apparently is hydrolyzed with difficulty. The N-terminal 8-residue portion has not been isolated from the hydrolysate and the C-terminal 9-residue portion has been isolated only in very small amount from Zone 13. The peptides in Zones 13 and 19 contain one residue each of leucine and isoleucine. It is still undetermined, however, which one occupies the indicated positions.

TABLE 2

AMINO ACID SEQUENCE IN SOLUBLE TRYPTIC PEPTIDES OF THE γ CHAIN

Zone no.	Peptide
1	val-aspNH_2-val-glu-asp-ala-gly-gly-glu-thr-leu-gly-arg
2	val-leu-thr-ser-leu-gly-asp-ala-ileu-lys
3	lysine
5	met-val-thr-gly-val-(ala,ser,ala,leu,ser,ser)arg
9	val-lys
10	his-leu-asp-asp-leu-lys
11	lys-(asp,thr,ser,gly,ala,val,ileu,leu_2)lys
12	tyr-his
13	ala-thr- (leu or ileu) -thr-ser- (ileu or leu) -try-gly-lys
16	ala-his-gly-lys
18	leu-leu-val-val-tyr-pro-try-(thr,glu)-arg
19	gly-his-phe-thr-glu-glu-asp-lys-ala-thr- (leu or ileu) -thr-ser- (ileu or leu) -try-gly-lys
21	ala-his-gly-lys-lys

The amino acid composition of the peptide in Zone 1 differs only slightly from a peptide of the β chains: in the γ chain peptide, one threonyl residue replaces a valyl residue. Hunt and Ingram[23] in a study of hemoglobin E have determined the sequence of the β chain peptide. Although the β and γ chain peptides differ in gross composition by a single amino acid residue, they differ in four ways in sequence, as the following comparison shows:

β chain: val-aspNH_2-val-asp-glu-val-gly-gly-glu-ala-leu-gly-arg

γ chain: val-aspNH_2-val-*glu-asp-ala*-gly-gly-glu-*thr*-leu-gly-arg.

The position of the amide group in both peptides was determined in the course of the present investigation.

From a chymotryptic hydrolysate of the γ chains (Zone 10, Figure 2), the peptide gly-lys-val-aspNH_2-val-glu(asp,ala,gly,gly,glu,thr,leu) has been isolated. This peptide establishes the connection between the N-terminal peptide in Zone 19 and the peptide in Zone 1.

The peptide in Zone 18 has been isolated only in rather small amount. How-

ever, it probably follows the peptide of Zone 1 in the sequence of the γ chains. The conclusion is derived from the isolation of gly-arg-leu-leu from Zone 23 of the chymotryptic hydrolysate.

These three tryptic peptides in the order 19, 1, and 18 provide the sequence of the first 40 residues from the N-terminus of the γ chain. The remaining tryptic peptides in Table 2 account for 37 additional residues whose placement in the chain can be inferred only indirectly at present. Because the amino acid analysis of γ chains as presented in Table 1 suggests a content of about 145 residues, the soluble tryptic peptides account for little more than half of the chain. Amino acid analysis of that portion of the γ chain which is insoluble at pH 6.5 after tryptic hydrolysis (the so-called "core") is in general agreement with the number and kind of residues as yet unaccounted for. The position of the remaining soluble tryptic peptides in the sequence of the γ chain is best considered in relation to information that is now available about the β chain.

Table 3 presents the sequence of soluble tryptic peptides of the β chain. The

TABLE 3

AMINO ACID SEQUENCE IN SOLUBLE TRYPTIC PEPTIDES OF THE β CHAIN

Zone no.	Peptide
1	val-aspNH$_2$-val-asp-glu-val-gly-gly-glu-ala-leu-gly-arg
2 + 3	phe-phe-glu-ser-phe-gly-asp-leu-ser-(ala,val,pro$_2$,asp$_2$,thr,met,gly)-lys
3	glu-phe-thr-pro-(tyr,val,ala$_2$,pro,glu$_2$)lys
4	lysine
8	val-val-ala-gly-val-ala-aspNH$_2$-ala-leu-ala-his-lys
9	val-lys
9	val-leu-gly-ala-phe-ser-asp-gly-leu-ala-(his,asp$_2$,leu$_2$)-lys
10	val-his-leu-thr-pro-glu-glu-lys
12	tyr-his
13	ser-ala-val-thr-ala-(leu,try,gly)-lys
15	val-leu-gly-ala-phe(ser,asp,gly,leu,ala,his,asp$_2$,leu$_2$)lys$_2$
16	ala-his-gly-lys
18	leu-(leu,val$_2$,pro,thr,gly,tyr,try)arg
20	val-val-(ala$_4$,asp,gly,his,leu,val,lys,tyr,his)
22	ala-his-gly-lys-lys

peptides were isolated by the methods that were applied also to the γ chain. The numbering of the zones coincides roughly with that of the zones in Figure 1.

Braunitzer and co-workers[24] have presented a partial formula of the β chain and have stressed[25] the homology between the α and β chains. The N-terminal sequences of the β and γ chains may be compared as follows:

10

β- val-his-leu-thr-pro-glu-glu-lys-ser-ala- val-thr-ala-
leu

γ- gly-his-phe-thr-glu-glu-asp-lys-ala-thr- *or* -thr-ser-
ileu

20

β- -leu-try-gly-lys-val-aspNH$_2$-val-asp-glu-val-gly-gly-glu-
ileu

γ- - *or* -try-gly-lys-val-aspNH$_2$-val-glu-asp-ala-gly-gly-glu-
leu

30 40

β- -ala-leu-gly-arg-leu-leu(thr,glu,pro,val,val,try,tyr)-arg-phe-etc.

γ- -thr-leu-gly-arg-leu-leu-val-val-tyr-pro-try(thr,glu)-arg-

The general homology of the two chains is apparent. Although the basic amino acids fall in identical positions, the other residues are subject to considerable substitution and rearrangement. Nevertheless, other peptides of the γ chain may be compared to those of the β chain on this basis. Clearly, val-lys, tyr-his, and ala-his-gly-lys-lys are present in the hydrolysate of both chains. The decapeptide of Zone 3 of the γ chains is N-terminal in val-leu- as is the hexadecapeptide of Zone 9 of the β chains, but other identity extends only to aspartic acid in the seventh position from the N-terminus. On the other hand, the C-terminal sequence of the β peptide is[24] -his-leu-asp-asp-leu-lys, a sequence identical with that of the peptide in Zone 10 of the γ chain chromatogram. One is tempted to suggest that the γ chain peptides in Zones 3 and 10 are adjacent in the chain. Braunitzer et al.[24] report a sequence from the 56th through the 85th residue of the β chain which may be fitted with sequences from the γ chain as follows:

	60	70
β-	-gly-asp-------pro-lys-val-lys-ala-his-gly-lys-lys-val-leu-gly-ala-phe-ser-	
γ-	-gly-aspNH_2-pro-lys-val-lys-ala-his-gly-lys-lys-val-leu-thr-ser-leu-gly-	
	80	
β-	-asp-gly-leu--ala-his-leu-asp-asp-leu-lys-gly-thr-phe-	
γ-	-asp-ala-ileu-lys-his-leu-asp-asp-leu-lys-gly-thr-phe-.	

Although their structures are incompletely determined, the isolation of certain peptides from the γ chain lends credence to this arrangement. The peptides gly-aspNH_2-(pro,val,lys_2) and gly(val,leu,lys_2) from the chymotryptic hydrolysate suggest the sequences from the 56th to the 61st, and from the 64th to the 68th residues. The link between the 66th and 67th also follows from the tryptic peptide of Zone 11. It differs in amino acid composition from that of Zone 2 (67th to 76th residues) only in the presence of an additional lysine which is known to be N-terminal. The final three residues of the β sequence are derived from the core: this same sequence may be isolated from a chymotryptic digest of the γ core and, in addition, the sequence lys-gly-thr-phe from a chymotryptic hydrolysate of the γ chains. (*Note added in proof:* More recently isolated peptides confirm the sequence suggested here for these 30 residues of the γ chain.)

The sequence tyr-his no doubt is derived from the C-terminus of the γ chain. The work of Naughton[26] suggests this as well as the fact that free histidine may be detected in a chymotryptic hydrolysate of the γ chain (Zone 11, Fig. 2). Tyr-his is also C-terminal in the β chain.[24]

There is a meager resemblance in the γ-chain peptide of Zone 6 and the β-chain peptide of Zone 8 as follows:

β val-val-ala-gly-val-ala-aspNH_2-ala-leu-ala-his-lys
γ met-val-thr-gly-val-(ala,ser,ala,leu,ser,ser)-arg.

The sequence within the parentheses is probable although not yet confirmed. A β chain peptide of this composition plus tyr-his make up the sequence of the last 14 residues from the C-terminus of the β chain according to Braunitzer *et al.*[24] Perhaps this γ chain peptide plus tyr-his also is C-terminal in the γ chain. It is of interest to note that our sequence for this β chain peptide differs much from that of Braunitzer *et al.*, who report it as val-val-val-ala-ala-gly-ala-asp-leu-ala-his-lys.

If the above conclusions are correct and the suggested homology exists between

the β and γ chains, then the sequences in the γ chain are known between residues 1 to 40, approximately 56 to 85, and approximately 137 to 150 and unknown sequences exist between residues 41 to 55 and 86 to 136. In the β chain, residues 41 to 59 are taken by the peptide in Zones 2 and 3 (Table 3) but no similar peptide has yet been detected in the soluble tryptic peptides of the γ chain. Presumably, it is present in the insoluble fraction. The presence of such a peptide in positions 41 to 59 of the γ chain is suggested by the isolation of the peptide gly-aspNH_2(pro, val,lys_2) from a chymotryptic hydrolysate which, as noted above, may coincide with positions 56 to 61 of the β chain. Certain sequences of yet undetermined position are known through the isolation of peptides from a chymotryptic hydrolysate of the γ core or the γ chain: these sequences are asp-ser-phe, ala-ser-try, ala-ileu-his-phe, and ala-gluNH_2-leu-ser-glu-leu.

Sequences in the α chains of hemoglobin A and F: The investigation of the γ chains also provides opportunity for the isolation of the α chain of hemoglobin F and of the peptides derived therefrom by tryptic hydrolysis. One can, therefore, begin to determine on a far more exact basis whether the α chains of hemoglobins A and F are identical. The tryptic peptides of the α chains of hemoglobin A are listed in Table 4. It is of interest that sequences in peptides of Zones 11 and 13

TABLE 4

AMINO ACID SEQUENCE IN SOLUBLE TRYPTIC PEPTIDES OF THE α^A CHAIN

Zone no.	Peptide
2	val-leu-ser-pro-ala-asp-lys
3	val-asp-pro-val-aspNH_2-phe-lys
3	lys
5	val-ala-asp-ala-(his,thr,ser,ala,val,leu)(ala_4,asp_5,val,leu_3,ser,pro,his_2,met)-lys
6	thr-aspNH_2-val-lys
7	val-leu-ser-pro-ala-asp-lys-thr-aspNH_2-val-lys
11	met-phe-leu-ser-phe-pro-thr-thr-lys
13	val-gly-ala-(his,ala,gly,glu)tyr-gly-ala-glu-ala-leu-glu-arg
14	leu-arg
16	gly-his-gly-lys
17	ala-ala-try-gly-lys
19	tyr-arg
20	thr-tyr-phe-pro-his-phe-asp-leu-ser-ser-gly-ala-(gluNH_2,his,val)-lys
21	gly-his-gly-lys-lys

are at variance with those reported by Braunitzer *et al.*[20] In the 6th to 8th positions of the peptide in Zone 11, they give the sequence as -thr-pro-thr- instead of -pro-thr-thr-; Hill and Konigsberg[27] agree with our sequence. In the 9th to 12th positions of the peptide in Zone 13, Braunitzer *et al.* give the sequence -gly-glu-ala-ala- instead of -gly-ala-glu-ala.

Only a limited investigation of the peptides from the α^F chains has yet been made. In particular, the peptides in Zones 7, 13, 17, and 21 have been studied. The amino acid composition is identical with that of similar peptides of α^A chains. The sequence is identical in the peptide from Zone 17 and also in the other peptides to the extent that it has been studied. These investigations are continuing.

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THERMODYNAMIC POTENTIALS OF SYMMETRICAL ELECTROLYTES

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The classical treatment of the thermodynamic properties of electrolytic solutions by Debye and Hückel[1] leads to the now familiar limiting square-root law which has been amply confirmed by experiment. At nonzero concentrations, however, positive deviations from the limiting law appear; there has been considerable speculation about their physical origin since 1923. The purpose of this communication is to show, by means of a more detailed study of the ionic potentials, that the properties of electrolytic solutions, specific for each electrolyte at nonzero concentrations, do not appear to be derivable by extension of the limiting solution of the Poisson-Boltzmann equation to higher concentrations, but rather must be sought in the projection of a theory of fused salts into and through the region of concentrated solutions.

We shall use the conventional model of charged spheres of diameter a in a dielectric continuum to represent the solution. Let there be n_i ions of charge ϵ_i in a