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The Enzymes of the Galactose Operon in *Escherichia coli*

III. THE SIZE AND COMPOSITION OF GALACTOKINASE*

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SUMMARY

The structure of the galactokinase in *Escherichia coli* has been analyzed with the aim of defining the size of its structural gene. To that end the molecular weight, amino acid composition, number of tryptic peptides, and amino-terminal residues of this protein were determined. The results indicate that this galactokinase consists of one polypeptide chain containing 368 residues, and hence that its structural gene contains about 1100 base pairs.

One of the conclusions that can be realized from the results of the previous two articles in this series (1, 2) is that the amino acid residues of uridine diphosphogalactose 4-epimerase are arranged into two equivalent sequences. As each sequence contains about 360 residues and is not divisible into multiple, identical subsequences of significant size, the DNA specifying the primary structure of this epimerase contains about 1080 base pairs.

In this article, we extend our analysis to the structure of galactokinase (3) with the same aim of defining the size of its structural gene (or genes). This analysis includes determinations of (*a*) molecular weight, (*b*) amino acid composition, (*c*) tryptic peptides, and (*d*) amino-terminal residues of this enzyme. The data from these determinations consistently indicate that galactokinase consists of one polypeptide chain containing 368 amino acid residues. We therefore infer that the structural gene for galactokinase is of approximately the same size as that for UDP-galactose 4-epimerase, each containing about 1100 base pairs. As recent experiments (4) on the remaining enzyme of the galactose operon, galactose-1-P uridyl transferase, yield preliminary evidence that its structural gene is of this same size, it appears that the galactose operon in *Escherichia coli* is divided into three segments of equivalent size, each segment specifying the structure of one enzyme.

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EXPERIMENTAL PROCEDURE

Materials

E. coli galactokinase was purified according to our procedure (3). The purified preparations had the maximum specific activities (4.3×10^3 units per mg) previously reported (3). Trypsin, obtained from Worthington, had been crystallized five times. Galactose-1-^{14}C, obtained from Volk Radiochemical Corporation, was further purified by chromatography (3). Urea was deionized by the procedure of Benesch, Lardy, and Benesch (5) prior to use. Other reagents were commercial preparations.

Methods

Assays—The activities of galactokinase and UDP-galactose 4-epimerase were determined by the methods we have described (3). One unit of either enzyme is that amount which catalyzes the formation of 1 μmole of product per hour under the conditions of each assay. Protein was measured according to the method of Lowry *et al.* (6), with crystalline bovine serum albumin as standard.

Chemical and Physical Analyses of Galactokinase—Amino acids were determined by the methods described in the preceding article (2), except that protein samples were hydrolyzed in 6 N HCl at 110° for four different time periods (20, 48, 70, and 96 hours) rather than three, and free sulfhydryl groups were determined only by Method A (2).

Amino-terminal residues were determined as before (2), except that the concentration of urea used during the carbamylation was 8.0 M rather than 4.0 M, and the duration of this reaction was 16 rather than 6 hours.

Galactokinase was modified prior to its tryptic hydrolysis by oxidation with performic acid. The protein was dialyzed against water and then lyophilized; 5.8 mg were dissolved in 0.50 ml of 98% formic acid, and 0.50 ml of performic acid was then added. Performic acid was prepared by mixing 1.0 ml of 30% hydrogen peroxide with 9.0 ml of 98% formic acid and allowing this mixture to stand for 1 hour at 25°. Oxidation of the protein proceeded at 0° for 2½ hours, after which 10 ml of water were added and the mixture was lyophilized. The resulting solids were taken up in 10 ml of water and the lyophilization was repeated. The tryptic peptides were formed from this oxidized protein, separated on filter paper, and allowed to react with

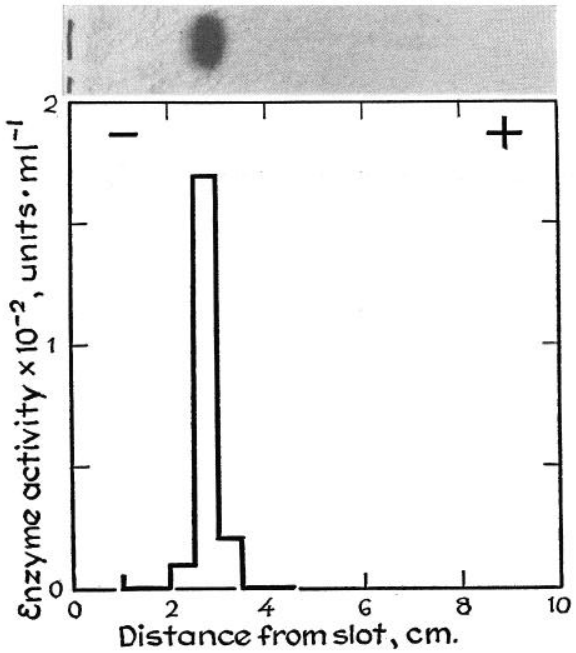

FIG. 1. Starch gel electrophoresis of purified galactokinase. Gels were made in 1.0 mM EDTA, 1.0 mM mercaptoacetic acid, and 0.05 M Tris-citrate buffer, pH 7.5. Electrophoresis was carried out and the gels were sectioned and stained as described in the legend to Fig. 1 of Reference 1, with the exception that the electrodes were immersed in reservoirs containing 0.02 M potassium phosphate buffer, pH 7.5. The *curve* represents the galactokinase activity eluted from 0.5-cm sections of the unstained half of the gel in 0.5 ml of 0.01 M potassium phosphate buffer, pH 6.5, after an 18-hour exposure at 0–4°.

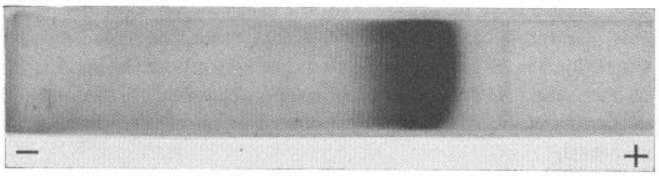

FIG. 2. Polyacrylamide gel electrophoresis of purified galactokinase. The method is that described by Ornstein (8) and Davis (9). The buffers in the stacking gel, main gel, and reservoir were 0.062 M Tris-HCl (pH 6.7), 0.038 M Tris-HCl (pH 8.9), and 0.005 M Tris-glycine (pH 9.5), respectively, the acrylamide concentration of the main gel being 7.5%. Galactokinase (70 μg in 10 μl of 0.01 M potassium phosphate, pH 6.5) was mixed with 10 μl of 30% sucrose and layered on the gel. After a 30-min exposure to a voltage gradient of 40 volts per cm at room temperature, the gel was stained with Amido black to yield the above photograph.

tert-butyl hypochlorite, ninhydrin, or an arginine-specific reagent according to the procedures described in the preceding article (2).

The sedimentation coefficient and molecular weight of galactokinase were determined in the Spinco model E ultracentrifuge according to the procedures described in the first article of this series (1). With regard to molecular weight, all determinations resulted from sedimentation to equilibrium by the method described for Fig. 6*b* of Reference 1, in which $(1/r) \cdot (dc/dr)$ is plotted against the protein concentration, c, at equilibrium. No significant curvature was observed in any of these plots, and the agreement of the points to the straight line calculated by the method of least squares was equivalent to that exhibited in Fig. 6*b* of Reference 1. Equilibrium was established within 20 hours of sedimentation of a 3-mm liquid column in a 12-mm, 2.5°, double sector cell at the speeds indicated in Table II. Except where noted otherwise the solvent for all the sedimentation experiments was 0.10 M potassium chloride-0.01 M potassium phosphate buffer, pH 7.0. The partial specific volume of galactokinase, computed from the amino acid composition given in Table III, was 0.735 ml per g (7).

RESULTS

Homogeneity of Purified Galactokinase

The procedure for the purification of galactokinase from *E. coli* was described (3) without presenting evidence regarding the purity of the resulting preparation. This evidence indicates that the purified preparations are highly homogeneous, and is given here because it is important to the interpretation of the results given in the succeeding sections.

Chromatography of the enzyme preparation on a column of DEAE-Sephadex is the last step in the purification procedure. The purified galactokinase (Fraction VIII of Reference 3) represents 90% of the enzyme activity eluted from this column as a single peak. Within this region of the peak, the activity and protein distributions are sufficiently coincident to maintain the specific activity within ±4% of the value for Fraction VIII (4.3×10^3 units per mg).

The results of electrophoresis of Fraction VIII on starch and polyacrylamide gels are shown in Figs. 1 and 2, respectively. Except for the very faint additional zone found in the polyacrylamide gel, these tests reveal the presence of only one protein in Fraction VIII and indicate that it is galactokinase.

Both boundary and zone sedimentation of Fraction VIII give further evidence of its homogeneity. The schlieren pattern resulting from the single sedimenting boundary is shown in Fig. 3. There is a slight asymmetry in the boundary peak which has not been analyzed as to whether it represents a low level (≤5%) of contaminants or of aggregation. However, the results shown in Fig. 4 for the zone sedimentation of Fraction VIII through a sucrose gradient do not indicate the existence of significant amounts of contaminating proteins; the distributions of protein and of galactokinase activity are unimodal and coincident within

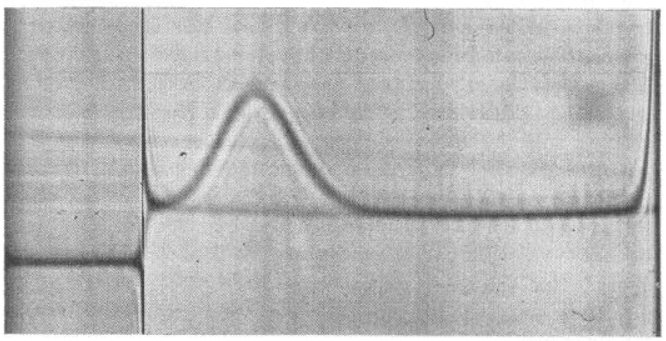

FIG. 3. Boundary sedimentation of purified galactokinase. This schlieren pattern was photographed at a phase angle of 75°, 76 min after a speed of 52,640 rpm was reached. The initial protein concentration was 9.0 mg per ml, and the temperature was 7.2°.

the accuracy of the assays. Hence, it appears likely that the asymmetry in the boundary peak results from a characteristic of galactokinase itself.

Physical and Chemical Properties of Galactokinase

Sedimentation Coefficient—The sedimentation coefficients of galactokinase calculated from schlieren patterns observed at 52,640 rpm under various conditions of concentration and temperature are given in Table I. The effect of temperature on the sedimentation velocity appears to be attributable to its effect on the viscosity and density of the solvent, since the values of $s_{20,w}$ are the same whether the measurement is made at 7.2° or 23.9°. The magnitude of the effect of protein concentration on the value of $s_{20,w}$ appears to be small, but the data are insufficient for its quantitative determination. We take the mean of the four results given in Table I, or 3.54 S, as the value for the $s_{20,w}$ of galactokinase in the concentration range, 2 to 10 mg per ml.

The sedimentation coefficient can also be calculated by the method of Martin and Ames (10) from the data obtained by zone sedimentation of galactokinase through a sucrose gradient (Fig. 4). This consists of measuring the distance sedimented by the zone of galactokinase relative to that sedimented by a reference protein of known sedimentation coefficient. We used the UDP-galactose 4-epimerase of *E. coli* as the reference protein ($s_{20,w}$ 5.02 S; (1)), and obtained a value of 3.52 S for the $s_{20,w}$ of galactokinase, in good agreement with the above mean.

Molecular Weight—The molecular weight of galactokinase was determined by sedimentation to equilibrium at 14° in 0.10 M KCl-0.01 M potassium phosphate buffer, pH 6.5, according to the procedures given under "Methods." The results and other conditions of centrifugation are given in Table II. There appears to be some decrease in the apparent value of the molecular weight with decrease in the protein concentration. When the apparent molecular weights are plotted against the initial concentrations of protein, linear extrapolation to zero concentration yields a value of 4.0×10^4 for the molecular weight of galactokinase; this is the value adopted here.

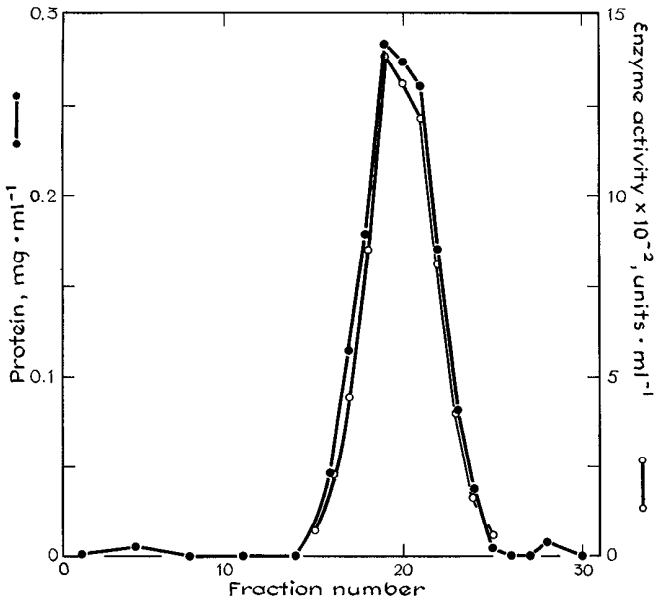

FIG. 4. Zone sedimentation of purified galactokinase. The protein (0.2 ml at a concentration of 1.7 mg per ml) was layered on top of a 5 to 20% constant sucrose gradient in 1.0 mM mercaptoacetic acid-0.01 M potassium phosphate buffer, pH 6.4. The total volume of 4.7 ml was centrifuged in a Spinco SW 39 rotor for 22 hours at 3.8×10^4 rpm and 6°; 31 fractions of equal volume were collected from the bottom of the tube and assayed for protein and galactokinase activity to yield the above results.

TABLE I

Sedimentation coefficient of galactokinase

Initial protein concentration	Temperature of rotor	$s_{20,w}$
mg/ml		*S*
9.0	7.2°	3.47
4.5	7.2	3.47
4.5	23.9	3.47
2.3	7.2	3.75

TABLE II

Molecular weight of galactokinase

Experiment	Initial concentration	Angular velocity	Molecular weight × 10^{-4}
	mg/ml	*rpm*	
1	4.0	12,590	4.3
2	4.0	24,630	4.3
3	1.2	20,410	4.2
4	1.0	20,410	4.0

Molecular Weight in 4 M Guanidine Hydrochloride—In seeking to answer the question of how many polypeptide chains contribute to this molecular weight of 4.0×10^4, we have determined the effect of guanidine hydrochloride on its value. Purified galactokinase was dissolved in 4 M guanidine hydrochloride, 0.1 M KCl and 0.01 M potassium phosphate buffer, pH 6.5, to a concentration of 1.0 mg per ml. A second, control solution, identical with the first except for the absence of guanidine hydrochloride, was also prepared. Both were centrifuged to equilibrium at 14.5° and 23,150 rpm, and the resulting schlieren patterns were analyzed as described above. In the case of the guanidine hydrochloride solution the density of this solvent was determined directly, but the partial specific volume ($\bar{v}$) of galactokinase was assumed to be unaffected by the guanidine hydrochloride. The ratio of the calculated molecular weight in the presence of guanidine hydrochloride to that in its absence was 1.01.

We draw two conclusions from this result. First, galactokinase does not contain multiple subunits which dissociate in 4 M guanidine hydrochloride. Second, galactokinase exhibits little, if any, preferential interaction between water and guanidine hydrochloride. If such preferential interaction existed, the assumption that $\bar{v}$ was unaffected by the presence of guanidine hydrochloride would be wrong and the above ratio of calculated molecular weights would differ, correspondingly, from unity. The argument for both conclusions is saved from circularity by the improbability of the extraordinarily large preferential interaction that would have to be assumed were galactokinase to be dissociable into two or more equivalent subunits in 4 M guanidine hydrochloride. In any case, the independent evidence given in succeeding sections that this enzyme consists of only one polypeptide chain confirms the first conclusion and thereby allows the second.

Amino Acid Composition—The frequencies of amino acid

TABLE III

Amino acid composition of galactokinase

Amino acid	Residues per molecule[a]	Standard deviation
Aspartic acid	36.9	0.3
Threonine	12.2[b]	
Serine	13.7[b]	
Glutamic acid	51.2	0.5
Proline	14.4	1.0
Glycine	33.0	0.3
Alanine	39.4	0.4
Valine	36.1[c]	0.4
Methionine	10.0[d]	0.3
Isoleucine	22.0[e]	0.6
Leucine	30.4[e]	0.7
Tyrosine	9.4	0.4
Phenylalanine	12.8	0.2
Lysine	15.8	0.2
Histidine	6.6	0.1
Arginine	13.1	0.5
Half-cystine	10.3[e]	
—SH groups	7.9[e]	
Tryptophan	1.9[f]	
Total	368[g]	

[a] The procedures are specified under "Methods." Except where noted, each value is the mean of four analyses, *i.e.* of the 20-, 48-, 70-, and 96-hour digests.

[b] Obtained by extrapolation to zero time of hydrolysis.

[c] The value for the 20-hour digest was not included in the mean.

[d] Since the values for methionine determined before and after performic acid oxidation were not significantly different, all values are included in this average.

[e] Average of two analyses.

[f] Single determination.

[g] This is the sum of the closest integral value for each residue number.

TABLE IV

Amino-terminal residues of galactokinase

The values are calculated on the basis of a molecular weight of 40,000 and the reported yields for each amino acid (11): serine, 20%; aspartic acid, 99%; glutamic acid, 60%; and glycine, 98%. In addition, blank values determined with the uncarbamylated protein (11) were subtracted prior to correction for the yields, such blanks being ≤ 0.04 residue per molecule.

Amino acid	Residues per molecule of galactokinase			
	Preparation 1		Preparation 2	Average
	Sample 1	Sample 2		
Serine	0.64	0.64	0.74	0.67
Aspartic acid	0.13	0.37	0.17	0.22
Glutamic acid	0.05	0.05	0.01	0.04
Glycine		0.02	0.10	0.06

residues in galactokinase are given in Table III as the number of residues per molecule of enzyme having a molecular weight 4.0×10^4. Were all polypeptide chains in galactokinase equivalent, the value for tryptophan would limit the maximum number of identical chains per molecule to two. Were it certain that the number of residues for the second and third least frequent amino acids, histidine and tyrosine, is odd (as indicated in Table III), this maximum could be reduced to one. However, a combined error in molecular weight and amino acid analysis sufficient to allow an even number of residues for each of these amino acids cannot be eliminated (*e.g.* were the calculated partial specific volume of galactokinase to be in error by 2%, the calculated molecular weight would suffer an error of 6%).

Tryptic Peptides—The maximum number of peptide bonds in a galactokinase molecule which can be hydrolyzed by trypsin is 29 ± 3. This is the sum of the lysine and arginine residues given

TABLE V

Amino acid residues in polypeptides derived from galactose operon

Amino acid	Residues per polypeptide derived from				
	Galactose operon			Precursor[a]	*E. coli* average[b]
	Kinase	Transferase[c]	Epimerase		
Asx	37	39	46	39	36
Thr	12	21	16	16	20
Ser	14	14	20	14	16
Glx	51	42	32	42	40
Pro	14	24	23	23	15
Gly	33	20	33	33	32
Ala	39	38	29	38	38
Val	36	24	31	31	26
Met	10	7	9	9	10
Ile	22	8	19	19	19
Leu	30	31	30	30	31
Tyr	9	10	14	10	10
Phe	13	15	10	13	12
Lys	16	14	15	15	23
His	7	13	10	10	7
Arg	13	17	13	13	19
Trp	2	11	6	6	
Cys	10		4		
Total	368	348 + Cys residues	360	361 + Cys residues	354 + Trp and Cys residues

[a] Each residue number in this column is either the intermediate of the values for the polypeptides of the kinase, transferase, and epimerase, or the number which is represented more than once among these three values.

[b] These residue numbers were calculated for a polypeptide having a molecular weight of 40,000 and the molar residue frequencies of the total protein in *E. coli*. The frequencies were the average of those measured by Sueoka (12) and given in Table II of his article. Tryptophan was not determined (the average frequency in the galactose enzymes was used to make the molecular weight equal 40,000). Sueoka's value for half-cystine yields two residues per polypeptide, but as this is only one-fourth the frequency found by Roberts *et al.* (13), it is not included in the table.

[c] The values for the transferase polypeptide were calculated from the data of Saito, Ozutsumi, and Kurahashi (4), assuming a molecular weight of 40,000 for the polypeptide. Our values differ from those calculated by Saito *et al.* (4) because of a trivial error in their calculation: they divided the mass contributed by each type of residue by the molecular weight of the amino acid, rather than by the molecular weight of the residue. They did not determine the half-cystine value.

in Table III, the range of values being given to account for a maximum error of 10%, combined from the measurements of molecular weight and amino acid frequencies. The maximum number of tryptic peptides resulting from tryptic hydrolysis of galactokinase is $1 + (29 \pm 3)/N$, where N represents the multiplicity of equivalent polypeptide chains making up the galactokinase molecule; for $N = 1$, the maximum number of tryptic peptides is 30 ± 3.

The procedures for the performic acid oxidation of galactokinase, its subsequent hydrolysis by trypsin, and the separation and staining of the resulting tryptic peptides on filter paper sheets are specified under "Methods." Four such two dimensional peptide maps were prepared, with the use of 2 mg of peptides per map. The positions of the peptides on two of these maps were revealed by reaction with *tert*-butyl hypochlorite, ninhydrin was used on the third map, and the fourth filter paper sheet was first exposed to the arginine-specific reagent and then to ninhydrin.

Comparison of all four sheets indicated 31 definite tryptic peptides, of which only 13 were stained with the arginine-specific reagent. Both values are consistent with a value of 1 for N, and are inconsistent with any other integer.

Amino-terminal Residues—The preceding evidence does not eliminate the possibility that two or more *different* polypeptide chains make up the galactokinase molecule, these chains being held in association by bonds that are not disrupted in guanidine hydrochloride. The number of polypeptide chains per molecule, whether identical or not, can be determined by end group analysis, providing of course that the ends are available to the reagent used in analysis. We have attempted to determine the number and kind of amino-terminal residues in galactokinase by the method of Stark and Smyth (11), in which these residues are carbamylated with cyanate (see "Methods"). The results from two independent preparations of purified galactokinase are given in Table IV, one of the two preparations having been analyzed twice.

Serine is the only amino acid recovered in which the calculated number of residues per galactokinase molecule approximates a nonzero integer; in this case, 1. We infer from these results that there is one amino-terminal serine residue per enzyme molecule and no other terminal residues with free amino groups. Subject to reservations of nonreactive, blocked amino-terminal residues, we conclude that galactokinase contains only one polypeptide chain.

Other reservations concerning this conclusion should be noted. Serine exhibits only a 20% recovery in this procedure (11), forcing the use of a large correction factor in arriving at the values given in Table IV. However, it is unlikely that an error in this correction factor could be sufficient to allow for either two or no amino-terminal serine residues (11).

The recovery of significant although variable amounts of aspartic acid is puzzling. We think it unlikely that it arises from a contaminating protein because of the rather stringent homogeneity tests that have been applied. If it arises from another type of polypeptide chain in galactokinase, then carbamylation of its amino-terminal aspartate residue must be difficult, since recovery of carbamylated aspartate is high (99%). The third possibility is that cleavage of the polypeptide chain adjacent to a nonterminal aspartate residue occurs with appreciable frequency during the carbamylation. Although there is no direct evidence or example for this, we have observed that the carbamylated protein yields at least six protein zones upon electrophoresis in polyacrylamide gel under conditions where the uncarbamylated protein yields a single zone. We have not determined whether the extra zones arise from cleavage of the polypeptide or from other causes, such as incomplete carbamylation or aggregation.

DISCUSSION

The arguments leading to the conclusion that galactokinase consists of one polypeptide chain containing 368 amino acid residues have been given in the preceding sections. This is the simplest structure indicated by the results. More complex structures which consist of two or more different polypeptides containing a total of 368 residues are allowed by the data only if two *ad hoc* assumptions are made: (*a*) all but one of the polypeptides contain blocked amino-terminal residues, and (*b*) the bonds connecting the polypeptides are not broken in guanidine hydrochloride.

The proposed structure containing but one polypeptide and the unlikely structures containing different polypeptides all demand that each of the 368 residues be specified by the structural gene (or genes) for galactokinase. Hence the segment of DNA determining the primary structure of galactokinase contains 3 times 368 or approximately 1100 base pairs, and is equivalent in size to that for UDP-galactose 4-epimerase (2).

The third enzyme of the galactose operon, galactose-1-P uridyl transferase, has recently been shown to have a molecular weight of 8.0×10^4 in the native form, and preliminary evidence indicates that it may consist of two equivalent subunits (4). If this is the case, the structural gene for this transferase would be equivalent in size to its nearest neighbors, the epimerase and kinase genes.

The equivalent size of two and probably all three galactose genes, their linkage in a single operon, and the linkage of the reactions catalyzed by the enzymes they generate all induce the speculation that the genes of this operon evolved from a common precursor gene. With this in mind, we have made a comparison of the amino acid frequencies in these three proteins, as indicated in Table V. Inspection of this table reveals few grounds for considering the polypeptides of the kinase (P_k), the transferase (P_t), and the epimerase (P_e) to be alike. Comparison of the residue numbers of each enzyme with those for a hypothetical precursor polypeptide ($P_{\min}$), constructed by minimizing the difference between it and the three enzymes, yields 39, 50, and 39 for the sum of the absolute differences between corresponding residue numbers (leaving out tryptophan and cysteine) for the pairs of $P_k - P_{\min}$, $P_t - P_{\min}$, and $P_e - P_{\min}$, respectively. These numbers are quite large, and are to be compared with the corresponding sum of 45 for the $P_{coli} - P_{\min}$ pair, where P_{coli} is another hypothetical polypeptide having a molecular weight of 40,000 and residue frequencies equal to the amino acid frequencies found for the total protein in *E. coli* (12). The only obvious similarities among P_k, P_t, and P_e not shared by P_{coli} are the lysine residue numbers and the sum of the lysine and arginine residue numbers.[1] These are clearly insufficient to extend the argument for a common precursor gene, and we must wait upon a

[1] Comparison of the positions of the tryptic peptides from P_k and P_e revealed only a few that were similarly disposed. However, P_k was oxidized with performic acid prior to digestion, whereas P_e was either reduced and alkylated or unmodified (2). The peptides containing half-cystine residues would be expected to exhibit different positions even if identical in composition.

more detailed comparison afforded by the amino acid sequences before this is possible.

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