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Effect of Histidine on the Enzyme Which Catalyzes the First Step of Histidine Biosynthesis in *Salmonella typhimurium**

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ABSTRACT: The enzyme, phosphoribosyltransferase, which catalyzes the first step of the pathway for histidine biosynthesis in *Salmonella typhimurium*, is inhibited by the end product of the pathway, histidine. In this report we present studies on the mechanism of the inhibition of phosphoribosyltransferase by histidine. These studies bring to light the following findings. (1) In the presence of histidine the enzyme displays a positive ultraviolet difference spectrum. Analysis of this effect by the solvent perturbation method indicates a burying of chromophores upon addition of histidine. Spectrophotometric titration of the ionization of tyrosyl hydroxyl groups discloses that histidine prevents ionization of 12 tyrosyl residues in the enzyme. (2) Addition of histidine to phosphoribosyltransferase causes a shift in the fluorescence emission spectrum of the enzyme. The extent of the shift is dependent upon histidine concentration. Titration of the shift with histidine reveals a nonlinear behavior, suggesting a cooperativity in this effect of histidine. (3) Addition of histidine to phosphoribosyltransferase does not significantly alter the circular dichroic spectrum of the enzyme. This finding indicates that histidine does not cause a significant change in the secondary structure of the enzyme. (4) Histidine-insensitive phosphoribosyltransferase, isolated from a feedback-resistant mutant, does not show the change in fluorescence which is produced by histidine in the wild-type enzyme. The mutant enzyme does, however, bind histidine, judging from the fact that it, like the wild-type enzyme, is protected by histidine against denaturation by urea. We conclude that inhibition of phosphoribosyltransferase by histidine is brought about by a change in the conformation of the enzyme. Although phosphoribosyltransferase from a feedback-resistant mutant is able to bind histidine, it is unable to undergo the conformational change characteristic of the wild-type enzyme. Thus, the failure of the mutant enzyme to be inhibited by histidine is probably due to an alteration of its structure which does not permit a conformational change to occur when it binds histidine.

The biosynthesis of histine is carried out in *Salmonella typhimurium* through a series of ten steps, each catalyzed by a specific enzyme. The information for the structures of these enzymes is encoded in a small segment of the *Salmonella* chromosome, the histidine operon (for the most recent review, see Brenner and Ames, 1970). Regulation of histidine biosynthesis is accomplished through two different mechanisms. One mechanism, feedback inhibition, acts at the level of enzyme activity; the end product of the pathway, histidine, inhibits the first enzyme of the pathway, *N*-1-(5′-phosphoribosyl)adenosine triphosphate:pyrophosphate phosphoribosyltransferase (EC 4.2.1c) or, more simply, phosphoribosyltransferase, thereby shutting off the whole pathway (Ames *et al.*, 1961; Martin, 1963; Klungsoyr *et al.*, 1968). The second mechansim, *repression*, acts at the level of enzyme synthesis; the rate of synthesis of the histidine enzymes is increased under conditions of histidine limitation and is decreased under conditions of histidine excess (Ames *et al.*, 1960; Ames and Hartman, 1963). It appears, however, that His-tRNA, rather than histidine itself, is the mediator of this effect (Schlesinger and Magasanik, 1964; Silbert *et al.*, 1966; Roth *et al.*, 1966). The mechanism by which His-tRNA affects the synthesis of the histidine enzymes is not known.

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The idea that phosphoribosyltransferase has some influence on repression of the histidine operon was recently proposed (Kovach *et al.*, 1969a,b). The basis for this idea was the finding that the kinetic pattern of repression of the histidine enzymes is modified by the state of the feedback-sensitive site of the enzyme (Kovach *et al.*, 1969a). Moreover, repression of the histidine operon by the histidine analog, 1,2,4-triazolalanine, is prevented when the feedback-sensitive site of phosphoribosyltransferase is altered either biochemically or genetically (Kovach *et al.*, 1969b). Thus, it appears that feedback inhibition and repression, the two most important mechanisms regulating the biosynthesis of histidine, may be interrelated. Because phosphoribosyltransferase appears to play a central role in regulation of histidine biosynthesis, we sought to obtain a deeper insight into the molecular mechanisms underlying the inhibition of the enzyme by the end product of the pathway, histidine. In this study, data are reported which show that the binding of histidine to the enzyme is accompanied by a conformational change. This conformational change correlates well with the inhibition of the catalytic activity and is absent from a mutant enzyme which has a much higher K_I for histidine.

Materials and Methods

Purification, Assay, and Properties of Wild-Type Phosphoribosyltransferase. The histidine auxotroph of *Salmonella typhimurium*, *hisEll*, was grown in a 300-l. fermenter under conditions in which the histidine operon is derepressed (Margolies and Goldberger, 1966). The enzyme was purified by a modification of the method of Voll *et al.* (1967) as described by Whitfield (1970). In addition, a hydroxylapatite step was included, as suggested by Dr. Robert Bell.

Enzymic activity was assayed by measuring the initial rate of phosphoribosyl-ATP synthesis from phosphoribosylpyrophosphate (PRPP) and ATP. The method employed was that of Voll *et al.* (1967), except that in the assay carried out in the presence of urea pyrophosphatase was omitted; although the linearity of the assay lasts for a shorter time, reproducible results were obtained by using the tracing of a Gilford recorder.

Upon electrophoresis in polyacrylamide gels, the purified enzyme gave one major band and one faint minor band. In 8 M urea (using the method described in the manual supplied by Canal Industrial Corp.), the enzyme gave one band.

Feedback-Resistant Phosphoribosyltransferase. Enzyme purified from the feedback-resistant mutant, *hisG1109hisIF135* (Sheppard, 1964), was a generous gift of Dr. Harvey Whitfield, Jr. Its purification and properties have been reported (Whitfield, 1970). This mutant enzyme had a K_I for histidine of 10^{-2} M (Whitfield, 1970).

Measurement of Protein Concentration. Protein concentrations were measured by the method of Lowry *et al.* (1951), using insulin standards.

Difference Absorption Spectra. Difference absorption spectra of phosphoribosyltransferase in the presence and absence of histidine were obtained at 25° with a Cary Model 14 spectrophotometer. Two sets of quartz cells (3-ml capacity, 1-cm light path) were set in tandem. A mixture of phosphoribosyltransferase in 0.025 M Tris-HCl buffer, pH 7.5, and L-histidine which had been neutralized with KOH, was placed in one of the sample cuvets; the other contained only buffer. In the reference beam, the enzyme was placed in one cell and histidine in the other. The protein concentration was 0.56 mg/ml, which corresponded to an A_{280} of 0.575. The cuvets containing the protein were set closest to the photomultiplier in all cases. The spectra were recorded immediately after addition of histidine. No time dependence was observed in the changes which occurred at 10^{-4} or 10^{-3} M histidine; time dependence was not studied in the case of 10^{-2} M histidine.

Solvent perturbation difference spectra were obtained by following the method of Herskovits and Laskowski (1962a,b). One of the sample cells contained the enzyme and the perturbant in the desired solvent (6 M guanidine·HCl in 0.025 M Tris-HCl buffer, pH 7.5; 10^{-3} M histidine in the same buffer; or plain buffer). In the reference beam the enzyme, in the appropriate solvent, was present in the first cell and the perturbant, in buffer, was present in the second cell. The protein concentration was 0.28 mg/ml and the A_{280} was 0.290. Glycerol (25%, v/v) was used as perturbant.

Spectrophotometric Titration of Phenolic Hydroxyl Groups. Spectrophotometric titration of the phenolic hydroxyl groups of phosphoribosyltransferase was carried out in 0.001 M Tris-HCl, pH 7.5, containing KCl (0.35 M); in the same buffer containing guanidine·HCl (6 M); or in the same buffer containing neutralized histidine (10^{-3} or 10^{-2} M). A Zeiss spectrophotometer was employed to follow the changes in absorbancy at 295 mμ. The cells (capacity 3 ml) contained a small Teflon magnet which allowed constant stirring during titration with 1 N KOH. The base was added from an Agla microsyringe at room temperature; pH measurements were done directly in the cell with a Radiometer SE 25 pH meter. A very small amount of precipitation occurred in the very alkaline pH range. This precipitation was evident from analyses of the ultraviolet spectra. A correction was therefore made by subtracting the value of A_{315} from that of A_{295}. This correction seems to be adequate, as judged by the measurement of the optical density at the isosbestic point. Appropriate corrections were made for dilution. The protein concentration varied from 0.15 to 0.2 mg per ml, corresponding to an A_{280} of 0.155 0.210 at neutral pH.

Determination of Tryptophan and Tyrosine Content. The amounts of tyrosine and tryptophan in phosphoribosyltransferase were determined on separate samples according to the procedures of Edelhoch (1967).

Ultraviolet Fluorescence. Fluorescence spectra of wild-type and feedback-resistant phosphoribosyltransferase were measured in a Turner Model 210 spectrofluorometer. Excitation was at 280 mμ. In all studies reported in this paper, the temperature was maintained at 25° and the enzyme concentration was 15 μg/ml. The solvent consisted of 0.01 M Tris-HCl buffer, pH 7.5, containing KCl (0.35 M). The effect of histidine on the fluorescence properties of the enzyme was investigated by adding the enzyme to the buffer containing histidine. In all reported spectra, the solvent fluorescence has been subtracted.

Circular Dichroism. Circular dichroic spectra in the far ultraviolet were obtained with a Cary Model 60 spectropolarimeter, equipped with a Pockels cell. The optical density of the wild-type enzyme solution was 0.105 at 280 mμ and 1.3 at 220 mμ, corresponding to a protein concentration of 100 μg/ml; the optical density of the feedback-resistant enzyme solution was 0.10 at 280 mμ and 1.32 at 220 mμ, corresponding to a protein concentration of 100 μg/ml. The enzyme was dissolved in 0.01 M Tris-HCl, pH 7.0, containing KCl (0.35 M). Cells of 0.2-cm light path were used. In the spectra reported, the solvent spectrum is already substracted. The data are reported as the ellipticities (θ) recorded on the instrument. Molecular ellipticity at 220 mμ, in deg cm² per dmole is given by the equation, $[\theta] = 100\theta/(\text{light path} \times \text{molarity})$. A mean residue weight of 115 for the peptide group, and a

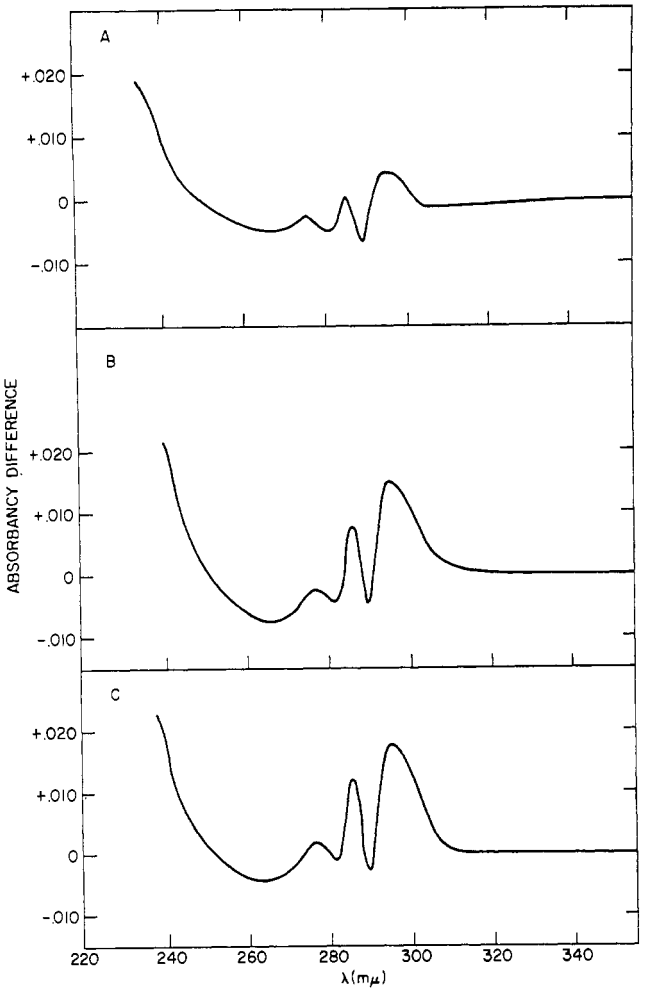

FIGURE 1: Difference absorption spectra of phosphoribosyltransferase and histidine: part A, 10^{-4} M histidine; part B, 10^{-3} M histidine; part C, 10^{-2} M histidine. The same enzyme sample was used for all three experiments, adding increasing amounts of histidine. For details see Materials and Methods.

molecular weight of 200,000 for the phosphoribosyltransferase, were used to calculate $[\theta]$.

Results

Ultraviolet Absorption Studies. It has been reported that phosphoribosyltransferase contains 48 tyrosyl residues/200,000 g, as measured by amino acid analysis (Voll *et al.*, 1967). However, the enzyme preparation that was used for that analysis was probably not so pure as the one used in this study. With the present preparation, measurement of the number of tyrosyl residues, in water and in 6 M guanidine·HCl according to the method of Edelhoch (1967), has yielded 58–59 tyrosyl residues per 200,000 g (Table I, parts A and B). We have found that the tryptophan content of the enzyme, not previously reported, is 36 residues/200,000 g (Table I).

Figure 1 shows the difference absorption spectra produced in wild-type phosphoribosyltransferase by the addition of histidine (10^{-4} M–10^{-2} M). A positive difference peak was observed at 295, 287, and 277 mμ, corresponding to a red shift. The heights of the difference peaks were dependent upon the histidine concentration. This dependence, however, was not the same for both the tyrosine and the tryptophan peaks. It appears that the difference peak at 295 mμ (tryptophan)

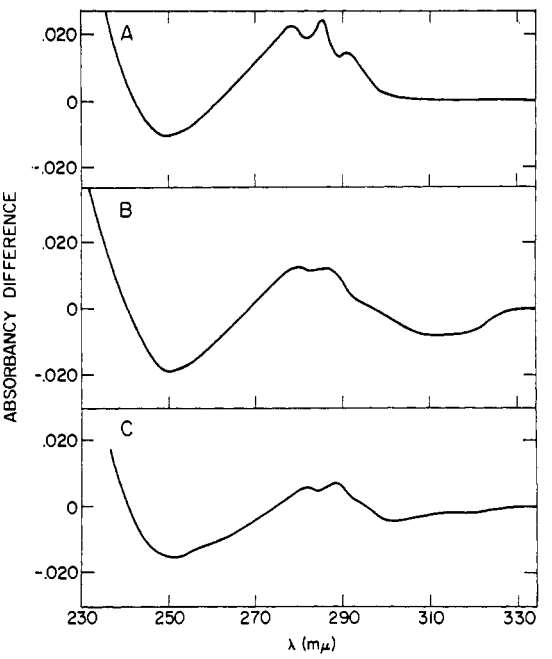

FIGURE 2: Effect of glycerol (25% v/v) on the difference absorption spectrum of phosphoribosyltransferase: part A, in 0.025 M Tris-HCl, pH 7.5, containing guanidine·HCl (6 M); part B, in 0.025 M Tris-HCl, pH 7.5; part C, in 0.025 M Tris-HCl, pH 7.5, containing histidine (10^{-3} M). For details see Materials and Methods.

increased only slightly between 10^{-3} and 10^{-2} M, whereas a much larger effect was seen in the difference peak at 287 mμ (tyrosine and tryptophan) (Table II). This difference is reflected in the ratio calculated in the fourth column of Table II.

The relative degree of exposure of the tyrosyl and tryptophanyl residues has been investigated, using the solvent perturbation method of Herskovits and Laskowski (1962a,b). The effect of glycerol (25%, v/v) on the difference absorption spectra of phosphoribosyltransferase in its native state and in 6 M guanidine·HCl has been studied and has been compared to the effect obtained in the presence of 10^{-3} M histidine. As

TABLE I: Determination of Tyrosine and Tryptophan Content of Phosphoribosyltransferase.[a]

Tryptophan		Tyrosine		
			$\Delta\epsilon_{295}$[b]	Tyrosyl Residues/Mole
ϵ_{280}	183,500	A, In water	133,600	58
ϵ_{288}	130,500	B, In guanidine·HCl (6 M)	150,000	57.6
Residues/mole	36.5	C, In histidine (10^{-3} M)	108,500	47
		D, In histidine (10^{-2} M)	108,000	47

[a] The methods of Edelhoch were employed (Edelhoch, 1967). [b] $\Delta\epsilon_{295}$ is the difference between the molar absorbtion coefficient (295 mμ) at pH 13 and at pH 7.5.

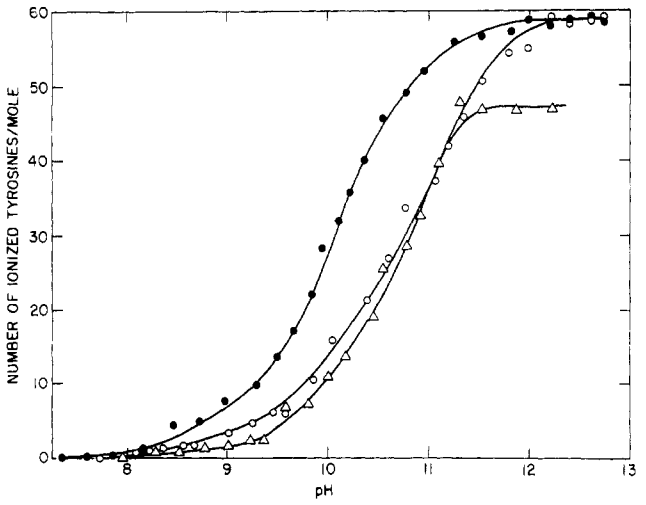

FIGURE 3: Spectrophotometric titration of tyrosyl hydroxyl groups of phosphoribosyltransferase at 295 mμ in 0.001 M Tris-HCl buffer, pH 7.5, containing KCl (0.35 M). The titration was carried out in the buffer without other additions (○—○), in the presence of 6 M guanidine·HCl (●—●), or in the presence of 10^{-2} M histidine (△—△). No time-dependent changes were observed. Values for $\Delta\epsilon_{295}$ of 2590 in guanidine·HCl and of 2300 in water were used to calculate the number of ionized tyrosyl hydroxyl groups. For details see Materials and Methods.

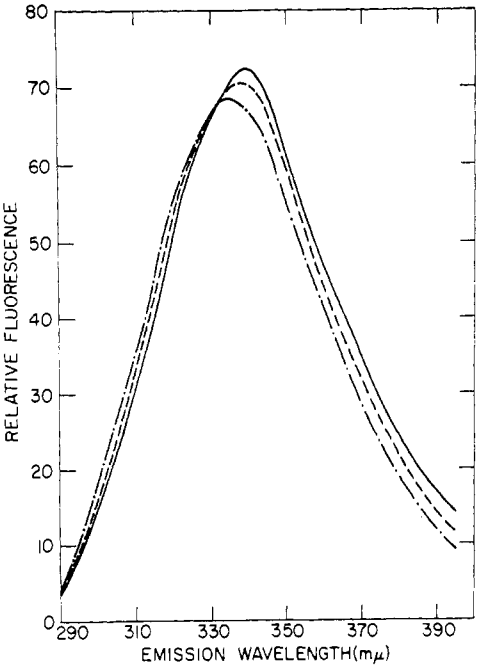

FIGURE 4: Fluorescence emission spectrum of phosphoribosyltransferase in the absence of histidine (—·—) and in the presence of histidine at 2×10^{-4} M (------) and at 6×10^{-4} M (——). The protein was dissolved in 0.01 M Tris-HCl buffer, pH 7.5, containing KCl (0.35 M). Excitation was at 280 mμ. For details see Materials and Methods.

shown in Figure 2, three major peaks appeared (at 277, 286, and 293 mμ, respectively), due to perturbation of tyrosyl and tryptophanyl residues (Herskovits and Laskowski, 1962a,b). Assuming that all residues were totally exposed to the neutral solvent in 6 M guanidine·HCl, and comparing the heights of the difference peaks under the various conditions, it can be calculated that in the native state both tryptophanyl and tyrosyl residues are 50% exposed. In the presence of histidine, the exposure to the aqueous environment was drastically reduced; tyrosyl residues were now 30% exposed.

It is well known that the data obtained by the solvent perturbation method (Herskovits and Laskowski, 1962a,b) does not necessarily represent the complete exposure or the complete burying of chromophores. For this reason, the only conclusion we may draw from our data is that the exposure of both tyrosyl and tryptophanyl residues was reduced by histidine. However, if one assumes that the effect of histidine does consist in completely burying previously totally exposed tyrosyl residues, then we can calculate that approximately 20% of the tyrosyl residues (*i.e.*, 12 residues) are involved in this effect. This possibility is suggested by the spectrophotometric titration data (see below). In the case of tryptophan, the resolution of the peak is not great enough to allow a detailed analysis of the data. Although perturbants may cause dissociation of enzyme–substrate and enzyme–inhibitor com-

TABLE II: Dependence of Difference Spectra of Phosphoribosyltransferase on Histidine Concentration.[a]

Histidine Concentration (M)	$\Delta\epsilon_{295}$	$\Delta\epsilon_{287}$	$\Delta\epsilon_{295}/\Delta\epsilon_{287}$
10^{-4}	1433	357	4.0
10^{-3}	5357	2678	2.0
10^{-2}	6023	4287	1.4

[a] Calculated from the data of Figure 1.

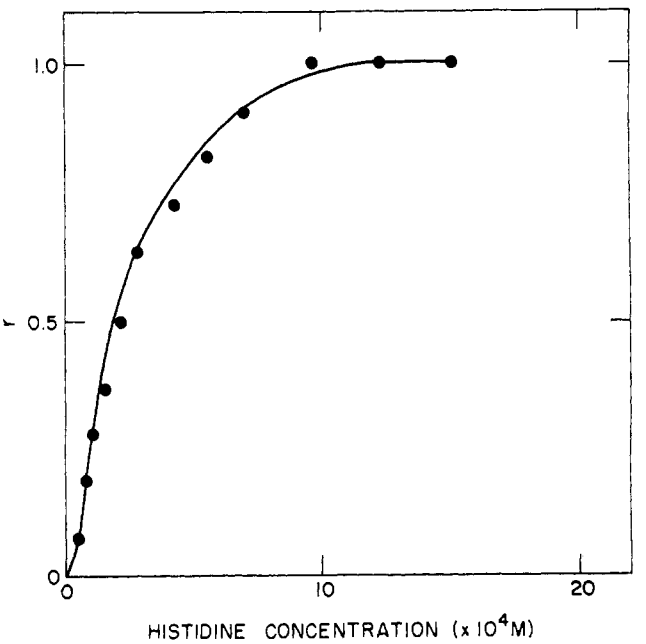

FIGURE 5: Fluorometric titration of phosphoribosyltransferase with histidine, monitored by the increase in fluorescence at 343 mμ. Excitation was at 280 mμ. The solvent was 0.01 M Tris-HCl, pH 7.5, containing KCl (0.35 M). For details see text.

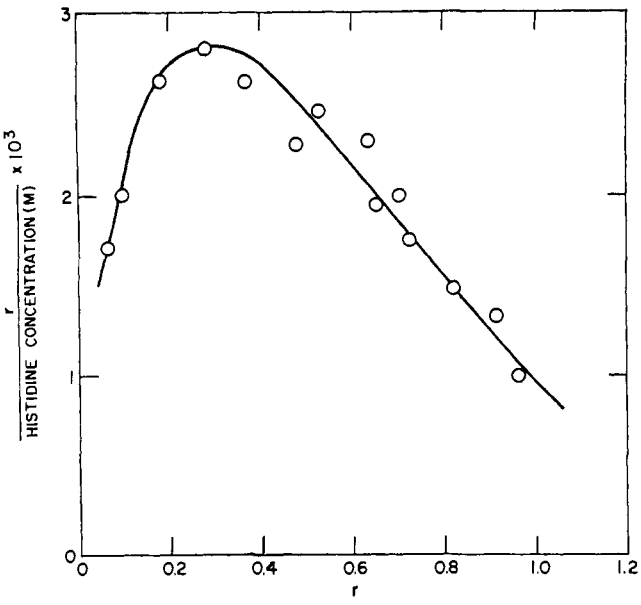

FIGURE 6: Scatchard plot of the data of Figure 5. For details see text.

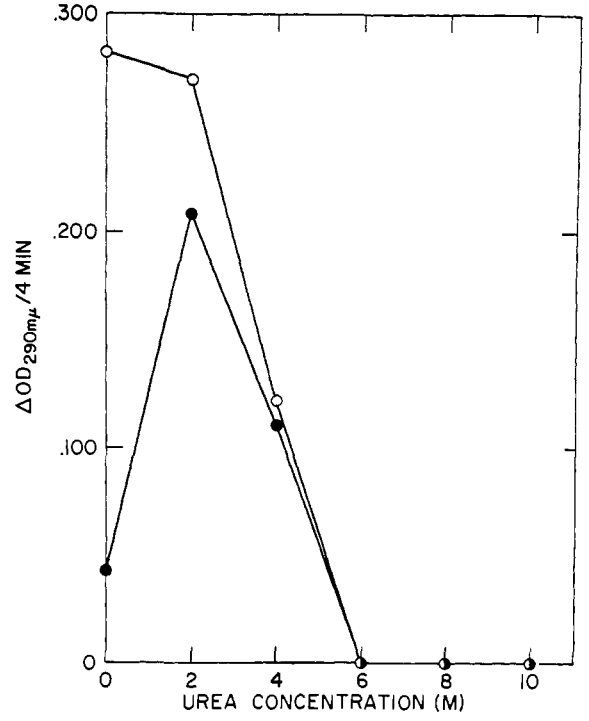

FIGURE 8: Effect of urea at various concentrations on the activity of phosphoribosyltransferase, in the absence of histidine (○—○) and in the presence of 5×10^{-3} M histidine (●—●). The activity is expressed as the change in absorbancy at 290 mμ in 4 min.

plexes, in these experiments this effect is probably not relevant because the enzyme was usually stored at −20° in 50% glycerol, in which solvent it appeared to be perfectly active, stable, and sensitive to inhibition by histidine. Moreover, the concentration of histidine was fivefold higher than that required for half-maximal effect on the catalytic activity.

Spectrophotometric Titration of Phenolic Hydroxyl Groups. Figure 3 shows the titration of the hydroxyl groups of the tyrosyl residues in phosphoribosyltransferase in water (in the presence and absence of histidine), and in 6 M guanidine·HCl. As shown in Table I, A–D, the total change in molar extinction coefficient at 295 mμ was 133,600 in water, 150,000 in 6 M guanidine·HCl, and 108,000 in 10^{-3} and 10^{-2} M histidine.

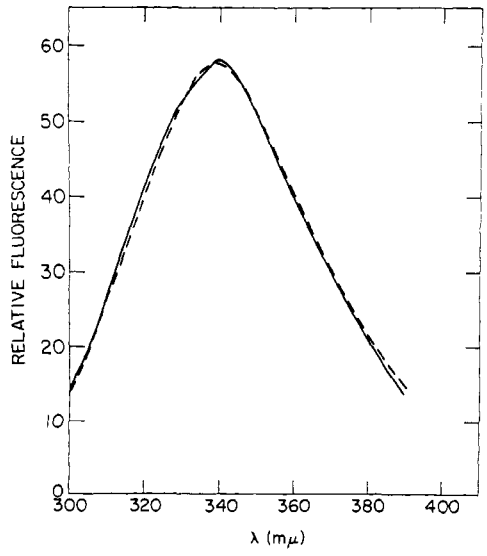

FIGURE 7: Fluorescence emission spectrum of phosphoribosyltransferase isolated from the feedback-resistant mutant, *hisG1109-hisIF135*, in the absence of histidine (——) and in the presence of 10^{-3} M histidine (-----). Conditions were otherwise identical with those described in the legend to Figure 4.

Using 2300 as the value for the $\Delta\epsilon_{295}$ of tyrosine in water (Crammer and Neuberger, 1943) and 2590 as the value for this parameter in 6 M guanidine·HCl (Edelhoch, 1967), it was calculated that in water or in guanidide·HCl 58–59 tyrosyl hydroxyl groups per 200,000 g could be titrated. In the presence of histidine, however, only 47 such groups/200,000 g could be titrated (Table I, parts C and D). The titrations were carried out several times, with reproducible results. The midpoints of the titrations were (Figure 3): 10.05 in 6 M guanidine·HCl (which is very close to the value obtained with amino acid mixtures in proportions present in some well defined proteins) (Edelhoch, 1967), 10.65 in water, and 10.60 in the presence of histidine (10^{-2} M). Moreover, in the presence of histidine the curve was shifted to higher pH values (by about 0.25 pH unit) in the pH range between 9 and 10. The titration of phenolic hydroxyl groups in the presence of histidine was studied also at a lower concentration of histidine (10^{-3} M) with similar results, *i.e.*, only 47 tyrosyl groups/200,000 g could be titrated. Moreover, no evidence of further ionization could be obtained, even when the pH was raised to 13.30.

Fluorescence Studies. Figure 4 shows fluorescence emission spectra of phosphoribosyltransferase in the presence of histidine at several concentrations. In the absence of histidine the peak of emission was at 335 mμ. It is clear that histidine caused a shift of the emission peak toward higher wavelength and that the extent of the shift was dependent upon the concentration of histidine. These results suggest that addition of histidine to the enzyme alters the environment of some of the tryptophanyl residues. As shown in Figure 5, the maximum effect was found at a histidine concentration of 10^{-3} M. At this concentration the emission maximum was shifted to approximately 343 mμ. The dependence of this effect on histidine concentration allowed a titration of fluorescence as a function of histidine concentration. Figure 5 shows such a

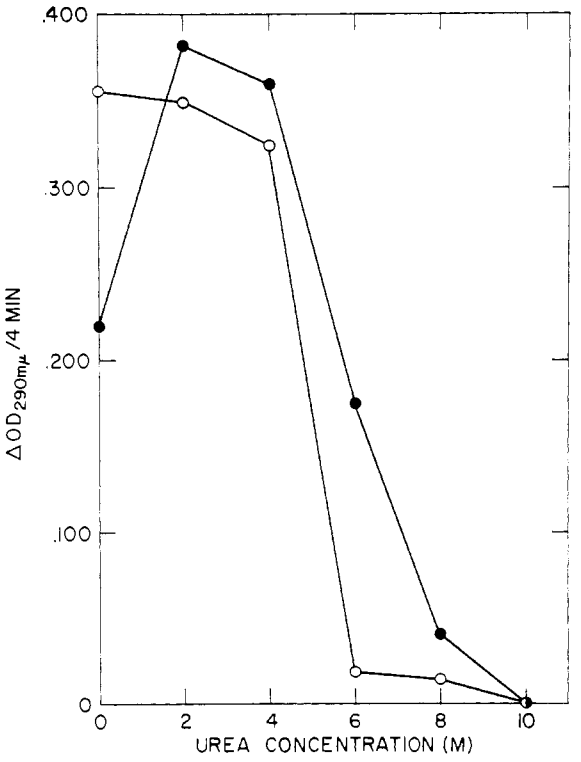

FIGURE 9: Effect of urea at various concentrations on the activity of phosphoribosyltransferase isolated from the feedback-resistant mutant, *hisG1109hisIF135*, in the absence of histidine (○—○) and in the presence of 5×10^{-3} M histidine (●—●). Data are expressed as described in the legend to Figure 8.

titration. Since no information is available on the amount of histidine bound per mole of enzyme, the symbol "*r*" here refers to the increase in fluorescence at a given histidine concentration divided by the increase in fluorescence at saturation. Half-maximum effect was obtained at a histidine concentration of 2×10^{-4} M. Although the K_I value for histidine reported by Whitfield (1970) is somewhat lower (7×10^{-5} M), the histidine sensitivity of the enzyme depends on the age of the preparation (Martin, 1963). With the enzyme preparation used for these experiments, 2×10^{-4} M histidine gave 49 ± 7.4% inhibition (average of nine determinations). The excellent agreement between the midpoint of the fluorescence and the K_I value suggests that the change observed in tryptophan fluorescence is directly related to the inhibition of catalytic activity by histidine.

In Figure 6 the data of Figure 5 are presented in the form of a Scatchard plot (Scatchard, 1949). The results suggest a cooperativity in the change which occurs in the environment of tryptophanyl residues upon addition of histidine. This is suggested by the nonlinearity of the Scatchard plot and is consistent with the findings of Whitfield (1970) which indicated cooperativity of histidine inhibition of enzymic activity.

The effect of histidine on the fluorescence of enzyme from the feedback-resistant mutant, *hisG1109hisIF135* (Sheppard, 1964), was investigated (Figure 7). In contrast to the data obtained with the wild-type enzyme (*cf.* Figure 4), no effect was observed, even at a rather high concentration of histidine (10^{-3} M).

Effects of Urea. Figure 8 shows the effect of urea at various concentrations on the activity of wild-type phosphoribosyltransferase, in the presence and absence of histidine. In the absence of histidine, the enzyme was almost completely active in 2 M urea, whereas in 6 M urea the activity was completely lost. In the absence of urea, 85% inhibition of the enzymic activity was produced by 5×10^{-3} M histidine. Urea was able to prevent this inhibition. At 2 M urea, histidine inhibited only 20% of the enzymic activity and at 4 M urea the inhibition had disappeared.

In Figure 9 the results of similar experiments are reported, in which the feedback-resistant enzyme was used. In the absence of histidine, the catalytic activity was stable in urea at concentrations up to 4 M, but at 6 M urea the activity was almost completely lost. In the absence of urea, the mutant enzyme was 38% inhibited by 5×10^{-3} M histidine. At 2 and 4 M urea, however, no inhibition by histidine was obtained, the activity being actually higher than that of the control (without histidine). At 6 M urea, moreover, a significant proportion of the activity was still retained. Thus, in the feedback-resistant enzyme urea prevented inhibition by histidine and histidine prevented denaturation by urea.

Optical Activity. Figure 10 shows the circular dichroic spectra in the far ultraviolet obtained with wild-type and feedback-resistant phosphoribosyltransferase, both under the same conditions. With wild-type enzyme, circular dichroic spectra were also recorded in the presence of histidine (7×10^{-4} M). The amounts of α helix in the wild-type and feedback-resistant enzymes were calculated to be 38 and 32%, respectively.[1] In the presence of 7×10^{-4} M histidine the amount of α helix in wild-type enzyme was calculated to be 35%. These values do not appear to be markedly different from one another, and in the last case the large positive circular dichroism peak of histidine itself in the far ultraviolet introduces a possible source of error.

Discussion

The phosphoribosyltransferase, which catalyzes the first step unique to the pathway for histidine biosynthesis in *Salmonella typhimurium*, was shown by Voll *et al.* (1967) to have a molecular weight of approximately 210,000. The enzyme appears to be composed of six subunits with a molecular weight of 35,000 (Voll *et al.*, 1967; Whitfield, 1970). Whether all the subunits are identical or not has not been definitely settled, although the following data are consistent with the idea that they are identical: (a) Voll *et al.* (1967) identified a single reactive amino-terminal residue; (b) electrophoresis in sodium dodecyl sulfate (Whitfield, 1970) and in 6 M urea (Voll *et al.*, 1967) showed mainly one band; and (c) genetic studies of the histidine operon have not revealed any intragenic complementation in the *G* gene (Loper *et al.*, 1964).

Histidine inhibits the catalytic activity of the enzyme by binding at a site distinct from the active site, as shown by both kinetic analyses (Martin, 1963; Whitfield, 1970) and by chemical modifications (Martin, 1963). The kinetic data of Whitfield (1970), when analyzed by the modified Hill plot, indicate the presence of at least two interacting binding sites for histidine. Several lines of evidence further support the existence of more than one binding site for histidine. Martin (1963) showed that phosphoribosyltransferase which had been rendered insensitive to feedback inhibition by chemical means still bound histidine; and wild-type enzyme (Martin, 1963) as well as feedback-resistant enzyme (Whitfield, 1970) were both protected by histidine against heat inactivation.

[1] The values for molecular ellipticity at 220 mμ which we used for these calculations are −35,300 (α helix) and +4400 (random coil), as given by Greenfield and Fasman (1969) for poly(L-lysine).

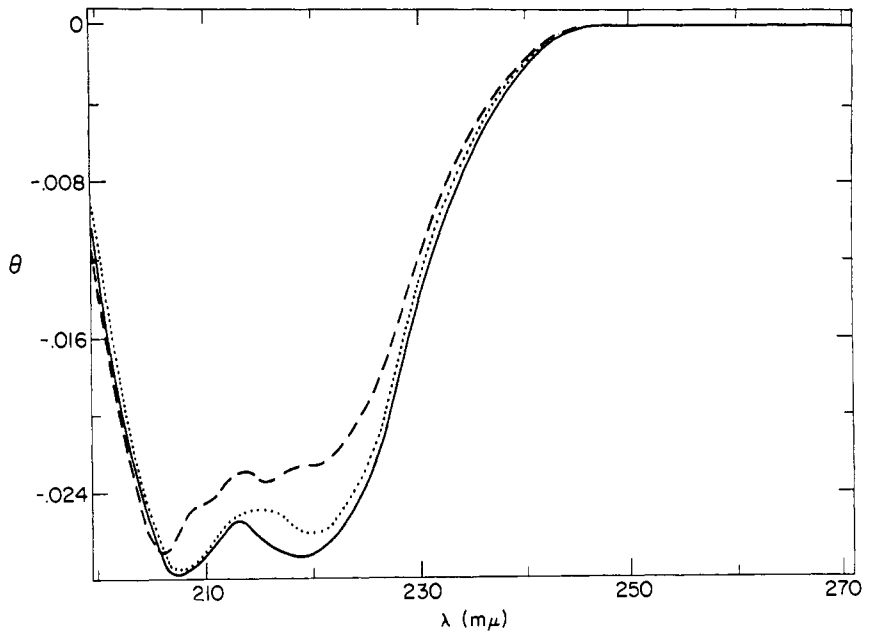

FIGURE 10: Circular dichroic spectra of phosphoribosyltransferase: wild-type enzyme (——); wild-type enzyme in the presence of 5×10^{-4} M histidine (· · · · ·); and enzyme isolated from the feedback-resistant mutant, *hisG1109hisIF135* (— —).

Since histidine inhibits the enzyme by binding at a site other than the catalytic site, one would expect that the binding of histidine to the enzyme is accompanied by a conformational change. Martin (1963) could not show any change in the sedimentation properties of the enzyme upon addition of histidine, although a conformational change was indicated by an increased sensitivity to trypsin in the presence of histidine. Whitfield (1970) observed a time-dependent increase in optical density at 280 mμ (up to 200% in 3 hr) upon addition of histidine at a very high concentration (10^{-2} M). This effect was not observed with the feedback-resistant enzyme. Although not apparently due to light scattering, the cause of this increase in optical density is not yet known.

In this paper we present more direct evidence for a conformational change induced by histidine in wild-type phosphoribosyltransferase. Several lines of evidence are presented.

(1) Upon addition of histidine, a red shift occurred in the absorption difference spectra. It is well known from the work of Yanari and Bovey (1960) that a blue shift occurs in the ultraviolet spectra of model indole and phenol compounds as the solvent is changed from lower to higher polarity. Therefore, the red shift in the absorption difference spectra of phosphoribosyltransferase produced by histidine could be attributed to a decrease in the polarity of the environment of the chromophores—that is, to a burying of certain amino acid residues. Apparently both tyrosyl and tryptophanyl residues are involved, as indicated by the presence of difference peaks at 295, and at 287 and 277 mμ (Donovan, 1969). The effect was dependent upon histidine concentration and appeared to be very complex. At the lowest concentration of histidine tested (10^{-4} M), the difference peak at 295 mμ was predominantly affected; at higher histidine concentrations (10^{-3} M and 10^{-2} M), the difference peak at 277 mμ increased more than did that at 295 mμ (Table II). Thus, histidine, at low concentration, produced a change in the enzyme which rendered it different in response to histidine at increasing concentrations. These findings suggest either the existence of intermediate forms of the enzyme in the cooperative binding of histidine, as predicted by the general model of Koshland (1969), or the existence of at least two structurally different histidine binding sites in the enzyme.

(2) The red shift in the absorption spectrum observed upon addition of histidine to the wild-type enzyme was due to the burying of some chromophores. Solvent perturbation data show that in the presence of histidine the degree of exposure of tyrosyl residues, or the number of exposed tyrosyl residues, was decreased. Assuming the latter interpretation to be correct, and on the basis of a tyrosine content of 60 residues/mole (Table I), 12 tyrosyl residues could be calculated to become buried in the presence of histidine.

(3) Spectrophotometric titration of the ionization of tyrosyl hydroxyl groups showed that in the presence of histidine 12 tyrosyl residues cannot be ionized. These data, together with the results of solvent perturbation and difference absorption spectroscopy, indicate that histidine induces a conformational change in which 12 previously exposed tyrosyl residues become buried. The possibility that the effects observed are due simply to a shielding of chromophores by histidine does not appear likely. Not only do the fluorescence data support the idea of a conformational change (see below), but also the number of tyrosyl residues (12) is too large to be reasonably accounted for on the basis of shielding. It may not be fortuitous that the number of tyrosyl residues involved in this phenomenon is a multiple of the number of subunits of which the enzyme is comprised.

(4) The fluorescence emission spectrum of wild-type phosphoribosyltransferase showed a red shift upon addition of histidine. This effect was dependent upon histidine concentration. The Scatchard plot of these data suggests a cooperativity in this effect which could be interpreted in terms of interaction between two different histidine-binding sites as the basis for the inhibition by histidine. These data agree well with the Hill plot of kinetic data previously reported by Whitfield (1970), which shows a slope higher than one.

In proteins, emission of fluorescence is mainly due to tryptophanyl residues and, to a lesser extent, to tyrosyl residues (Chen *et al.*, 1969). The emission maximum of the indole side chain of model compounds is shifted toward lower

wavelengths when the polarity of the solvent is reduced (Van Duren, 1961). The emission maximum in proteins is at wavelengths lower than in the model compounds, presumably due to the hydrophobic environment surrounding the indole side chains of tryptophanyl residues (Teale, 1960). Therefore, a red shift in fluorescence is usually interpreted as an exposure of the chromophore to the aqueous phase. The emission peaks of a large group of proteins do, in fact, display this red shift when exposed to 8 M urea (Teale, 1960). We have verified that this is also the case with the phosphoribosyltransferase (unpublished). The effect of histidine on the fluorescence of the wild-type enzyme (red shift) can therefore be interpreted as an exposure of some tryptophanyl residues to the aqueous solvent. This exposure, however, is either incomplete or affects only a few of the tryptophanyl residues, since a much larger red shift was observed in 10 M urea (unpublished).

The data from absorption spectroscopy suggest that histidine causes the burying of certain residues, whereas the data from fluorescence experiments suggest that histidine causes the exposure of certain residues. Therefore, it appears likely that the two techniques monitor effects taking place in different portions of the molecule. However, we cannot exclude the possibility that the data from fluorescence and absorption experiments do actually reflect the same phenomenon. In fact, there is no reason to assume that a shift in fluorescence must be in the opposite direction from that in absorption, provided that the type of electronic transition be the same (Turro, 1967). The finding of a red shift both in absorption and in fluorescence upon binding of a ligand to an allosteric enzyme has been reported previously (Janin and Cohen, 1969). If, indeed, the red shift in absorption and fluorescence which occurred in response to histidine both indicate a change of the same kind, we would be inclined to suggest that they indicate a burying of chromophores rather than an exposure of chromophores, since evidence from another kind of experiment, spectrophotometric titration of the ionization of tyrosyl residues, indicates that histidine causes a burying of chromophores in the enzyme.

The conformational change induced by histidine is not accompanied by gross changes in the structure of phosphoribosyltransferase. This conclusion is based on the observation that the secondary structure of the enzyme, as judged by measurements of circular dichroism, did not appear to be significantly altered by exposure to histidine.

In contrast to its effect on the wild-type phosphoribosyltransferase, histidine did not produce any conformational change in the feedback-resistant enzyme, as judged by fluorescence spectroscopy. This suggests that the changes observed with the wild-type enzyme are directly related to the effect of histidine on the catalytic activity of the enzyme. The question arises of whether the feedback-resistant enzyme fails to undergo a conformational change because it cannot bind histidine or because the interaction between its subunits is altered. The data of Whitfield (1970), which show that histidine is able to stabilize the mutant enzyme against heat denaturation, support the latter possibility. The results of our studies with urea also strongly support this conclusion. Even though histidine could not promote a discernible change in the conformation of the mutant enzyme, it was able to efficiently protect the enzyme from denaturation by urea. Furthermore, although 2 M urea did not affect the catalytic activity of either the wild-type or mutant enzyme, the sensitivity to inhibition by histidine was strikingly affected in both enzymes. Thus, the results of a number of different studies all support the view that the mutant enzyme does bind histidine and that the failure of this enzyme to undergo the conformational change observed with the wild-type enzyme is due to an alteration in its structure which prevents the phenomenon if histidine binding from being translated into conformational change.

The spectroscopic data reported in this paper indicate that the inhibition of phosphoribosyltransferase by histidine is brought about by a conformational change in the enzyme. It appears that upon binding histidine the enzyme assumes a more compact structure.

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Synthesis and Characterization of 1-*O*-Alkyldihydroxyacetone Phosphates and Derivatives*

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ABSTRACT: This report describes the chemical synthesis of the cyclohexylammonium salt of 1-*O*-hexadecyl-2,2-dimethoxypropane 3-phosphate and its octadecyl analog. The key reaction in the synthetic route is oxidation of benzoylated chimyl or batyl alcohol with dimethyl sulfoxide in the presence of dicyclohexylcarbodiimide and trifluoroacetic acid to the corresponding keto intermediate; the latter compound is subsequently phosphorylated and isolated as the cyclohexylammonium salt. The organically synthesized *O*-alkyldihydroxyacetone phosphates and those synthesized by microsomal enzymes of mammalian cells were found to have identical properties.

The *O*-alkyldihydroxyacetone phosphates are central intermediates in the pathway that produces diacyl-*O*-alkylglycerol, *O*-alkylacylglycerylphosphorylcholine, and *O*-alkylacylglycerylphosphorylethanolamine.

In a recent communication (Piantadosi *et al.*, 1970) we reported preliminary studies directed toward the synthesis of *O*-alkyldihydroxyacetone and its derivatives. Although the biosynthesis of alk-1-enylacyl phospholipids has not been elucidated, Snyder and coworkers (Wykle and Snyder, 1969; Snyder *et al.*, 1969, 1970a–c; Wykle and Snyder, 1970) recently described a microsomal enzyme complex that synthesizes *O*-alkyl ether bonds in normal and neoplastic cells. The intact phospholipids have been synthesized enzymically (Snyder *et al.*, 1970a; Wykle and Snyder, 1970) *via* *O*-alkyldihydroxyacetone phosphate, which is the first ether-containing intermediate formed in the new pathway proposed (Snyder *et al.*, 1969, 1970c); *O*-alkyldihydroxyacetone is also formed by the enzyme system.

The ketone intermediates are reduced enzymically by NADPH and can subsequently serve as acyl acceptors, thereby emphasizing the importance of these new and metabolically important ether-linked keto lipids. The microsomal enzymes synthesize *O*-alkyldihydroxyacetone phosphate from long-chain fatty alcohol and dihydroxyacetone phosphate and require ATP, CoA, and Mg^{2+} as cofactors. The results of similar studies by others (Hajra, 1969, 1970; Kapoulas and Thompson, 1969) have demonstrated the presence of the *O*-alkyl ether synthesizing enzymes in other systems. In this paper, we describe the complete organic synthesis and the characterization of the keto intermediates; the intermediates isolated from enzyme systems have been previously characterized chemically and chromatographically. The organically synthesized compounds are of great importance in confirming the findings of the earlier studies and for use in further metabolic studies.

We have prepared the cyclohexylammonium salt of 1-*O*-octadecyl-2,2-dimethoxypropane 3-phosphate (VIII) and 1-*O*-hexadecyl-2,2-dimethoxypropane 3-phosphate (see Scheme I). In this synthetic route, both 1-*O*-octadecylglycerol and 1-*O*-hexadecylglycerol were used as starting materials. Compound I was benzoylated in the presence of pyridine at $-10°$, resulting in a crude mixture of mono- and dibenzoates (II) in which the monobenzoate predominated approximately 2:1 as determined by thin-layer chromatography. However, II was not isolated but converted into III with Me_2SO and DCC[1] in the presence of trifluoroacetic acid. Compound III was ketalized to afford IV, then hydrolyzed to V with NaOH and subsequently phosphorylated with diphenyl phosphorochloridate; the cyclohexylammonium salt, VIII, was finally isolated. The cyclohexylammonium salt was shaken with dilue HCl or Dowex 50W ion-exchange resin at room temperature to release *O*-alkyldihydroxyacetone phosphate.

We have compared the chemical and chromatographic be-

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[1] Abbreviation used is: DCC, dicyclohexylcarbodiimide.