Comparative Structural Properties of Honeybee and Rabbit α-Glycerophosphate Dehydrogenases*

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ABSTRACT: Several structural properties of honeybee and rabbit diphosphopyridine nucleotide dependent α-glycerophosphate dehydrogenases (EC 1.1.1.8) have been compared. Both carboxypeptidase hydrolysis and hydrazinolysis were used to determine the carboxyl-terminal residues; 2 moles of C-terminal methionine can be released from 77,000 g of the rabbit enzyme, indicating not only that the enzyme is probably a dimer but also that its molecular weight is around 77,000 and not 60,000 g as has been suggested. At least one of the penultimate residues is histidine. The honeybee dehydrogenase contains two C-terminal alanine residues; the terminal sequence in each chain is probably -Thr-Gln-Asp-Ala. The N-terminal is apparently blocked in both proteins. Because of tryptic-resistent cores, peptide maps could not unequivocally demonstrate identical subunit amino acid sequences in either of the dehydrogenases; however, the tryptic peptide maps did not give any indication that the subunits might differ. In addition, the maps revealed that the primary sequences of the tryptic-digestible portions of the rabbit and honeybee enzymes are markedly different. Gel filtration methods demonstrated that both dehydrogenases bind 2 moles of reduced diphosphopyridine nucleotide and do not bind any free adenosine diphosphate ribose. Removal of a chromophore from the native rabbit dehydrogenase did not affect DPNH binding or the optical rotatory dispersion spectrum. The gross conformations of the rabbit and honeybee enzymes, as evidenced by optical rotatory dispersion in the region from 225 to 470 mμ, are very similar. The average hydrophobicity for the rabbit, honeybee, and rat enzymes was calculated from the known amino acid compositions; this value is essentially identical in all three cases. The results reveal that honeybee and rabbit α-glycerophosphate dehydrogenases share several similar properties, such as dimeric structure, blocked N-terminals, DPNH binding, optical rotatory dispersion, and hydrophobicity. There are, however, other properties which differ markedly, such as C-terminal sequence and amino acid sequence as illustrated by tryptic peptide maps.

Extramitochondrial DPN^+-dependent GPDH[1] (EC 1.1.1.8) has been cystallized from rabbit skeletal muscle (Baranowski, 1963) and from honeybee flight muscle (Marquardt and Brosemer, 1966). Of the metazoan tissues that have been investigated, the flight muscle of many insects has by far the highest GPDH specific activities; this is related to the role of the enzyme in the glycerophosphate shunt (Zebe *et al.*, 1959; Sacktor, 1965). The specific activity in mammalian skeletal muscle is also relatively high. Unlike the situation in insect flight muscle, the GPDH reaction in skeletal muscle is not part of the main pathway for carbohydrate degradation, but rather is involved in maintaining a balanced cellular redox state (Bücher and Klingenberg, 1958). Since the rabbit and honeybee enzymes diverged more than 7×10^8 years ago and since their metabolic functions are quite different, a comparative study of their structures has been undertaken.

The molecular weight of the rabbit GPDH protein has been reported to be 77,000 (van Eys *et al.*, 1964); the contribution of a bound chromophore, which must be added to give the native molecular weight, is probably less than 1000. This molecular weight has been confirmed by physical and fluorescence studies (van Eys *et al.*, 1959; Ankel *et al.*, 1960). The enzyme dissociates in 9 M guanidine hydrochloride into polypeptides with molecular weight around 40,000 (Deal and Holleman, 1964). Recently, however, T. P. Fondy, C. R. Ross, and S. Sollohub (1968, personal communication) have reexamined the molecular weight of the rabbit enzyme using several physical and chemical methods; their results indicate a value of about 60,000. The large discrepancy in molecular weight estimations has not yet been explained. Resolution of this uncertainty is important not only in terms of the rabbit enzyme itself, but also in terms of phylogenetic variations in GPDH. The molecular weight of the rat enzyme is 58,000 (Fondy *et al.*, 1968) and of the honeybee enzyme 65,000 (Brosemer and Marquardt, 1966). If the molecular weight of rabbit GPDH is 60,000, there is probably no significant difference in size of the three

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[1] Abbreviations used are: GPDH, DPN^+-dependent α-glycerophosphate dehydrogenase; ADPR, adenosine diphosphate ribose.

GPDHs. If, on the other hand, the rabbit molecular weight is 77,000, it would appear that there exist at least two classes of GPDH based upon molecular weight. The phylogenetic and taxonomic implications of such classes would be of interest.

Most, but not all, evidence indicates that the rabbit enzyme is a dimer. Dissociation in guanidine (Deal and Holleman, 1964), kinetic analysis of acid inactivation, acetyl determinations, and amino acid analysis (van Eys *et al.*, 1964) are compatible with a dimeric structure. Fluorescence studies indicate rabbit GPDH binds 1 mole of DPNH/70,000 g (Ankel *et al.*, 1960), whereas gel filtration methods suggest 2 moles of DPNH bound/mole of enzyme (Pfleiderer and Auricchio, 1964). A tetrameric structure has not been rigorously excluded; in fact, some evidence is consistent with such a structure (van Eys *et al.*, 1964). It is also unknown whether subunits have identical primary structures, although the recent experiments of T. P. Fondy, C. R. Ross, and S. J. Sollohub (1968, personal communication) suggest very similar if not identical sequences.

Rabbit, but not honeybee, GPDH binds one or more ultraviolet-absorbing compounds (van Eys *et al.*, 1959; Ankel *et al.*, 1960). This group(s) can be removed without significantly affecting enzyme activity or physical properties. Adenosine diphosphate ribose is recovered after removal of the protein, but it is not clear whether it is bound as such to the native enzyme or is a degradation product of the chromophore(s) (Celliers *et al.*, 1963).

This report describes comparative studies on primary, conformational, and subunit structural properties of rabbit and honeybee GPDHs using the techniques of end-terminal determination, peptide mapping, nucleotide binding, optical rotatory dispersion, and hydrophobicity calculations.

Materials and Methods

Honeybee GPDH was crystallized from frozen thoraces (Marquardt and Brosemer, 1966); rabbit GPDH was obtained from Sigma Chemical Co. Carboxymethylation of cysteine residues with iodoacetic acid (Mann Research Laboratories, recrystallized from petroleum ether, bp 30–60°) was performed essentially according to Hirs (1967); aminoethylation with ethylenimine (Matheson Coleman and Bell) was performed according to Cole (1967). Urea was obtained from J. T. Baker Chemical Co. and recrystallized from ethanol; fresh solutions were always used.

The honeybee GPDH assay using dihydroxyacetone phosphate has been described (Marquardt and Brosemer, 1966); the assay for the rabbit enzyme was the same, except that 50 mM Tris–5 mM EDTA (pH 7.6) was the assay buffer. Protein was determined either by the method of Lowry *et al.* (1951) using the respective dehydrogenase dried at 105° as standard or by amino acid analysis after 24-hr hydrolysis at 107° in 6 N HCl. Amino acid analyses were performed on a Technicon AutoAnalyzer equipped with a 3-mm diameter column or on a Beckman Model 120C amino acid analyzer.

Proteolytic digests for peptide maps were prepared by incubating rabbit or honeybee carboxymethyl-GPDH at 37° in 0.2 M NH_4HCO_3 (pH 8.1) with L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin (Worthington Biochemical Corp.). The initial substrate to trypsin weight ratio was 60:1; after 6-hr incubation, more trypsin, equal to one-half of the original amount, was added and the digestion continued for another 16 hr. The peptide mapping method described by Bennett (1967) was employed. The chromatographic solvent was 1-butanol–acetic acid–H_2O (12:3:5, v/v); electrophoresis was conducted at pH 3.6. Ninhydrin, Sakaguchi (arginine), Pauly (tyrosine plus histidine), Ehrlich (tryptophan), and tyrosine color reactions were described by Easley (1965).

Carboxypeptidase A (Worthington Biochemical Corp.) and carboxypeptidase B (Sigma Chemical Co.) were both treated with diisopropyl fluorophosphate. Carboxypeptidase digestions of GPDHs were performed either in 50 mM Veronal (pH 8.1) or 0.2 M *N*-ethylmorpholine acetate (pH 8.5). Weight ratios, incubation temperatures, and incubation times are given in the Results section. The reaction was terminated by adding trichloroacetic acid to a final concentration of 1 mg/ml; the precipitate was washed once with trichloroacetic acid (0.5 mg/ml); the supernatants were combined, extracted with ether, and filtered through glass wool. One-half was applied directly to the amino acid analyzer. In order to determine glutamine and asparagine, the other half was hydrolyzed 3 hr at 107° with 3 N HCl before applying to the amino acid analyzer; the increase in glutamate and aspartate after acid hydrolysis was equivalent to glutamine and asparagine present in the original sample. For each time sample, a carboxypeptidase blank (lacking protein substrate) was also analyzed in the same manner. Control experiments showed that the protein substrates lacked detectable free amino acids.

Hydrazinolysis of rabbit GPDH was carried out as described by Akabori *et al.* (1952); hydrazine (95+%) from Eastman Organic Chemicals was used without further purification. Hydrazinolysis of bee GPDH was carried out as described by Braun and Schroeder (1967) using distilled hydrazine; extraction of the hydrazinolytic mixture was performed according to Korenman *et al.* (1966). Attempts to identify the N-terminal amino acid were made using the dansyl technique in the presence or absence of 8 M urea (Gray, 1967); the thin-layer chromatographic systems of Morse and Horecker (1966) and Nedkov and Genov (1966) were used for detection of dansylamino acids.

The gel filtration method of Hummel and Dreyer (1962) was used in nucleotide-binding studies. A 1.5 × 10 cm column of medium grade Sephadex G-50 (Pharmacia) was equilibrated with 0.1 M Tris, 10 mM EDTA, and 1 mM dithiothreitol (pH 7.8) containing nucleotide at the concentration used for that experiment. The enzyme was dialyzed overnight at 2° *vs.* the Tris–EDTA–dithiothreitol buffer; a concentrated aliquot of the nucleotide dissolved in the same buffer was added to the enzyme solution at room temperature to give the desired concentration. A 1.0-ml aliquot

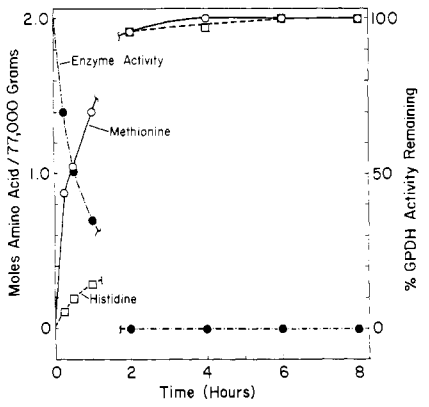


FIGURE 1: Carboxypeptidase A digestion of rabbit α-glycerophosphate dehydrogenase. The data are from two separate experiments. Proteolytic digestions during the first hour were performed in the Veronal buffer at 37°; digestions from 2 to 8 hr were performed in the *N*-ethylmorpholine acetate buffer at 30°. α-Glycerophosphate dehydrogenase enzymic activity remaining is expressed as per cent of activity in a control incubated without carboxypeptidase A.

containing about 20 nmoles of enzyme was then applied to the column, and the column was eluted with Tris–EDTA–dithiothreitol–nucleotide buffer. Absorbance at 260 mμ was monitored with a Beckman flow-through cell; when the appearance of a peak or trough in absorbance was observed, the effluent was collected until the base-line absorbance was again attained. The amount of DPNH in the peak, trough, and base-line pools was determined by decrease in absorbance at 340 mμ upon addition of dihydroxyacetone phosphate. Adenosine diphosphate ribose was measured by absorbance at 259 mμ, assuming identical molar absorbancy index as adenosine diphosphate (15.4×10^3 M^{-1} cm^{-1}). In experiments where the nucleotide was not added to the eluting buffer, a 0.6×32 cm column of medium grade Sephadex G-50 was used.

Optical rotatory dispersion measurements were made with a Durrum-Jasco ORD/UV-5 spectropolarimeter at ambient temperature (28 ± 0.5°). Measurements between 225 and 300 mμ were made on solutions with GPDH concentrations between 0.3 and 0.7 mg per ml; measurements between 280 and 470 mμ were made on solutions containing 0.5–0.8 mg of protein/ml. The buffer was 0.1 M Tris–1 mM β-mercaptoethanol (pH 7.5). In all cases, rotation was independent of protein concentration. Calculations of reduced mean residue rotations included a mean residue weight of 107 for rabbit GPDH (van Eys *et al.*, 1964) and 110 for honeybee GPDH (Brosemer and Marquardt, 1966); the index of refraction for water, including dispersion corrections (Yang, 1967), was used. Values from 300 to 470 mμ were used for the Moffitt–Yang plot (Moffitt and Yang, 1956; λ_0 arbitrarily set at 212 mμ) and for the modified two-term Drude plot (Shechter and Blout, 1964). A FORTRAN IV program written for the IBM 360-67 computer was used for linear least-squares analysis of the data.

Results

Carboxyl Terminal of Rabbit GPDH. Carboxypeptidase A released amino acids from native rabbit GPDH, so that it was possible to relate decrease in enzymic activity with release of carboxyl-terminal residues. The data presented in Figure 1 clearly show that methionine is a carboxyl-terminal amino acid with histidine a penultimate residue. After 4 hr of carboxypeptidase A digestion, 2.0 mole of both methionine and histidine was released from 77,000 g of GPDH; these values remained constant during a further 4-hr digestion. The results suggest that rabbit GPDH is a dimer with a C-terminal sequence in both subunits of -His-Met. There was a good correlation between release of methionine and loss of enzymic activity.

Carboxypeptidase A plus B digestions demonstrated that arginine or lysine is not released as a C-terminal amino acid from rabbit GPDH (Table I). In expt 1 of the table free lysine did not appear until extensive digestion of the dehydrogenase had already proceeded; no arginine was detected even after prolonged digestion.

TABLE I: Amino Acids Released from Rabbit α-Glycerophosphate Dehydrogenase by Treatment with Carboxypeptidases A Plus B.

	Expt 1[a]			Expt 2[b]
Incubation Time (hr):	1.1	5.3	8.3	2.5
Amino Acid[c]				
Met	0.81	1.7	1.8	1.4
His	0.26	0.80	0.90	0.71
Ala	0.12	1.2	2.6	1.1
Leu	0.10	1.3	2.4	1.3
Ile	0.11	1.1	2.1	1.1
Val	0.06	0.96	1.8	0.76
Thr	0.0	0.88	1.2	<0.95
Lys	0.0	0.80	0.99	0.43
Asn	0.0	0.38	1.0	
Gln	0.0	0.18	0.88	
Ser	0.0	0.14	0.63	<0.4
Phe	0.0	0.43	0.58	0.43
Arg	0.0	0.41	0.58	0.00
Gly	0.0	0.25	0.55	0.33
Tyr	0.0	0.40	0.51	0.36
GPDH activity remaining (per cent of control incubated without carboxypeptidase)	100	53	20	54

[a] Incubation temperature 30°. Carboxypeptidase A to GPDH weight ratio 1:80 until 3.3 hr, then 1:34; carboxypeptidase B to GPDH weight ratio 1:45 until 3.3 hr, then 1:20. [b] Incubation temperature 30°. Carboxypeptidase B to GPDH weight ratio 1:20. Glutamine, asparagine, threonine, and serine were not determined after acid hydrolysis. [c] Amount released in moles/77,000 g of protein.

TABLE II: Amino Acids Released from Rabbit α-Glycerophosphate Dehydrogenase by Treatment with Carboxypeptidase A.[b]

	Expt 1[c]	Expt 2	Expt 3		
Incubation Time (hr):	7	8	2	4	8
Amino Acid[b]					
Met	1.9	2.0	1.3	1.4	1.5
His	*a*	0.68	0.77	0.82	0.80
Ala	0.78	2.1	0.62	0.75	1.5
Ile	0.48	1.2	0.30	0.48	0.87
Leu	0.39	1.5	0.25	0.36	0.66
Thr	*a*	1.2	*a*	*a*	0.73
Val	0.33	1.1	0.30	0.36	0.59
Asn	*a*	0.76	0.0	0.0	0.41
Ser	*a*	0.56	0.0	0.0	0.27
Phe	0.0	0.46	0.0	0.0	0.11
Gln	*a*	0.43	0.0	0.0	0.20
Tyr	0.0	0.32	0.0	0.0	0.0
Gly	0.0	0.25	0.34	0.16	0.23
Lys	0.0	0.25	0.0	0.0	0.0
GPDH activity remaining (per cent of control incubated without carboxypeptidase)	7	*a*	22	25	2

[a] Not determined. [b] The weight ratio of substrate to carboxypeptidase A was 10:1 and the incubation temperature was 32°. The amount of amino acid released is given in moles/77,000 g of protein. [c] Temperature 30°.

In expt 2, only carboxypeptidase B was added to GPDH; the results indicate, however, that the protease was contaminated with carboxypeptidase A. Again, very little lysine and no arginine were released. In these experiments, there was no correlation between loss of GPDH enzymic activity and release of methionine residues. This is especially apparent in the first two samples of expt 1; although 0.81 and 1.7 moles of methionine per 77,000 g had been hydrolyzed, respectively, 100 and 53% enzyme activity remained.

Quantitative hydrazinolysis confirmed that methionine is the C-terminal residue. After hydrazinolysis, 1.0 mole of methionine sulfoxide/77,000 g of enzyme was found; in addition, traces of serine, alanine, and glycine were also present. These latter three amino acids are generally found as hydrazinolysis artifacts (Braun and Schroeder, 1967). Since a 50% yield of methionine as the sulfoxide is near the range of reported yields, these data are consistent with two C-terminal methionines per GPDH molecule. The terminal amino acids that would not have been detected by this method are asparagine, glutamine, and possibly tryptophan and arginine; the carboxypeptidase experiments indicated that these were not present.

Results from other carboxypeptidase A digestions of rabbit GPDH are presented in Table II. These data, as well as those of carboxypeptidase A plus B digestions presented in Table I, are consistent with two C-terminal methionine residues and with a penultimate histidine. However, at the longer incubation times no more than 0.9 mole of histidine was hydrolyzed, whereas 1 or more moles of alanine, leucine, isoleucine, valine, threonine, lysine, and asparagine had been released (expt 1 in Table I and expt 2 in Table II). The most plausible explanation of these results, if viewed alone, is that there are two nonidentical subunits, both with C-terminal methionine, one with penultimate histidine, the second with another penultimate residue, probably alanine. The results in Figure 1, though, indicate identical sequences of -His-Met. A possible explanation of the apparent discrepancy is that, in some but not all cases for unknown reasons, the release of histidine is incomplete and that the amino acid sequence immediately adjacent to the histidine in each subunit contains two or more residues of all the amino acids mentioned above. These results are not due to use of different commercial lots of GPDH, since different lots gave similar results.

The possibility that rabbit GPDH is composed of a single polypeptide chain with sequence -Met-His-Met is unlikely. In both the 1-hr sample in Figure 1 and the sample in expt 2 of Table II, the difference between free methionine and histidine is greater than 1.0 mole/77,000 g of GPDH.

Carboxyl-Terminal Residue of Honeybee GPDH. Determination of the carboxyl-terminal residue of honeybee GPDH proved to be more difficult. Preliminary experiments indicated that the native enzyme was refractory to carboxypeptidase A or B digestion; therefore, honeybee carboxymethyl-GPDH was used in further studies. Even with the denatured carboxymethylated derivative, initial experiments showed no release of any amino acid. Only after digestions with high levels of carboxypeptidase A for very long periods

TABLE III: Amino Acids Released from Honeybee Carboxymethylated α-Glycerophosphate Dehydrogenase by Treatment with Carboxypeptidase A.

	Expt 1			Expt 2		Expt 3	Expt 4		Expt 5
Weight Ratio of Substrate to Carboxypeptidase A:	12:1			10:1		6:1	6:1		12:1 until 24 hr, then 6:1
Incubation Temp (°C):	37			32		33	32		32
Incubation Time (hr):	0.5	1.0	2.0	1.8	3.5	6.0	2.5	23.5	48
Amino Acid[a]									
Ala	0.41	0.55	0.80	1.0	1.7	1.6	1.2	1.8	2.0
Asp	0.24	0.30	0.48	*b*	1.3	2.0	1.3	2.2	2.0
Gln	0.17	0.28	0.53	0.65	1.3	1.5	1.0	1.3	*b*
Thr	0.0	0.0	0.0	0.0	0.0	1.5	0.62	1.7	*b*
Gly	0.0	0.0	0.0	0.0	0.0	1.0	0.47	1.4	1.3
Ile	0.0	0.0	0.0	0.0	0.0	0.93	0.43	1.0	1.2
Leu	0.0	0.0	0.0	0.0	0.0	0.52	0.30	0.79	*b*

[a] The amount of amino acid released is given in moles/65,000 g of protein. [b] Not determined.

could free amino acids be observed. The data in Table III show that alanine was the first amino acid released by carboxypeptidase A digestion; 2 moles of alanine/mole of honeybee GPDH could be released, but only after a 48-hr incubation. Aspartate and glutamine appeared at identical initial rates after alanine. Aspartate is probably the penultimate amino acid, since its release by carboxypeptidase A is generally much slower than that of glutamine. This sequence would also account for the long incubation periods and high protease levels required for release of alanine, since penultimate aspartate residues slow or completely inhibit liberation of amino acids which are otherwise susceptible to carboxypeptidase-catalyzed hydrolysis (Ambler, 1967).

In two cases (expt 3 and 4, Table III), more aspartate was released than alanine; this may reflect an aspartate residue near the carboxyl terminal in addition to the penultimate residue. The data do not suggest that, if honeybee GPDH is composed of two subunits, these subunits might be dissimilar.

The terminal sequence appears to be -Thr-Gln-Asp-Ala, with glycine, isoleucine, and leucine also near the carboxyl terminus. Carboxypeptidase B did not release any arginine or lysine from honeybee carboxymethyl-GPDH.

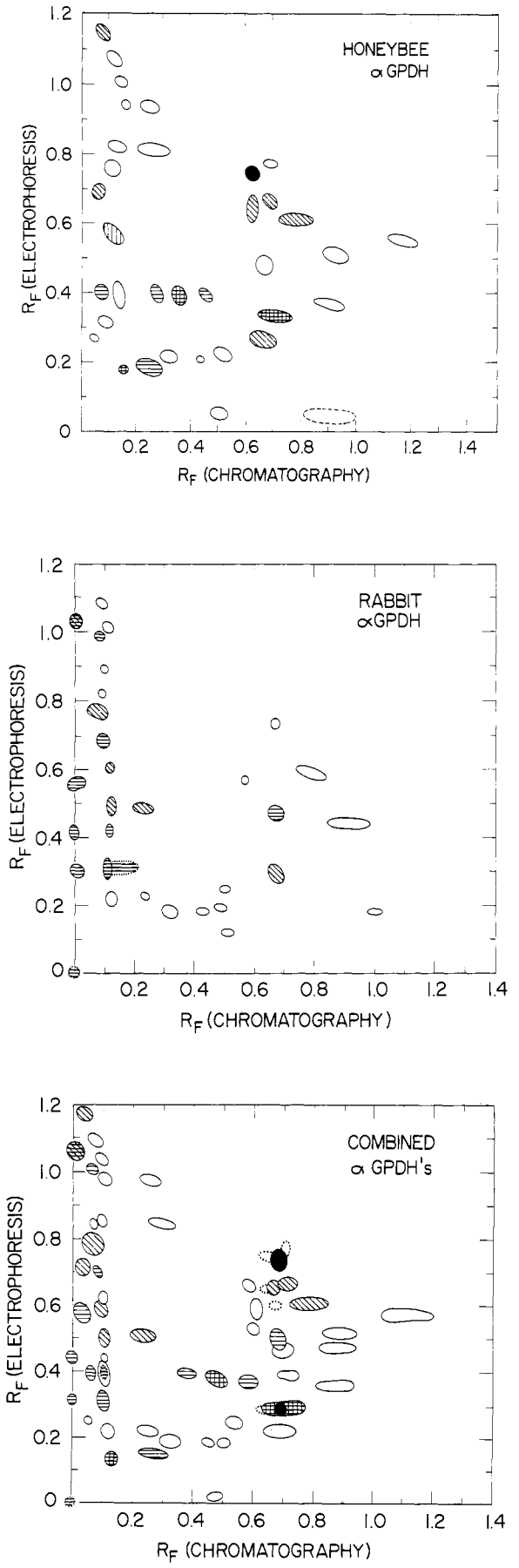
Hydrazinolysis of honeybee GPDH confirmed the above results; 1.0 mole of alanine/mole of GPDH was found, in addition to the usual traces of serine and glycine, after both 20- and 60-hr hydrazinolysis; 50% yield of alanine is within the range of reported yields (Braun and Schroeder, 1967), so that these data are consistent with two carboxyl-terminal alanines per mole of honeybee GPDH.

Under conditions of partial and acid hydrolysis resulting in selective cleavage of aspartate peptide bonds, a terminal sequence of -Asp-Ala should result in rapid release of free alanine, since only one bond must be cleaved (Schultz, 1967). Honeybee aminoethyl-GPDH was hydrolyzed for 6 hr at 105° in 0.03 M HCl, as described by Schultz; free amino acids in the hydrolysate were then determined with the amino acid analyzer. The only free amino acids present at levels greater than 1 mole/mole of GPDH were alanine (2.7 moles/mole), glycine (1.2 moles/mole), and aspartate (19.1 moles/mole). Thus alanine is released at the expected initial rapid rate. This, of course, does not prove the -Gln-Asp-Ala C-terminal sequence, since alanine could be arising from other segments (such as -Asp-Ala-Asp-) that may be especially labile in acid. Since 2.7 moles of alanine/mole of enzyme was released, at least some of the alanine must have arisen from these internal sequences. However, the experimental results are consistent with the suggested C-terminal structure.

Amino Terminals. The dansyl technique failed to indicate presence of a free N-terminal amino acid residue in either honeybee or rabbit GPDH. van Eys *et al.* (1964) reported that they could find no free amino terminal in the rabbit enzyme using the dinitrofluorobenzene method. They also found acetyl groups in the enzyme and suggested that the amino terminal is acetylated. Further studies are required to determine whether the blocking group is the same in both rabbit and honeybee GPDH.

Peptide Maps. There are 30 arginine and 40 lysine residues per mole of honeybee GPDH (Brosemer and Marquardt, 1966) and 16 arginine and 60 lysine residues per mole of rabbit GPDH (van Eys *et al.*, 1964). Peptide maps of tryptic digests might indicate whether either enzyme is composed of identical subunits. In order to eliminate artifacts arising from disulfide bond formation during digestion and peptide mapping, the carboxymethylated derivatives were used.

After tryptic digestion of either enzyme, ninhydrin-positive material was found at the origin of the peptide maps. It is probable that this material contained more than just undigested whole protein, since the Ehrlich spray indicated the tryptophan residues in both digests were located only at the origins. This core material also



stained very strongly for arginine and histidine plus tyrosine. All attempts to render the cores susceptible to tryptic digestion failed. The aminoethylated derivatives of each GPDH also showed tryptic-resistant material. Thus, the cores were either peptide material containing trypsin-resistant lysine or arginine bonds or a large peptide(s) having an arginine or lysine at the carboxyl terminus. It was not possible to differentiate between these two alternatives since not enough core material was available for fractionation. Therefore, peptide mapping could not be used to unequivocally establish the presence of identical subunits.

This method could be used, however, to compare primary structural features of rabbit and honeybee enzymes. The peptide maps for the rabbit, honeybee, and combined GPDHs are shown in Figure 2; in each case, insoluble core material was removed before sample application to the paper. The number of peptides and of specific amino acids found on each map is shown in Table IV. Almost all of the peptides on the combined map could be unequivocally assigned to either rabbit or bee GPDH. Out of the 54–60 peptides in the combined map, only 2 appeared to overlap; these were the two fastest peptides in the electrophoretic direction of the rabbit map and the second and third fastest peptides of the bee map. Neither of these spots was free lysine or arginine. Thus, the peptide maps indicated that there are very few if any extended regions with identical amino acid sequences in the trypsin-sensitive portion of rabbit and honeybee GPDHs.

Although presence of the core in digests of both honeybee and rabbit carboxymethyl-GPDH precluded unequivocal demonstration of sequence identity of the respective subunits, the number of peptide and specific amino acid spots in each case is consistent with identical subunit structures. As shown in Table IV, in no case did the number of peptides or specific amino acids exceed the maximum possible number calculated on the assumption of two identical subunits. A molecular weight of 77,000 was used for calculating the number of rabbit peptides; however, a molecular weight of 60,000 would not alter these conclusions. T. P. Fondy, C. R. Ross, and S. J. Sollohub (1968, personal communication) developed peptide maps after combined tryptic–chymotryptic digestion of rabbit GPDH; their results indicated two identical subunits.

Nucleotide Binding. The gel filtration technique of Hummel and Dreyer (1962) as applied by Pfleiderer

FIGURE 2: Reconstructed tryptic peptide maps of honeybee and rabbit carboxymethyl-α-glycerophosphate dehydrogenases. Lysine and phenol red served as reference standards in the electrophoretic and chromatographic directions, respectively. Insoluble digest material was removed before peptide mapping. The combined peptide map was produced by applying equal amounts of each tryptic digest. Dotted outlines indicate very light ninhydrin-positive spots; diagonal lines indicate Sakaguchi-positive (arginine) spots; horizontal lines indicate Pauly-positive (histidine or tyrosine) spots; vertical lines indicate tyrosine-positive spots; solid areas indicate Sakaguchi-, Pauly-, and tyrosine-positive spots.

TABLE IV: Number of Peptides Staining for Specific Amino Acids in Tryptic Peptide Maps of Rabbit and Honeybee Carboxymethyl α-Glycerophosphate Dehydrogenase.

Stain	Rabbit		Honeybee		Combined	
	Obsd[a]	Calcd[b]	Obsd[a]	Calcd[b]	Obsd[a]	Calcd[b]
Ninhydrin	30–32	39	34–36	36	54–60	75
Arginine	6	8	7	15	13	23
Histidine + tyrosine	9	13	8	10	17	23
Tyrosine	0	4	5	5	4	9
Tryptophan	0	2	0	1	0	3

[a] Peptide maps are shown in Figure 2. [b] Calculations are based upon amino acid compositions and molecular weights reported for rabbit (van Eys *et al.*, 1964) and honeybee (Brosemer and Marquardt, 1966) enzymes. Recent results in our laboratory indicate that the tyrosine content of honeybee GPDH is 5 instead of 4 residues per 33,000 g.

and Auricchio (1964) to DPNH binding by dehydrogenases was employed to measure binding of DPNH and of ADPR by rabbit and honeybee GPDHs. The results with DPNH are shown in Table V. Ankel *et al.* (1960) reported that the DPNH and rabbit GPDH spectra are additive; we, however, observed that binding of DPNH to either rabbit or honeybee GPDH resulted in a significant decrease in the 340-mμ absorbance of the nucleotide. Therefore, estimations of DPNH bound to GPDH were based solely on the area under the nucleotide trough; the experimental error in the number of DPNH molecules bound per mole of enzyme is about ±10%. Both honeybee and native rabbit GPDHs bind close to 2.0 moles of DPNH/mole of enzyme; this confirms the report by Pfleiderer and Auricchio on the native rabbit enzyme. Increasing the DPNH concentration from about 67 μM to about 195 μM did not significantly alter the number of nucleotide molecules bound; therefore, the binding constant for DPNH must be markedly lower than 50 μM. Removal of bound chromophore from native rabbit GPDH by charcoal treatment did not significantly affect DPNH binding. Thus, the chromophore either binds at a site distinct from the DPNH binding site or is displaced by DPNH. Attempts in our laboratory to demonstrate release of a chromophore from native rabbit GPDH by addition of DPNH have failed, although some evidence for such an exchange has been reported (Ankel *et al.*, 1960).

The chromophore attached to native rabbit GPDH binds quite tightly (van Eys *et al.*, 1959; Ankel *et al.*, 1960). Removal of this substance by charcoal treatment followed by incubation of the enzyme with free chromophore should result in rebinding. Using the gel filtration method, the binding of ADPR to native rabbit GPDH, charcoal-treated rabbit GPDH, and honeybee GPDH was measured; the concentration of ADPR in all experiments was 94 μM. In no case was any binding observed, since the absorbance measured in the flow-through cell remained constant throughout the run (limits of detection about 0.1 mole of ADPR/mole of enzyme). It is possible that the binding constant for ADPR might be greater than 100 μM, especially since the inhibition constant of ADPR for reaction of α-glycerophosphate and DPN^+ is about 160 μM (Kim and Anderson, 1968). It was not feasible to run similar ADPR binding experiments with very high ADPR concentrations because of problems of sensitivity and expense. Since binding of chromophore to rabbit GPDH is so tenacious, it should be possible to add an excess of the chromophore to the enzyme and then separate free and bound chromophore on a Sephadex G-50 column. This experiment was performed with native rabbit, charcoal-treated rabbit, and honeybee

TABLE V: DPNH Binding by α-Glycerophosphate Dehydrogenase.

GPDH	DPNH Concn (μM)	ADPR Concn (μM)	Moles of DPNH Bound/Mole of GPDH[a]
Honeybee	66	0	1.9
	197	0	2.0
	73	94	1.5
Native rabbit	68	0	2.0
	191	0	1.8
	74	94	2.0
Charcoal-treated rabbit	72	0	1.9
	193	0	2.0
	73	94	1.9

[a] Based on molecular weights of 77,000 for rabbit GPDH and 65,000 for honeybee GPDH.

TABLE VI: ADPR Binding by α-Glycerophosphate Dehydrogenase.

GPDH	280/260[a] −ADPR	280/260[a] +ADPR[b]
Honeybee	1.53	1.60
Native rabbit	1.44	1.45
Charcoal-treated rabbit	1.57	1.57

[a] $A_{280\,m\mu}/A_{260\,m\mu}$ of protein (about 3 mg) after passage through 0.6 × 32 cm column of Sephadex G-50 at 24°. Sample dissolved and eluted with 0.1 M Tris, 10 mM EDTA, and 1 mM dithiothreitol (pH 7.8). [b] Protein incubated 30 min at 24° with 10 mM ADPR and then applied to column.

GPDHs. In no case was the 280/260 ratio of the enzyme altered after incubation of 20 μM enzyme with 10 mM ADPR at 24° for 30 min followed by chromatography on Sephadex G-50 (Table VI). Thus, there is no evidence that rabbit or honeybee GPDH binds ADPR in a manner analogous to the binding by rabbit GPDH of its native chromophore.

The data in Table V show that 94 μM ADPR slightly inhibits DPNH binding by honeybee GPDH but not by rabbit GPDH. This slight inhibition probably simply reflects competition of a structural analog (ADPR) for the pyridine nucleotide-binding site; such a competition has been observed in kinetic studies with the rabbit enzyme (Kim and Anderson, 1968).

Optical Rotatory Dispersion. As a measure of comparative conformation, the optical rotatory dispersion of rabbit and honeybee GPDHs was observed in the range from 225 to 470 mμ. Since the native honeybee enzyme lacks bound chromophore, both the native and charcoal-treated rabbit enzymes were measured in order to account for any effect of the chromophore. In Table VII are presented the reduced mean residue rotations at 233 mμ, $[m']_{233}$, as well as a_0 and b_0 values from the Moffitt–Yang equation; in addition, the effective per cent α helix was calculated from the $[m']_{233}$ value, from b_0, and from the modified two-term Drude equation. In view of the large uncertainty in calculating the amount of α helix from ORD data, the helix values given in Table VII should be viewed only as experimental estimates. What is of prime concern here are the comparative values of the various parameters for the three proteins.

Removal of the chromophore from native rabbit GPDH did not significantly alter any of the ORD parameters; thus the protein conformation is apparently independent of chromophore binding. The dispersion parameters of the honeybee enzyme are slightly greater than for the rabbit protein, but the differences are not significant. The ORD spectra for all three GPDHs are similar to those of simple proteins, except that the first minimum occurs at 235 mμ instead of 233 mμ. The calculated values of the effective α helix in rabbit GPDH range from 24 to 39%; the values for honeybee GPDH range from 27 to 40%.

In all cases H_{193} differs significantly from H_{225}. Carver *et al.* (1966) pointed out that such a difference probably reflects presence of conformational structures in addition to random coil and α helix. This may explain the rather large discrepancies in each case between the effective α helix as calculated from $[m']_{233}$ and from the 300- to 470-mμ dispersion spectrum.

Hydrophobicity. Bigelow (1967) has suggested that the average hydrophobicity of protein is a structural parameter related, in many cases, to some of the physical properties of the protein. The average hydrophobicity is based on free energies of transfer of amino acid side chains from an organic to an aqueous environment; this parameter is calculated from the amino acid composition of the protein and the hydrophobicity value for the individual amino acid residues. Since the amino acid compositions of the rabbit (van Eys *et al.*, 1964), the rat (Fondy *et al.*, 1968), and the honeybee (Brosemer and Marquardt, 1966) GPDHs have been reported, it was possible to calculate the average hydrophobicities in order to determine whether this parameter may have been conserved despite phylogenetic variation of the GPDH molecule. (T. P. Fondy, C. R. Ross, and

TABLE VII: Optical Rotatory Dispersion Parameters of Honeybee and Rabbit α-Glycerophosphate Dehydrogenases.

Protein	$[m']_{233}$ (deg)	Helix[c] (%)	Moffitt–Yang Equation[a] a_0	b_0	Helix Y[d] (%)	Helix CSB[e] (%)	MTTDE[a,b] Helix[f] H_{193} (%)	H_{225} (%)	$H_{193-225}$ (%)
Honeybee GPDH	−5240	27	−180	−200	32	38	40	32	38
Native rabbit GPDH	−4820	24	−113	−181	29	35	39	27	35
Charcoal-treated rabbit GPDH	−5060	26	−125	−184	29	36	39	28	36

[a] For the range from 300 to 470 mμ. [b] Modified two-term Drude equation (Shechter and Blout, 1964). [c] $(-[m']_{233} - 1770)/128.3$ (Carver *et al.*, 1966). [d] $-b_0/6.3$ (Yang, 1967). [e] $(-b_0 + 100)/8.0$ (Carver *et al.*, 1966). [f] Helix calculated from A_{193}, A_{225}, $(A_{193} - A_{225})$, respectively (Carver *et al.*, 1966).

S. Sollohub (1968, personal communication) have redetermined the amino acid composition of the rabbit enzyme and have found slightly different values than those reported by van Eys; the average hydrophobicity is not altered by using the newer values.) The calculated values are the following (in kilocalories per residue): 1.14 for rabbit, 1.15 for rat, 1.14 for honeybee; thus all three values are essentially identical.

Discussion

The C-terminal results are consistent with a dimeric structure for both rabbit and honeybee GPDH. The data for the honeybee enzyme indicate that both polypeptide subunits have identical sequences in the last three or four C-terminal residues. As discussed above, the data for the rabbit enzyme are somewhat ambiguous. There is no doubt that both rabbit subunit polypeptides contain a C-terminal methionine and that histidine is the penultimate residue in at least one of the monomers. The data in Figure 1 suggest that histidine is the penultimate residue in both subunits, but the most logical explanation of the data in Tables I and II is that the penultimate residue in one of the chains is not histidine. The only valid conclusion which is consistent with both sets of data was described above, *i.e.*, that in some cases carboxypeptidase hydrolyzes histidine from only one of two identical (in the C-terminal portion) chains and that two or more residues of alanine, leucine, isoleucine, valine, threonine, lysine, and asparagine are then released from this one polypeptide chain.

The C-terminal sequence of honeybee GPDH appears to be -(Leu-Ile-Gly)-Thr-Gln-Asp-Ala; that of the rabbit enzyme (assuming identical subunits) is -(Thr-Val-Ile-Leu)-Ala-His-Met. The sequence of residues in parentheses is tentative. Except for the presence of threonine, leucine, and isoleucine in each case, there appears to be no homology in the C-terminal sequences of these two dehydrogenases. In fact, the three terminal residues have completely different functional groups: alanine-glutamine, histidine-aspartate, and methionine-alanine. If the rabbit enzyme is composed of two different subunits (-His-Met and probably -Ala-Met), the antipenultimate residues would likely be leucine, isoleucine, threonine, or valine. Again, there is no apparent homology with the honeybee enzyme. Since the molecular weights of the honeybee and rabbit enzymes are quite different (65,000 *vs.* 60,000 or 77,000), a deletion or extension of the subunit polypeptide at the C terminus may have occurred during evolutionary change.

The C-terminal studies, especially those shown in Figure 1, are a compelling indication that the molecular weight of rabbit GPDH is 77,000. The GPDH determination for the 2-, 4-, 6-, and 8-hr incubations shown in Figure 1 was made by amino acid analysis after acid hydrolysis for 24, 48, and 72 hr; this is by far the most accurate method for protein estimation. Individual values for moles of methionine released per 77,000 g of protein were: 1.92 at 2 hr, 1.99 at 4 hr, 2.03 at 6 hr, and 2.02 at 8 hr; the corresponding values for the penultimate histidine were 1.95, 1.95, 1.98, and 1.98. Fresh carboxypeptidase A was added after 2 hr of incubation. If the molecular weight of rabbit GPDH is 60,000 g, then carboxypeptidase A released less than 1.6 moles of methionine and histidine per mole of enzyme and the hydrolysis then ceased, despite addition of fresh carboxypeptidase. Final resolution of the discrepancy in molecular weight determinations for rabbit GPDH must, however, await further studies.

Hydrolysis of native rabbit GPDH by carboxypeptidase A showed a good correlation between release of C-terminal methionine and decrease in dehydrogenase activity; hydrolysis by carboxypeptidase A plus B, on the other hand, resulted in a slower decrease in enzyme activity relative to methionine hydrolysis. Probably this methionine is not directly involved in the catalytic activity. Removal of the C-terminal residue may result in a conformational change which, in turn, affects enzyme function. The rate of conformational change may depend on subtle environmental factors, such as temperature, pH, buffer, and interaction with other proteins. Such factors may have been sufficiently different in the carboxypeptidase A and A plus B experiments to account for the varying results.

The fact that the tryptic peptide maps of honeybee and rabbit GPDH showed only two overlapping peptides would indicate that the primary structures of the two enzymes are markedly different. However, caution must be exercised in interpreting such data. First, it is unknown how much of each dehydrogenase is represented in the tryptic core. Secondly, unexpected proteolytic cleavages or lack of expected cleavages can lead to artifacts; this was strikingly illustrated by the studies of Harris and Hindley (1965). Thirdly, Harris and Perham (1968) have reported that peptide maps of lobster and pig glyceraldehyde 3-phosphate dehydrogenases exaggerate the few sequence differences due to the relatively high frequency of different arginine and lysine dispositions in the proteins.

Both honeybee and rabbit GPDHs bind 2 equiv of DPNH. Ankel *et al.* (1960), using fluorescence titration methods, reported that only one DPNH is bound by the rabbit enzyme. Preliminary fluorescent titrations in our laboratory show about two DPNHs bound by the honeybee enzyme. It is still necessary to confirm whether there is indeed a discrepancy in the number of DPNHs bound by the rabbit enzyme as measured by gel filtration and by fluorescence titration. The gel filtration technique certainly gives true binding values. It is possible that only one of the two DPNHs binds at a site where its fluorescence is enhanced; this site might, for example, be at the active center and the other DPNH binding site might be involved in some other function, such as controlling conformation. Since the fluorescence of both DPNHs bound by the honeybee enzyme is apparently increased, there may be a significant difference in catalytic activity of the honeybee and rabbit GPDHs.

Native or charcoal-treated rabbit GPDH as well as honeybee GPDH do not bind free ADPR. It thus appears that ADPR is not one of the ultraviolet-absorbing compounds bound to the native rabbit

enzyme, but rather an artifact produced from the bound chromophore. Celliers *et al.* (1963) have detected release of ADPR and a smaller ultraviolet-absorbing compound from rabbit GPDH. Our results and those of Celliers *et al.* would indicate that the chromophore bound to rabbit GPDH is a degradation product of DPN^+ or DPNH which had been originally attached to the protein. Degradation could occur during the purification procedure. The chromophore probably plays no significant physiological role, since its removal does not affect enzymic activity, crystal form, sedimentation coefficient (Ankel *et al.*, 1960), DPNH binding, or ORD spectrum.

Calculations of protein hydrophobicity as described by Bigelow (1967) do not take into consideration conformation of the protein; that is, they do not include correction factors for the buried or exposed nature of individual residues. However, Bigelow has demonstrated there does exist a relationship between hydrophobicity of many proteins and some of their physical properties. For example, proteins with higher hydrophobicity values than "normal" tend to show self-association and lower solubility. Honeybee GPDH aggregates at moderate protein concentrations and has a relatively low solubility (Marquardt and Brosemer, 1966); the rabbit enzyme does not aggregate (van Eys *et al.*, 1959) and has the typical high solubility of most globular proteins. These differing properties are not reflected in the calculated hydrophobicity, since this value is identical for each GPDH, 1.14 kcal/mole.

Hydrophobicity calculations for the rat enzyme gave essentially the same value, 1.15 kcal/mole. Although the amino acid compositions of the three GPDHs are similar, there are more than enough differences to allow for amino acid interchanges which could greatly alter the hydrophobicity. If hydrophobicity calculations are indeed a meaningful reflection of some aspects of protein structure, the essentially identical values for rabbit, rat, and honeybee GPDH would suggest that during evolutionary development of this protein there has been a strict conservation of the hydrophobicity parameter.

The similar hydrophobicity and ORD parameters for the rabbit and honeybee enzymes might suggest that a specific conformation is required for catalyzing the GPDH reaction and that this conformation can be conserved only if hydrophobicity is maintained essentially constant. The GPDH molecule has undergone many primary structural changes during the course of evolution. This is documented not only by the studies reported here but also by comparative immunochemical and electrophoretic investigations of insect GPDHs (Brosemer *et al.*, 1967). Unpublished observations in our laboratory have shown that there are many enzymic variants of GPDH even within the single genus *Bombus* (bumblebees). However, despite the significant rate of phylogenetic change in primary structure, the conformation necessary for catalytic activity in the GPDH reaction may have remained essentially invariable.

Many of the comparative physical, chemical, and enzymic properties of rabbit and honeybee GPDH not described in this report are mentioned in the papers by Marquardt and Brosemer (1966) and Brosemer and Marquardt (1966). Many properties are similar enough to indicate that the rabbit and honeybee enzymes are homologous; for example, amino acid composition, dimeric structure, blocked N terminals, ORD spectrum, average hydrophobicity, DPNH binding, reaction with DPN^+ analogs, inhibition by sulfhydryl reagents, turnover number, and inhibition by substrate. Many properties, on the other hand, are quite different; for example, C-terminal sequence, tryptic peptide maps, electrophoretic behavior, aggregation at moderate protein concentrations, solubility, molecular weight, binding of chromophore, and pH optimum. Those properties that are different in the two GPDHs may reflect not only the fact that the molecules diverged over 7×10^8 years ago but that the metabolic function of each is now quite distinct.

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Added in Proof

The data cited in the text as personal communication from T. P. Fondy, C. R. Ross, and S. Sollohub have recently appeared (Fondy *et al.*, 1969). Kim and Anderson (1969) reported fluorescence titrations of rabbit GPDH which indicate binding of 2 moles of DPNH/78,000 g of enzyme.

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Synthesis of 14α-Hydroxyprogesterone and Its Isolation from Human Late-Pregnancy Urine*

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ABSTRACT: Nonlabeled and high specific activity [4-^{14}C]-14α-hydroxyprogesterone was synthesized from 14α-hydroxydeoxycorticosterone, prepared by microbiological hydroxylation of deoxycorticosterone. Purified [4-^{14}C]-14α-hydroxyprogesterone of known specific activity was added as a recovery marker to a large pool of late-pregnancy urine (82 l.) and the steroid was isolated and identified. The 14α-hydroxyprogesterone excreted in the urine was quantitated by the isotope derivative procedure by first reducing it to 14α,20β-dihydroxypregn-4-en-3-one with 20β-hydroxysteroid dehydrogenase followed by acetylation with [^{3}H]acetic anhydride.

From the ^{3}H/^{14}C ratio of the purified derivative the excretion of 14α-hydroxyprogesterone was found to be 0.52 μg/day. Identification of the isolated 14α-hydroxyprogesterone was confirmed by infrared analysis.

The microbiological hydroxylation at C_{15} and C_{16} of the steroid nucleus has been described and the subject has been extensively reviewed (Dorfman and Ungar, 1965). In recent years these hydroxylations have also been demonstrated to occur in mammalian tissues. A number of neutral 16α- and 15α-hydroxy steroids have been isolated from human urine, (Hirschmann and Hirschmann, 1945, 1950; Lieberman *et al.*, 1953; Fotherby *et al.*, 1957; Neher *et al.*, 1959; Hirschmann *et al.*, 1961; Fukushima *et al.*, 1961; Bongiovanni, 1962; Reynolds, 1965, 1966; Ruse and Solomon, 1966a,b; YoungLai and Solomon, 1967; Giannopoulos and Solomon, 1967). These findings prompted us to initiate a study to determine whether 14α-hydroxylated steroids could also be formed by mammalian tissues. We therefore set out to synthesize labeled 14α-hydroxyprogesterone[1] in order to use it as an aid in its isolation.

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[1] The following trivial names have been used: progesterone = pregn-4-ene-3,20-dione; pregnanediol = 5β-pregnane-3α,20α-diol; deoxycorticosterone = 21-hydroxypregn-4-ene-3,20-dione; deoxycorticosterone acetate = 21-acetoxypregn-4-ene-3,20-dione; 14α-hydroxyprogesterone = 14α-hydroxypregn-4-ene-3,20-dione; testosterone = 17β-hydroxyandrost-4-en-3-one; 14α-hydroxytestosterone = 14α,17β-dihydroxyandrost-4-en-3-one; estrone = 3-hydroxyestra-1,3,5(10)-trien-17-one; 14α-hydroxyestrone = 3,14α-dihydroxyestra-1,3,5(10)-trien-17-one; 17β-estradiol = estra-1,3,5(10)-triene-3,17β-diol; 14α-hydroxyestradiol = estra-1,3,5(10)-triene-3,14α,17β-triol.