R values for these systems was therefore more adequate. The derived values of R as a function of p_0 for perylene in glycerol–propylene glycol (1:1) at 4° are shown in Figure 8. It can be clearly seen that the changes in R with p_0 of perylene in the tested micelles and in the glycerol–propylene glycol system qualitatively follow the same pattern. For both systems the lowest values of R appear around p_0 corresponding to the out-of-plane rotation, and the highest values of R at p_0 corresponding to the in-plane rotation. R values at $p_0 = 0.5$ are somewhat lower than the respective average of the in- and out-of-plane R values. A comparison of the obtained values for R in the micelles and in the glycerol–propylene glycol system at $p_0 = 0.5$, $p_0 = -0.25$, and at the minima of the curves, shows that all three ratios of R fall in the range of 3–5. The variation of the rate of rotation of perylene in propylene glycol at −14° resembles in shape the curves given in Figure 8. An analogous comparison of the ratios of the R values of the micelles and the propylene glycol system gives values in the range of 2–3.

The above findings indicate that in the anisotropy of rotation observed for perylene molecules in CTABr–HSNa micelles, the solvent structure plays little, if any, part and therefore that the interior of these micelles is nearly isotropic and resembles in nature an aliphatic hydrocarbon solvent.

The considerable anisotropy of rotation ($R_p/R_{op} > 10$) observed in all the media must be attributed to the shape of the molecule and its relation to the nearby solvent molecules. It appears that the molecule can "slip" in its own plane much more easily than rotate out of it. Certainly the dissolved molecule cannot be conceived as anything resembling an oblate ellipsoid of revolution in a totally isotropic medium. Such a situation would demand $R_p \simeq R_{op}$ (Perrin, 1934, 1936), which is far from the present case. There is reason to believe that the assumption of a hydrodynamic ellipsoid having roughly the shape of the molecule is a valid one when the particle is large compared to the solvent molecules (10^2:1 or 10^3:1), but our present observations show that this assumption is no longer tenable when the ratio is of the order of 5:1.

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Comparative Structural Properties of Insect Triose Phosphate Dehydrogenases*

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ABSTRACT: Triosephosphate dehydrogenase (EC 1.2.1.12) was isolated from honey bees, four species of bumblebees (*Bombus nevadensis*, *Bombus occidentalis*, *Bombus appositus*, and *Psithyrus suckleyi*), leaf-cutting bees, fleshflies, and screwworm flies. The amino acid compositions and, except for the screwworm fly, tryptic peptide maps of these proteins were compared to one another and with the published data for lobster and pig triose phosphate dehydrogenase. The structural relationships of most of the dehydrogenases correlate with the known phylogeny of the species; however, the honeybee enzyme differs much more from the other enzymes than is expected from the phylogeny. The structure of the *Psithyrus* dehydrogenase is very similar to that of the three Bombus species; this may suggest that the genus *Psithyrus*, which comprises the bumblebees with inquiline (parasitic like) behavioral patterns, probably arose after lineages leading to at least some modern *Bombus* species had diverged from the common ancestral stock.

Although insects comprise the largest and most diverse of all taxonomic classes, there have been very few studies on the comparative structure of insect proteins. The primary sequences of only four insect proteins, all cytochromes *c*, have been published (McLaughlin, 1969). The present report describes studies on the comparative structures of triose phosphate dehydrogenases (D-glyceraldehyde 3-phosphate + phosphate + DPN^+ = 1,3-diphospho-D-glyceric acid + DPNH, EC 1.2.1.12) from six species of bees and two species of flies. The methods employed include enzyme purification, amino acid composition, and peptide mapping. This enzyme was chosen since it could be easily isolated from every insect species we tried and the primary sequences of the lobster (Davidson *et al.*, 1967) and pig (Harris and Perham, 1968) enzymes have been reported. All triose phosphate dehydrogenases yet investigated are tetramers with subunit molecular

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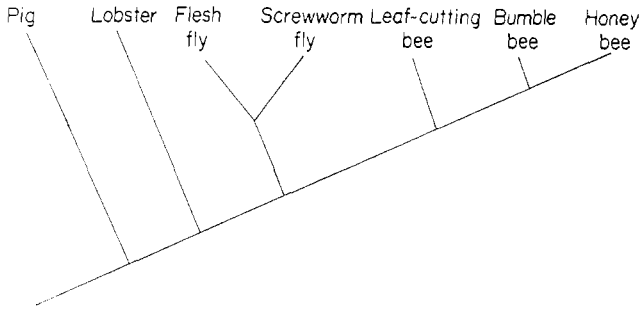


FIGURE 1: Phylogenetic relationships of seven organisms. Only the relative order of divergences, not the times of divergence, are represented.

weights around 36,000 (Allison and Kaplan, 1964; Harris and Perham, 1965; Harrington and Karr, 1965).

As a point of reference, the accepted phylogeny of the organisms discussed in this paper is shown in Figure 1; this figure does not attempt to indicate at what time in evolutionary history each branching occurred, but rather it illustrates only the order of branching.

Four species of bumblebees (tribe *Bombini*) are considered in this report. There are two genera of bumblebees, the common *Bombus* and the inquiline (parasitic like) *Psithyrus*. *Psithyrus* queens invade and take over a nest already established by a *Bombus* queen and force the *Bombus* workers to forage and care for the *Psithyrus* brood. *Psithyrus* lacks a worker caste, since it "enslaves" the *Bombus* workers.

The three *Bombus* species used represent all three taxonomic sections of the genus *Bombus* found in North America north of Mexico (Burks, 1951).

In this report we use the term "overall rate of evolutionary change" of a protein to indicate the total number of amino acid substitutions that have occurred per amount of time since divergence of the protein from some given ancestral protein. The substitutions may have occurred at an approximately constant rate during the whole time interval (Margoliash *et al.*, 1969; Wilson and Sarich, 1969; Sarich, 1969), they may have occurred during a relatively short time interval (perhaps when selective pressure was high), or they may have occurred at rates intermediate to these two extremes (Kirsch, 1969). The data presented here do not allow definite conclusions in this regard.

Materials and Methods

Materials. Trypsin treated with L-(1-tosylamido-2-phenyl)-ethyl chloromethyl ketone was purchased from Worthington. Dithiothreitol was from Calbiochem; D-fructose-1,6-diP and aldolase were from Sigma. Urea (J. T. Baker Co.) was recrystallized from ethanol. Iodoacetic acid-2-^{14}C (83 μCi/mg) was obtained from Amersham–Searle; 100 μCi of the labeled compound was diluted with 100 mg of unlabeled iodoacetic acid. CM- and DEAE-celluloses were purchased from Bio-Rad.

Italian honey bees (*Apis mellifera*) were purchased from C. G. Wenner and Sons, Glenn, Calif. Fleshflies (*Sarcophaga bullata*) were reared in the laboratory. Screwworm flies (*Callitroga hominivorax*) were kindly supplied by the USDA Screwworm Eradication Program, Mission, Texas. Leaf-cutting bee (*Megachile rotundata*) larvae were purchased from Dority Bee Boards, Nyssa, Ore., and were reared to 1 week of adult age in the laboratory. Bumblebees (*Bombus nevadensis*, *Bombus appositus*, *Bombus occidentalis*, and *Psithyrus suckleyi*) were caught in the field and stored frozen.

Carboxymethylation. The method is a modification of the procedure described by Harris and Perham (1965). The enzyme (20–80 mg) was dialyzed exhaustively at 2° against 0.1 M Tris-HCl–10 mM EDTA–1 mM dithiothreitol, (pH 8.7 measured at 23°). The dialyzed solution (2–8 ml) was centrifuged to remove insoluble protein. The absorbance of the solution at 280 nm was measured; it was assumed that 1 mg of enzyme/ml had an absorbance of 1.0 at 280 nm (Fox and Dandliker, 1956). One-half volume of 1 M Tris-HCl–0.1 M EDTA (pH 8.7 measured at 23°) was added to the solution; solid urea was added to a final concentration of 8 M. After incubation for 3 hr at 37°, iodoacetic acid-2-^{14}C dissolved in 0.5 ml of 1 N NaOH was added; the amount added was 75 μg/mg of enzyme plus 185 μg/μmole of dithiothreitol in the enzyme solution. The mixture was incubated a further 60 min in the dark at 37°. Excess β-mercaptoethanol was added and the resulting solution dialyzed several days against many changes of 1 mM HCl. If a major precipitate formed, the protein was lyophilized; if most of the protein remained in solution, this solution was stored frozen.

Peptide Mapping. The [^{14}C]carboxymethyltriose phosphate dehydrogenase was suspended in water; the pH was adjusted to about 8 by adding 1 M NH_3 and using phenol red as indicator. The incubation temperature was 37°. At zero time, L-(1-tosylamido-2-phenyl)ethyl chloromethyl ketone treated trypsin dissolved in 1 mM HCl was added at a substrate to trypsin weight ratio of 200:1. After 1.5 hr, an equal amount of trypsin was again added. The pH was kept constant by further additions of 1 M NH_3. After a total incubation time of 3 hr, the samples were frozen and lyophilized to dryness. The peptide-mapping method for the material soluble in water has been described (Brosemer and Kuhn, 1969). Combined peptide maps, in which material from two digests was run on the same paper, were also run.

Ninhydrin, arginine, and Pauly (histidine + tyrosine) color reactions have been described (Easley *et al.*, 1969); the Pauly reaction was more successful if the arginine reaction had not been first used. The Ehrlich test (Easley, 1965) located tryptophan. [^{14}C]Carboxymethylcysteine was located with autoradiography; Kodak Medical X-Ray film (No Screen) was placed in contact with the peptide map for 3 weeks and then developed.

Amino Acid Composition. Protein samples in hydrolysis vials were suspended in distilled constant-boiling HCl. The vial was evacuated and flushed with nitrogen twice, evacuated to at least 0.01 mm, and sealed. After hydrolysis at 108° the samples were dried in a rotary evaporator and analyzed on a Beckman 120C amino acid analyzer. Except as noted below, duplicates, and sometimes triplicates, were run for each time sample. Samples of carboxymethyltriose phosphate dehydrogenase were hydrolyzed for 24, 48, 72, and 96 hr; samples of the untreated enzyme were hydrolyzed for 24 hr and, in some cases, for 48 and 72 hr. Serine and threonine were determined by extrapolation to zero time (Downs and Pigman, 1969); valine and isoleucine levels were calculated from the maximum values reached by 72 and 96 hr. Single determinations of tryptophan in the honey bee and *B. nevadensis* proteins were run after hydrolysis in the presence of thioglycolic acid (Matsubara and Sasaki, 1969); using proteins with known tryptophan contents, we confirmed the reported yield of tryptophan to be around 85%.

TABLE I: Amino Acid Composition of Triose Phosphate Dehydrogenases.

Amino Acid	Honey Bee	Bumblebee				Leaf-Cutting Bee	Fleshfly	Screw-worm Fly	Lobster	Pig
		B. nevadensis	*B. appositus*	*B. occidentalis*	*P. suckleyi*					
Lysine	30	26	26	26	27	26	26	25	28	26
Histidine	6	6	6	6	6	6	5	5	5	11
Arginine	9	11	11	11	11	11	10	10	9	10
Aspartic acid	36	38	38	38	38	40	40	38	32	38
Threonine	18	16	17	16	16	16	22	23	20	22
Serine	19	21	19	20	21	23	24	25	25	19
Glutamic acid	23	20	20	20	21	19	16	17	24	18
Proline	11	15	14	14	14	14	13	14	12	12
Glycine	30	30	31	30	30	31	30	30	30	32
Alanine	37	36	38	38	37	34	37	35	32	32
Cysteine	5	3	3	3	3	3	3	3	5	4
Valine	32	32	33	34	31	30	36	37	38	34
Methionine	5	5	5	5	5	5	5	4	10	9
Isoleucine	24	26	25	25	25	27	20	21	18	21
Leucine	20	21	20	20	21	20	19	19	18	18
Tyrosine	12	11	11	11	11	11	9	8	9	9
Phenylalanine	12	12	12	12	12	13	14	16	15	14
Tryptophan	3	3	3[b]	3[b]	3[b]	3[b]	3[b]	3[b]	3	3
Total[a]	332	332	332	332	332	332	332	333	333	332

[a] The values for the individual amino acid residues of the insect dehydrogenases were adjusted to give a total of 332–333, which are the totals for a single subunit of the tetrameric pig and lobster enzymes, respectively. [b] Not measured, but assumed to be 3. See text for explanation.

There was only sufficient screwworm fly triose phosphate dehydrogenase to run single 24-, 48-, 72-, and 96-hr samples of the untreated enzyme and duplicate 24-hr samples which had been oxidized with performic acid (Moore, 1963) for cysteic acid determination. The combined composition data, however, were very good and are probably as reliable as that for the other insect triose phosphate dehydrogenases.

Except as noted below, where three or more values for a single amino acid residue were available, the standard deviation was either under 3% or under 0.5 residue/subunit. The standard deviations for methionine in the leaf-cutting bee, fleshfly, and screwworm proteins were ±0.76, 1.15, and 0.84 residues per subunit, respectively, and for lysine in fleshfly ±3.6 per cent.

Calculations. The deviation function comparing amino acid compositions was calculated using a FORTRAN IV program written for the IBM 360-67 computer.

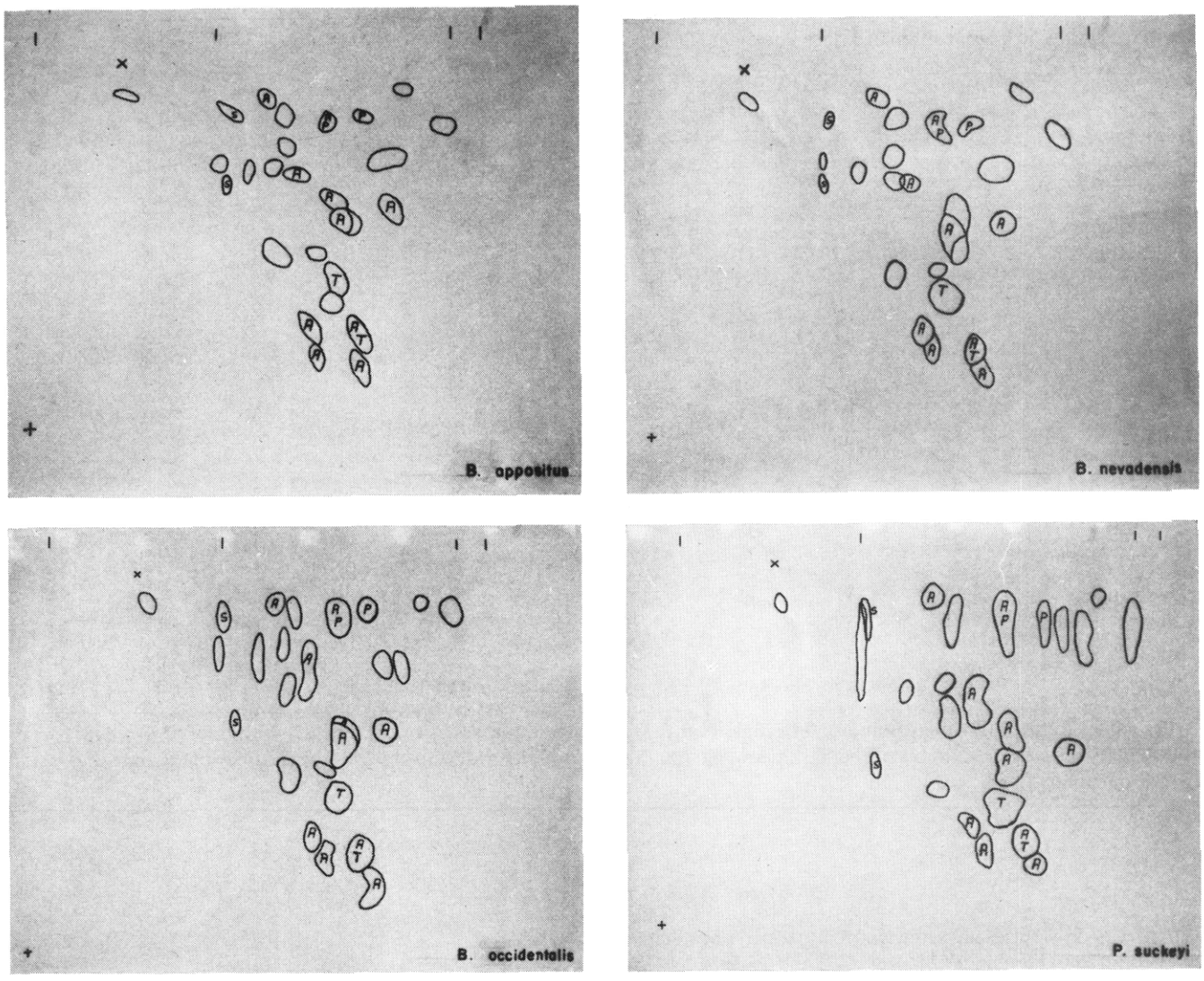
Enzyme Assay. The method previously published (Carlson *et al.*, 1971) was used.

Enzyme Purification. The procedure, which was identical for all insect species, has been described (Carlson *et al.*, 1971). The criteria of purity for the honey bee enzyme have been reported (Marquardt *et al.*, 1968) and included a single band disc gel electrophoresis, a single peak in sedimentation velocity ultracentrifugation, and specificity in an immunological reaction. The sole criteria of purity applied to the other insect triose phosphate dehydrogenases (each of which had been recrystallized three to four times) was presence of a single band in the same disc gel electrophoretic system used with the honey bee enzyme.

Results

Amino Acid Compositions. Table I lists the amino acid compositions of six bee and two fly triose phosphate dehydrogenases. The total number of amino acid residues was adjusted to 332 or 333, which are the values per 36,000 molecular weight subunit of the pig and lobster enzymes, respectively (Harris and Perham, 1968). Inspection of the table reveals obvious similarities in the compositions of all the proteins, although there are some significant differences. The compositions of the lobster and pig enzymes are included for comparison.

In order to simplify digestion of this large mass of amino acid composition data, a slight modification of the deviation function suggested by Harris *et al.* (1969) was applied. This approach compares composition data for two proteins by the deviation function, $[\Sigma(P_{1,n} - P_{2,n})^2]^{1/2}$, where $P_{1,n}$ is the mole per cent of amino acid n in protein-1 and $P_{2,n}$ is the mole per cent of the same amino acid in protein-2. Harris *et al.* used mole fraction instead of mole per cent. (A typographical error resulted in the absence of the square sign in the original reference.) The smaller the value for the deviation function, the more similar are the amino acid compositions. This function has been found to quite accurately reflect comparative structural similarities in proteins where primary sequences were known. Of course, there is no substitute for determination of primary sequences for estimating relative similarities, but the deviation function appears to provide useful information in the absence of such sequence data. The deviation function values for the various insect



triose phosphate dehydrogenases are listed in Table II; the lobster and pig enzymes are included for comparison. In those cases where tryptophan was not determined, a value of three residues per subunit was assumed, since this is the value for the enzyme from pig and lobster (Davidson *et al.*, 1967; Harris and Perham, 1968), from several other vertebrates (Allison and Kaplan, 1964), and from the other insects in Table II.

The four bumblebee triose phosphate dehydrogenases have very similar compositions, as would be expected. When honey bee triose phosphate dehydrogenase is the reference protein (deviation function 0.0), the order of increasing deviation function is qualitatively consistent with the accepted phylogeny, *i.e.*, honey bee, bumblebees, leaf-cutting bee, fleshfly $\simeq$ screwworm fly. However, if any of the bumblebee triose phosphate dehydrogenases is the reference, the order is bumblebee, leaf-cutting bee, honeybee, fleshfly $\simeq$ screwworm fly. With leaf-cutting bee as reference the order is leaf-cutting bee, bumblebees, honey bee, fleshfly $\simeq$ screwworm fly. With fleshfly as reference, the order is fleshfly, screwworm fly, leaf-cutting bee $\simeq$ bumblebees, honey bee.

The comparison of the insects to lobster gives values for the deviation function which range from 3.9 to 5.3; the comparison of the insects to pig gives values from 3.3 to 4.1. These broad ranges probably represent the approximate upper limits in the deviation function which occur when comparing any two animal triose phosphate dehydrogenases. Interpretation of comparative triose phosphate dehydrogenase structures to this method is certainly questionable when the deviation function values are within this upper range, since the amino acid composition differences become so large that they no longer accurately reflect primary structural differences. For example, the deviation function value for insect–pig is in each case smaller than that for insect–lobster, although lobsters are phylogentically more closely related to insects than are pigs. (A convergent evolutionary relation between insect and pig triose phosphate dehydrogenases is of course possible but unlikely.)

The unexpectedly large deviation function for honey bee is significant when either bumblebee or leaf-cutting bee is the reference. The significance of the larger honey bee value is questionable when fleshfly or screwworm fly is the reference, although in both cases the honey bee value is greater than that of any of the other insects, which at least suggests that the greater honey bee dissimilarity may extend even to the comparison with flies.

The most likely explanation of these amino acid composition data is that the overall rate of evolutionary change of the triose phosphate dehydrogenase molecule in the honey bee phyletic branch has been faster than that in the other bee branches and possibly also than that in the fly branch.

Tryptic peptide maps strengthen the above explanation.

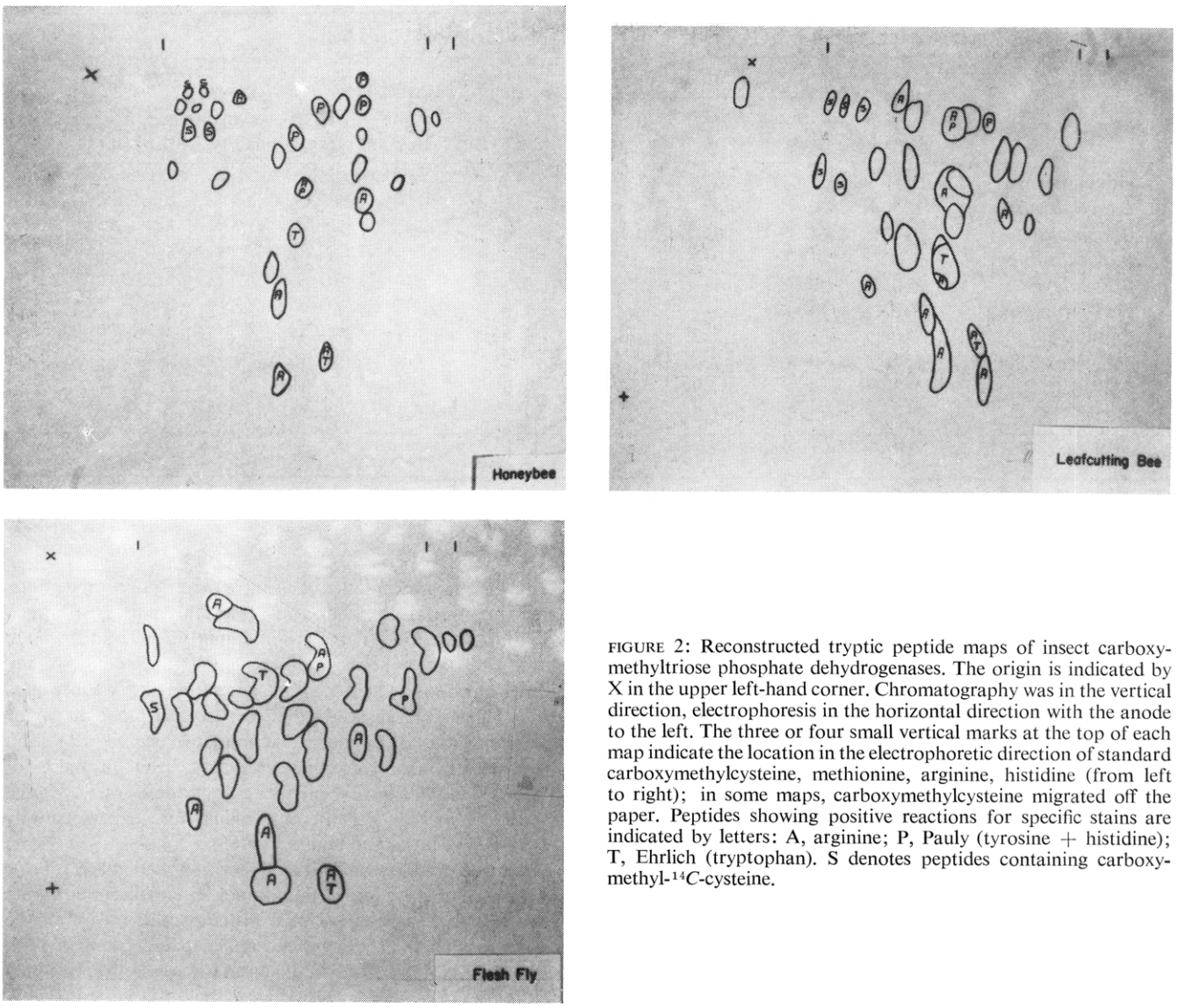


FIGURE 2: Reconstructed tryptic peptide maps of insect carboxymethyltriose phosphate dehydrogenases. The origin is indicated by X in the upper left-hand corner. Chromatography was in the vertical direction, electrophoresis in the horizontal direction with the anode to the left. The three or four small vertical marks at the top of each map indicate the location in the electrophoretic direction of standard carboxymethylcysteine, methionine, arginine, histidine (from left to right); in some maps, carboxymethylcysteine migrated off the paper. Peptides showing positive reactions for specific stains are indicated by letters: A, arginine; P, Pauly (tyrosine + histidine); T, Ehrlich (tryptophan). S denotes peptides containing carboxymethyl-^{14}C-cysteine.

(The amount of screwworm fly triose phosphate dehydrogenase isolated was too small to analyze by peptide mapping.) The peptide maps for each of the carboxymethyltriose phosphate dehydrogenases are shown in Figure 2. Only the major peptide spots are shown. There were 5–10 minor peptides observed for each of the maps, but these were arbitrarily disregarded. These minor peptides, some of which were not always detected, were probably products of incomplete tryptic hydrolysis or of chymotryptic-type cleavages. In every case the number of major peptide spots is less than the maximum expected number calculated from the arginine and lysine content of the triose phosphate dehydrogenases (Table I); that is, there is a tryptic-resistant core. Such a core has been reported for carboxymethylated rabbit triose phosphate dehydrogenase (Bond *et al.*, 1970). Presence of the core precludes comparisons of the whole triose phosphate dehydrogenase molecule; only the tryptic-sensitive portions can be compared. But since the number of major tryptic peptides found is in the range from 64% (*P. suckleyi*) to 82% (leaf-cutting bee) of the maximum predicted number, it is apparent that most of the protein molecule is being analyzed and compared in each case.

In Table III the peptide maps for triose phosphate dehydrogenases from honey bee, bumblebee (*B. nevadensis*), leaf-cutting bee, and fleshfly are compared. In Table IV the peptide maps for four bumblebees (*B. nevadensis*, *B. appositus*, *B. occidentalis*, and *P. suckleyi*) are compared. For each pair of proteins in these two tables, a combined peptide map with digests from both proteins was run in order to confirm differences observed on separate maps. The tables show both the number of common tryptic peptides and the number of dissimilar peptides for each pair of proteins; absence of a peptide in one of a pair of maps is treated as a dissimilar peptide.

With honey bee triose phosphate dehydrogenase as reference protein, the order of decreasing similarity of peptide maps is honey bee, bumblebee, leaf-cutting bee, fleshfly; however, the bumblebee and leaf-cutting bee proteins are almost as different from honey bee triose phosphate dehydrogenase as is the fleshfly protein. With bumblebee as reference, the order of decreasing similarity is bumblebee, leaf-cutting bee, fleshfly, honey bee. With leaf-cutting bee as reference, the order is leaf-cutting bee, bumblebee, fleshfly, honey bee. With fleshfly as reference, the order is fleshfly, bumblebee $\simeq$ leaf-cutting bee, honey bee.

With the one exception of honey bee, all of the above comparisons are consistent with the accepted phylogeny of these insects. The most likely explanation for the aberrant

TABLE II: Deviation Functions for Amino Acid Compositions of Triosephosphate Dehydrogenases.

		Bumblee								
	Honey Bee	*B. nevadensis*	*B. appositus*	*B. occidentalis*	*P. suckleyi*	Leaf-Cutting Bee	Fleshfly	Screw-worm Fly	Lobster	Pig
Honey bee	0									
B. nevadensis[a]	2.5	0								
B. appositus[a]	2.2	1.1	0							
B. occidentalis[a]	2.3	1.0	0.6	0						
P. suckleyi[a]	2.0	0.7	1.1	1.1	0					
Leaf-cutting bee	3.2	1.4	2.2	2.2	1.6	0				
Fleshfly	4.0	3.5	3.3	3.2	3.6	3.7	0			
Screwworm fly	4.4	3.8	3.7	3.6	3.9	3.9	1.4	0		
Lobster	4.3	4.9	4.9	4.8	4.9	5.3	4.3	3.9	0	
Pig	3.9	3.8	3.6	3.8	3.9	3.9	3.3	3.5	4.1	0

[a] Bumblebee.

honey bee values is a faster overall rate of evolutionary change for triose phosphate dehydrogenase in the honey bee lineage than in the other insect lineages tested.

Table IV shows that the bumblebee triose phosphate dehydrogenases from three *Bombus* and one *Psithyrus* species are all very similar to one another. This confirms the conclusion drawn from the amino acid composition data.

Discussion

The only unequivocal method for comparing the primary structure of various proteins is to sequence all of them; this is impractical at present for insect triose phosphate dehydrogenases. Quantitative immunochemical techniques are also very useful for comparing primary sequences, but these methods have not been successful with the proteins covered in this report (Marquardt *et al.*, 1968). Therefore comparison of amino acid compositions and peptide maps is probably the best-available method for obtaining within a reasonable length of time quantitative information on the relative primary structures of insect triose phosphate dehydrogenases.

The major disadvantage of using amino acid compositions for such comparisons is that many amino acid substitutions will not be reflected as changes in composition. For example, at one site of two homologous proteins amino acid X may substitute for amino acid Y, while at another site Y substitutes for X. The major disadvantages of using peptide maps for these comparisons include incomplete tryptic digestion (core), nontryptic hydrolytic cleavages, and presence of different peptides with identical migration rates. Some of these difficulties with peptide maps were experimentally illustrated by Harris and Hindley (1965).

Despite the limitations of the methods applied in this report, the data strongly suggest that honey bee triose phosphate dehydrogenase has evolved at a faster overall rate than the enzyme in the other insects tested. The differences in both amino acid compositions and peptide maps between honey bee and the other insects tested is so striking that it is highly unlikely that these differences are due to artifacts.

There are five tryptic peptides that have identical primary sequences in the lobster and pig triose phosphate dehydrogenases (Harris and Perham, 1968); this is exclusive of free

TABLE III: Comparison of Tryptic Peptide Maps for Carboxymethyltriose Phosphate Dehydrogenases from Four Insects.

	Honey Bee	Bumblebee (*B. nevadensis*)	Leaf-Cutting Bee	Fleshfly
Honey bee	32[a]			
	0			
Bumblebee	11	27		
(*B. nevadensis*)	*36*	*0*		
Leaf-cutting bee	10	21	31	
	42	*16*	*0*	
Fleshfly	8	13	13	28
	43	*28*	*34*	*0*

[a] The upper value is the number of common peptide map spots; the lower value in italics is the number of different peptide map spots.

TABLE IV: Comparison of Tryptic Peptide Maps for Carboxymethyltriose Phosphate Dehydrogenase from Four Bumblebee Species.

	B. nevadensis	*B. occidentalis*	*B. appositus*	*P. suckleyi*
B. nevadensis	27[a]			
	0			
B. occidentalis	23	26		
	7	*0*		
B. appositus	26	24	27	
	2	*5*	*0*	
P. suckleyi	23	23	24	25
	6	*5*	*4*	*0*

[a] The upper value is the number of common peptide map spots; the lower value in italics is the number of different peptide map spots.

lysine which is recovered in tryptic digests of both proteins. (None of the peptide maps of the insect triose phosphate dehydrogenases showed free lysine or arginine.) In addition there are up to five peptides with such small sequence differences (*e.g.*, valine to isoleucine) that they might have identical positions on peptide maps. Thus the minimum number of eight common tryptic peptides found for the insect enzymes (honey bee–fleshfly comparison) probably is at or near the lower limit defined by the identical or near identical regions conserved in all animal triose phosphate dehydrogenases.

Triose phosphate dehydrogenase is, in the evolutionary sense, a very conservative protein, since 72% of all residues is identical in the pig and lobster enzyme (Harris and Perham, 1968). However, from the data presented here plus the amino acid composition for vertebrate triose phosphate dehydrogenases (Allison and Kaplan, 1964), it is evident that the variable regions of the dehydrogenase molecule have undergone appreciable evolutionary alteration.

Recently some investigators have postulated that most evolutionary changes in proteins are primarily due to neutral mutations and random genetic drift (King and Jukes, 1969; Kimura, 1969). One of the supporting observations is that the rates of amino acid substitution for many homogeneous proteins have apparently remained essentially constant during long periods of evolutionary time (Margoliash *et al.*, 1969; Wilson and Sarich, 1969). There are some known exceptions to constant amino acid substitution rates, for example, guinea pig insulin (King and Jukes, 1969), and primitive primate hemoglobin (Margoliash *et al.*, 1969). These exceptions have been attributed to selective pressure (King and Jukes, 1969) or to statistical probability (Sarich, 1969). The great importance attributed to neutral mutations and random genetic drift has been challenged (Clarke, 1970).

It appears that honey bee triose phosphate dehydrogenase may be another exception to the class of proteins with constant evolutionary rates. We have reported evidence that honey bee glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) may have also evolved faster than the same protein in other bee lineages (Brosemer *et al.*, 1971). In addition, we have preliminary evidence[1] from amino acid composition data that honey bee cytochrome *c* may have changed faster than the cytochrome in other insects. It is possible, then, that many, if not a majority, of honey bee proteins may have evolved at an overall rate significantly greater than that in other insects. If this is indeed true, it is not immediately apparent what factors might be involved in accelerating the evolutionary rate. One obvious difference between honey bees and most other insects is the highly specialized social behavior.

The similarity of the three *Bombus* triose phosphate dehydrogenases among themselves is no greater than the similarity of each to the *Psithyrus* dehydrogenase (Tables II and IV). This suggests that the time of divergence of these species may have been relatively recent and that the *P. suckleyi* lineage branched off the main bumblebee phyletic line after lineages leading to at least some modern *Bombus* species had diverged from the common ancestral stock. These conclusions would, of course, be invalid if the overall rates of evolutionary change of triose phosphate dehydrogenase were not essentially constant in all bumblebee species tested. However, a similar comparative study of bumblebee glycerol 3-phosphate dehydrogenases also indicated that three *Psithyrus* species (including *suckleyi*) had branched quite recently from the main bumblebee line (Brosemer *et al.*, 1971). Our working hypothesis is, therefore, that the genus *Psithyrus* and, by implication, its inquiline behavioral pattern evolved only after the basic social behavior of *Bombus* had been established. This is the first case in which molecular studies may have suggested when in evolutionary time a particular behavioral pattern developed in an invertebrate line.

[1] 8th International Congress of Biochemistry, Symposium 9, Interlaken, Switzerland, 1970.

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