Mutant Studies of Yeast Phosphofructokinase†

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ABSTRACT: A phosphofructokinase mutant (*pfk1-1* mutation) previously reported [Clifton, D., Weinstock, S. B., & Fraenkel, D. G. (1978) *Genetics 88*, 1–11] grows well on glucose. We show here that its levels of hexose monophosphates are high during glucose metabolism, it produces normal fermentation products, the pentose phosphate pathway is not its major catabolic route, an additional block in phosphoglycerate kinase causes accumulation of fructose 1,6-bisphosphate, and antigen cross-reactive with wild-type phosphofructokinase is present in some conditions. Another phosphofructokinase mutant strain [Navon, G., Shulman, R. G., Yamane, T., Eccleshall, T. R., Lam, K.-B., Baronofsky, J. J., & Marmur, J. (1979) *Biochemistry 18*, 4487–4499] resembles the *pfk1-1* mutant in some of the above respects, but contains phosphofructokinase with lower affinity for fructose 6-phosphate, which has been purified and is a mutant form of the known enzyme. Its mutation, now called *pfk2-1*, is not allelic with *pfk1-1*, and haploid strains carrying both mutations do not grow on glucose. New alleles of *pfk2* have been selected. Like *pfk2-1*, they cause glucose negativity only in the presence of the *pfk1-1* mutation, but their effect on the enzyme is less severe. Yeast phosphofructokinase is known to be composed of nonidentical subunits, α and β, which in the present work have been identified in crude extracts with antibody after sodium dodecyl sulfate gel electrophoresis. Wild-type strains and *pfk2* mutants contained α and β, while strains carrying *pfk1-1* were deficient in α. A clone carrying wild-type gene *PFK1* on the plasmid YEp13 was identified. Cells carrying this hybrid plasmid contained excess α subunit. We conclude that the *PFK1* gene probably controls, or specifies, α. *PFK2*, therefore, might specify β, the mutations being missense. The present results would accord with β having catalytic function and possibly being adequate alone.

This paper is about gene–enzyme relationships in phosphofructokinase of *Saccharomyces*. The enzyme has a molecular weight of ca. 800 000 (Kopperschläger et al., 1977; Tamaki & Hess, 1975a) and is an octamer, $\alpha_4\beta_4$ (Kopperschläger et al., 1977; Tamaki & Hess, 1975a; Plietz et al., 1978; Tijane et al., 1979), with the two subunits α and β differing antigenically (Herrmann et al., 1973) and being of slightly different size (Kopperschläger et al., 1977; Tamaki & Hess, 1975b). The relationship between physical and functional structure is not well understood, and studies of kinetics and ligand binding have been interpreted as indicating four (Laurent et al., 1979) or eight (Reuter et al., 1979) protomers. It has been proposed that catalytic and regulatory domains might reside on different subunits (Laurent et al., 1978), and, based on the value of four protomers, Tijane et al. (1980) have suggested that β might be the catalytic subunit, since fructose 6-phosphate protects against enzyme inactivation by sulfhydryl group reagents and the protected sulfhydryl groups are on β. Some of the data presented below also point to β having essential catalytic function.

We earlier reported (Clifton et al., 1978) a mutant of *Saccharomyces cerevisiae* which, by enzyme assay, lacked phosphofructokinase. The original strain was obtained from a mutant (*pyk*) lacking pyruvate kinase as a derivative slightly resistant to growth inhibition by glucose. As expected, like its *pyk* parental strain, the double mutant (*pfk pyk*) also failed to grow on glucose. However, segregants carrying the *pfk* mutation alone did grow on glucose, and that surprising result led to the present work.

Experimental Procedures

Strains. The strains (see Table I) wild type in phosphofructokinase were DFY1 (*a, lys1-1*; Clifton et al., 1978), DFY22 [Clifton et al., 1978; strain MC6A, *a ino1-13 ino4-8* (Henry et al., 1975)], DFY56 (*α leu2-1 trp1-1 tyr7-1*; Clifton et al., 1978), and DFY308 (strain 20B12 from E. W. Jones, *α gal2 pep4-3 trp1 SUC2 CUP1*; Jones, 1977).

Table I: Summary of Strains Used[a]

strain	mutations	strain	mutations
DFY1	wild type	DFY250	*pfk2-1*
DFY22	wild type	DFY315	*pfk2-1*
DFY56	wild type	DFY346	*pfk2-2*
DFY308	wild type	DFY291	*pfk1-1 pfk2-1*
DFY70	*pfk1-1*	DFY344	*pfk1-1 pfk2-2*
DFY199	*pfk1-1*	DFY319	*pfk1-1 pgi*
DFY201	*pfk1-1*	DFY337	*pgk*
DFY338	*pfk1-1*	DFY339	*pfk1-1 pgk*
DFY158	*pfk2-1 pykl-10*	DFY242	*PFK1/pfk1-1 PFK2/pfk2-1*

[a] Only phosphofructokinase (*pfk*) or other glycolysis mutations (*pgi*, phosphoglucose isomerase; *pgk*, phosphoglycerate kinase; and *pyk*, pyruvate kinase) are shown; see Experimental Procedures for derivations and for other markers.

The prototype *pfk1-1* mutant strain is DFY70 (*a ino leu2-1*; Clifton et al., 1978). The same mutation, *pfk1-1*, was placed in other strain backgrounds, e.g., DFY199 (*a pfk1-1 leu2-1 trp1-1*), DFY201 (*a ino pfk1-1 lys1-1 trp1-1*), and DFY338 (*ino pfk1-1 leu2-1 his4C712*).

The *pfk2-1* mutation comes from strain DFY158 [455-7d (Navon et al., 1979) *α ino pfk pyk*?], courtesy of K.-B. Lam. This strain was known (Navon et al., 1979) to also contain a mutation causing loss of pyruvate kinase activity. A series of crosses established that the latter defect was allelic with the known gene for pyruvate kinase (*PYK*) and segregated from the phosphofructokinase deficiency. The pyruvate kinase mutation in DFY158 (here called *pyk1-10*) is unusual, for it does not cause glucose negativity (the enzyme may be functional in vivo but labile to extraction). Nonetheless, all the experiments using the *pfk* mutation of DFY158 (now called *pfk2-1*) employed strains wild type for pyruvate kinase (*PYK*), constructed by crosses, e.g., DFY250 (*a ino lys1-1 pfk2-1 trp1-1*) (see below) and DFY315 (*pfk2-1 pep4-3*), a segregant from a cross between DFY250 and DFY308.

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Table II: Growth and Enzyme Levels [a]

strain	growth, colony size (mm)			phosphofructokinase (units/mg) at fructose-6-P concn in assay	
	no added C source	pyruvate	glucose	5 mM	10 mM
DFY1 (wild type)	0.8	1.5	3.0	0.150 [b]	0.158 [b]
DFY22 (wild type)	0.7	1.7	2.5 [c]	0.189	0.197
DFY56 (wild type)	0.7	1.1	3.0	0.153	0.148
DFY70 (*pfk1-1*)	0.5	1.3	2.0 [c]	0.002	0.003
DFY201 (*pfk1-1*)	0.7	1.4	2.0	0.001	0.003
DFY250 (*pfk2-1*)	0.8	1.8	3.4	0.005	0.022
DFY346 (*pfk2-2*)	0.7	1.3	2.7	0.175	0.192
DFY291 (*pfk1-1 pfk2-1*)	0.5	1.2	0.0	0.001 [d]	0.001 [d]
DFY344 (*pfk1-1 pfk2-2*)	0.2	1.3	0.0	0.001 [d]	0.003 [d]
DFY291/pGK9 (*pfk1-1 pfk2-1/PFK1*)	ND [f]	0.8 [e]	1.8	0.011	0.026

[a] Growth is shown as average colony size after 4 days of incubation at 30 °C on enriched medium without supplementation, or supplemented with 1% pyruvate, or 3 days of incubation on medium supplemented with glucose. Phosphofructokinase in crude extracts was assayed at two concentrations of fructose-6-P, 5 and 10 mM. The cultures for assays were harvested from logarithmic phase (A_{580} of 10) in medium with 2% glucose unless indicated otherwise. [b] Values from growth in pyruvate were 0.124 (5 mM fructose-6-P) and 0.144 (10 mM). [c] Growth on minimal medium with glucose (4 days incubation), DFY22, 1.0 mm; DFY70, 0.8 mm. [d] Grown in pyruvate. [e] Three days incubation. [f] ND, not determined.

The glucose-negative double mutant *pfk1-1 pfk2-1*, strain DFY291 (*α ino leu2-1 pfk1-1 pfk2-1 trp1-1*), was obtained by a series of crosses (see below). The other glucose-negative double mutant carrying a different *pfk2* mutation, strain DFY344 (*a ino lys1-1 pfk1-1 pfk2-2 trp1-1*), was obtained from DFY201 by ethyl methanesulfonate mutagenesis and inositol starvation (Henry et al., 1975) in minimal medium with glucose and maltose, followed by screening for the inability either to grow in glucose or to complement with DFY291 for growth on glucose. (The glucose-negative mutant strain carrying *pfk1-1* and *pgi*, DFY319, was obtained from DFY201 in the same mutagenesis as gave DFY344.) The *pfk2-2* mutation in a *PFK1* background was DFY346 (*a ino leu2-1 lys1-1 pfk2-2 trp1-1*), a segregant of a diploid formed between DFY344 and DFY56.

The phosphoglycerate kinase mutation was from a strain from G. Fink, 7249-3B (DFY332, *a pgk his4C712*). The strain was crossed with DFY70 (*pfk1-1*), and DFY337 (*pgk*) is one segregant, DFY338 (*pfk1-1*, see above) is another, and DFY339 (*his4C712 leu2-1 pfk1-1 pgk*) is a third, all from a single tetrad.

Some details of the strain derivations are as follows. DFY138, a *pfk1-1* segregant from diploid DFY69 (Clifton et al., 1978), was crossed with DFY1 (above), giving diploid DFY142, of which DFY199 (*pfk1-1*, see above) and DFY241 (*pfk1-1*) were segregants. DFY201 (another *pfk1-1* strain, see above) was a segregant from a cross between DFY199 and DFY158, as was DFY250 (a *pfk2-1* strain, see above). DFY193 (*a ino leu2-1 pfk2-1*) came from a cross between DFY158 and DFY155 (*a ade1 leu2-1 trp1-1 tyr7-1*). The double-mutant strain DFY291 (*pfk1-1 pfk2-1*) is segregant 7a (see Table VII) from the diploid, DFY242, formed by crossing DFY241 and DFY193. Phenotypes and phosphofructokinase levels of the main strains are given in Table II.

Media and Enzyme Assays. Growth was in the enriched medium described earlier (Clifton et al., 1978): minimal medium 63 supplemented with 1% Bactotryptone, 0.4% yeast extract, and an additional carbon source, 1% or 2%, as specified. Enzyme assays were also described earlier (Clifton et al., 1978).

Purification of Phosphofructokinase. The procedure was slightly modified from that of Tamaki & Hess (1975a), the steps being disruption of cells with a French pressure cell, protamine sulfate, ammonium sulfate, DEAE, Cibacron blue Sepharose 4B (elution of the enzyme with 1 mM ATP), and Sepharose 6C. The strain used for preparation of "wild-type" enzyme was DFY308, because it carries the *pep4-3* mutation causing deficiency in certain proteases (Jones, 1977). Nonetheless, 2 mM phenylmethanesulfonyl fluoride was included in all buffers, as was 5 mM fructose-6-P. The same procedure was used for partial purification of the "K_m" activity from a *pfk2-1* strain, DFY315 (also carrying the *pep4-3* mutation).

Antiserum to Phosphofructokinase. The immunogen was the enzyme purified from the wild-type strain (see Figure 1).

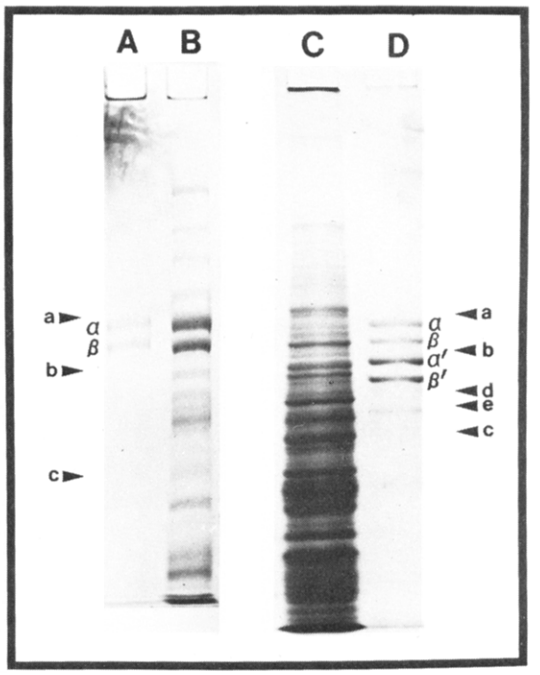

FIGURE 1: Purified phosphofructokinase. (A) Enzyme (5 μg of protein) purified from wild-type strain DFY308 and used for immunization (subunits labeled α and β). (B) Partially purified K_m enzyme (30 μg) from strain DFY315 (*pfk2-1*). (C) Crude extract (200 μg) from wild-type strain DFY1. (D) The same preparation as in (A), but after 18 months storage (subunits α, β, α′, and β′). $NaDodSO_4$ gel electrophoresis and protein staining by Coomassie blue as in Clifton & Fraenkel (1981). (A) and (B) used a 7.5% gel and no stacking gel; (C) and (D) (as well as the gels of Figures 4–6) used a 5–10% separating gel and a 5% stacking gel. Migration of known proteins [see Clifton & Fraenkel (1981)] is indicated by arrows: (a) β-galactosidase; (b) phosphorylase; (c) albumin; (d) (rabbit muscle) phosphofrutokinase; (e) transketolase.

Table III: Metabolite Concentrations in Acid-Soluble Pool [a]

strain	nmol per mg dry wt for metabolites									
	glucose-6-P		fructose-6-P		fructose-1,6-P_2		dihydroxyacetone-P		ATP	
	−[d]	+	−	+	−	+	−	+	−	+
DFY1 (wild type)	2.4	5.3	0.4	0.4	0.3	13.6	0.2	1.8	ND	
DFY56 (wild type)	1.5	4.6	0.4	1.1	0.5	12.0	ND		4.7	4.0
DFY70 (*pfk1-1*)	2.7	22.3	0.5	3.4	<0.2	2.2	0.3	0.3	3.3	ND
DFY201 (*pfk1-1*)	0.7	24.3	<0.1	4.4	0.3	3.2	ND		4.6	ND
DFY338 (*pfk1-1*)	0.6	17.3	0.2	3.1	<0.2	<0.2	0.2	0.4	3.4	1.4
DFY250 (*pfk2-1*)	0.9	13.8	0.2	2.3	<0.2	6.1	ND		4.3	4.0
DFY346 (*pfk2-2*)	ND[c]	16.3	ND	2.8	ND	9.4	ND		ND	5.6
DFY291 (*pfk1-1 pfk2-1*)	3.9	108	1.0	26.3	<0.2	<0.2	ND		4.2	ND
DFY344 (*pfk1-1 pfk2-2*)	3.9	98	0.6	23.0	<0.2	<0.2	ND		4.9	3.3
DFY337 (*pgk*)	2.5	1.1	1.2	0.5	3.1	56	ND		3.9	0.9
DFY339 (*pfk1-1 pgk*)	4.4	5.0	0.9	1.3	1.2	54	0.4	4.1	3.0	0.8
DFY339 (*pfk1-1 pgk*)[b]	2.1	7.4	0.4	1.4	ND	54	0.6	4.0	5.9	1.7

[a] Samples were obtained from cultures in growth in medium with lactate plus glycerol, and 2 h after addition of 1% glucose to such cultures. See Experimental Procedures. [b] These incubations were in minimal medium with lactate. [c] ND, not determined. [d] (+) or (−) indicates the presence or absence of glucose in the incubation.

An amount of 0.5 mg in Freund's complete adjuvant (Difco) was injected subcutaneously into a male rabbit, followed after 4 weeks by a second treatment with 0.25 mg (without adjuvant), and the serum was obtained 1 week later.

Metabolite Determinations. Fermentation products in medium were determined as follows: glucose, by glucose oxidase (Sigma); glycerol, with glycerol kinase and glycerol-3-P dehydrogenase (Wieland, 1974); and ethanol, with alcohol dehydrogenase (Sigma).

Metabolites in the acid-soluble pool were determined as in Orozco de Silva & Fraenkel (1979).

Detection of Subunits with Antibody. The method was adapted from Towbin et al. (1979). After sodium dodecyl sulfate ($NaDodSO_4$)–polyacrylamide gel electrophoresis, the gel was washed twice (5 min each) in water and 3 times (20 min each) in transfer buffer [25 mM Tris, 192 mM glycine, and 20% (v/v) methanol, pH 8.3]. The assembly for blotting contained, in order, cathode (−) to anode (+), a Scotch-Brite pad, one sheet of Whatman 2MM filter paper, the washed gel, two sheets of nitrocellulose paper (11 × 14 cm, Schleicher & Schuell, BA85), one sheet of Whatman 3MM, and a Scotch-Brite pad. Electrophoretic transfer was for 60 min at ca. 60 V, 100 mA. The nitrocellulose sheet closest to the gel was used for immunological detection of the proteins. This sheet was washed for 30 min with Tris–saline (10 mM Tris–0.9% NaCl, pH 7.4), then for 3 h at 37 °C in Tris–saline containing freshly dissolved 3% bovine albumin and 0.01% NaN_3, and once more for 30 min with Tris–saline. It was then exposed overnight on a rotor at 30 °C to 5 mL of the above bovine albumin solution containing 0.1 mL of antiserum to phosphofructokinase (this amount of antiserum would precipitate 8 units of enzyme). This step was followed by six washes (1 h total) with Tris–saline supplemented with 0.05% Triton X-100. The sheet was then exposed 3 h at 30 °C to 5 mL of a 1:2000 dilution of horseradish peroxidase conjugated to goat anti-rabbit IgG (Miles Laboratories, no. 61-202) in the buffer containing bovine albumin and again washed 6 times with Tris–saline–Triton. The bound peroxidase was detected by 20–30-min exposure to 100 mL of a solution of *o*-dianisidine dihydrochloride (Sigma 510-50), 45 μg/mL, and 0.006% H_2O_2, in Tris–saline, and the reaction was stopped by washing with water.

Results

Glucose Metabolism in the pfk1 Mutant. Several characteristics of strains carrying the various mutations are shown in Tables II–V. The prototype strain with the original phosphofructokinase mutation (now called *pfk1-1*) is DFY70. As seen in Table II, DFY70 contained no phosphofructokinase activity, but growth on glucose was almost normal, in either enriched medium or minimal medium. In these characteristics, the *pfk1-1* mutant differed from other known single gene yeast glycolysis mutants, e.g., in phosphoglucose isomerase, phosphoglycerate kinase, phosphoglycerate mutase, and pyruvate kinase, which do not grow on glucose-containing media [reviewed in Fraenkel (1982)].

Table IV: Fermentation Products from Glucose [a]

strain		μmol/mL			ethanol/ glucose	glycerol/ glucose
		glucose	ethanol	glycerol		
DFY22 (0 h)		117	2.7	0.5		
DFY22 (2 h)		0	162	15.1		
	net:	117	159	14.6	1.36	0.12
DFY70 (0 h)		119	1.2	0.3		
DFY70 (2 h)		0	170	14.8		
	net:	119	169	14.5	1.42	0.12

[a] Cells of strain DFY22 (*PFK*) and DFY70 (*pfk1-1*) grown in glucose were washed and resuspended at an A_{580} value of 100, flushed with N_2, and supplemented with cycloheximide, 100 μg/mL, and glucose, 20 mg/mL. Culture supernatants from 0- and 2-h incubations were assayed for glucose, ethanol, and glycerol. (In similar incubations, no pyruvate was detected, but an amount of acetate ca. two-thirds the amount of glycerol was found in both strains.)

Table III shows the profile of intracellular metabolites. The wild-type strains were typical [e.g., see Gancedo & Gancedo (1979) and Ciriacy & Breitenbach (1979)] in showing hexose monophosphate levels somewhat higher and fructose-1,6-P_2 levels much higher in a medium with glucose than in a gluconeogenic medium. In the strains with the *pfk1-1* mutation, incubation with glucose gave unusually high levels of hexose monophosphates but much lower levels of fructose-1,6-P_2 than wild type. Analogous data have already been presented for strain DFY70 (*pfk1-1*) (Gancedo & Gancedo, 1979) as well as for another glucose-positive phosphofructokinase mutant allelic with *pfk1-1* (Ciriacy & Breitenbach, 1979).

Although the pattern of metabolites is consistent with a blocked phosphofructokinase reaction, several lines of evidence suggest that the reaction may be functioning quite well in vivo. First, the pattern of fermentation products formed by incubation of resting cells with glucose was normal, with ethanol being the major product (Table IV).

Second, experiments on the metabolism of radioactive glucose by resting cells gave similar overall patterns in wild-

Table V: Metabolism of Radioactive Glucose [a]

strain	fraction	labeled glucose substrate (% input) 1-^{14}C- labeled	2-^{14}C- labeled	3,4-^{14}C- labeled	6-^{14}C- labeled
DFY1	cells	27	30	27	25
(wild type)	medium	75	69	16	74
	CO_2	−2	3	57	0
DFY56	cells	27	26	22	29
(wild type)	medium	68	62	9	64
	CO_2	4	12	70	7
DFY70	cells	50	48	48	41
(*pfk1-1*)	medium	44	48	14	56
	CO_2	6	3	37	3
DFY201	cells	28	27	24	24
(*pfk1-1*)	medium	60	58	19	65
	CO_2	11	15	57	11
DFY250	cells	28	27	24	24
(*pfk2-1*)	medium	53	56	15	62
	CO_2	12	15	63	7
DFY346	cells	40	33	34	30
(*pfk2-2*)	medium	62	63	17	70
	CO_2	−2	4	50	3

[a] Glucose-grown cells were inoculated, 25 A_{580} units, to cold 1-mL (final volume) mixtures containing minimal medium 63, cycloheximide (50 μg/mL), and [1-, 2-, 3,4-, or 6-^{14}C]glucose (1 mg containing 0.2–2 μCi). The tubes were flushed with N_2, closed with screw caps, and incubated 140 min on a shaker at 30 °C, after which total counts per minute and counts per minute remaining in medium after removal of the cells were determined, the difference being radioactivity in cells. The difference between total counts per minute after incubation and original input counts per minute is given as radioactivity in "CO_2". Values are expressed as percent of original input. Glucose utilization was complete.

type strains and *pfk1-1* mutants (Table V): almost equal fates of the 1, 2, and 6 positions of glucose (partly assimilated and partly accumulated in the medium, presumably as ethanol), and substantial loss of the 3 and 4 positions (presumably as carbon dioxide). There was no preferential loss of the 1 position in the *pfk1-1* strains, which loss would have been expected had the pentose phosphate pathway been their major route of glucose metabolism.

Third, double mutants also lacking phosphoglucose isomerase (DFY319, *pfk1-1 pgi*) or phosphoglycerate kinase (DFY339, *pfk1-1 pgk*) were both, like the *pgi* and *pgk* single gene mutants, completely glucose negative. Thus, the *pfk1-1* mutation does not make a metabolic route available which bypasses the phosphoglucose isomerase and phosphoglycerate kinase reactions. Some data on metabolite levels are included in Table III. A *pgk* single gene mutant (strain DFY337), as is known (Ciriacy & Breitenbach, 1979), accumulated from glucose very high levels of fructose-1,6-P_2. In the double mutant *pfk1-1 pgk* (strain DFY339), the accumulation pattern resembled the *pgk* mutant rather than the *pfk1-1* mutant. Similar results (Table III, last line) were obtained with incubations in buffer with lactate, as in enriched medium with lactate and glycerol, confirming that the accumulated metabolites came from glucose.

The above results together would fit with the pathway of glucose metabolism in the *pfk1-1* mutant being either glycolysis or, if using a bypass reaction to phosphofructokinase, the bypass reentering glycolysis before the phosphoglycerate kinase reaction.

Cross-Reacting Material and Competence for Glucose Metabolism. Since the results above suggested that in a *pfk1-1* mutant a phosphofructokinase activity was likely active in vivo, although not readily revealed by usual assay in vitro, it was necessary to determine whether antigen cross-reacting with

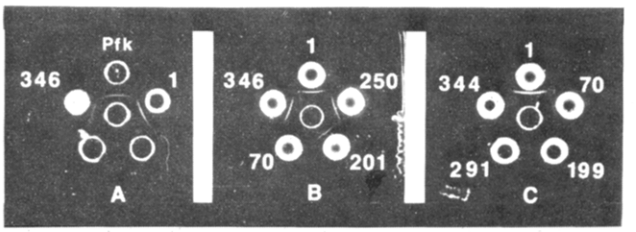

FIGURE 2: Ouchterlony plates. Center wells contained 8 μL of antiserum to phosphofructokinase. Sample wells contained (8 μL) 2 μg of the purified phosphofructokinase (panel A, Pfk) or (in other wells) crude extracts from the indicated strains (DFY1, wild type; DFY70, -199, and -201, *pfk1-1*; DFY250, *pfk2-1*; DFY346, *pfk2-2*; DFY291, *pfk1-1 pfk2-1*; DFY344, *pfk1-1 pfk2-1*; except for the latter two, cultures were harvested from active growth on glucose.) The crude extracts were prepared by a French press in the usual extraction buffer (Clifton et al., 1978) supplemented with 2 mM phenylmethanesulfonyl fluoride and 5 mM fructose-6-P and were concentrated to ca. 60 mg/mL (Bradford, 1976) by dialysis vs. poly(ethylene glycol). Immunoplates were from Hyland Laboratories, and incubation was 48 h at 30 °C in a moist chamber.

Table VI: Competence for Glucose Utilization [a]

expt	strain	growth conditions [b]	glucose utilization [μmol h^{-1} (A_{580} unit)$^{-1}$]
1	DFY1	glucose, logarithmic	1.57
2	DFY1	glucose, stationary	0.53
3	DFY1	lactate + glycerol, logarithmic	0.92
4	DFY1	lactate + glycerol, stationary	0.50
5	DFY70 (*pfk1-1*)	glucose, logarithmic	0.93
6	DFY70	glucose, stationary	0.01
7	DFY70	lactate + glycerol, logarithmic	0.25
8	DFY70	lactate + glycerol, stationary	0.02

[a] The cells were grown in medium with 1% glucose or 1% lactate + 1% glycerol, to logarithmic or stationary phase (see footnote *b*), harvested, washed, and resuspended in 1-mL volumes containing M63, ca. 60 A_{580} units of cells, 0.1 M glucose, and cycloheximide, 50 μg/mL. Glucose was assayed periodically over a 4-h incubation at 30 °C, and rates of utilization were obtained from plots vs. time. [b] Experiment 1, 19-h incubation, from A_{580} of 0.01 to 18; 2, 68 h, from 0.3 to 31; 3, 19 h, from 0.4 to 14; 4, 68 h, from 0.3 to 34; 5, 19 h, from 0.01 to 18; 6, 68 h, from 0.3 to 43; 7, 19 h, from 0.02 to 13; 8, 68 h, from 0.3 to 34.

wild-type phosphofructokinase was present. The enzyme was purified from a wild-type strain (Figure 1) and antiserum obtained. On Ouchterlony plates, the antiserum formed a band of identity with the pure enzyme and with a crude extract of the wild-type strain (Figure 2). With extracts of *pfk1-1* strains (DFY70, 199 and 201), results of the double-diffusion experiments were initially somewhat inconsistent, for a precipitin band showing at least partial identity with wild type might or might not be seen, depending on the strain or particular culture. However, the band was usually seen if extracts were prepared from cultures in active growth on glucose, as was the case for those strains in the experiment of Figure 2.

Tests of glucose utilization (Table VI) revealed that strain DFY70 (*pfk1-1*), by contrast with wild-type strain DFY1, used glucose rapidly when obtained from cultures growing on glucose but not from cultures in stationary phase. [The phenomenon of loss of competence for glucose utilization was also

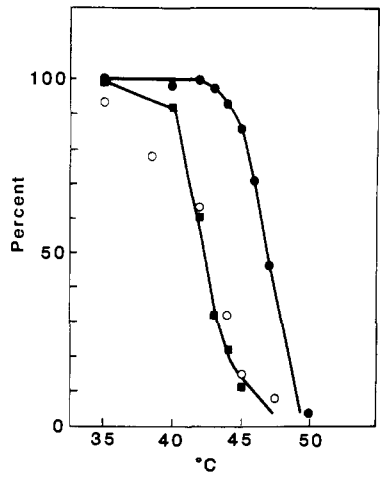

FIGURE 3: Heat inactivation of phosphofructokinase. Crude extracts were diluted to give 0.16 unit/mL in extract buffer (Clifton et al., 1978) supplemented with 10 mg of bovine plasma albumin per mL. Samples (0.35 mL) were placed 2 min in a water bath at the temperature indicated and chilled in ice, and 0.1-mL samples were assayed. DFY1 (wild type), closed circles; DFY250 (*pfk2-1*), open circles; DFY346 (*pfk2-2*), closed squares.

observed with two other *pfk1-1* mutant strains (DFY199 and DFY201), but not in two other wild-type strains (DFY22 and DFY56).] Resumption of growth on glucose in liquid culture, by cultures unable to use glucose according to the protocol of Table VI (i.e., cultures "incompetent" for glucose utilization), involved a lag of at least 12 h, and, in fact, on plates, *pfk1-1* strains lagged in colony development on glucose by about 1 day.

These results, as well as data on subunits presented below, show that a protein related to phosphofructokinase is present in *pfk1-1* mutants growing on glucose, but not necessarily present in other conditions. Nonetheless, the same result of no readily assayable phosphofructokinase activity (Table II) was obtained whether or not extracts were prepared from cultures competent for glucose utilization.

pfk2-1. Navon et al. (1979) reported another phosphofructokinase mutant which lacked the enzyme, grew on glucose, and accumulated hexose monophosphates. The strain was also found to lack pyruvate kinase activity in vitro (Navon et al., 1979), but the latter characteristic segregated from the phosphofructokinase deficiency (see Experimental Procedures), and we have compared strains (e.g., DFY250) carrying only the phosphofructokinase mutation (now called *pfk2-1*) with *pfk1-1* mutant strains. They were similar in growth (Table II), metabolite accumulations (Table III), and metabolism of radioactive glucose (Table V).

However, unlike *pfk1-1* strains, *pfk2-1* strains had a moderate phosphofructokinase activity if assayed with 10 mM instead of 5 mM fructose-6-P (Table II). This high K_m activity is a mutant phosphofructokinase, for (a) on Ouchterlony plates (Figure 2, strain DFY250) there was a band of identity with wild-type enzyme, (b) the antiserum removed the high K_m activity from an extract (not shown), and (c) although the activity was somewhat labile (a heat inactivation curve is in Figure 3) a partial purification was obtained with the same scheme used for the wild-type enzyme, and the enriched material contained two prominent protein bands of the same size as in wild-type enzyme (Figure 1, and also see below).

The Two Phosphofructokinase Mutations Are in Different Genes. When *pkf1-1* and *pfk2-1* mutant strains were crossed with each other, the diploid, by assay, contained phosphofructokinase activity of normal K_m for fructose-6-P. A total of 22 complete tetrads from such diploids gave the following

Table VII: *pfk1-1* × *pfk2-1* [a]

spore	growth, colony size (mm) glucose	growth, colony size (mm) pyruvate	phosphofructokinase [b] at fructose-6-P concn 5 mM	phosphofructokinase [b] at fructose-6-P concn 10 mM	genotype
7a	0.0	1.2	0.004	0.004	*pfk1-1 pfk2-1*
7b	4.0	1.5	0.158	0.167	*PFK1 PFK2*
7c	3.5	1.0	0.002	0.003	*pfk1-1 PFK2*
7d	5.0	1.5	0.015	0.061	*PFK1 pfk2-1*

[a] DFY241 (α *pfk1-1*) was crossed with DFY193 (*a pfk2-1*), giving diploid DFY242 (see Experimental Procedures). Tetrad 7 was a usual tetratype ascus. Growth was on enriched medium with glucose or pyruvate, with colony size measured after 5 days of incubation. Phosphofructokinase was assayed in cells treated with toluene (Clifton et al., 1978). Genotypes were determined by appropriate backcrosses. Spore 7a is strain DFY291. [b] Units are μmol min^{-1} (A_{580} unit)$^{-1}$ of culture.

pattern of growth on glucose: 3 contained 4 spores which grew on glucose, 5 contained 2 glucose-positive and 2 glucose-negative spores, and 14 contained 3 glucose-positive and 1 glucose-negative spore. Such a pattern would be typical of two nonallelic mutations segregating from each other, both of which must be present to confer glucose negativity. Assay showed that the 3:1 tetrads typically contained one spore with normal phosphofructokinase activity, one with the high K_m activity, and two with no detectable activity, with the glucose-negative spore invariably being one of the latter [e.g., spore 7a (strain DFY291), Table VII]. These results are consistent with it being the two phosphofructokinase mutations which are nonallelic and which, together, but not singly, confer inability to grow on glucose.

When the glucose-negative double mutant (strain DFY291) was incubated with glucose (Table III), the accumulation of the hexose monophosphates was substantially greater than even in the single gene mutants, and fructose-1,6-P_2 was not detected. The strain showed little if any cross-reacting material in the Ouchterlony test (Figure 2).

Thus, the two phosphofructokinase mutations proved to be in different genes, and in vivo functional loss of the reaction required both mutations.

Additional pfk2 Mutations. It seemed likely that *pfk2-1* was a structural gene mutation for one of the enzyme subunits. Further mutations were sought to determine whether the enzyme would always be altered in the same way (altered K_m) or whether other mutations in the *PFK2* gene might give a phenotype of total loss of enzyme function. For this purpose, we selected glucose-negative derivatives from a *pfk1-1 PFK2* mutant strain (DFY201). Mutations in other genes causing the glucose-negative phenotype would complement with DFY291 (*pfk1-1 pfk2-1*) while new mutations in *pfk2* (e.g., *pfk1-1 pkf2-2* etc.) would not. Four such noncomplementing derivatives were obtained.

In several respects, the new strains resembled DFY291 (*pfk1-1 pfk2-1*). Thus, crossed with a wild strain, seven of nine complete tetrads segregated three glucose-positive and one glucose-negative spores, rather than the 2:2 segregation expected if the mutations conferred glucose negativity alone. Also, as in strain DFY291, in DFY344 (*pfk1-1 pfk2-2*) phosphofructokinase antigen was absent by the Ouchterlony test (Figure 2), and hexose monophosphates accumulated to very high concentrations from glucose (Table III).

However, the new *pfk2* mutations proved to not be identical with *pkf2-1*, for phosphofructokinase assay of 3:1 tetrads from crosses of the new double mutants with a wild type showed two of the glucose-positive segregants to have enzyme of apparently normal affinity for fructose-6-P (rather than one

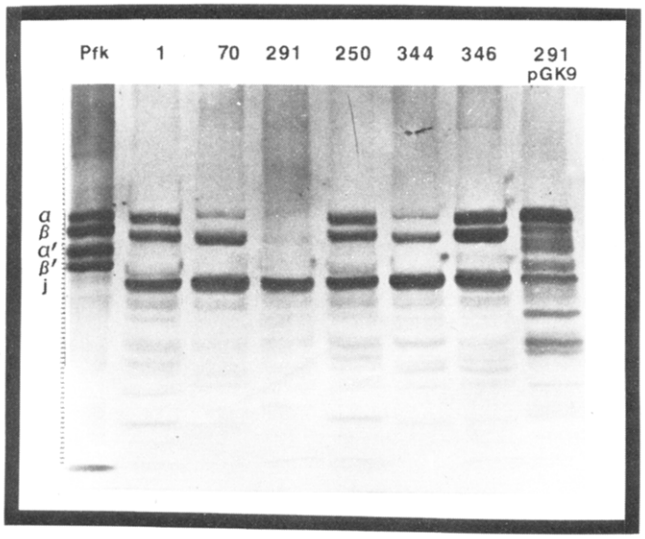

FIGURE 4: Subunits as detected with antibody. The samples were, in left-hand lane, purified phosphofructokinase [as in (D) of Figure 1] and, in the other lanes, crude extracts (200 μg of protein) of the strains indicated (DFY1, wild type; DFY70, *pfk1-1*; DFY291, *pfk1-1 pfk2-1*; DFY250, *pfk2-1*; DFY344, *pfk1-1 pfk2-2*; DFY346, *pfk2-2*; DFY291/pGK9, *pfk1-1 pfk2-1/PFK1*). The cells, with the exception of DFY291 and DFY344, were harvested from growth on glucose. See Experimental Procedures.

having altered K_m). The identification of a *PFK1 pfk2-2* segregant (DFY346), therefore, was first made on genetic grounds: backcrossed with DFY291 (*pfk1-1 pfk2-1*), 17 of 19 tetrads contained 2 glucose-positive and 2 glucose-negative spores. The phosphofructokinase in strain DFY346 (*pfk2-2*) was abnormal in being more labile than the wild-type activity (Figure 3). In other respects, the *pfk2-2* strain, DFY346, resembled the *pfk2-1* strain, DFY250: by the Ouchterlony test (Figure 2), it gave a band of identity with wild-type enzyme, and in growth with glucose, hexose monophosphate concentrations were higher than normal (Table III).

Thus, *pfk2-2* resembles *pfk2-1* in causing glucose negativity in the presence of *pfk1-1*, in which case phosphofructokinase seems to be absent functionally, but alone it has a lesser effect on the enzyme.

Identification of Subunits. Yeast phosphofructokinase has shown a complex pattern of protein subunits, but there is agreement that with rapid purification and care to avoid proteolysis the native octameric enzyme consists of nonidentical subunits (Herrmann et al., 1973), α and β, of slightly different size. In *S. cerevisiae*, the native molecular weight is 835 000, with subunit molecular weights being ca. 104 000 according to equilibrium sedimentation in the presence of sodium dodecyl sulfate (Kopperschläger et al., 1977), and proteolysis causes a characteristic pattern of smaller subunits [α′, β′, etc. (Huse et al., 1976)]. In *S. carlsbergensis*, the native molecular weight is ca. 720 000, and the subunit molecular weights are ca. 90 000 according to sedimentation in guanidine hydrochloride (Tamaki & Hess, 1975b). However, in *S. carlsbergensis*, uncorrected sizes of subunits in $NaDodSO_4$ gel electrophoresis are α 140 000, β 130 000, α′ 92 000, and β′ 87 000, with the differences between α and α′ and between β and β′ not ascribed to proteolysis (Tamaki & Hess, 1975a).

For immunological identification of the subunits, after $NaDodSO_4$ gel electrophoresis, the proteins were electrophoretically transferred to nitrocellulose paper and incubated with antiserum, and the complexes were visualized by a peroxidase assay (Towbin et al., 1979). The first lane of Figure 4 shows the same enzyme preparation which gave four bands when stained by Coomassie blue (Figure 1). The same four bands were revealed by the antiserum test, showing that the antiserum recognized both subunits. [The identical migration of the bands by the two tests could be observed by staining with sudan black (Towbin et al., 1979) of the material passing through to a second nitrocellulose sheet.]

The other lanes of Figure 4 show various crude extracts tested the same way. In the wild-type strain DFY1, as well as the two different *pfk2* mutants DFY250 and DFY346, both the α and β bands were prominently revealed. The *pfk1-1* mutant strain DFY70 showed β but little if any α. One of the glucose-negative double mutants, strain DFY291 (*pfk1-1 pfk2-1*), showed neither band, while the other, strain DFY344, contained β.

In addition, all the crude extracts contained a prominent band (labeled j) with migration corresponding to a size of 80 000 daltons. We believe band j to be unrelated to phosphofructokinase and its presence to reflect antibody to an unknown minor contaminant in the immunogen, for preabsorption of the antiserum with crude extract from the double mutant strain DFY291 considerably reduced band j by comparison with the known phosphofructokinase subunits (Figure 5). A shortened period of exposure to antiserum had the same effect (not shown). (A control serum from the rabbit before immunization gave no staining in this test.)

In Figure 6, extracts from logarithmic and stationary phase cultures are compared. For the wild-type strain DFY1, the same α and β bands were seen from both cultures, but for the *pfk1-1* mutant strain DFY70, although the logarithmic phase culture had β but not α (as in Figure 4), the stationary phase culture contained neither band. If the phenomenon of competence for glucose utilization is recalled, the latter result suggests that it is the β subunit which is needed for in vivo activity in the *pfk1-1* mutant.

These immunological tests give no indication of which subunit is affected by the *pfk2* mutations, since both subunits were present in the two mutants. However, the results do

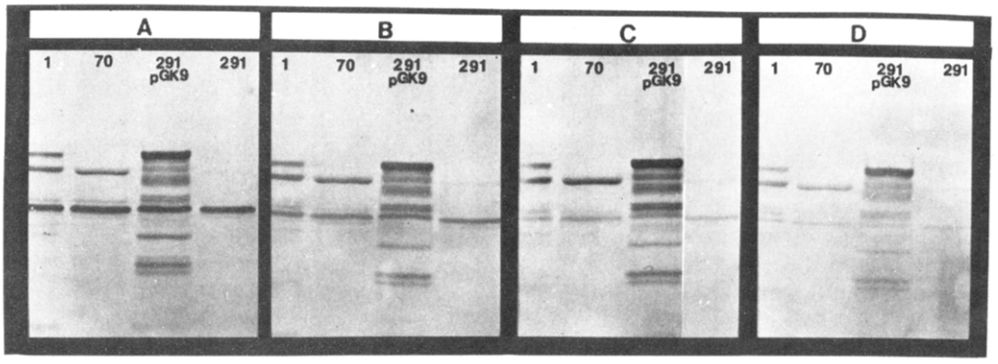

FIGURE 5: Effect of preabsorption on subunit detection. Antibody was pretreated before use by overnight exposure to 0 (panel A), 5 (B), 15 (C), or 45 mg of protein (D) of the crude extract from strain DFY291 (*pfk1-1 pfk2-1*). Strains as in Figure 4.

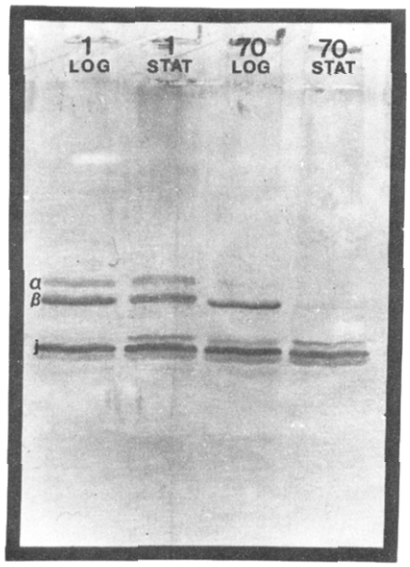

FIGURE 6: Subunits in logarithmic and stationary phase cultures. DFY1, wild-type strain; DFY70, *pfk1-1* mutant.

indicate that the *pfk1-1* mutation may affect α. This model is supported by results of gene cloning.

Cloning PFK1. G. Kawasaki has used a random pool of yeast DNA on the vector YEp13 (Broach et al., 1979) to transform a variety of yeast glycolytic mutants, selecting for complementation of function (to be reported). Thus, glucose-positive transformants were obtained in a selection using strain DFY291 (*pfk1-1 pfk2-1*). One might expect complementation of either mutation but not both simultaneously, giving strains of genotype *pfk1-1 pfk2-1* (chromosome)/*PFK1* or *PFK2* (plasmid). To date, one clone (strain DFY291/pGK9, Table II) has been identified as likely carrying a plasmid with *PFK1*. It contains the activity with altered K_m. When the plasmid from this strain was introduced by transformation into a *pfk1-1 PFK2* strain (DFY70), the transformants [*pfk1-1 PFK2* (chromosome)/*PFK1* (plasmid)] contained apparently normal phosphofructokinase (specific activity of 0.14); in a *PFK1 pfk2-1* strain, on the other hand, the transformants [*PFK1 pfk2-1* (chromosome)/*PFK1* (plasmid)] had activity of altered K_m.

Antibody labeling of subunits in an extract from strain DFY291/pGK9 is shown in Figures 4 and 5. There was unusually prominent staining of material corresponding to the α subunit, and β was also found, as well as a definite smear to lower molecular weight material, which might be degradation products of excessive α subunit. These results would fit with the *PFK1* gene somehow determining the α subunit.

Discussion

At least two separate genes govern yeast phosphofructokinase, *PFK1* and *PFK2*. Mutation in either affects the activity, strains with *pfk1-1* containing no measurable activity in vitro and strains with *pfk2-1* or other alleles having an activity different from that of wild type. The *pfk1-1* mutation probably affects the α subunit: when tested with antibody, strains carrying *pkf1-1* lacked α; when the wild-type gene *PFK1* was on a plasmid, a high level of α was present. Such plasmids are apparently carried in multiple copies, which would account for the high level of product, either if *PFK1* were the structural gene for α or if it somehow regulated the expression of the structural gene.

What the *PFK2* gene does is less certain. If *PFK1* is the structural gene for the α subunit, then it seems unlikely, but not impossible, that the same subunit is also affected by *PFK2*. It is more likely *PFK2* affects the other subunit, β, the mutations being missense and thus not changing the amount or size of the subunit. Isoelectric focusing experiments might confirm β-subunit alterations in *pfk2* mutants, and *PKF2* clones would also be informative.

The present work also bears on the relationship between the functional and physical structure of the enzyme. If *PFK2* affects β, the simplest possibility would be that β is necessary for catalysis, for in all the situations where the reaction is functional, β is present. Perhaps the strongest correlation was in the *pfk1-1 PFK2* strain, where β was only found in cells competent for glucose metabolism. The apparent absence of α in the latter strain would accord with the suggestion of Tijane et al. (1980) that β alone might determine the catalytic functions of this enzyme, i.e., be the "catalytic subunit". It should be remarked, however, that although α seems to be dispensable when β is normal (the *pfk1-1 PFK2* strains grow on glucose), it is needed when β is mutant (for the *pfk1-1 pfk2* strains do not grow on glucose). It may be that the necessity for α in the latter case reflects protection, in vivo, of the altered β subunit. Nonetheless, it is not known whether the α subunit is entirely dispensable even in *PFK2* strains, for there might be a low level of α not clearly observed by the present methods. Deletion of the *PFK1* gene would afford a better test of this question.

There are other unresolved observations. First, regardless of whether one subunit or both are required for catalysis, it is not clear why single gene mutants have not been found yet which both destroy phosphofructokinase activity *and* cause glucose negativity. All the mutations in this paper were obtained indirectly. Thus, *pfk1-1* was selected on the basis of the slight resistance it afforded to glucose toxicity in a pyruvate kinase mutant (Clifton et al., 1978). A mutation allelic to *pfk1-1* was obtained as a glucose-negative clone, but the defective growth segregated independently from the phosphofructokinase lesion (Ciriacy & Breitenbach, 1979). The *pfk2-1* mutation was reported in association with a mutation affecting pyruvate kinase (Navon et al., 1979). P. K. Maitra (personal communication) has also found phosphofructokinase mutants to grow on glucose.

Two entirely different speculations might be offered as to why the single gene mutants in question have not been readily obtained. It might be that either subunit alone is adequate for catalysis, so that double mutants are needed for loss of function. The results of Tijane et al. (1980) do tend to argue against the α subunit having catalytic function alone, but studies of the separated subunits have not yet been reported. Or, it is conceivable that mutations completely inactivating either *PFK1* or *PFK2* would result in total loss of phosphofructokinase function but that such mutants do not, for some unknown reason, grow on the "permissive" media used to date in the selections.

Second, there is the problem of the failure to reveal phosphofructokinase activity in the *pfk1-1* strains, even with variations in assay conditions and phosphate donors. Nonetheless, an activity depending on β subunit, functional in vivo, seems to best fit the results. For if an activity unrelated to phosphofructokinase were functioning in the *pfk1-1* strains, that activity would have to be also governed by the *PFK1* and *PFK2* genes.

Acknowledgments

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Metabolism of 5,6-Epoxyretinoic Acid in Vivo: Isolation of a Major Intestinal Metabolite†

Joseph L. Napoli,* Hoda Khalil, and Anne M. McCormick

ABSTRACT: The major metabolite in the small intestinal mucosa of vitamin A deficient rats dosed intrajugularly with 5,6-epoxy[^{3}H]retinoic acid has been identified as 5,6-epoxyretinoyl β-glucuronide. The assignment was based on the metabolite's chemical, spectral, and chromatographic properties. Incubation of the metabolite with β-glucuronidase released 5,6-epoxyretinoic acid. Incubation of 5,6-epoxyretinoic acid with rat liver microsomes in the presence of uridine-5′-diphospho-1α-D-glucuronic acid produced the metabolite. 5,6-Epoxy[^{3}H]retinoyl β-glucuronide was observed in the liver, small intestinal mucosa, and intestinal contents but not in kidney of vitamin A deficient rats. Its concentration was greatly diminished in liver and small intestinal mucosa, and it was not observed in kidney of vitamin A deficient rats dosed orally with retinoic acid for several days before administration of 5,6-epoxy[^{3}H]retinoic acid. Generally, oral retinoic acid treatment accelerated 5,6-epoxyretinoic acid metabolism and enhanced accumulation of highly polar metabolites. Moreover, 5,6-epoxyretinoic acid metabolism was more rapid than that of retinoic acid and did not result in production of retinoic acid.

Retinoic acid is a quantitatively significant metabolite of retinol under physiological conditions (McCormick & Napoli, 1981). It is also more active than retinol in directing differentiation in vitro, by at least an order of magnitude (Sporn et al., 1976; Strickland & Mahdavi, 1978), but unlike retinol, it does not support vision or mammalian reproduction (Dowling & Wald, 1960; De Luca, 1978; Goodman, 1980). Retinoic acid undergoes rapid metabolism in vivo (Fidge et al., 1968; Geison & Johnson, 1970) to several more polar metabolites which include glucuronides (Dunagin et al., 1965, 1966; Lippel & Olson, 1968a,b) in a tissue-specific manner (Napoli & McCormick, 1981). These facts suggest that retinoic acid, or one of its metabolites, rather than retinol, is the form of vitamin A that directs epithelial differentiation.

5,6-Epoxyretinoic acid was the first target-tissue metabolite of retinoic acid isolated and identified (Napoli et al., 1978; McCormick et al., 1978). Like retinoic acid, 5,6-epoxyretinoic acid has been established as a metabolic product of retinol under physiological conditions (McCormick & Napoli, 1981). Moreover, synthetic racemic 5,6-epoxyretinoic acid is equipotent with retinoic acid in inhibiting the induction of ornithine decarboxylase activity, and the promotion of skin tumors by phorbol esters (Verma et al., 1980). The epoxide is also equipotent with retinoic acid in the induction of differentiation

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