A component of polyphloretin phosphate (PPP) may be an alternative substrate for ATP-glycerol transferase

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Summary. A low molecular weight fraction of a polyphloretin phosphate (PPP) preparation simulated glycerol in a milieu designed for enzymatic assay of glycerol. It is suggested that a component of this PPP mixture is able to act as an alternative substrate for the ATP-glycerol transfer enzyme; it becomes phosphorylated and thus initiates the series of reactions resulting in a increased conversion of NADH to NAD and the observed changes in absorbance.

Polyphloretin phosphates (PPPs) are high-mol-wt polyanionic polyesters of phloretin and phosphoric acid first synthesized by Diczfalusy et al.[3]; they probably contain structures of the type shown in the figure. A low-mol-wt fraction, Leo Compound (LC) 1259, commonly referred to as PPP, has been shown to enhance basal and hormone-stimulated glycerol release from isolated fat cells of the rat, in vitro[5]. This lipolytic activity was presumed to be a result of antagonism of prostaglandins[6] inhibiting lipolysis, or inhibition of cAMP phosphodiesterase activity[7]. However, we have observed recently that PPP itself may behave like glycerol and produce falsely high estimates of glycerol concentration when this is assayed by the enzymatic procedure of Eggstein and Kreutz[8]. This linked series of 3 reactions is initiated by glycerol kinase or ATP: glycerol 3-phosphotransferase (E.C. 2.7.1.30) in the presence of ATP, resulting in the formation of glycerophosphate. The ADP produced in this reaction in used next by pyruvate kinase on phosphoenol pyruvate (PEP). The pyruvate product provides the substrate for the 3rd and final reaction of the series, when it is reduced to lactate by lactate dehydrogenase, and NADH converted to NAD. The resultant decrease in absorbance at 340 nM is monitored as an index of the original glycerol concentration. The system may be summarized as follows:

Glycerol + PEP + NADH → Glycerol-1-phosphate + lactate + NAD.

We have investigated, therefore, the effects of the low-mol.-wt fraction (LC 1259) and 2 other mixtures of polyphloretin phosphate on this glycerol assay system.

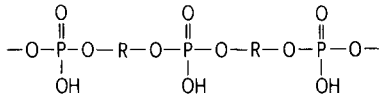
Methods. The compounds examined (see figure for structural formula) were a low-mol.-wt fraction of PPP (LC 1259), a high-mol.-wt fraction (LC 1261) and a crude mixture of PPP (LC 101k).

Glycerol was assayed manually using the Boehringer Mannheim GmbH diagnostic kit 15747 on a Pye-Unicam SPI800 spectrophotometer, in quartz cuvettes having a 1-cm light path. To 2.6 ml of 'reagent' (containing 6 mM NADH, 33 mM ATP, 11 mM PEP and approximately 800 units ml^{-1} LDH and 130 units ml^{-1} PK) was added 0.5 ml of aqueous glycerol solution or solution of compound under investigation (1.0, 0.5 or 0.25 mg ml^{-1}). When glycerol and compound were examined simultaneously, then 0.5 ml of each solution was added to 2.1 ml of reagent. Samples were agitated and incubated for 10 min at 20 °C prior to determination of initial absorbance, 20 µl of GK (150 units ml) was added, samples mixed and incubated for a further 10 min at 20 °C; reagent blanks were included. Application of the Lambert-Beer law to the conditions described indicates an expected absorbance of 0.498 for the glycerol standard solution (0.5 µmoles ml^{-1}).

Results. The low-mol.-wt fraction of PPP (LC 1259) is prepared by dialysis of the crude mixture (LC 101k) against distilled water; at no time is glycerol involved in its production and thus there is no possibility of the observations being explained by glycerol contamination of the compounds (H. Fex, personal communication, 1976). Table 1 summarizes the results obtained. It is evident that there is a linear relationship between the concentration of LC 1259 and the apparent concentration of glycerol in the measuring cuvette, in the presence or absence of actual glycerol.

Compounds LC 1261 and LC 101k were without effect on the assay system at the concentrations examined.

Discussion. Dihydroxyacetone (but not monohydroxyacetone) and dl-glyceraldehyde are other, known alternative substrates for ATP-glycerol transferase[9]. However, the critical structural features determining the substrate specificity are not readily obvious. A primary alcohol on carbon



Structural formula of a generalized polyanionic polyester of phloretin and phosphoric acid where R is the parent dihydrochalcone, phloretin (see Eakins[4]).

Effects of phosphorylated and non-phosphorylated phloretin-like compounds on glycerol estimation

Group	Sample	ΔE	Apparent glycerol concentration in cuvette (µmoles $ml^{-1} \times 10^3$)	% of actual glycerol
1	Aqueous glycerol standard solution	0.49*	78	98
2	LC1259 160 µg/ml^{-1}	0.33	53	-
3	LC1259 80 µg/ml^{-1}	0.16	26	-
4	LC1259 40 µg/ml^{-1}	0.08	13	-
5	1+2	0.81	130	162
6	1+3	0.66	106	132
7	1+4	0.58	93	116
8	1+LC1261 (160 µg/ml^{-1})	0.50	80	100
9	1+LC101K (160 µg/ml^{-1})	0.47	75	94

* Mean of 3-6 observations.

number 1 is a common feature of the 3-carbon aliphatic substrates, with a 2nd primary alcohol on carbon atom number 3, and separated by a secondary alcohol (as in the case of glycerol) or a ketone group (as in the case of dihydroxyacetone). Alternatively, an aldehyde may replace the 2nd secondary alcohol group (as in diglyceraldehyde). Neither an acid nor a methyl group permits substrate activity as seen by the absence of such activity with glyceric acid and propanediol, respectively[9]. Thus, the active component of PPP awaiting identification is likely to be a small molecule of the order of mass probably of the monomer or dimer, but possibly with several sites for accepting ATP, since from the table the presence of a concentration of LC 1259 of 80 μg ml^{-1} produced an apparent concentration of glycerol of 26 μmoles ml^{-1} or approximately 240 μg ml^{-1}.

In view of these findings, the previously-reported lipolytic effects of PPP are being re-examined.

It is possible that the inhibitory action of PPP on (ATP-dependent) glucose uptake by fat cells[5] may be associated also with phosphorylation of PPP.

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3 E. Diczfalusy, O. Ferno, H. Fex, B. Hogberg, T. Linderot and Th. Rosenberg, Acta chem. scand. *7*, 913 (1953).
4 K.E. Eakins, in: The Prostaglandins: Progress in Research, p. 263 Ed. S.M.M. Karim. MTP, Oxford 1972.
5 P.B. Curtis-Prior, Adv. exp. Med. Biol. *63*, 43 (1975).
6 K.E. Eakins, Ann. N.Y. Acad. Sci. *180*, 386 (1971).
7 P.B. Curtis-Prior and Yin-Ha Chan, Pharmac. Res. Commun., in press (1981).
8 M. Eggstein and F.H. Kreutz, Klin. Wschr. *44*, 262 (1966).
9 C. Bublitz and E.P. Kennedy, J. biol. Chem. *211*, 951 (1954).

Identification of both fucosterol and isofucosterol in the silkworm, *Bombyx mori*

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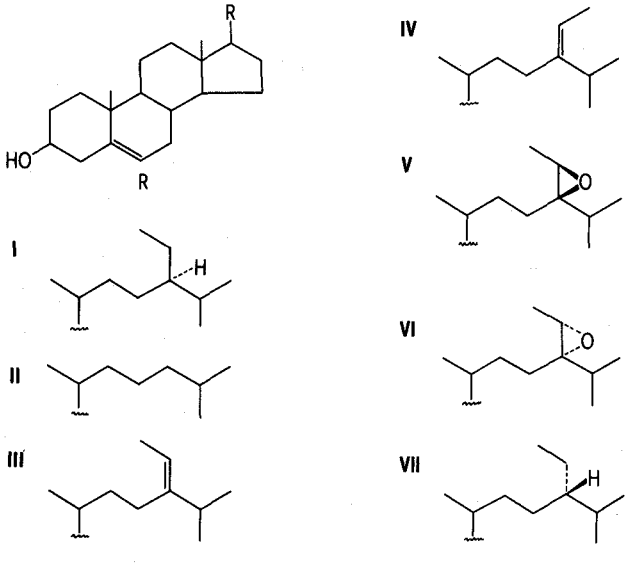
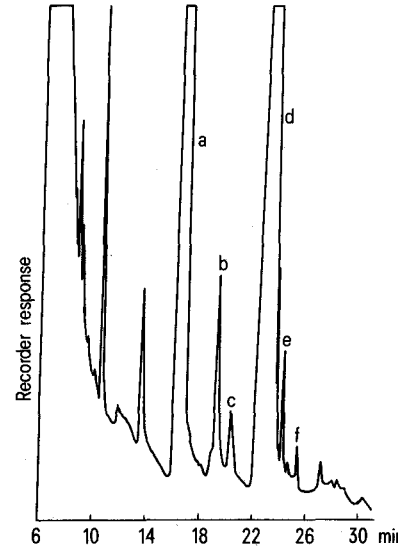
Summary. Fucosterol and isofucosterol were identified in the silkworm, *Bombyx mori.*

Since phytophagous insects have no ability to synthesize sterols de novo, they utilize plant sterols, e.g. sitosterol (I), by converting them into cholesterol (II) for their growth and development. Therefore, the C-24, 28 carbon-carbon bond cleavage reaction of I is an important metabolic process for insects. Although fucosterol (III) seems to be established as an intermediate in the conversion of sitosterol to cholesterol[1-4], a similar role for isofucosterol (IV) has remained obscure[3-5]. Recent observations by Nicotra et al.[6] that *Tenebrio molitor* transforms [^{3}H]-sitosterol into both fucosterol and isofucosterol prompted us to describe our successful identification of fucosterol and isofucosterol in the silkworm, *Bombyx mori.*

The 5th instar larvae (50 species) of *B. mori* reared on mulberry leaves were extracted with chloroform-methanol (1:1). The extract was fractionated with column chromatog-





Sterols in the silkworm, *Bombyx mori* as analyzed with their trimethylsilyl ethers by gas chromatography. An open-tubular glass capillary column (40 m) coated with OV-17 was used at 270 °C. Peaks at retention times of 17.0, 19.4, 20.5, 23.6, 24.4 and 25.5 min were assigned by comparison with the authentic samples, to cholesterol (a), campesterol (b), stigmasterol (c), sitosterol (d), fucosterol (e) and isofucosterol (f), respectively.