Metabolism of Unsaturated Fatty Acids in Protozoa*

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ABSTRACT: *Tetrahymena pyriformis*, when grown on a fatty acid-free medium, contains only two polyunsaturated fatty acids, 9,12-octadecadienoate and 6,9,12-octadecatrienoate. When grown in the presence of 11,14-eicosadienoate, 8,11,14-eicosatrienoate, 11-eicosenoate, or 11-octadecenoate, the protozoa incorporated the fatty acids into their neutral lipids and phospholipids.

Despite profound changes in fatty acid composition, the protozoa were normal in growth rate, appearance, and cell motility. Some of these fatty acids were desaturated or elongated. *T. pyriformis* incorporated 5,8,11,14-eicosatetraenoate into its lipids with nonreproducible effects on growth rate, appearance, and motility. 9,12,15-Octadecatrienoate, 6-octadecenoate, and 6,9,12-octadecatrienoate, but not 9,12-octadecadienoate, were very toxic to *T. pyriformis*. *Acanthamoeba* sp., which normally contains only ω-6-polyunsaturated fatty acids converted 9,12,15- octadecatrienoate into several ω-3-polyunsaturated fatty acids which were incorporated into the lipids of the amoebae with no apparent toxicity.

Many recent publications have demonstrated the widely varying capabilities of protists to synthesize polyunsaturated fatty acids. These differences are systematic, however, and provide supporting evidence for establishing phylogenetic relationships (Erwin and Bloch, 1964; Korn *et al.*, 1965). The fatty acid composition of protists grown on media free of fatty acids and the results of experiments in which protists were grown in media containing radioactive fatty acids have also helped delineate biosynthetic pathways (Haines *et al.*, 1962; Erwin and Bloch, 1963; Hulanika *et al.*, 1964; Davidoff and Korn, 1963; Korn, 1964a,b; Korn and Greenblatt, 1963).

The data to be presented in this paper illustrate the potential usefulness of protists in obtaining the answer to two other questions: the specificities of the enzymes involved in the biosynthesis of polyunsaturated fatty acids, and the physiological functions of polyunsaturated fatty acids.

Materials and Methods

Organisms. T. pyriformis (Kidder strain W) was obtained from the American Type Culture Collection (No. 10542). The cells were maintained on a medium recommended by Dr. V. Dewey which contained proteose–peptone (Difco Laboratories, Detroit, Mich.), 20 g/l.; yeast hydrolysate (Mann Research Laboratories, N. Y.), 5 g/l; glucose, 10 g/l.; $CaCl_2 \cdot 2H_2O$, 3.4×10^{-1} mM; $CuCl_2 \cdot 2H_2O$, 2.9×10^{-2} mM; $MnCl_2 \cdot 4H_2O$, 2.5 μM; $ZnCl_2$, 3.7×10^{-1} μM; $MgSO_4 \cdot 7H_2O$, 4.1×10^{-1} mM; $FeSO_4 \cdot 7H_2O$, 6.0 μM; KH_2PO_4, 7.4 $\times 10^{-1}$ mM; K_2HPO_4, 5.8×10^{-1} mM. Adjustment of the pH (*ca.* 6.8) was not necessary. Neither the proteose–peptone nor the yeast hydrolysate contained detectable fatty acids before or after saponification. The cells were maintained in test tubes containing 5 ml of medium and were subcultured every 2 days. Larger quantities of cells were grown in 100 ml of medium in a 1-l. Erlenmyer flask at room temperature without shaking. The pattern of fatty acid esters as judged by gas–liquid partition chromatography, and the yield of cells were essentially the same in flasks grown with and without shaking.

Acanthamoeba sp. was grown on a medium containing proteose–peptone and glucose, as previously described (Korn, 1963).

Fatty Acids. Methyl petroselinate, methyl vaccenate, and methyl 11-eicosenoate (all 99+% pure) were obtained from the Hormel Institute, Austin, Minn. Linoleic acid, α-linolenic acid (both 99+% pure) and methyl arachidonate were obtained from Applied Science, State College, Pa. Analysis by gas-liquid partition chromatography indicated that the methyl arachidonate was 89% pure, containing 7% methyl eicosatrienoate and 4% methyl eicosapentaenoate. No impurities were detected in the other compounds obtained commercially. Methyl α-linolenate was also isolated from flax seed by preparative gas–liquid partition chromatography (Korn, 1963); reanalysis by analytical gas–liquid partition chromatography demonstrated that it was pure. Methyl γ-linolenate was isolated from primrose seed by column chromatography on silica gel G (E. Merck, Darmstadt, Germany) impregnated with silver nitrate (de Vries, 1963). Analysis by gas–liquid partition chromatography indicated that the preparation contained 2% methyl linoleate. Methyl 11,14-eicosadienoate and methyl 8,11,14-eicosatrienoate were isolated from *Acanthamoeba* as mercuric acetate adducts, as previously described (Korn, 1963), as well as by column

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chromatography on silica impregnated with silver nitrate. Gas–liquid partition chromatographic analysis showed that the methyl eicosadienoate contained a very small amount of an ester with a somewhat earlier retention time, presumably methyl 11-eicosenoate; no impurities were detected in the methyl eicosatrienoate. All methyl esters were converted to fatty acids by saponification in 0.5 N NaOH in 50% methanol for 1 hr at 80°. The mixtures were acidified and the fatty acids were extracted with petroleum ether (bp 30–60°).

Fatty acids were added to the media as sodium salts prepared by addition of one and one-half times the equivalent amount of 0.5 N NaOH in aqueous methanol to the fatty acid. The solution was evaporated to dryness under a stream of nitrogen, distilled water was added, and the resulting solution was autoclaved before adding it to the medium. Autoclaving had no effect on the fatty acids as determined by gas–liquid partition chromatography. In some experiments, the sterile fatty acid soap was complexed with sterile fatty acid free albumin (Goodman, 1957) before addition to the medium. The albumin had no noticeable effect.

Experimental Procedures. T. pyriformis was grown for 2 days and *Acanthamoeba* for 7–10 days before harvesting by centrifugation at 0°. The appearance and activity of cells were observed under a microscope just prior to harvesting. Cells were washed twice in a buffer containing 0.15 M NaCl and 0.02 M KH_2PO_4, pH 7.0, and then were extracted with 20 volumes of chloroform–methanol, 2:1 (v/v), for 24 hr at room temperature. The mixture was filtered, the residue was washed thoroughly with the solvent, and the combined filtrates were evaporated to dryness on a rotary vacuum evaporator at 55°. Ethanol was added and the evaporation repeated to remove any residual water.

In some experiments the lipids were separated into neutral lipids and phospholipids by column chromatography on silicic acid; the fatty acids and neutral lipids were eluted with chloroform, and the phospholipids were eluted with methanol. On one occasion the fatty acids were separated from the neutral lipids (Weisman and Korn, 1966). Esterified fatty acids were converted to methyl esters by reacting with 0.5 N NaOH in absolute methanol at 40° for 0.5 hr. Alkaline methanolysis converts a maximum of 5% of unesterified fatty acids (if present) and a minimum of 85% of esterified fatty acids to methyl esters. Unesterified fatty acids were methylated with boron trifluoride reagent at 80° for 2 min.

Analytical gas–liquid partition chromatography was carried out on a stationary phase of 17% ethylene glycol succinate polyester, unless otherwise noted, using an argon ionization detector. Preparative gas–liquid partition chromatography was performed as previously described (Korn, 1963), using the same stationary phase and detector.

The following control experiment demonstrated that the fatty acid analysis reported for the total lipids represents the composition of the esterified fatty acids of the cells. *T. pyriformis* was grown in the presence of 1.4 mg of α-linolenic acid (0.05 μmole/ml). The cells were harvested, and the lipids were extracted and fractionated as described. Of the total fatty acids recovered, 55% were phospholipids, 37% were neutral lipids, and only 8% were unesterified fatty acids. Of the total α-linolenic acid added to the growth medium, 46% was recovered in the cell lipids of which only 8% was in the unesterified fatty acids; the remainder was approximately evenly divided between the neutral lipids and phospholipids. Even the α-linolenic acid recovered in the unesterified fatty acids was undoubtedly derived from cell lipids since the composition of the unesterified fatty acids was identical with that of the phospholipid fatty acids (26% α-linolenate and 8% 6,9,12,15-octadecatetraenoate). In any event, only 5% of the small amount of unesterified fatty acids would be converted to methyl esters by the alkaline methanolysis procedures used in the experiments in which total lipids were analyzed. As a further control, it was determined that the fatty acid compositions of the phospholipid and neutral lipid fractions were the same when methyl esters were formed by either alkaline methanolysis or by reaction with boron trifluoride–methanol. Therefore, no esterified fatty acids were present in those fractions. When α-linolenic acid was added to the lipids extracted from a control batch of *T. pyriformis*, 86% was recovered after fractionation as described above, and all as unesterified fatty acid.

All thin layer chromatography was carried out on plates coated with silver nitrate impregnated silica gel G (Morris, 1963). This procedure separates positional isomers as well as *cis–trans* isomers and fatty acids with different numbers of double bonds (Lees and Korn, 1966).

Oxidative degradation of individual fatty acid methyl esters was accomplished by the permanganate–periodate procedure of von Rudloff (1956). The resulting acids were methylated either with BF_3–methanol reagent or with freshly prepared diazomethane and qualitatively and quantitatively analyzed by gas–liquid partition chromatography.

Results

T. pyriformis

In the first experiments, the total lipid extracts were fractionated into phospholipids and neutral lipids. Control cells contained 3.5–4 times more phospholipid than neutral lipid. Polyunsaturated fatty acids were found predominantly in the phospholipids. When cells were grown in the presence of an exogenous fatty acid, the new fatty acid was incorporated into both the neutral lipids and the phospholipids. The composition of the unesterified fatty acids was the same as that of the neutral lipids in control and experimental cells in the one experiment in which the fatty acids were analyzed separately. In most experiments, the lipids were not fractionated; where the lipids were fractionated, only the data from the phospholipids are given.

In agreement with Erwin and Bloch (1963), linoleic acid (9,12-octadecadienoate) and γ-linolenic acid (6,9,12-octadecatrienoate) were the only polyunsatu-

TABLE I: Per Cent Composition of Fatty Acids of Phospholipids of *T. pyriformis* Grown in the Presence of Polyunsaturated 20-Carbon Fatty Acids.[a]

	Fatty Acid Added to Growth Medium (0.1 μmole/ml)			
	None[b,c]	20:2[11,14][d,e]	20:3[8,11,14][d,f]	20:4[5,8,11,14][d,g]
12:0	2.6	5.0 (6.7)[h]	3.5 (5.9)	2.8 (4.6)
14:0	12.7	12.9 (17.5)	12.7 (21.0)	11.4 (19.3)
16:0	7.4	7.6 (10.1)	7.9 (13.0)	11.0 (18.5)
18:0	1.8	0.8 (1.1)	1.9 (3.1)	2.0 (3.3)
18:1[9]	4.1	1.9 (2.6)	1.8 (2.9)	3.3 (5.5)
18:2[9,12]	26.9	11.4 (15.4)	7.0 (11.4)	13.9 (23.3)
18:3[6,9,12]	29.1	26.9 (36.3)	19.3 (31.7)	7.5 (12.7)
20:2[11,4]	0	19.2	0	0
20:3[8,11,14]	0	6.3	39.3	2.6[i]
20:4[5,8,11,14]	0	0	0	36.8
Others[j]	15.5	7.9 (10.7)	6.8 (11.5)	9.0 (13.1)

[a] In the designation of fatty acids, the first number refers to the number of carbon atoms in the molecule, the second number refers to the number of double bonds in the molecule, and the numbers in brackets refer to the positions of the double bonds within the molecule. [b] Average of three experiments. [c] Packed cell volume, 1.0 ml. [d] Average of two experiments. [e] Packed cell volume, 0.9 ml. [f] Packed cell volume, 0.8 ml. [g] Packed cell volume, 0.5 ml. [h] Values in parentheses are the per cent composition of the fatty acids normally present in *T. pyriformis*. [i] Present as a contaminant in the added arachidonate. [j] Several odd-numbered and branched-chain fatty acids (Erwin and Bloch, 1963).

rated fatty acids found in *T. pyriformis* grown on a fatty acid free medium. In the experiment shown in Table I, *e.g.*, those two acids made up more than half the fatty acids of the phospholipids. But, as will be shown, the fatty acid composition of *T. pyriformis* can be profoundly altered by the addition of a fatty acid to the growth medium.

11,14-Eicosadienoate was taken up by growing *T. pyriformis* and incorporated into its lipids (Table I) to a major extent. The fatty acid was also desaturated, in part, to 8,11,14-eicosatrienoate which was also incorporated into cell lipids. The new fatty acid was identified by its gas–liquid partition chromatographic retention time, by the retention time of the product of its hydrogenation, by its R_F in thin layer chromatography, and by identification of the products of its oxidative degradation. Together, 11,14-eicosadienoate and 8,11,14-eicosatrienoate comprised 25% of the fatty acids of the phospholipids. The incorporation of the new fatty acids into cell lipids was mainly at the expense of linoleic acid. This became more apparent when the per cent composition of the fatty acids normally present in *T. pyriformis* was recalculated, omitting from the calculation the two fatty acids of exogenous origin. Calculated in this manner, there was (Table I) a relative increase in the concentration of the saturated fatty acids and γ-linolenic acid and a decrease in linoleic and oleic acids.

Despite the significant changes in fatty acid composition, no ill effects were noted; cell growth was normal, as was the appearance and the motility of the cells.

8,11,14-Eicosatrienoate, when present in the growth medium, was incorporated extremely well into the lipids of *T. pyriformis* (Table I). No evidence was found for conversion of the fatty acid into any other fatty acids. The new fatty acid was incorporated into phospholipids in place of linoleate and γ-linolenate. The major effect was on the concentration of linoleate, as is emphasized by recalculation of the per cent composition of fatty acids other than eicosatrienoate. No abnormalities of cell growth or motility were observed, despite the fact that 39% of the fatty acids of these cells were abnormal.

Arachidonate (5,8,11,14-eicosatetraenoate) was also incorporated well into the cellular lipids (Table I), resulting in a decreased concentration of linoleate and γ-linolenate. In this case, however, the new fatty acid mainly replaced γ-linolenate. In these experiments, arachidonate greatly reduced cell growth. The cells were abnormally spherical and exhibited an abnormal swimming pattern. Usually the ellipsoidal cells dart to and fro in straight lines with abrupt directional changes, whereas these cells moved very slowly and sometimes remained in one place spinning on their axes. In subsequent experiments these physiological effects could not be reproduced, however, and their significance is unclear.

The addition of *α-linolenate* (9,12,15-octadecatrienoate) to the growth medium provides an interesting situation because this compound contains a double bond three carbons removed from the methyl terminus. The two polyunsaturated fatty acids normally synthesized by *T. pyriformis* are in the ω-6 group with the terminal double bond six carbons from the methyl end of the molecule. There is a physiological difference between these two classes of fatty acids in higher animals whose fatty acid requirement is satisfied by members of the ω-6 group but not by the ω-3 group.

When added to the growth medium in the concentration previously used (0.1 μmole/ml), α-linolenate was lethal. A very small amount of dead cells was collected and their lipids were found to contain 60% α-linolenic acid. When present in the growth medium at a concentration of 0.05 μmole/ml, α-linolenate still depressed growth. The cells that were present seemed to be normal except for a somewhat granular appearance due to black clumps in, or on, the cells. The α-linolenate was found in the cell lipids (Table II) replacing mainly linoleate and γ-linolenate. α-Linolenate was also desaturated to 6,9,12,15-octadecatetraenoate which was characterized by gas–liquid partition and thin layer chromatography and by oxidative degradation. Cells grown in the presence of less α-linolenate (0.025 μmole/ml) incorporated less of the fatty acid into their lipids (Table II) and were apparently normal.

TABLE II: Per Cent Composition of the Fatty Acids of the Total Lipids of *T. pyriformis* Grown in the Presence of α-Linolenate (18:3[9,12,15]).[a]

	Concn of α-Linolenate in Growth Medium (μmoles/ml)		
	None[b]	0.025[c]	0.05[d]
12:0	3.5	3.5 (3.9)[e]	2.0 (3.8)
14:0	17.5	15.7 (17.6)	12.2 (22.8)
16:0	9.1	8.8 (9.9)	9.1 (16.9)
18:0	3.6	3.5 (3.9)	3.5 (6.5)
18:1[9]	4.8	5.9 (6.7)	6.4 (11.8)
18:2[9,12]	19.7	17.4 (19.6)	4.2 (7.9)
18:3[6,9,12]	19.1	17.7 (19.9)	7.5 (14.0)
18:3[9,12,15]	0	6.7	39.0
18:4[6,9,12,15]	0	4.3	7.3
Others[f]	22.6	16.7 (18.7)	8.8 (16.3)

[a] See footnote *a*, Table I. [b] Packed cell volume, 1.1 ml. [c] Packed cell volume, 1.1 ml. [d] Packed cell volume, 0.35 ml. [e] See footnote *h*, Table I. [f] See footnote *j*, Table I.

We have shown elsewhere (Lees and Korn, 1966) that commercial α-linolenate contains appreciable amounts of two mono-*trans*, di-*cis* isomers. To eliminate the possibility that the toxic effects were due to the *trans* isomers, *T. pyriformis* was grown on the all *cis* isomer isolated from flax seed. It too was lethal at a concentration of 0.1 μmole/ml.

As a control on the toxicity of α-linolenate, *T. pyriformis* was grown in the presence of *γ-linolenate* (6,9,12-octadecatrienoate). Despite the fact that γ-linolenate is one of the two major fatty acids normally found in *T. pyriformis*, it was even more toxic than α-linolenate. At a concentration of γ-linolenate of 0.1 μmole/ml no growth occurred; at 0.025 μmole/ml, growth was only 25% of normal and the cells were distinctly granular in appearance; at 0.02 μmole/ml, growth was only 33% of normal but the cells were apparently normal in size, shape, and motility. The lipids of these cells contained as much as 61% γ-linolenate (twice normal levels).

In contrast to the two triunsaturated 18-carbon fatty acids, the diunsaturated fatty acid, *linoleate* (9,12-octadecadienoate), had no deleterious effects on *T. pyriformis* when present in the growth medium at a concentration of 0.07 μmole/ml. The concentration of linoleate in the phospholipids was increased to 31% from a normal value of 22%.

Petroselinate (6-octadecenoate) was, however, very toxic. When it was present at a concentration of 0.06 μmole/ml, cell growth was very poor (Table III). The

TABLE III: Per Cent Composition of Fatty Acids of Total Lipids of *T. pyriformis* Grown in the Presence of Petroselinate (18:1[6]).[a]

	Concn of Petroselinate in Medium (μmoles/ml)		
	None[b]	0.04[c]	0.06[d]
12:0	2.4	2.5	1.8
14:0	15.5	13.3	10.8
16:0	7.7	8.4	7.5
18:0	2.1	1.4	+[e]
18:1	3.3[f]	22.2[g]	49.5[g]
18:2[9,12]	23.8	16.2	4.1
18:3[6,9,12]	24.7	20.2	8.2
(20:1[8])[h]	0	1.5	2.6
Others[i]	20.4	14.3	15.4

[a] See footnote *a*, Table I. [b] Packed cell volume, 1.2 ml. [c] Packed cell volume, 0.04 ml. [d] Packed cell volume, 0.06 ml. [e] A shoulder on the octadecenoate peak. [f] All 18:1[9]. [g] Mainly 18:1[6]. [h] Not fully characterized. [i] See footnote *j*, Table I.

percentages of linoleate and γ-linoleate in the lipids were very low, while there was a large increase in the concentration of octadecenoate due to the incorporation of petroselinate. Furthermore, a small amount of petroselinate was probably elongated by two carbons to 8-eicosenoate. This new fatty acid was partially identified by gas–liquid partition and thin layer chromatography but insufficient quantities were available to locate definitely the position of the double bond by oxidative degradation.

Tetrahymena grown in the presence of *11-eicosenate* were apparently normal. The fatty acid was incorporated into the lipids (Table IV) and was, in part, desaturated to 11,14-eicosadienoate which was also incorporated into the lipids. Of the normal fatty acids, the concentration of linoleate was somewhat depressed.

Tetrahymena grown in the presence of *vaccenate*

TABLE IV: Per Cent Composition of Fatty Acids of Total Lipids of *T. pyriformis* Grown in the Presence of Vaccenate (18:1[11]) or 11-Eicosenoate.[a]

	Fatty Acid Added to Medium (0.1 μmole/ml)		
	None[b]	18:1[11][c]	20:1[11][d]
12:0	2.5	2.8	3.3
14:0	21.2	17.2	16.0
16:0	7.6	8.0	6.4
18:0	2.0	2.9	1.2
18:1	4.4[e]	28.2[f]	2.5[e]
18:2[6,11]	0	10.0	0
18:2[9,12]	19.9	7.1	11.3
18:3[6,9,12]	23.0	11.8	26.4
20:1[11]	0	0	9.3
20:1[13]	0	0.5	0
20:2[11,14]	0	0	8.7
Others[g]	19.4	11.3	15.0

[a] See footnote *a*, Table I. [b] Packed cell volume, 1.4 ml. [c] Packed cell volume, 1.4 ml. [d] Packed cell volume, 1.3 ml. [e] All 18:1[9]. [f] Mostly 18:1[11]. [g] See footnote *j*, Table I.

(11-octadecenoate) were also apparently normal despite a profound alteration in fatty acid composition (Table IV). The normal polyunsaturated fatty acids, linoleate and γ-linolenate, were greatly reduced in concentration and, in addition to vaccenate, two other fatty acids were present in the lipids. Both new fatty acids were completely identified by gas–liquid partition chromatography, thin layer chromatography, and oxidative degradation. One, 13-eicosenoate, would arise by chain elongation of vaccenate. The other was identified as 6,11-octadecadienoate. It was well separated by thin layer chromatography on $AgNO_3$–silica gel from linoleate (Lees and Korn, 1966) and had a shorter retention time on ethylene glycol succinate. Upon hydrogenation of this fatty acid, stearate was formed. The fatty acid was converted to the ethyl ester and then oxidized by periodate–permanganate. The products were then esterified by diazomethane and analyzed by gas–liquid partition chromatography (Davidoff and Korn, 1963). Ethyl methyl adipate, dimethyl glutarate, and methyl heptanoate were the only products, thus establishing the structure.

Acanthamoeba

These amoebae, as normally grown, contain polyunsaturated fatty acids only of the ω-6 group: 9,12-octadecadienoate, 11,14-eicosadienoate, 8,11,14-eicosatrienoate, and 5,8,11,14-eicosatetraenoate. When grown in the presence of *α-linolenate* (9,12,15-octadecatrienoate), *Acanthamoeba* incorporated the fatty acid into its lipids so that it accounted for 29% of the fatty acids of the amoebae. α-Linolenate was also converted to 11,14,17-eicosatrienoate, 8,11,14,17-eicosatetraenoate, and 5,8,11,14,17-eicosapentaenoate which accounted for 2–7%, 3%, and 4% of the total fatty acids of the amoebae, respectively. Quantitation of 11,14,17-eicosatrienoate is complicated by the fact that it has a retention time very near that of arachidonate. There was no detectable conversion of α-linolenate to 6,9,12,15-octadecatetraenoate which, conversely, was the only reaction found in *T. pyriformis*. Despite the incorporation of appreciable quantities of these ω-3 group fatty acids into their lipids, no abnormal effects on the *Acanthamoeba* were noted in contrast to the marked toxicity of α-linolenate in *T. pyriformis*.

Petroselinic acid (6-octadecenoate) was similarly nontoxic to the amoebae. Although it was incorporated into the cell lipids, petroselinate was not converted into any other fatty acids.

Discussion

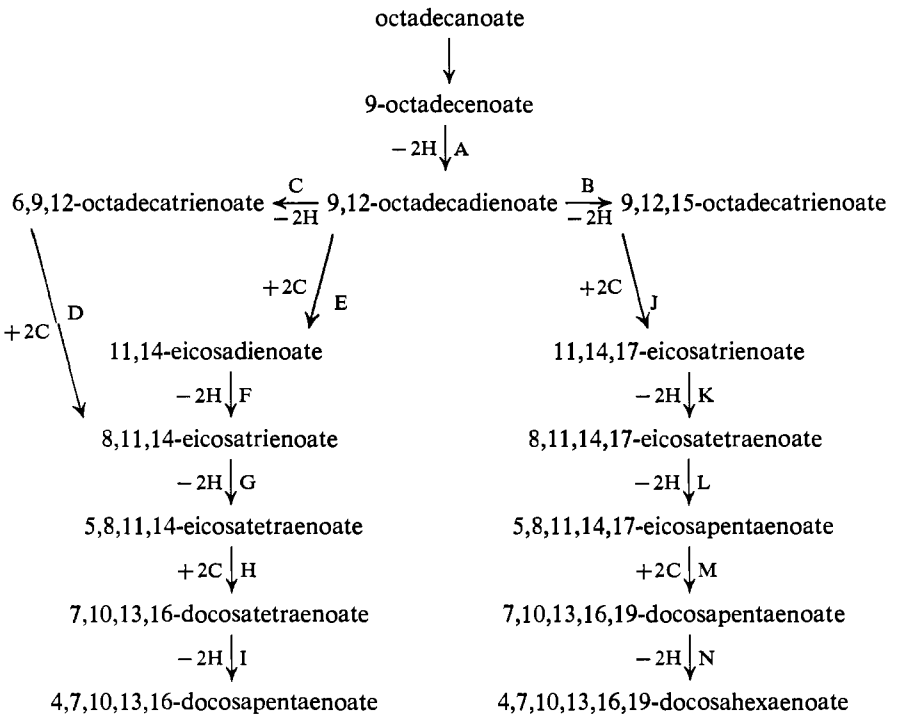
The known biosynthetic relationships among the common polyunsaturated fatty acids are summarized in Scheme I. The reactions fall into three categories: (1) introduction of double bonds toward the methyl terminus (steps A and B); (2) introduction of double bonds toward the carboxyl group (steps C, F, G, I, K, L, N); and (3) chain elongation by two carbons (steps D, E, H, J, and M).

Several questions concerning enzyme specificity immediately arise. Does one enzyme catalyze reactions A and B? Are the enzyme or enzymes that catalyze reactions E–I the same as those that catalyze reactions J–N? (These two reaction sequences are identical except for the presence of an additional double bond in one of the initial substrates.) What other fatty acids can serve as substrates for these enzymes?

Some of these questions can probably be answered from the fatty acid composition of organisms grown on a medium free of fatty acids. Both *T. pyriformis* and *Acanthamoeba* sp., *e.g.*, synthesize linoleate but not α-linolenate and, therefore, reactions A and B are presumably catalyzed by different enzymes; at least in these two protozoa the enzyme catalyzing A cannot catalyze B. Similarly, *Acanthamoeba* is capable of reaction E, but not H and, therefore, in this organism, the elongating enzyme must show some specificity for chain length of the substrate.

Other tentative answers can be obtained from experiments such as those described in this paper. The fact that *Acanthamoeba* grown in the presence of α-linolenate converts it to the ω-3 tri-, tetra-, and pentaunsaturated fatty acids suggests that reactions J–L can be catalyzed by the same enzymes that catalyze reactions E–G. It follows logically that *Acanthamoeba* would normally synthesize the ω-3 group of polyunsaturated fatty acids as well as the ω-6 group except that the enzyme catalyzing reaction B is not present. If reactions E–G and J–L are catalyzed by the same enzymes in higher animals it would explain the observations that dietary α-linolenate and linoleate each inhibit the conversion of the other

SCHEME I



to more unsaturated fatty acids of greater chain length (Holman, 1964).

When grown on a fatty acid free medium, *T. pyriformis* carries out only reactions A and C. But *T. pyriformis* is also able to desaturate 11,14-eicosadienoate to 8,11,14-eicosatrienoate and 9,12,15-octadecatrienoate to 6,9,12,15-octadecatetraenoate. One may presume that both reactions are catalyzed by the same enzyme that catalyzes reaction C. This would suggest that one important feature of the substrate for that enzyme is the number of carbons separating the double bond closest to the carboxyl group from the methyl terminus. This distance is nine carbon atoms in 9,12-octadecadienoate, 9,12,15-octadecatrienoate, and 11,14-eicosadienoate, all of which were desaturated. The enzyme appears also to act only on polyunsaturated fatty acids since 6,9-octadecadienoate is not synthesized from oleate and 11-eicosenoate was not converted to 8,11-eicosadienoate.

Similarly, the substrate specificity for the enzyme catalyzing reaction A in *T. pyriformis* may involve the distance of the double bond from the methyl terminus. 11-Eicosenoate could apparently substitute for 9-octadecenoate but neither 6-octadecenoate nor 11-octadecenoate were able to do so.

The observed elongation of 6-octadecenoate and 11-octadecenoate to the corresponding 20-carbon fatty acids is surprising since *T. pyriformis* does not seem to synthesize 20-carbon fatty acids when grown on a fatty acid free medium. Similarly, the conversion of 11-octadecenoate to 6,11-octadecadienoate is a unique reaction for *Tetrahymena*. This reaction is reminiscent of, although different from, a reaction typical of the cellular slime mold *Dictyostelium discoideum* which desaturates 11-octadecenoate to 5,11-octadecadienoate (Davidoff and Korn, 1963).

The physiological effects of the changes in fatty acid composition cannot be concluded from the present data. Only the most obvious effects were sought, changes in cell growth, cell appearance, or cell motility. The data do suggest that wide variations in fatty acid composition are compatible with relatively normal existence. It is also obvious that long-chain fatty acids are readily ingested by protozoa and incorporated into their lipids. Thus, the fatty acid composition of protozoa will probably vary greatly in their natural environment.

The toxicity of α-linolenate for *T. pyriformis* may be due to the fact that it is an ω-3 fatty acid. Most intriguing is the toxicity of γ-linolenate (6,9,12-octadecatrienoate). Since 9,12-octadecadienoate is not toxic while 6-octadecenoate is, one may tentatively assume that the toxicity is due to the double bond at position 6, and, perhaps, not unrelated to the fact that γ-linolenate is the major fatty acid of *Tetrahymena*.

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Immunochemical Studies on the Tobacco Mosaic Virus Protein. III. The Degradation of an Immunologically Active Tryptic Peptide of Tobacco Mosaic Virus Protein and the Reactivity of the Degradation Products with Antibodies to the Whole Protein*

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ABSTRACT: Tobacco mosaic virus protein (TMVP) tryptic peptide 8 representing residues 93–112 of the protein subunit and having the amino acid sequence Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg has previously been shown to exhibit immunological activity related to the whole tobacco mosaic virus protein. TMVP tryptic peptide 8 has been degraded by enzymes and by N-terminal stepwise cleavage, yielding desarginated peptide 8 (Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr), peptide 8 (−5) (Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg), and desarginated peptide 8 (−5) (Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr). The latter two peptides were further degraded by digestion with carboxypeptidase A. The immunological activities of the isolated peptides were tested by their capacity to inhibit the fixation of complement by TMVP and anti-TMVP, by their ability to compete with radioactive peptide 8 for antibodies to TMVP, or by their direct combination with anti-TMVP.

Such tests showed that the removal of five amino acids from the N-terminus of peptide 8 and the removal of at least two amino acids from the C-terminus of the peptide yielded an immunologically active peptide having the sequence Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala. The binding to anti-TMVP was somewhat reduced by the removal of five amino acids from the N-terminus of peptide 8 and was reduced to a greater extent by the removal of amino acids from the C-terminus of the peptide. Both pepsin and subtilisin digestion of the peptide destroyed its immunological activity.

Studies on the immunological activity of tryptic peptides of TMVP[1] showed that tryptic peptide 8 having the amino acid sequence: Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg (Gish, 1961; Funatsu *et al.*, 1964) specifically inhibited the fixation of complement by TMVP and anti-TMVP (Benjamini *et al.*, 1964). Acetylation of peptide 8 with 2-[^{14}C]acetic anhydride to yield [^{14}C]-acetyl peptide 8 ([^{14}C]peptide 8) resulted in no loss of its inhibitory activity as judged by inhibition of complement fixation. Furthermore [^{14}C]peptide 8 has been shown to bind specifically to anti-TMVP (Benjamini *et al.*, 1965).

In an effort to elucidate the antigenic determinant area contained in peptide 8, studies on the degradation of the peptide have been undertaken. The present paper reports the degradation of peptide 8 by digestion with

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[1] Abbreviations used in this work: TMVP, tobacco mosaic virus protein; [^{14}C]peptide 8, 2-[^{14}C]acetyl TMVP tryptic peptide 8; peptide 8 (−4), peptide 8 after four stepwise cleavages from the N-terminal end; peptide 8 (−5), peptide 8 after five stepwise cleavages from the N-terminal end; desarginated peptide 8, peptide 8 after removal of the C-terminal arginine; desarginated peptide 8 (−5), peptide 8 (−5) after removal of the C-terminal arginine; [^{14}C]desarginated peptide 8, 2-[^{14}C]acetyl peptide 8 after the removal of the C-terminal arginine.