HIGH INCREMENT OF TRIGLYCEROLS WITH ETHER LINKAGES IN THE RETINA DURING ANOXIA.

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SUMMARY. In the bovine retina incubated during 90 min in a medium lacking glucose without preoxygenation and gassed with O_2-free N_2 a striking rise in triglycerides with ether bonds was found. NaF and 2,4-DNP decrease the production of these molecules while cycloheximide (1.78mM) completely abolishes the phenomenon. The free fatty acid pool increases about six-fold and this rise is not affected by the inhibitors. Bovine serum albumin stimulates the free fatty acid and triglycerols production. The amount of these lipids decrease by further incubation under normoxia in the presence of glucose.

INTRODUCTION. Ether-linked neutral glycerides have been found only in very small quantities in mammalian tissues with the exception of tumors(1) and preputial gland(2,3) where they comprise a relatively abundant fraction.

The total triglycerols of the neural tissue are scarce (4-6), and they increase in the brain of rats undergoing convulsions by methionine sulfoximine(5) and are partly hydrolyzed at the onset of brain ischemia(6).

The central nervous system is irreversibly damaged by brief periods of oxygen deprivation. The retina is a part of the nervous system containing a highly compartmentalized cellular layer of photoreceptors. In the retina in vitro 30 min of anoxia in the absence of glucose cause irreversible changes(7). Furthermore, in the present study it was surprising to find production of triglycerols with ether bonds in the retina incubated under anoxia in the absence of added glucose. In addition, we show that the bovine retina contains only trace amounts of ether-bonded triglycerols before incubation or when incubated under normoxia plus added glucose. Moreover the effect of metabolic inhibitors and the changes in the free fatty acid levels are also described.

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A preliminary communication of this work has been presented(8).

MATERIALS AND METHODS. Bovine eyes were brought from the slaughterhouse packed in crushed ice. About 2 hours later the eyes were dissected and the retinas were removed and weighed. The incubations were done in the medium described by Ames(9) at 37°C with continous shaking under different conditions. Normoxia denotes preoxygenated media, addition of 2mg/ml of glucose and continuos flowing with O_2-CO_2(95-5) and anoxia indicates absence of glucose and the presence of an inert atmosphere of oxygen-free N_2 throughout the incubation time. A set of retinas in normoxia and in anoxia were also incubated with metabolic inhibitors as shown in Table I. An experiment was also conducted in the presence of free fatty acid-free bovine serum albumin under anoxic conditions.

At the end of the incubation period the retinas were homogenized in chloroform-methanol 2:1 (v/v) with a motor driven teflon pestle(10) and then passed through sintered-glass filters. The residue remaining on the filter was thoroughly washed with chloroform-methanol 2:1(v/v), then the crude total lipid extract was dried down under N_2, resuspended in a known volume and directly spotted for thin-layer chromatography.

All the incubation media were spun down at 6.500 x g at 4°C during 10 min after removing the retina and the pellet was combined with the rest of the retina. When albumin was included in the incubation media this was extracted by the procedure of Dole (11) after centrifugation as described above.

The total triglycerols and the free fatty acids were quantified by gradient-thickness thin-layer chromatography and photodensitometry(12). Development was carried out by hexane-diethyl ether-acetic acid 65:35:2,3(v/v/v). The quantification of the ether-bonded triglycerols was accomplished by photodensitometry of 250μm or 500μm layer of Silica Gel G developed with hexane-diethyl ether 95:5(v/v)(13). Charring of the plates was done with the copper acetate reagent (14).

RESULTS AND DISCUSSION. Incubation of the bovine retina under normoxia does not produce modifications in the free fatty acid level as compared with the unincubated retinas(Table I). Anoxia, on the contrary, greatly stimulates the production of free fatty

TABLE I.

MODIFICATIONS OF FREE FATTY ACIDS AND TRIGLYCEROLS IN THE RETINA INCUBATED IN ANOXIA AND THE EFFECTS OF METABOLIC INHIBITORS.

Triglycerols with ether linkages includes components with Rf of 0.31, 0.51 and 0.62 as indicated in Figure 1. The concentrations used of metabolic inhibitors were as follow: NaF 20 mM, 2,4-DNP 0.33 mM and cycloheximide 1.78 mM. The retinas in anoxia were incubated during 90 min in the absence of glucose under oxygen-free N_2. The figures for unincubated samples are means of three retinas, all others contained one retina. BSA, free fatty acid free-bovine serum albumin. Other details are described in Materials and Methods.

CONDITION	FREE FATTY ACIDS		TRIGLYCEROLS	
	Tissue	Media	Triacyl	With ether linkages
	µg/gram of fresh tissue			
Unincubated	111	-	122	21
Normoxia				
Control	141	-	123	17
+ NaF	270	-	82	24
+ Cycloheximide	144	-	116	7
Anoxia				
Control	496	-	159	109
+ NaF	409	-	100	30
+ 2,4-DNP	402	-	185	29
+ Cycloheximide	479	-	144	11
Anoxia + 0.5% BSA				
Control	341	343	162	128
+ NaF	352	207	103	46
+ 2,4-DNP	310	168	162	60
+ Cycloheximide	379	240	133	16

acids. These results suggest that the isolated retina in anoxia enlarges its free fatty acid pool as was described to occur during rat brain ischemia(6). The addition of 0.5% of bovine serum albumin to incubation media in combination with anoxia stimulates the free fatty acid production, and about half of the total free fatty acids are found outside the tissue(Table I).

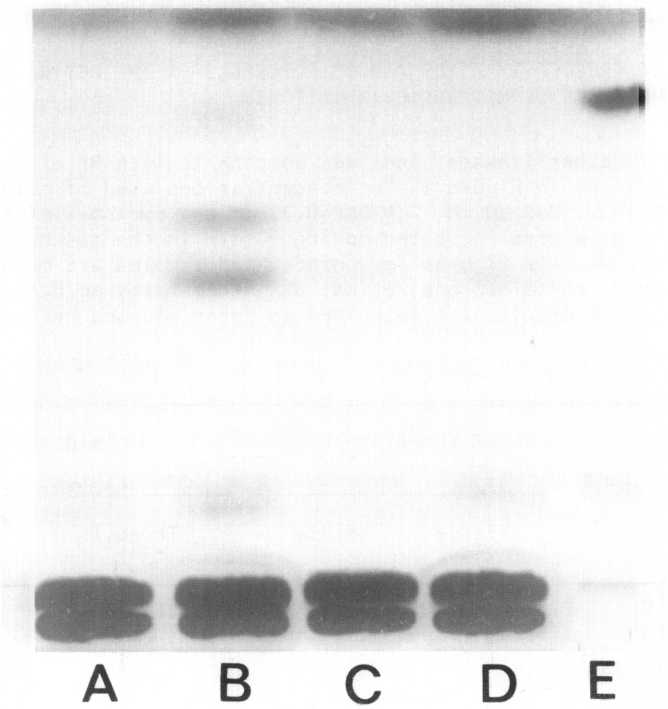

FIGURE 1.- Thin layer chromatogram of lipid extracts from retinas in different conditions. The layer was made of 500 µm with Silica Gel G and was developed with hexane-diethyl ether 95:5 (v/v). The lipid extracts spotted in all lanes was equivalent to 100 mg of retina fresh weight. Lane A, unincubated retina; Lane B, retina incubated in medium deprived of glucose and with an atmosphere of oxygen-free N_2 during 90 min; Lane C, retina incubated during 45 min as in B and then transfered to normoxia in the presence of 2% bovine serum albumin and incubated for a further period of 45 min; Lane D, similar experiment as in C with the difference being that the incubation in anoxia was done during 90 min and the recovery period in normoxia was for a further period of 90 min, and Lane E, contains from the top to the bottom standards of cholesterol esters, triolein and free fatty acids. The triglycerols with ether bonds have an Rf of 0.62, 0.51 and 0.31. The latter is very small and do not show in the picture.

Unincubated retinas as well as incubated in normoxia during 90 min show only trace amounts of triglycerides other than triacylglycerols. In Figure 1 a typical chromatographic separation of neutral lipids of the retina incubated under different conditions is presented. Retinas deprived of glucose and oxygen for 90 min show a striking accumulation of triglycerols with ether bonds. This comprises three fractions with the following Rf, 0.31, 0.51 and 0.62. The fraction with a Rf of 0.31 is vanishingly small. On the leading edge of the middle fraction there seems to be a fourth component. These bands are comparable with

the triglycerols with ether bonds as described by Snyder(15). At the present time we are engaged in the detailed characterization of these ether-bonded triglycerols.

Figure 1 also shows the reversibility of the described phenomena. Further incubation under normoxia for 45 and 90 min after incubation in anoxia markedly decreases the amount of the ether-linked triglycerols.

The content of the triacylglycerols as well as that of the triglycerols with ether linkages was unchanged during incubation in the presence of glucose and oxygen during 90 min (Table I). When both oxygen and glucose were omited during 90 min of incubation a large increase occurs in the level of triacylglycerols and of triglycerols with ether linkages. The latter rises about 400%. If 0.5% of bovine serum albumin is included in the media under anoxic conditions the change in the ether-bonded triglycerols is even greater.

Since the retina, as other parts of the nervous tissue, is irreversibly altered within short periods of time in anoxia, the marked increment in triglycerols with ether linkages was a surprising finding. The increased production of free fatty acids may reflect catabolic changes in acyl containing lipids while the rise in triglycerols with ether bonds may imply de novo biosynthesis or inhibition of the degradative pathway. Because the unincubated retinas contain only trace amounts of these molecules the former posibility seems to be likely. Furthermore, because the energy stores are known to be rapidly depleted in neural tissues in anoxia and since anaerobic glycolysis is a very active process in the retina we also studied the effect of metabolic inhibitors.

The addition of NaF(20mM), 2,4-DNP(0.33mM) and cycloheximide (1.78mM) markedly decreases the accumulation of triglycerols with ether bonds under anoxic conditions both in the presence and in the absence of 0.5% bovine serum albumin(Table I). Cycloheximide completely abolishes the production of ether-bonded triglycerols. The nature of this effect is currently being studied in our laboratory. In this connection it is interesting to note that cycloheximide at concentrations higher than those effective in the inhibition of protein biosynthesis and similar to those used in the present experiments(1.78mM) hinder energy transfer at site I of the respiratory chain decreasing the rate of hepatic

gluconeogenesis(16). Cycloheximide causes an opposite effect in the liver accumulating total triglycerides(17). In addition it inhibits very low density lipoprotein release, it decreases the inflow of free fatty acids to the liver and it decreases diglyceride acyltransferase activity (17).

During anoxia both NaF and 2,4-DNP reduced by about 70% the accumulation of triglycerols with ether bonds. The inhibitory action of these subtances in anoxia and in the presence of 0.5% bovine serum albumin is about 60% for NaF and 2,4-DNP. The effect of NaF may imply that metabolites such as dihydroxyacetone phosphate (18-19) and/or anaerobic glycolysis-derived energy are needed for the formation of the ether-bonded triglycerols. Whether or not the effect of 2,4-DNP represents uncoupling of the respiratory chain from the oxydative phosphorylation or a different action of the chemical remains an open question. In accordance with the first possibility, the ATP content of the retina decreases at a much slower rate than that of brain during ischemia remaining at about 40% one hour after the blood supply was interrupted (20).

It is interesting to note that the incorporation of (^{14}C) acetate into triglycerides in the perfused rat heart is greatly increased during anaerobiosis(21). When free fatty acids are added in the perfused turtle heart under hypoxia or anoxia a decrease in their oxidation to CO_2 and a rise in their incorporation into triglycerides was reported (22). Triglycerides biogenesis from (2-^{14}C) glucose in atherosclerotic rabbit aortas is also increased by hypoxic incubation (23). All these studies (21-23) have beary only to the total triglycerol fraction, and therefore we do not know if the changes taking place in the heart and in the aorta deprived of oxygen are analogous to the rise in the ether-bonded triglycerols of the retina or if they are only related to the triacylglycerols. Moreover, the data presented in this communication, to the best of our knowledge, are the first study describing such changes in the isolated retina in extreme anoxic conditions. Very recently we have also found analogous modifications in the cerebral grey and white matter (24).

Research on the described phenomena may be useful not only to further our knowledge about the regulation and the detailed mechanism of the biosynthesis of the ether-bonded glycerols, but also to gain information about unknown metabolic resources of the retina.

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