EFFECT OF NON-GLYCERIDE MINOR SUBSTANCES FROM AN ERUCIC ZERO RAPESEED OIL ON RAT HEART MITOCHONDRIA

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The suggestion according to which the incidence of myocardial focal degeneration in animals might only be due to the high proportion of erucic acid in rapeseed oils, does not seem quite conclusive.

In fact, our previous experiments indicated:

1. The rapid transformation of [14-^{14}C] erucic acid (ER) into [10-^{14}C] oleic acid (OL) in the rat liver (1). Only 2 hrs after the radioactive injection, half a part of ER was already converted into OL, and 26 hrs after injection, only a few traces of ER can be found in the liver;
2. The erucic acid does not seem to be selectively incorporated in the myocardia. When administered in solution in rapeseed oil by gastric probe, OL behaved like ER - radioactivity of the heart being much stronger than when these fatty acids were solubilized in olive oil (2);
3. Moreover, a slow oxidative rate of ER was observed in cardiac mitochondria (3) : 48 hrs after radioactive injection, 50 per cent of the total radioactivity was still found in ER molecules.

Moreover, Houtsmueller (4) and Clandinin (5) described the inhibition of oxidative phosphorylations in mitochondria after feeding rapeseed oil. In an experiment by Hsu and Kummerow (6) erucoyl-, oleoyl- or elaidoyl-carnitine, pyruvate or glutamate were used as substrates. The results indicated slow oxidation of all these compounds by mitochondria isolated from hearts of rats fed on rapeseed oil. Christiansen, Christophersen and Bremer (7) emphasized that the reason why animals fed on a diet containing high per cent oils with C 22-monounsaturated fatty acids develop fatty heart, but no fatty liver, is not definitely established and requires further studies.

Non-glyceride compounds have not yet been considered as possible agents responsible for myocardial pathological symptoms, although Prabucki and Schurch (8) indicated that the weight of hearts which increased after long term feeding on rapeseed oil was not due to the presence of erucic acid, and could be present in the non-saponifiable fraction of the oil. Therefore, we decided to study the influence of rapeseed oil non-glyceride compounds in vitro on the rat heart mitochondria respiratory rate and oxidative phosphorylations (9).

Before describing the methodology employed, it seems necessary to insist on the results expected from these studies.

We have not attempt to ascertain if the non-glyceride compounds might have an effect different to that produced by the erucic acid or by any other monounsaturated fatty acid with 22 carbon atoms. Numerous pharmacologically active substances have been described in animal and vegetable oils.

Our aim is to investigate whether minor compounds can be found in cruciferae oils acting in trace state and which might explain the pathological symptoms observed in the young animal fed a diet rich in rapeseed oil.

METHODOLOGY

Materials

CBU (non-glyceride concentrate from refined canbra oil) was obtained by molecular distillation (235°C) under 1-2 μHg pressure. CBU still contained triglycerides and non-polar substances which were removed by passage through silica gel columns. Subsequently, non-glyceride compounds were also separated through silica gel columns (Merck G60) and eluted with a gradient of hexane, diethylether, methanol and formic acid.

The 60-80 obtained fractions (from 1-1.5 g CBU) were pooled after visualization on thin layer plates. Every pooled fraction was tested further for study of its biological effect on RHM (rat heart mitochondria).

Isolation of rat heart mitochondria

The hearts were collected from developing rats (7-25 day old) which were kept on a lab chow diet after weaning. Fat and large vessels were removed, and opened hearts were rinsed with ice cold buffer: sucrose 0.3 M, triethanolamine HCl 20 mM, EDTA 2 mM, pH 7.2. The pooled hearts were then cut into pieces, and rinsed 4 times before homogenization (1w/10v) with the same buffer, in a loosely fitting pestle Potter-Elvehjem homogenizer (3 stocks). Nuclei and debris were eliminated by centrifugation at 300 xg. RHM were obtained by sedimentation at 5000 xg for 10 min. The sediment was washed, recentrifuged, and resuspended in 1.1 - 1.5 ml buffer, pH 7.4 (H_2KPO_4 3 mM, HK_2PO_4 13 mM, NaCl 26 mM, KCl 58 mM, NaF 12 mM, $MgCl_2$ 6 mM, sucrose 16 mM) with the help of a small Dounce homogenizer. The protein concentration was determined by a biuret procedure following solubilization with 4 per cent sodium cholate.

Determination of RHM respiratory rate

Respiratory rate was measured on 0.85 - 1.20 mg proteins per reaction chamber, by polarography with a Clark oxygen electrode, in 1.6 ml final volume. The medium contained 240 μmoles O_2 per ml.

Determination of ADP : O and respiratory control ratios

ADP : O ratios (equivalent to P : O ratios) were directly calculated from oxygen electrode tracings (micromoles ADP added/microatoms oxygen utilized). Respiratory control ratios were calculated from the respiratory rate in the presence of added ADP with respect to the rate obtained following its expenditure.

State 4: buffer pH 7.4, RHM in buffer pH 7.4 (30 μl), substrate (pyruvate 2.5 mM + malate 2.5 mM) 25 μl, sample in ethanol, 10 μl.

State 3: buffer pH 7.4, ADP (150 μM) 25 μl, RHM in buffer pH 7.4, 30 μl, tested sample in ethanol 10 μl, and respiration started upon addition of substrate (pyruvate 2.5 mM + malate 2.5 mM) 25 μl.

Effectors: effectors were tested by addition of 30 μg CBU fractions into the respiratory chamber.

RESULTS

Composition materials

Fig. 1a and 1b indicate Rf values for the 17 pooled CBU fractions obtained through silicagel column chromatography and further tested in mitochondria respiratory chamber. None of them was pure; all contained 2 or several compounds. A few spots were visible in U.V. light, more polar than the

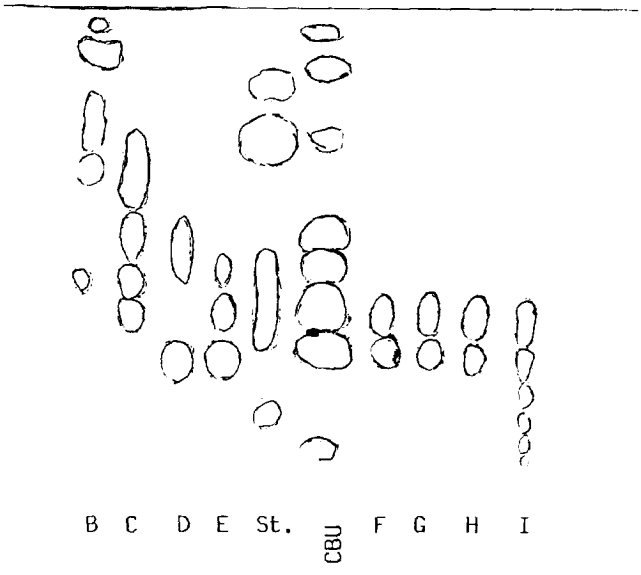

FIG. 1 a

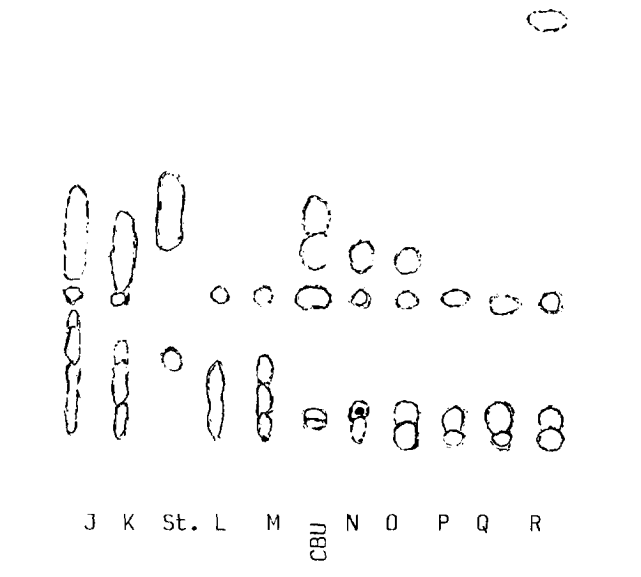

FIG. 1 b

Thin layer chromatography on Silica gel plates (20 x 20 cm x 0.25 mm)
Migration: hexane-diethylether (50:50)
Reagent: H_2SO_4 50 per cent
Standards (St.):
Fig. 1 a - triglycerides, tocopherol, oleic acid, rotenone
Fig. 1 b - oleic acid, rotenone
CBU: non-glyceride compounds before fractionation

sterols. Carotenes (Rf 0.92) and polar carotenoids (Rf 0.06) appeared in orange spots. Other spots were visible by carbonization after spraying H_2SO_4 (50 per cent). Cycloartenone (Rf 0.81) which is present in CBU has already been described (10), α and γ tocopherols (Rf 0.75 and 0.67) were found in the first fractions. CBU sterols which were known to contain brassicasterol in large proportion, migrated at the same Rf (0.27-0.30) as stigma- or sitosterol used as standard. These sterols were especially concentrated in fractions D, E and F.

Spots which appeared on TLC plates at the same Rf as sterols, or close to them (above and below) in fractions G to R were visible in brown colour by spraying H_2SO_4 and charring, while sterols were visible in pink and violet.

Respiratory rates

Carotenes, tocopherols and sterols were without any significant effect on RHM respiratory rate. Several other fractions isolated from CBU were very active, and namely fractions which appeared on thin layer plates at Rf identical with fatty acids, or smaller than fatty acids and sterols. Many of these fractions stimulated RHM respiratory rate so strongly that they seemed to function like uncoupling agents when ADP was added. However, the well known uncoupling effect of fatty acids can be suspected, although fractions were analysed on thin layer plates and did not necessarily show compounds at the fatty acid Rf.

Nevertheless, the respiratory rate stimulation was not obtained with I fraction, and was obtained with the J fraction whose constituents migrated at about the same Rf (stimulation was also obtained with the K and N fractions). Curiously, when fraction J was separated on chromatoplates:

- the compounds more polar than fatty acids did not have any significant effect on the RHM respiratory rate, and
- the compounds which migrated at fatty acid level appeared as strong respiratory inhibitors, and also inhibitors of oxidative phosphorylations.

The same inhibitory effect was also produced with the M fraction, whose migration on chromatoplates was entirely included below the sterols.

When albumin was added to the buffer used for RHM incubation in order to trap fatty acids, the stimulatory effect of several samples was reduced. However, when fraction J was tested after separation into 2 samples (one appearing at fatty acid level, and the 2nd below fatty acids), no influence of albumin addition in the respiratory chamber was found.

DISCUSSION

The respiratory rate stimulation obtained in the absence of albumin in the medium, and the lack of any effect when albumin was added, indicate that several CBU fractions still contained fatty acids, which were more or less easily complexed to albumin and no longer acted as uncoupling agents on RHM.

Other fractions again stimulated respiratory rate, even in the presence of albumin, when added in toto to the respiratory chamber. After separation on chromatoplates, an inhibitory effect can be observed with compounds collected at the fatty acid level, as well as with fractions which migrated below the sterol Rf.

One explanation of the inhibitory effect could be provided by the hypothesis according to which such substances might act at the transport level.

Fuel molecules must pass from the cytosol through the inner membrane, via specific transport systems, into the matrix compartment of mitochondria. Namely, phosphate carrier renders possible equimolar exchange of external ADP^{3-} for internal ATP^{4-}. Thus, one complementary attempt was carried out to determine RHM swelling speed, in order to study whether M and/or J fraction constituents could act on the phosphate entrance into cardiac mitochondria. A few preliminary results indicated significant variations when RHM were incubated in the presence of CBU fractions which inhibited respiratory rate.

CONCLUSION

We did not intend to reach any definite conclusion. Nevertheless, our recent experiment indicates that one or several non-glyceride compounds from cruciferae, still existing in CBU, might be active. The origin of these substances might be endogenous, but they might also be due to chemical pollutants such as pesticide degradation products and metabolites.

The present study suggests that, if our observations were not artefacts produced by contamination or compounds formed by degradation of glucosinolates during oil extraction and processing (11), several substances are possibly present in CBU which inhibit the respiratory rate. These compounds also seem to stimulate the respiratory rate when used in association - i.e. before chromatographic separation.

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