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THE INCORPORATION OF [^{14}C]FORMATE INTO EHRLICH ASCITES TUMOR LIPIDS

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SUMMARY

The time course of incorporation of [^{14}C]formate into the lipids of the nuclear and cytoplasmic fractions of Ehrlich ascites tumor cells was investigated. By paper-chromatographic analysis and hydrolysis studies it was shown that phosphatidylserine, and to a lesser extent, phosphatidylethanolamine were the most active phosphatides. However, a ninhydrin-negative, phosphorus-free lipid contained most of the radioactivity after 3 h incubation. This lipid releases fatty acids and ninhydrin-positive, water-soluble labeled compounds only after prolonged hydrolysis. These lipids moreover have paper-chromatographic properties which differentiate them from all the known common neutral lipids and phosphatides. They are tentatively identified as fatty acid amides of amino acids or peptides.

The data in these experiments indicate that the decarboxylation of phosphatidylserine to phosphatidylethanolamine and the direct methylation of phosphatidylethanolamine to lecithin are not major metabolic pathways in Ehrlich ascites tumor cells.

INTRODUCTION

The present experiments were designed to study simultaneously the incorporation of a metabolite into the lipids, proteins and nucleic acids of tumor cells. Since formate is converted to several precursors, which are in the metabolic pathways leading to the synthesis of the aforementioned biochemical compounds it was selected for use in this study. The experiments reported in this paper provide data on the incorporation of [^{14}C]formate into the lipids of Ehrlich ascites tumor cells. The incorporation of [^{14}C]-formate into the proteins and nucleic acids of these same systems will be reported elsewhere[1]. It is also noteworthy that relatively little is known concerning the incorporation of [^{14}C]formate into lipids[2]. Since formate is known to be incorporated into serine, ethanolamine and choline it permits the study of the simultaneous biosynthesis of the analogous parent phosphatides which contain these nitrogen components.

METHODS

The tumor used in this study was the Ehrlich-Hettre hyperdiploid strain ascites carcinoma. The cells were harvested from tumor bearing mice of the CFW strain.

For the maintenance of the tumors, cells and fluid (0.2 ml) from stock tumor bearing mice were injected intraperitoneally into mice of the above strain at periods of between 7 and 10 days of tumor growth.

In the present experiments fluid from tumor bearing mice was drained from the peritoneum by making a small incision through the abdominal wall of the anesthetized mice. The pooled fluids from 3–4 mice were used.

The incubation system consisted of the following: 1.0 ml of 0.25 M sucrose in 0.1 M phosphate buffer (pH 7.4), 0.8 ml of 0.1 M glucose in 0.25 M sucrose, 0.2 ml of [^{14}C]formate (8 μC) in water and 2.0 ml of freshly collected pooled fluid containing the tumor cells. These experimental conditions have been shown[3] to be satisfactory for the incorporation of formate into the nucleic acids of these cells. Incubations were carried out in 10-ml Erlenmeyer flasks at 37° using a Dubnoff shaking incubator.

At the desired time intervals the contents of the flasks were transferred to 15-ml conical centrifuge tubes and the tumor cells precipitated by centrifugation at 3000 rev./min for 10 min. The packed cell volume amounted to 0.5 ml. The supernatant fluid was discarded. The nuclear and cytoplasmic fractions were prepared as previously described[4]. The isolated fractions were then precipitated with trichloroacetic acid (final concentration of trichloroacetic acid was 10 %). The nuclear and cytoplasmic precipitates were used for extraction of the total lipids. (The residues remaining after lipid extraction were used for the isolation of the proteins and nucleic acids[1].)

The lipids in each fraction were obtained by sequential extraction with acetone, methanol, methanol–ether (1:1) and ether. These combined extracts were treated with a 2-fold volume of petroleum ether and then diluted with an excess of isotonic NaCl. The ethyl ether–petroleum ether upper phase was washed four times with isotonic saline. The centrifuging of the cells, the precipitation with trichloroacetic acid, and the saline washes were very effective in removing water soluble contaminants. The washed ether extracts were evaporated to dryness under nitrogen at 37° and the lipid residues dissolved in 0.4 ml of chloroform–methanol (1:1). 20 and 40 μl (containing 1–4 μg of lipid P) were used for paper chromatography[5–7]. 100 μl of each were plated on stainless steel planchets and the radioactivity determined with an automatic Nuclear-Chicago gas flow counter (Micromil window). The lipid residues were then eluted and digested in order to determine total lipid P. The specific activities of the lipids were calculated as counts/min/μg of P.

Autoradiograms of the chromatograms were prepared on X-ray film. The exposure time was 2 months.

The remaining lipids were hydrolyzed in 1 N HCl for 2 h under reflux. The fatty acids and non-saponifiable lipids were removed by extraction with ether. The aqueous phase was concentrated to a small volume and aliquots were taken for paper chromatographic analysis of ethanolamine, choline and amino acids. Two dimensional chromatography for amino acids was carried out according to HARDY[8]. Autoradiograms were also prepared on X-ray film.

RESULTS

Two experiments were performed in order to study the time course of incorporation of [^{14}C]formate into the nuclear and cytoplasmic (the latter includes mitochondria and microsomes) lipid fractions. In Expt. I, the systems were incubated for 2, 5, 15 and

30 min and 1 h. In Expt. II, the systems were incubated for 5, 15 and 30 min, and 1, 1.5 and 3 h. The rates of incorporation for Expt. II are shown in Fig. 1. The rates of incorporation for Expt. I were the same as those shown by the first hour interval in Fig. 1. Since it is more accurate to analyze for lipid P than to weigh very small amounts of lipids, the specific activities are given as counts/min/μg of lipid P. In these experiments cholesterol was not labeled and triglycerides and other glycerides contained very little radioactivity. It can be seen that the specific activity and rates of incorporation for the first hour are essentially the same for the nuclear and cytoplasmic lipids. However, after the first hour the rate of incorporation of ^{14}C into, and the specific activity of, the cytoplasmic lipids were slightly greater than those of the nuclear lipids. The significance of this finding is not known.

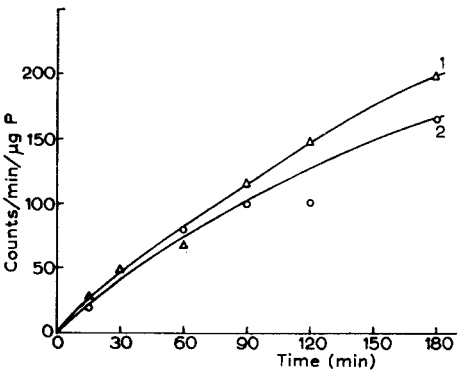

Fig. 1. Rates of incorporation of [^{14}C]formate into Ehrlich ascites tumor cells. The cytoplasmic and nuclear lipids are shown by curves 1 and 2 respectively. The details of this experiment (Expt. II) are given in the text. The per cent of added counts incorporated after 3 h was as follows: nuclear lipids, 0.5; cytoplasmic lipids, 1.0; the total lipid P extracted from each incubation (average of six values) was 16.7 μg for the nuclear lipids and 50.1 μg for the cytoplasmic lipids.

Autoradiograms showing the time sequence of incorporation into the cytoplasmic lipids of Expt. II (similar results were obtained in Expt. I) are shown in Figs. 2 and 3. The separation of the phosphatides is shown in Fig. 2 and the separation of the neutral lipids is shown in Fig. 3. It was clearly evident by autoradiography that the exposed spots on the film were superimposed on the spots on the chromatograms, the latter spots having been detected by staining with Rhodamine 6G. The spots were discrete with essentially no trailing. The characterization of the individual phosphatides was based on their mobility and chemical tests[5–7].

Since the same amount of lipid phosphorus was used for each time interval the increase in radioactivity in phosphatidylserine and in the fast moving component which moves with the non-phosphatides can be visually ascertained by inspection of Fig. 2. The radioactivity in the other phosphatides is not visible in this reproduction. The spots corresponding to phosphatidylserine and phosphatidylethanolamine were ninhydrin positive and had mobilities characteristic for these phosphatides.

The autoradiogram and chromatogram shown in Fig. 3 clearly reveals that most (but not all) of the radioactivity occurred in the origin spot which contains predominantly the phosphatides but which also contains other polar neutral lipids (such as monoglycerides and bile acids). Hence it is clear that the fast moving component in Fig. 2 which moves with the non-phosphatides cannot be triglycerides, cholesterol esters, free cholesterol, diglycerides and free fatty acids. The fast moving lipids must be more polar compounds than these common neutral lipids. Evidence to be presented later in this paper indicates that they have the properties of fatty acid amides of amino acids or peptides.

The lipid spots on chromatograms for the 3-h interval (Expt. II) of both the nuclear and cytoplasmic lipids (chromatograms were similar to that shown in Fig. 2) were cut off and placed on aluminum planchets and their radioactivities were determined. The radioactivities of both the front and back side of the spots were measured and the average values of these were determined. In Table I is shown the relative distribution of ^{14}C-radioactivity in the phosphatides.

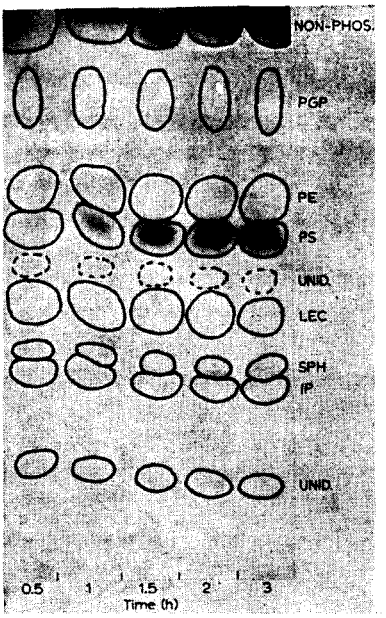

Fig. 2. Superimposed autoradiogram and chromatogram of ^{14}C-labeled Ehrlich ascites tumor phosphatides. The cytoplasmic lipids of Expt. II are shown. Paper chromatography was carried out on silicic acid impregnated paper[5-7]. NON-PHOS., non-phosphatides; PGP, polyglycerolphosphatides (cardiolipin); PE, phosphatidylethanolamine; PS, phosphatidylserine; LEC, lecithin; SPH, sphingomyelin; IP, inositol phosphatides; UNID., unidentified components. The identification of PGP and IP is tentative. The neutral lipid chromatogram is shown in Fig. 3.

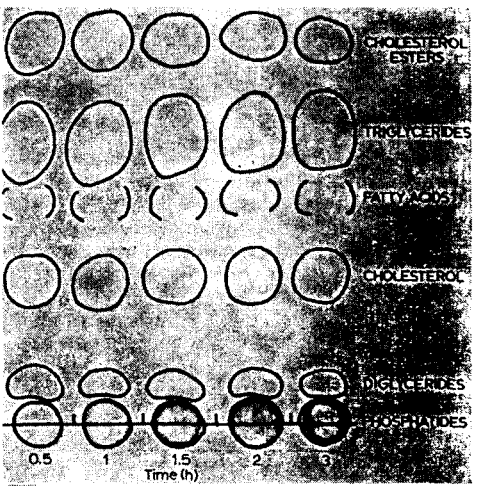

Fig. 3. Superimposed autoradiogram and chromatogram of ^{14}C-labeled Ehrlich ascites tumor non-phosphatides. The cytoplasmic lipids of Expt. II are shown. Paper chromatography was carried out on silicic acid impregnated paper as described previously[6,7]. The solvent system was *n*-heptane–diisobutylketone–acetic acid (96:6:0.5). The fatty acid fraction may also contain CoQ (ubiquinone) or α-tocopherol. The phosphatides at the origin also contain the unidentified radioactive neutral lipids.

After 3 h most of the radioactivity occurred in the unidentified fast moving lipid and in the phosphatidylserine in both the nuclear lipids and the cytoplasmic lipids.

Supporting evidence that phosphatidylserine and phosphatidylethanolamine were labeled was obtained by hydrolysis studies in which the liberated serine and ethanolamine were separated by paper chromatography and measured for radioactivity. The lipids of the 1-h sample of Expt. II were used. Only these 2 N-bases were counted although other ninhydrin positive compounds were observed on the chromatograms. It can be seen in Table II that in both the nuclear and cytoplasmic lipids about 80 % of the radioactivity in these 2 compounds occurred in serine and 20 % in ethanol-

amine. KAY has previously shown the ability of these tumor cells to incorporate formate into serine[3].

When the aqueous hydrolysates of the 3-h sample in Expt. II were analyzed for radioactivity, serine and ethanolamine were active, however, considerably more radioactivity occurred in other water soluble ninhydrin positive components. In the 2-dimensional system of HARDY these ninhydrin positive compounds give the pattern shown in Fig. 4. Spot b has the mobility of serine. The other spots were not identified.

TABLE I

DISTRIBUTION OF ^{14}C-RADIOACTIVITY IN THE PHOSPHATIDES OF THE NUCLEAR AND CYTOPLASMIC FRACTIONS OF ASCITES TUMOR INCUBATED WITH [^{14}C]FORMATE

	*Per cent of total counts in the phosphatides**				
	PS	*PE*	*LEC*	*SPH***	*Unidentified****
Nuclear§	21	14	14	8	42
Cytoplasmic§	35	7	9	7	36

* A small amount of activity was observed in the other phosphatide spots. PS, phosphatidylserine; PE, phosphatidylethanolamine; SPH, sphingomyelin; LEC, lecithin.

** May also include lysophosphatidylethanolamine.

*** These lipids represent the fast moving components in Fig. 2. Chromatograms of the nuclear and cytoplasmic lipids were run in duplicate, one set being used for autoradiography and the other set for elution of the various lipid spots.

§ Lipids taken from the 3 h incubated samples of Expt. II. The total counts/min applied to the paper was as follows: nuclear lipids, 400; cytoplasmic lipids, 600.

TABLE II

DISTRIBUTION OF ^{14}C-RADIOACTIVITY IN SERINE AND ETHANOLAMINE ISOLATED BY HYDROLYSIS OF THE PHOSPHATIDES OF THE CYTOPLASMIC FRACTION OF ASCITES TUMOR CELLS INCUBATED WITH [^{14}C]FORMATE

Solvent A, *n*-propanol–conc. ammonia–water (60:30:10); solvent B, phenol saturated with water. The serine and ethanolamine were completely resolved in these systems and were detected by the ninhydrin reagent. The total counts/min applied to the paper was 400.

	Per cent of radioactivity	
	Serine	*Ethanolamine*
Solvent A	79	21
Solvent B	79	21

The cytoplasmic lipids contained more of these radioactive components and furthermore they were more active in the cytoplasmic fractions than in the nuclear fraction. This was easily ascertained by autoradiographic analysis.

Another interesting finding was observed by autoradiographic analysis of the 3-h sample of both the nuclear and cytoplasmic fractions in Expt. II. A small but significant amount of radioactivity occurred in the neutral lipids, particularly the triglycer-

ides and diglycerides. Hydrolysis of these neutral lipids revealed that some of this radioactivity was in the fatty acid fraction. On the other hand radioactivity in cholesterol and cholesterol esters was not detectable. Autoradiograms in Fig. 3 do not show these findings since the radioactivity in the triglycerides and diglycerides was too low to allow for good photographic reproduction.

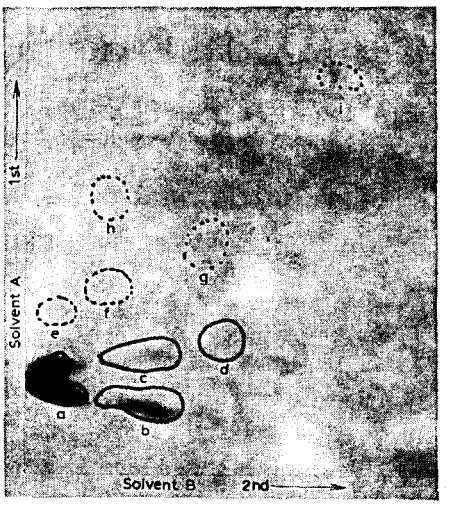

Fig. 4. Two dimensional chromatogram of the water soluble hydrolysis products of the ^{14}C-labeled Ehrlich ascites tumor lipids. The cytoplasmic lipids of Expt. II (3-h interval) were used. Chromatography was carried out as described by HARDY[8]. Solvent A, ethanol–*n*-butanol–water–propionic acid (10:10:5:2); Solvent B, *n*-butanol–acetone–water–dicyclohexylamine (10:10:5:2). All spots outlined were ninhydrin positive. The dotted spots were minor components (*i.e.* spots e, f, g, h and i). Spots a, b, c and d were major components. Spot b is believed to be serine.

The individual radioactive lipid components on the chromatograms of the 3-h incubation sample (Expt. II) were eluted and hydrolyzed with 1 N aqueous HCl at 95° for 4 h. The hydrolysates were extracted with ether. The ether extracts were washed with water. The radioactivity in the ether phase and in the water phase was measured. The results are given in Table III. It can be seen that the major part of the radioactivity of the glycerolphosphatides occurred in the aqueous phase. This was expected since most of the radioactivity in these lipids occurred in serine and ethanolamine (and possibly choline). The data on the unidentified fast moving component

TABLE III

HYDROLYSIS OF LIPID COMPONENTS OBTAINED FROM CHROMATOGRAMS OF ^{14}C-LABELED CYTOPLASMIC LIPIDS

Lipid components were obtained from the chromatogram shown in Fig. 2. Hydrolysis was carried out in 1 N aqueous HCl for 4 h at 95°. The counts/min in the various fractions ranged from 30 to 306.

Lipid component	*Per cent of total ^{14}C-radioactivity*	
	Water phase	*Ether phase*
Phosphatidylethanolamine	73	27
Phosphatidylserine	88	12
Lecithin	70	30
Unidentified lipids	15	85

(see Fig. 2) is of special interest since most of the activity remained in the ether phase. The chromatographic mobility of this fast moving component was not altered by this 4-h acid hydrolysis. Prolonged hydrolysis (18 h) of this fraction did yield fatty acids and radioactive water soluble ninhydrin-positive compounds which most likely represent amino acids. The ninhydrin-positive compounds released gave a similar pattern to that shown in Fig. 4. Hence these lipids are appreciably more stable to acid hydrolysis than the typical phosphatides. Inasmuch as these unidentified lipids are radioactive, contain no phosphorous, are more acid stable than the glycerol-lipids and have chemical and chromatographic properties which differentiate them from all the known common lipids, they represent a new type of lipid. Since these compounds release fatty acids and amino acids only after prolonged hydrolysis they have the properties of fatty acid amides of amino acids or peptides.

DISCUSSION

The results of these experiments demonstrate that Ehrlich ascites tumor cells can incorporate [^{14}C]formate into several lipids. The per cent of added isotope incorporated into the nuclear and cytoplasmic lipids was 0.5 and 1.0 respectively after 3 h. However, the cytoplasmic lipids contained three times as much lipid P as the nuclear lipids. By chromatographic separation of the intact lipids, radioactivity was found in phosphatidylserine, phosphatidylethanolamine, lecithin, sphingomyelin and in unidentified lipids. These findings confirm the well known metabolic conversion of formate to serine and ethanolamine.

However, after 3 h incubation the major lipids which were labeled were fast moving unidentified components (Fig. 2). The exact nature of these lipids is unknown but on the basis of their chromatographic mobility and their chemical properties they may be fatty acid amides of amino acids or peptides. Fatty acid amides and lipid–amino acid complexes have been reported by HENDLER[9,10], FRIEDMAN AND GABY[11] and FUKUI AND AXELROD[12]. The high activity of these components points to their metabolic importance. Indeed their possible relationship to protein synthesis has been pointed out by the aforementioned workers[9,10].

The present observation that after 3 h incubation the triglycerides and diglycerides contained a small but measurable amount of radioactivity is noteworthy. Hydrolysis studies on both the phosphatide and neutral lipids showed that some activity was in the fatty acid fraction (see Table III). The manner of entry of formate into the fatty acids remains to be elucidated. One possible pathway is as follows: formate → serine → pyruvate → acetate → fatty acids. If this pathway prevails it is noteworthy that in these experiments cholesterol does not become labeled.

KENNEDY *et al.*[13,14] have reported the presence of phosphatidylserine decarboxylase in liver and in *Escherichia coli*. In *E. coli* this enzyme plays an important role in the quantitative conversion of phosphatidylserine to phosphatidylethanolamine. However, in the present system this metabolic pathway cannot play such a predominant role since even after 3 h phosphatidylserine contains much more radioactivity than phosphatidylethanolamine. Furthermore the very small amount of radioactivity in lecithin demonstrates that the direct methylation of phosphatidylethanolamine to lecithin[15–17] does not occur in these systems to any appreciable extent.

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REFERENCES

1 E. R. M. KAY, to be submitted for publication.
2 H. J. DEUEL, Jr., *The Lipids*, Vol. III, Interscience, N. Y., 1957.
3 E. R. M. KAY, *Federation Proc.*, 19 (1960) 393.
4 R. M. S. SMELLIE, G. F. HUMPHREY, E. R. M. KAY AND J. N. DAVIDSON, *Biochem. J.*, 60 (1955) 177.
5 G. V. MARINETTI, *J. Lipid Research*, 3 (1962) 1.
6 G. V. MARINETTI AND E. STOTZ, *Biochim. Biophys. Acta*, 37 (1960) 571.
7 G. V. MARINETTI, J. ERBLAND AND J. KOCHEN, *Federation Proc.*, 16 (1957) 837.
8 T. L. HARDY, D. O. HOLLAND AND J. H. C. NAYLER, *Anal. Chem.*, 27 (1955) 971.
9 R. W. HENDLER, *J. Biol. Chem.*, 234 (1959) 1466.
10 R. W. HENDLER, *Federation Proc.*, 19 (1960) 346.
11 H. FRIEDMAN AND W. L. GABY, *Federation Proc.*, 19 (1960) 199.
12 T. FUKUI AND B. AXELROD, *Federation Proc.*, 19 (1960) 6.
13 E. P. KENNEDY, *Federation Proc.*, 20 (1961) 934.
14 J. N. KANFER AND E. P. KENNEDY, *Federation Proc.*, 21 (1962) 296.
15 D. P. GROTH, S. A. BAIN AND C. C. PFEIFFER, *J. Pharmacol. Exptl. Therap.*, 124 (1958) 290.
16 C. ARTOM, *Biochem. Biophys. Research Commun.*, 3 (1960) 244.
17 C. ARTOM, *Federation Proc.*, 21 (1962) 297.