Total Plasmalogens and *O*-(Acylalkylglycerophosphoryl)ethanolamine from Labelled Hexadecanol and Palmitate during Hypoxia and Anoxia in Rat Heart

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By the use of the Langendorff technique, surviving isolated rat hearts were perfused with [$1\text{-}^{14}\text{C}$]palmitate, [$1\text{-}^{14}\text{C}$]hexadecanol or [$1\text{-}^{14}\text{C}$, $1\text{-}^3\text{H}$]hexadecanol under normal or anoxic conditions. After perfusion for 30 min with either precursor, when oxygenated or in an hypoxic condition, or when 1 mM-KCN was included in the system, the heart tissues showed no significant chemical changes in their content of total lipids, total phospholipids or total ethanolamine-containing phospholipids. Changes were observed in the ratio of alkyl- to alk-1-enyl-glycerophosphorylethanolamine in the tissue perfused with $\text{N}_2 + \text{CO}_2$ plus CN^- . A slight increase from 4.0 ± 0.3 to $4.9 \pm 0.2\%$ in alkyl derivatives and a decrease from 11.2 ± 0.4 to $9.4 \pm 0.3\%$ in alk-1-enyl derivatives was observed. The incorporation of the [^{14}C]palmitate and the [^{14}C]hexadecanol into the recovered phospholipids and plasmalogens was severely decreased in the tissues perfused with CN^- : in the hypoxic state only a mild inhibition was observed compared with the oxygenated systems. Considerable ^3H from [$1\text{-}^{14}\text{C}$, $1\text{-}^3\text{H}$]hexadecanol was retained (25–35%) in the alk-1-enyl ether chains of plasmalogens under both the oxygenated conditions and with CN^- , suggesting that the same mechanism of incorporation is operational at high or low O_2 concentrations. The results are consistent with an O_2 -dependent, CN^- -sensitive step in the biosynthesis of plasmalogens in the rat heart.

There are several reports that the synthesis of plasmalogens from long-chain fatty alcohols is through *O*-(2-acyl-1-alkyl-*sn*-glycero-3-phosphoryl)-ethanolamines, followed by aerobic desaturation of the monoalkoxyglycerol (Wood & Healy, 1970; Snyder, 1972; McMurray & Magee, 1972; Wykle *et al.*, 1972; Paltauf & Holasek, 1973; Paltauf *et al.*, 1974; Van den Bosch, 1974; Wykle & Lockmiller, 1975). No evidence is available for this pathway in the rat heart.

Studies in systems that permit changes in the degree of oxygenation would provide information on this step. Some of the effects of hypoxia have been reported for perfused isolated rat hearts utilizing the Langendorff technique with labelled lipids (Rabinowitz & Hercker, 1974). The object of the present study was to determine whether the biosynthesis of lipids, ethanolamine-containing phospholipids and plasmalogens may be impaired by hypoxia and the presence of CN^- . Surviving isolated rat hearts were perfused with a lipid-free albumin complex containing either [$1\text{-}^{14}\text{C}$]palmitate, [$1\text{-}^{14}\text{C}$]hexadecanol or [$1\text{-}^{14}\text{C}$, $1\text{-}^3\text{H}$]hexadecanol with equilibrated gas mixtures of $\text{O}_2 + \text{CO}_2$ (95:5) and $\text{N}_2 + \text{CO}_2$ (99:1) in the presence or absence of CN^- .

Materials and Methods

Heart perfusion

Wistar male rats weighing 350–375 g with continuous access to food were used. The apparatus, the solutions and the Langendorff technique used have been well described (Rabinowitz & Hercker, 1974). The perfusate solution contained either [$1\text{-}^{14}\text{C}$]palmitate, [$1\text{-}^{14}\text{C}$]hexadecanol or [$1\text{-}^{14}\text{C}$, $1\text{-}^3\text{H}$]hexadecanol at a concentration of 0.2 mM, with a radioactivity of 1–15 μCi . The perfusate was filtered before use through a Millipore Millex filter (pore size 0.45 μm). These solutions were then reassayed for specific radioactivity. At the end of the perfusion (30 ± 5 min) the heart was washed with buffer solution containing no radioactivity and was then frozen with special clamps kept at -20°C . A mixture of $\text{O}_2 + \text{CO}_2$ (95:5) was the gas phase used for the oxygenated system. The hypoxic gas mixture was $\text{N}_2 + \text{CO}_2$ (99:1). The anoxic gas mixture was $\text{N}_2 + \text{CO}_2$ (99:1) with the perfusion solution containing 1 mM-KCN.

Chemical assays

All solvents and chemicals were purified before use. Washed binder-free precoated 250 μm -thick silica

gel G and M glass plates (Analtech Inc., Newark, DE, U.S.A.) were used for the chromatographic separations. All lipids, or solutions which contained lipids, were kept under N_2 . The hearts usually weighed between 1.0 and 1.2 g wet wt. and were homogenized in 21 ml of chloroform/methanol (2:1, v/v) by using a glass homogenizer until no large particles were visible. The technique followed was described by Rabinowitz *et al.* (1971) and Hirsch & Ahrens (1959). Total phospholipid was determined by charring or by phosphorus content (Bartlett, 1959; Marsh & Weinstein, 1966). Portions of this material containing 300 μ g of lipid were spotted on pre-washed t.l.c. plates coated with silica gel H (250 μ m thick). The methods used have been described by Rabinowitz *et al.* (1971).

Dimethylacetal derivatives

For the determination of total ethanolamine-containing plasmalogens the method of Dittmer & Wells (1969) was used. The recovered dimethylacetal derivatives were then chromatographed on a silica gel G plate with toluene/diethyl ether/acetic acid (90:10:1, by vol.) as solvent. Standards were used to determine R_F values. Material was detected with I_2 vapour. The plate was scraped and the extract rechromatographed on the same system. The material obtained from the scrapings was assayed for total content of the dimethylacetal derivative by charring with H_2SO_4 (Marsh & Weinstein, 1966) and by comparison with a standard. Portions from the chromatography plate were used to assay ^{14}C radioactivity (Chase & Rabinowitz, 1972).

N-Dinitrophenyl O-methyl derivatives

Another portion of the total ethanolamine phospholipids obtained from t.l.c. was used to prepare the N-dinitrophenyl O-methyl derivative by the method of Renkonen (1968). By use of authentic materials it was possible to obtain correction curves on the yields. These curves were used to obtain quantified and reproducible results.

The areas corresponding to the methylated dinitrophenyl derivatives of O-(acylalkenylglycerophosphoryl)ethanolamine and acylalkylglycerophosphoryl)ethanolamine were scraped. The material obtained from the t.l.c. scrapings was extracted into chloroform and placed in a small volumetric flask. Portions were taken for the assays. (1) U.v. spectrophotometry at 236 nm was used to quantify the derivatives by comparison with standards made from authentic materials that had undergone the same manipulations. (2) Another portion was evaporated to dryness and charred.

The acid hydrolysis method of Viswanathan *et al.* (1968a) was used to hydrolyse the O-(acylalk-1-

enylglycerophosphoryl)ethanolamine. The hydrolysed and unhydrolysed material were then separated by t.l.c. in the second dimension. This was followed by alkaline methanolysis of the resulting O-(alk-1-enylglycerophosphoryl)ethanolamine and unhydrolysed diacyl ethanolamine phospholipids, which permitted separation and quantitative determination by the use of an internal methyl ester standard and g.l.c. (Viswanathan *et al.*, 1968b; Viswanathan, 1974).

Radioactivity assays

New England Nuclear Corp. (Boston, MA, U.S.A.) supplied the radioactive materials. A Packard Tri-Carb spectrometer was used for the radioactive assays. Gas chromatography and radioactive assay of the products was by oxidation and subsequent assay in a proportional radioactivity counter (Packard 894). The [^{14}C]palmitate and [^{14}C] and [^{14}C , 3H]hexadecanol were authentic and pure by chromatographic techniques. All materials containing 3H and ^{14}C were assayed by the use of Oxymat-oxidator (Intertechnique, Paris, France) after conversion into $^{14}CO_2$ and 3H_2O .

Results and Discussion

No significant differences were found in the composition of rat heart tissue for total lipids nor in the phospholipid distribution before or after perfusion. The chemical composition observed in this study is similar to data previously reported (Simon & Rouser, 1969).

Table 1 shows the percentage composition of the ethanolamine-containing group of lipids found in the rat heart. This group of phospholipids is quantitatively important, for it represents one-quarter of the total phospholipid content. These results were obtained from the ethanolamine phospholipid fraction isolated by t.l.c. from the phospholipid samples: (a) by charring of the dimethylacetal compound formed from the ethanolamine-containing phospholipids

Table 1. Composition of ethanolamine-containing phospholipids of rat heart under different oxygenation states

Results are means $\pm$ S.E.M. of eight analyses and were analysed by Student's *t* test.

	Composition (%)		Significance of difference	
	$O_2 + CO_2$	$N_2 + CO_2 + KCN$	<i>t</i>	<i>P</i>
Plasmalogens				
Acylalk-1-enyl	11.2 $\pm$ 0.4	9.4 $\pm$ 0.3	3.6	<0.005
Diacyl	82.3 $\pm$ 0.2	83.7 $\pm$ 0.3	3.9	<0.005
Acylalkyl	4.0 $\pm$ 0.3	4.9 $\pm$ 0.2	2.5	<0.05
Unidentified	2.5	2.0	—	—

Table 2. Incorporation of [^{14}C]palmitate and [^{14}C]hexadecanol into phospholipids and plasmalogens of the perfused rat heart under different oxygenation states

Results are the means $\pm$ S.E.M. for six perfusions. Statistical significances: between columns (1) and (3), and (4) and (6): line (a), $P < 0.001$; line (b), $P < 0.001$; line (c), $P < 0.001$; line (d), $P < 0.001$; line (e), $P < 0.025$; line (f), $P < 0.001$; between columns (1) and (2), and (4) and (5): line (a), NS; line (b), $P < 0.001$; line (c), $P < 0.001$; line (d), NS; line (e), $P < 0.025$; line (f), NS; between columns (2) and (3), and (5) and (6): line (a), $P < 0.001$; line (b), $P < 0.001$; line (c), $P < 0.001$; line (d), $P < 0.001$; line (e), $P < 0.05$; line (f), $P < 0.001$ (NS, not significant).

	Incorporation (nmol/h per g wet wt.)					
	[^{14}C]Palmitate			[^{14}C]Hexadecanol		
	$\text{O}_2 + \text{CO}_2$ (1)	$\text{N}_2 + \text{CO}_2$ (2)	$\text{N}_2 + \text{CO}_2 + \text{KCN}$ (3)	$\text{O}_2 + \text{CO}_2$ (4)	$\text{N}_2 + \text{CO}_2$ (5)	$\text{N}_2 + \text{CO}_2 + \text{KCN}$ (6)
Total lipids	(a) 1492 ± 29	1574 ± 35	683 ± 44	(d) 1462 ± 42	1346 ± 47	631 ± 74
Total phospholipids	(b) 365 ± 14	260 ± 12	79 ± 8	(e) 390 ± 12	341 ± 14	168 ± 74
O-(Acylalk-1-enyl-glycerophosphoryl)ethanolamine*	(c) 164 ± 9	100 ± 8	9 ± 1	(f) 54 ± 3	49 ± 5	10 ± 3
Ratio of label in total phospholipids to ethanolamine-containing plasmalogens	2.2	2.6	8.8	7.2	7.0	16.8

* Includes alk-1-enyl and acyl groups isolated as the dinitrophenyl derivative.

obtained from the sample and (b) by charring of the separated fractions obtained by the Viswanathan (1974) technique. These data, after correction for blank values, permitted the determination of the percentage of diacyl and acylalkenyl lipids. Assays of the dinitrophenyl derivatives provided results that were not as reliable as the above methods, but, if yield corrections were applied, the general trend was the same.

The values obtained for the content of the diacyl derivatives showed significant differences in percentage between the perfused rat heart kept under oxygenated conditions compared with that of the heart kept under $\text{N}_2 + \text{CO}_2$ in the presence of CN^- . The percentage value for plasmalogens was higher (11.2 ± 0.4) for the oxygenated tissue than for the anoxic tissue with CN^- (9.4 ± 0.3). On the other hand, the value for the acylalkyl fraction was lower (4.0 ± 0.3) for the oxygenated tissue than that obtained under $\text{N}_2 + \text{CO}_2$ with CN^- (4.9 ± 0.2). Student's t test suggests a statistical significance for these data. These results are compatible with the reported KCN-sensitive synthesis of plasmalogen (Paltauf & Holasek, 1973; Wykle & Lockmiller, 1975).

Table 2 presents data on the incorporation of [^{14}C]palmitate and [^{14}C]hexadecanol into lipids. (a) Total lipid incorporation value from [^{14}C]palmitate was $1492 \text{ nmol/h per g}$ of wet tissue under good oxygenation. Under hypoxia with CN^- the incorporation dropped to 683 nmol/h per g . (b) Incorporation into total phospholipids was 365 nmol under the best conditions; hypoxia decreased the value somewhat, but in the presence of CN^- incorporation was decreased to about 20% of the

original. (c) Incorporation into plasmalogens was 164 nmol in O_2 , with some decrease for hypoxia, but in the presence of CN^- the value dropped to 9 nmol/h per g . (d) The ratio of incorporation of the ^{14}C into all phospholipids compared with that found in O-(acylalk-1-enylglycerophosphoryl)ethanolamines was 2.2–2.6 for oxygenation or hypoxia and 8.8 for conditions involving CN^- . Table 2 also gives the statistical significance of the data. (The products formed may have been synthesized during the early period of incubation, and the data are not intended to indicate actual rates.) The ^{14}C results observed in (a), (b) and (c) indicate that, in the hypoxic tissue perfused with CN^- , the labelling of the plasmalogens from labelled palmitate decreased to a negligible value. Under $\text{N}_2 + \text{CO}_2$ conditions, the known reserves of myoglobin in heart tissue may account for the effects observed, and, even in the presence of CN^- , there still was oxidative capacity capable of the dehydrogenation step. In our previous work of involving assays of ATP, ADP and AMP in heart and lung tissue, it was shown that, even after severe hypoxia, stores of oxidative capacity were still available in these tissues (Rabinowitz & Hercker, 1974; Bassett *et al.*, 1974). Although we believe that this residual (previously found) oxidative capacity is sufficient to account for the formation observed of the alk-1-enols, the possibility that non- O_2 -dependent pathways exist cannot be excluded. Data on the incorporation of labelled hexadecanol are also shown in Table 2 and parallel the palmitate data.

Heart perfusions were also performed with doubly labelled [^{14}C , ^3H]hexadecanol. Thus changes that may occur in the ratio of $^3\text{H}/^{14}\text{C}$ in the recovered

Table 3. Incorporation of [$1\text{-}^3\text{H}$, $1\text{-}^{14}\text{C}$]hexadecanol into phospholipids and plasmalogens under different oxygenation states

Results are the mean $\pm$ S.E.M. of six perfusions. The blank was obtained from perfusions of heat-denatured rat hearts. *O*-(Diacylglycerophosphoryl)ethanolamines were obtained after HCl hydrolysis and t.l.c. to remove the unhydrolysed diacylglycerol phosphates. *O*-(Acylalk-1-enylglycerophosphoryl)ethanolamines were isolated as the dinitrophenyl derivatives. The alk-1-enyl chains were isolated as the dimethylacetals after treatment of phospholipids with methanolic HCl.

	Ratio of $^3\text{H}/^{14}\text{C}$	
	$\text{O}_2 + \text{CO}_2$	$\text{N}_2 + \text{CO}_2 + \text{KCN}$
Original	30 ± 0.1	30 ± 0.1
In <i>O</i> -(diacylglycerol-phosphoryl)ethanolamines	3 ± 1	4 ± 1
In <i>O</i> -(acyl-alk-1-enyl-glycerophosphoryl)ethanolamine	6 ± 2	7 ± 2
In the alk-1-enyl chains	10 ± 2	11 ± 2
Blank	26 ± 3	27 ± 2

phospholipid and plasmalogen fraction yield information on the dehydrogenation step in alk-1-enyl ether synthesis under good oxygenation compared with total anoxia. (Control experiments, using a heat-denatured heart, showed that the [$1\text{-}^3\text{H}$]hexadecanol lost 2–3% of its total ^3H , the ^{14}C label of either [$1\text{-}^{14}\text{C}$]palmitate or [$1\text{-}^{14}\text{C}$]hexadecanol did not show exchange or loss.) The results are presented in Table 3. For incorporation into diacyl phospholipids, the hexadecanol must be first oxidized to palmitoyl-CoA. Therefore, about 95–98% of the $1\text{-}^3\text{H}$ should be lost. Loss may not be complete, perhaps owing to the unusual property of some ^3H -labelled compounds of readily exchanging their ^3H for ^1H with other molecules or within the molecule (Chase & Rabinowitz, 1972). The results showed only a 90% loss of ^3H . In the formation of alk-1-enyl chains, the observed values indicate a loss of about 70% of the ^3H label. A 50% loss of ^3H was observed in Ehrlich ascites cells, which was attributed to ^3H of the alkyl group (Wood & Healy, 1970). An appreciable quantity of the doubly labelled hexadecanol may have been first oxidized to palmitic acid and then reduced to [$1\text{-}^{14}\text{C}$]hexadecanol, resulting in the greater than 50% ^3H loss observed in the perfused heart.

All of our results obtained for the rat heart were consistent with a CN^- -sensitive step in the enzymic synthesis of ethanolamine-containing plasmalogens.

This metabolic step, even when feeble (with CN^-), did not appear to differ in mechanism from that in O_2 as judged by the utilization of doubly labelled hexadecanol. The drastic decrease of palmitic acid and hexadecanol incorporation into total lipids and total phospholipids can be attributed to the consequences of anoxia for energy-generating systems in general, whereas the greater effects on plasmalogen synthesis may be the result of the unique requirements of the mixed-function oxidase (Paltauf, 1972; Wykle *et al.*, 1972; Paltauf & Holasek, 1973; Paltauf *et al.*, 1974).

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