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Specific ^{3}H-Labelling by diimide reduction of unsaturated bonds

II. Methods and applications of labelling

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ABSTRACT

Methods for specific labelling of alkyl groups on a mCi/mmole level are described, in which double bonds are hydrogenated with labelled diimide and in which tritiated water is used as the source of the label. In one method, labelled diimide is obtained by exchange labelling and oxidation of hydrazine in acetonitrile and in the other, potassium azodicarboxylate is decomposed with labelled hydrogen ions in a pyridine medium. Molar specific activities of 8 and 20 % of the original water are obtained. The latter method is preferred for reproducible quantitative conversions of unsaturated fatty acids and methyl esters on a 10-100 mg scale.

By partial oxidation with permanganate in acetone, both the specificity of diimide labelling and the usefulness of permanganate degradation for the localization of tritium labels in fatty acids were demonstrated. The possible application of a combination of diimide labelling and oxidative degradation for the determination of the structure of polyunsaturated acids is illustrated for arachidonic acid.

INTRODUCTION

Until now one of the most common methods for the introduction of a hydrogen label in organic molecules is catalytic hydrogenation of double bonds with labelled hydrogen gas. High specific activities can be obtained but appreciable migration and *cis-trans* isomerization of the double bonds occur. The contributions of these complicating reactions are, in general, irreproducible and dependent on the type and method of preparation of the catalyst and on the conditions of the reaction [1].

No migration or *cis-trans* isomerization has been found in reductions with diimide. Moreover, the exclusive reduction of symmetrical double bonds, the absence of discrimination between different double bonds in the absence of interfering steric factors and the stereo-specific *cis*-addition [2-4] are all attractive factors for specific labelling by reduction with diimide. A large number of diimide sources are known [3, 4], but only exchange labelling of hydrazine with tritiated water and decomposition of azodicarboxylate salts with labelled protons are promising for labelling of diimide. The use of potassium azodicarboxylate for labelling has recently also been reported by Paliokas and Schroepfer [5]. It must be emphasized, however, that this type of hydrogenation reactions can be applied without special precautions only for the preparation of compounds with specific activities in the mCi/mmole range.

Experimental

Exchange labelling in small scale reductions with hydrazine.

Principles of the method.

Specific labelling with deuterium by reduction with N_2D_4 in a completely deuterated medium of hydroxylic solvents has been reported before [6-8]. The method is laborious, however, and certainly not applicable for efficient labelling with tritium, in which case highly active reaction products can only be obtained, if no other exchangeable hydrogen atoms are present. We therefore studied the possibility to use aprotic solvents in reductions with hydrazine. The experimental results and their implications for the mechanism of reductions with diimide have been discussed in a separate paper [9].

Acetonitrile was shown to be the best reaction medium and optimal results for methyl oleate were obtained with a slight excess of anhydrous hydrazine on a 1/30 molar scale in 60 ml solvent at 60-80° C in the presence of a catalytic amount of 40 µl anhydrous acetic acid and a continuous stream of oxygen of 60 ml/min.

The labelling procedure is based upon the exchange of hydrogen between hydrazine and small amounts of tritiated water :

$$N_2H_4 \xrightarrow[H^+]{^3H_2O} N_2{}^3H_4 \xrightarrow{O_2} N_2{}^3H_2 + > C = C < \rightarrow N_2 + > \underset{C\text{——}C}{^3H\ \ ^3H} < \quad (1)$$

Under equilibrium conditions and if no isotope effects occur in the reaction steps the theoretical specific activity of stearate from oleate (β) will be related to the specific activity of water (α in mCi/mmole) and the amounts of water (*a* mmole) and hydrazine (*b* mmole) by the formula :

$$\beta = \frac{a}{a + 2b}\alpha \text{ mCi/mmole} \quad (2)$$

The contribution of acetic acid to the total pool of exchangeable hydrogen can be neglected under the experimental conditions.

To obtain optimal specific activities the amount of hydrazine must be small with respect to the amount of tritiated water ($b << a$). In practice, these optimal conditions cannot be attained, however. It will be shown that the yield of the reaction is adversely influenced by the presence of water. On the other hand, a two molar excess of hydrazine had to be taken as a minimum amount for a reasonable conversion of the substrate.

To avoid excessive amounts of radioactive compounds and to make the method applicable to synthetical and analytical problems downscaling of the reaction to amounts of 100 mg and less was investigated.

Method and results.

The reactions were carried out in small test tubes in 1 ml acetonitrile in an atmosphere of oxygen. It was found that the continuous renewal of the gaseous phase was essential for good reaction yields. Satisfactory results were obtained without evaporation of the solvent by leading a continuous stream of O_2-gas over the top of the reaction tubes, which were vigorously vibrated during the reaction in a thermostat at 60° C.

In preliminary experiments up to 1/3 mmole of methyl oleate was treated with varying amounts of tritiated water (specific activity 31.7 μCi/mmole) and hydrazine, which contained 20 μl acetic acid per ml hydrazine. After different reaction times, the samples were transferred into a separatory funnel with a few ml of light petroleum, washed successively with 8% HCl, 10% sodium carbonate and three times with distilled water. The organic layer was made up to 10 ml in a glass-stoppered volumetric flask and dried with a small amount of anhydrous sodium sulfate. Samples of 100 μl were taken for counting in a Tricarb liquid scintillation counter Model 314A and the amounts of stearate were determined by GLC. Quantitative recovery was controlled by internal standardization with known amounts of methyl palmitate.

The reaction yields decrease by reducing the scale of the reaction. Moreover, the rate of the reaction and the final yields of stearate are lowered by the admixture of water (Fig. 1). In other experiments with different amounts of oleate and larger excesses of hydrazine in the presence of 50 μl ^3H_2O it was shown that a 100 % conversion of the oleate is not easily attainable (Table 1).

The calculated specific activities of methyl stearate were found to be only 20-30 % of the values obtained from formula 2, due to an isotope effect in one of the reaction steps. Different amounts of ^3H_2O, N_2H_4 and oleate did not influence the efficiency of ^3H-incorporation. The labelling efficiency is also independent of the degree of conversion of oleate up to conversions of 70 %. These results are all in agreement with the observation [9] that oxidation of hydrazine is the rate-determining step in the reaction. No interpretation

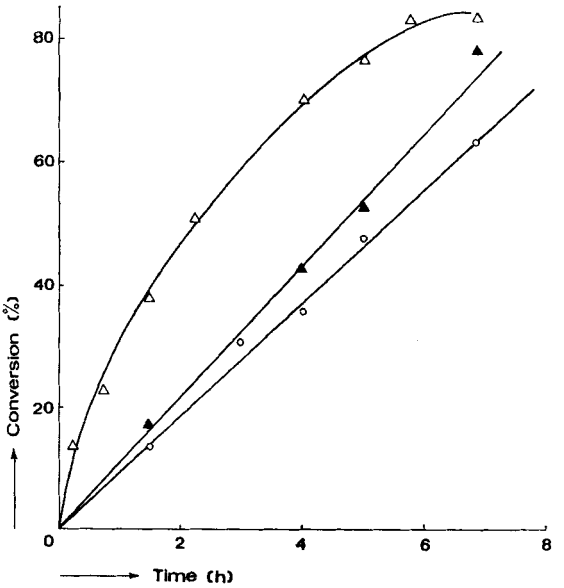

FIG. 1. Influence of water on the reduction at 60° C of 110 μl methyl oleate (0.326 mmole) in 1 ml acetonitrile with 20 μl anhydrous hydrazine (0.63 mmole), containing 20 μl acetic acid per ml.

△ no water
▲ 10 μl water
○ 20 μl water

TABLE 1. Reduction of different amounts of methyl oleate with a large excess of N_2H_4 (containing 20 μl CH_3COOH/ml) in the presence of variable amounts of ^3H_2O (specific activity 31.7 μCi/mmole).

Oleate (mmole)	N_2H_4 (mmole)	H_2O (mmole)	Reaction time (h)	Conversion (%)	Spec. act. in % of theoretical value
0.326	0.315	1.10	3	30.5	27.5
0.326	0.315	1.10	6	69	29.5
0.326	0.63	0.55[5]	3	42.5	31.7
0.326	0.63	0.55[5]	6	78	23.5
0.326	0.63	1.10	3	35.5	31.3
0.326	0.63	1.10	6	63	28.0
0.326	1.57	2.78	3	17	30.0
0.326	3.15	2.78	3	33.5	30.0
0.326	3.15	2.78	6	52	28.2
0.0296	1.57	2.78	3	55	27.2
0.0296	3.15	2.78	3	78.5	20.5
0.0296	3.15	2.78	6	88.5	17.0
0.00296	1.57	2.78	3	61	28.6
0.00296	3.15	2.78	3	83	17.4
0.00296	3.15	2.78	6	89	17.0

can be given for the deviations in the labelling efficiency for conversions of oleate above 70 %.

The difficulty to attain a quantitative conversion of double bonds and the influence of the conversion of the substrate on the specific activity of the product are serious drawbacks in analytical applications. For preparative purposes, however, a modification of the procedure can be applied, in which inactive anhydrous hydrazine is added in subsequent portions until the double bonds are completely saturated. To avoid great variations in the reaction rates it is preferred to use a somewhat larger scale (0.5-1 mmole) in 10-20 ml acetonitrile, through which oxygen gas is passed during the reaction at 60-80° C.

In a representative experiment, 0.67 mmole methyl oleate (200 mg) was converted with 3.15 mmole anhydrous hydrazine in the presence of 2.78 mmole ^3H_2O (specific activity 2.63 mCi/mmole) in 20 ml refluxing acetonitrile. At this level the reaction has to be carried out in a fume cupboard. In 4 h, a conversion of 80 % was achieved. By reaction with a second portion of 3.15 mmole hydrazine, the yield of stearate increased to ca. 95 %. The overall labelling efficiency was ca. 8 %.

Diimide reductions with potassium azodicarboxylate.

Principles of the method.

The adverse influence of small amounts of water on the diimide reduction with labelled hydrazine, the drop in the specific activity by exchange labelling and the loss of label by the intermediate oxidation of hydrazine to diimide prompted us to look for another source of diimide. Decomposition of potassium azodicarboxylate with labelled protons offers an attractive alternative :

$$KOOC{-}N{=}N{-}COOK + 2\ {}^3H^+ \rightarrow 2\ CO_2 + N_2\ {}^3H_2 + 2\ K^+ \quad (3)$$

One may also expect a better reproducibility in microscale reductions, since the influence of inefficient oxygen supply is avoided.

Diimide reductions with potassium azodicarboxylate have been described by a number of authors [10-14]. In all cases, the reactions were carried out at room temperature using a number of protic and aprotic media. Most favourable results were obtained by Hamersma and Snyder [14] in anhydrous pyridine and it was shown that in the case of aprotic solvents, water had to be avoided. The presence of oxygen was not found to be deleterious [14], but in the present experiments the reaction mixture was flushed with nitrogen to avoid complications. A comparison was made between pyridine and acetonitrile, since the latter had been successful in hydrazine reductions. Furthermore, the temperature of the reaction medium was found to influence the labelling efficiency.

In a recent article Paliokas and Schroepfer [5] also applied the decomposition of azodicarboxylate with CH_3COO^3H, but no experimental details are given.

Method and results.

Potassium azodicarboxylate was prepared according to the procedure described by Thiele and King [15]. The salt was stored at 0° C in a closed container under nitrogen and it was found that fresh preparations had to be made regularly for optimal results.

For the reduction of samples of methyl oleate, a solution of the ester in 2-4 ml solvent was stirred magnetically with an appropriate amount of azodicarboxylate in a two-necked tube with a flat bottom, which was provided with a reflux condenser and a dropping funnel. The reaction was controlled by adding an equivalent amount of anhydrous acetic acid in 4-6 ml solvent as slowly as possible. For labelling purposes, anhydrous acetic acid was exchanged in a solvent with a known amount of tritiated water and dried overnight on anhydrous sodium sulfate. No isotope effect occurs during this exchange procedure.

It was found that the best results were obtained by waiting until nitrogen evolution stopped before a new drop of the acid solution was added. This is in agreement with the consideration that the bimolecular disproportionation reaction will be suppressed by low concentrations of diimide :

$$\textit{reduction reaction}: N_2H_2 + >C=C< \rightarrow N_2 + \overset{H}{>C}\!\!-\!\!-\overset{H}{C}< \qquad (4)$$

$$\textit{disproportionation reaction}: 2\,N_2H_2 \rightarrow N_2 + N_2H_4 \qquad (5)$$

With freshly prepared azodicarboxylate, the theoretical amount of acid was needed for complete conversion of the reagent.

At the end of the reaction, the mixture was diluted with light petroleum and washed with diluted HCl, Na_2CO_3-solution and three times with distilled water and dried over Na_2SO_4. The conversion was determined by GLC and known aliquots were counted with a Tricarb liquid scintillation counter.

Representative results for acetonitrile and pyridine solutions at different temperatures are given in Table 2.

The reaction was markedly accelerated by increasing the temperature without any significant decrease in the yield. At 80-120° C, the reaction with methyl oleate proceeds at least 20 times as fast as at room temperature and the reaction time is completely determined by the rate at which acetic acid is added. Above 100° C, a decrease in the reaction yield was observed, probably due to decomposition of the azodicarboxylate [15].

The yield of tritium incorporation is increased from ca. 5 % at 20° C to ca. 20 % at 100° C. No significant differences in the results were obtained by using pyridine instead of acetonitrile as solvent, but as a temperature of 100° C is most favourable for labelling purposes, pyridine was chosen as the most attractive medium. Under these conditions, the tritium label could be simply introduced by simultaneous addition of an equivalent amount of tri-

tiated water and a slight excess of pure acetic anhydride (freshly distilled over P_2O_5) in pyridine from the dropping funnel to the azodicarboxylate in the reaction mixture.

When quantitative conversion of the unsaturated substrate is desired, the best results are obtained by adding successive amounts of azodicarboxylate to the reaction mixture and subsequent reaction by slow addition of equivalent

TABLE 2. Reduction of methyl oleate (1/3 mmole) with potassium azodicarboxylate (2/3 mmole).

Medium	T (°C)	Reaction time (h)	Conversion (%)	Spec. act. H_2O (μCi/mmole)	Spec. act. stearate (μCi/mmole)	^3H-incorporation (% of theory)[a]
Acetonitrile	20	40	45.5	25.0	0.9	5 (1)
Acetonitrile	80	3	45	25.0	1.9	10.4 (1)
Pyridine	20	50	46.5	25.0	1.6	8.8 (1)
Pyridine	80	2.5	44.7	25.0	3.4	13.4 (2)
Pyridine	102	2-3	50 ± 2.5	25.0	5.0 ± 0.5	20 ± 2 (2)
Pyridine	120	2-3	32	25.0	4.5	18 (2)

[a] In experiments (1) acetic acid was labelled by exchange with ^3H_2O, in experiments (2) by hydrolysis of acetic anhydride (mixture of 12 μl ^3H_2O and 80 μl acetic anhydride in 4-6 ml pyridine per experiment).

TABLE 3. Quantitative conversion of methyl oleate and oleic acid by repeated addition of azodicarboxylate.

Substrate	Azodicarboxylate (mmoles)	Conversion (%)	^3H-incorporation (% of theory)
Methyl oleate (1/3 mmole)	2/3	61.8	19.5
	+2/3	90.0	19.7
	+2/3	98-100	18.2
Oleic acid (1/3 mmole)	2/3	24.4	29.2[a]
	+2/3	73.3	19.2
	+2/3	83	15.7
	+2/3	96	14.4
	+2/3	99	14.9
Oleic acid (1/30 mmole)	2/3	76	
	+2/3	98	
	+2/3	100	35

[a] Figures are corrected for exchange with carboxyl hydrogen of oleic acid.

amounts of tritiated water and acetic anhydride. An example is given in Table 3. Instead of methyl esters, acids can be hydrogenated although the specific activity of the final product will be lowered by exchange of the carboxyl hydrogen. Oleic acid itself is not able to decompose the azodicarboxylate at an appreciable rate and labelled acetic acid has to be added for high yields of hydrogenation. Downscaling of the reaction to amounts of 9 mg (1/30 mmole) has also been investigated (Table 3).

For preparative purposes, tritiated water with a specific activity of 2.63 mCi/mmole was used and the reaction was carried out in a fume cupboard.

Specificity of labelling by diimide

The specific activity of carbon atoms in the alkyl chain of carboxylic acids can be determined in one step by incomplete oxidation with potassium permanganate in acetone [16] according to the procedure described by Murray [17] for the determination of the site of the substituent in branched fatty acids.

In this procedure, part of the original acid is oxidized to acids with shorter chain length and the specific activity at the different positions can be deduced from the differences in the specific activities of the components. Since specific labelling with diimide has been reported before [6-8] it seemed worthwhile to check at one time this specificity for tritium labelling and the applicability of the degradation method for the determination of a tritium label.

For this purpose, 1/3 mmole of methyl oleate was hydrogenated with three successive portions of 2/3 mmole potassium azodicarboxylate by dropwise addition of a mixture of 80 µl pure acetic anhydride and 12 µl ^3H_2O (spec. act. 2.63 mCi/mmole) in dry pyridine. The ester was freed from solvent and saponified with alcoholic KOH (400 mg KOH in a mixture of alcohol and water (9 : 1) by standing overnight at room temperature. After addition of water the reaction mixture was extracted with light petroleum to remove unsaponified product. After acidification with 1 N HCl, the aqueous layer was extracted with ether, the ethereal layer washed with distilled water and dried over Na_2SO_4.

After evaporation of the solvent 85.4 mg stearic acid-^{3}H was obtained with a specific activity of 0.547 mCi/mmole (20.7 % of theoretical value). For the degradation reaction, 10.2 mg of the acid was dissolved in 5 ml dry acetone, followed by addition of 0.5 g pulverized $KMnO_4$. The mixture was refluxed until all permanganate had been consumed (ca. 24 h). After evaporation of the acetone, the residue was treated with 10 ml 10 % aqueous H_2SO_4 and a small excess of $NaHSO_3$ was added until the solution was colourless. Subsequently, the fatty acids were extracted with light petroleum (b. p. 40-60° C) esterified with diazomethane and analysed with a radiogaschromatograph. For this purpose, an F & M Model 700 gaschromatograph with thermal conductivity detectors was combined with a Nuclear Chicago high temper-

ature proportional internal flowcounter Model 4998. Helium was used as the carrier gas and methane was admixed before entering the counter (CH_4 : He = 2 : 1). The fatty acid methyl esters were separated on a column of 10 % PEGA on silanized Chromosorb W; temperature programming from 80 to 210° C was applied.

The specific activity of the methyl esters was determined by area integration. The relative response of the thermal conductivity detector was obtained from a calibration curve. The specific activity per carbon atom can be calculated by subtraction. In complex cases, the best results were obtained by averaging over at least three chromatographic runs. In Tables 4 and 5 the specific activities of the degraded acids with respect to the original acid and the calculated distribution of the ^{3}H-activity over the carbon chain are given for the product of the reduction of methyl oleate with labelled diimide and for a commercially available ^{3}H-stearic acid (ex Radiochemical Centre, Amersham; specific activity 332 mCi/mmole), which was nominally labelled at the 9,10-positions and which was prepared by catalytic hydrogenation of elaidic acid with tritium gas.

The results for the product from the diimide reduction are in perfect agreement with specific labelling at the 9,10-positions, indicating that permanganate degradation will also be a valuable method for the localization of tritium label in a fatty acid chain. In the same way it was found that in the

TABLE 4. Specific activities of degradation products in % of value for stearic acid.

Chain length of degraded acids	Theoretical spec. act. for 9,10-^{3}-stearic acid	Stearic acid from diimide reduction	Stearic acid ex. Amersham
18	100	100	100
17	100	98	100.3 ± 4.1[a]
16	100	99	95.3 ± 3.0
15	100	100	98.6 ± 4.5
14	100	99	97.0 ± 3.3
13	100	101	96.3 ± 1.1
12	100	96	96.0 ± 1.6
11	100	97.5	91.0 ± 1.4
10	50	48.5	70.3 ± 1.5
9	0	0	46.1 ± 0.5
8			35.0 ± 0.4
7			25.8 ± 1.7
6			19.8 ± 0.9
5			10 ± 1.2

[a] Average values with standard deviations of the mean were obtained from three chromatographic runs.

TABLE 5. Distribution of radioactivity in alkyl chain of labelled stearic acids.

Position of ^{3}H-label	Theoretical values for 9.10-^{3}H-stearic acid	Stearic acid from diimide reduction	Stearic acid ex. Amersham
1	—	—	—
2	0	+2	—0.3
3	0	−1	+5.0
4	0	−1	−3.3
5	0	+1	+1.6
6	0	−2	+0.7
7	0	+5	+0.3
8	0	−2	+5.0
9	50	+49	+20.7
10	50	+48.5	+24.2
11	0	0	+11.1
12	0		+9.2
13	0		+6.0
14	0		+9.8
15-18	0		+10.0

commercial sample, which had a much higher specific activity, only 45 % of the total activity is present at the 9,10-positions. The other half of the activity is distributed all over the alkyl chain, particularly towards the methyl group.

DETERMINATION OF THE STRUCTURE OF POLYUNSATURATED ACIDS

The results in the preceding section suggest that labelling with diimide in combination with permanganate oxidation may be used for the determination of the structure of polyunsaturated acids. As an example 27.9 mg (0.0875 mmole) of the methyl ester of 5,8,11,14-eicosatetraenoic acid (methyl arachidonate) was reduced with four portions of 130 mg potassium azodicarboxylate (2/3 mmole) in pyridine at 100° C by dropwise addition of 3 ml portions of pyridine containing 80 µl acetic anhydride and 12 µl ^3H_2O (spec. act. 2.63 mCi/mmole).

In this way, an almost complete conversion to labelled arachidic acid was achieved with a labelling efficiency of 16.8%. The arachidic ester (spec. act. 1.76 mCi/mmole) was purified by TLC (silicagel G-20% $AgNO_3$; eluant benzene-ether, 93/7 v/v).

26.3 mg pure methyl arachidate was saponified, the arachidic acid was recovered and oxidized with 1.5 g $KMnO_4$ in 15 ml refluxing acetone as described above. An amount of 14.6 mg of a mixture of acids was obtained, which was esterified with diazomethane and analysed by radiogaschromatography as before. The average results of four chromatograms are given in Table 6.

TABLE 6. Specific activities (%) of degraded acids from arachidic acid.

Chain length of degraded acids	Theor. value for arachidic acid from arachidonic acid	Expt. value
6	0	0
7	12.5	16.7 ± 1.7
8	25	29.4 ± 3.3
9	25	24.4 ± 2.1
10	37.5	38.2 ± 2.2
11	50	54.2 ± 5.2
12	50	46.8 ± 3.9
13	62.5	62.6 ± 2.1
14	75	72.9 ± 1.4
15	75	68.4 ± 1.1
16	87.5	88.3 ± 1.9
17	100	110.3 ± 4.1[a]
18	100	94.1 ± 2.0[a]
19	100	95.9 ± 2.0[a]
20	100	100.7 ± 1.7[a]

[a] Average value from the C^{17}-C^{20}-acids was taken as the 100 % activity standard.

From these results the distribution of the ^3H-label in arachidic acid can be calculated :

C-C-C-C-C—C—C—C—C—C—C—C—C—C—C—C—C—C—C—C—COOH
0 0 0 0 0 16.7 12.7 5.0 13.8 16.0 7.4 15.8 10.3 4.5 19.9 22.0 16.2 1.8 4.8

These results indicate that, even in the case of a tetraenoic acid, a reasonable impression of the location of the double bonds is obtained.

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