A Simple Procedure for Preparation and Assay of Small Quantities of ^{14}C-Labeled Long-Chain Aliphatic Aldehyde

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SUMMARY

(1-^{14}C)-dodecanal was synthesized from (1-^{14}C)-dodecanoic acid. The two-step synthesis beginning with 0.9 mg of the acid resulted in an overall yield of 42 % after purification. The final product was separated from the starting material and side products by silicic acid column chromatography. Purity was verified by thin layer chromatography and stimulation of light emission catalysed by bacterial luciferase.

1. INTRODUCTION.

Long-chain aliphatic aldehydes stimulate bacterial luminescence *in vitro* [1] and *in vivo* [2]. Whether they participate in the light-emitting reaction as catalysts or as reactants that eventually get consumed is not known. The difference—if there is indeed any—in the amount of aldehyde before and after a reaction *in vitro* is far below the limits of chemical detection. To determine the fate of the aldehyde molecule and thus elucidate its role in light emission, it was imperative that aldehyde of a high specific radioactivity be prepared. Accordingly, a method was devised for synthesis of ^{14}C-dodecanal from less than 1 mg of commercially available (1-^{14}C)-dodecanoic acid. Methods for purification and assay of small quantities of ^{14}C-aldehyde were also devised.

2. MATERIALS AND METHODS.

2.1 *Synthesis.*

The procedure for synthesis involved two steps :

(1) $CH_3(CH_2)_{10}{}^{14}COOH + SOCl_2 \longrightarrow CH_3(CH_2)_{10}{}^{14}COCl + SO_2 + HCl$

(2) $CH_3(CH_2)_{10}{}^{14}COCl + H_2 \xrightarrow[Pd]{} CH_3(CH_2)_{10}{}^{14}CHO + HCl$

The reactions were made to take place one after the other in the same vessel. The synthesis of dodecanal by the above procedure (synthesis of the acyl halide followed by Rosenmund reduction) has not been reported in the literature. For the synthesis of the acid chloride, the method of Martin and Fieser [3] served as model. The Rosenmund reduction of acid chlorides to aldehydes has been reviewed by Mossetig and Mozingo [4].

The apparatus was assembled from a Bantam-ware semi-micro kit from Kontes Glass Co. (Vineland, N. J.). To a 25-ml three-neck pear-shaped flask was attached a reflux condenser at the center hole, a thermometer, and a Pasteur pipette through which a stream of gas from a cylinder could be allowed in. The course of the reactions was followed by collecting and titrating the HCl evolved from the top of the condenser.

A drop of $SOCl_2$ was added to 0.9 mg of radioactive dodecanoic acid (22 curies.mole^{-1}, Calbiochem, Los Angeles, Calif.) in the reaction flask. The mixture was refluxed for 30 min at 50-60° C by intermittent application of heat from a Bunsen burner. Unreacted $SOCl_2$ was driven off with N_2 at the end of the reaction. 5 mg of palladium asbestos (5 %, Fisher Scientific Co.), and 0.3 ml xylene were added, and the reaction flask was flushed with H_2.

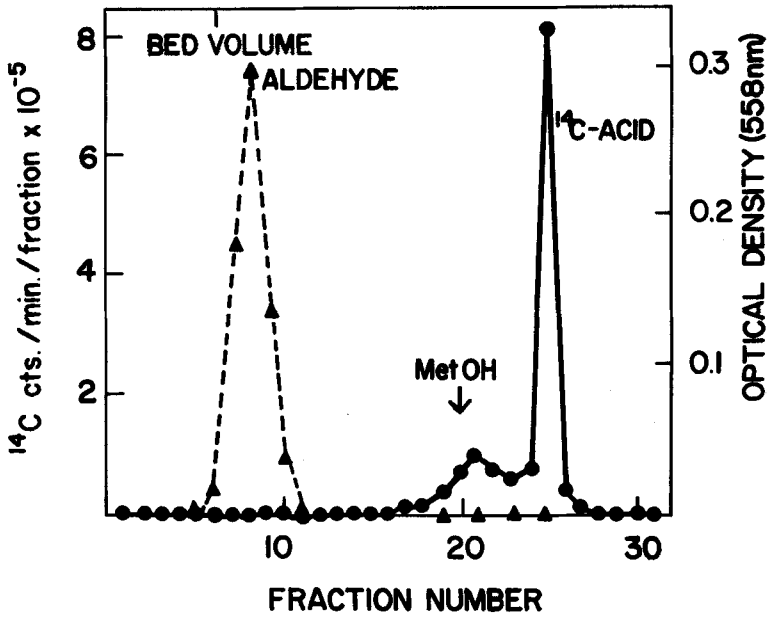

FIG. 1. Separation of dodecanal from ^{14}C-dodecanoic acid by silicic acid column chromatography. 0.25 mg dodecanal was mixed with 14 μg ^{14}C-dodecanoic acid (2.9 μc) in 1 ml benzene and applied to the column (bed volume = 30 ml). Elution with 100 ml benzene was followed with 100 ml methanol. 5 ml fractions were collected and assayed : (▲). Schiff test; and (●) radioactivity.

After oxygen was driven off, the temperature of the mixture was raised by means of an electric heating mantle and maintained at 142-145° C for 1 hr.

After the mixture had cooled to room temperature, the inside of the reflux condenser was washed with diethyl ether. The ether was allowed to evaporate, and the reaction products were solubilized in benzene and transferred onto a column of silicic acid for chromatography.

2.2 *Purification.*

The column was prepared in the following manner. A 30-ml slurry of silicic acid (Bio-sil-ha, minus 325 mesh, Bio-Rad Laboratories, Richmond, Calif.) in benzene was packed forming a 2 × 10 cm column in a cylindrically-shaped separatory funnel.

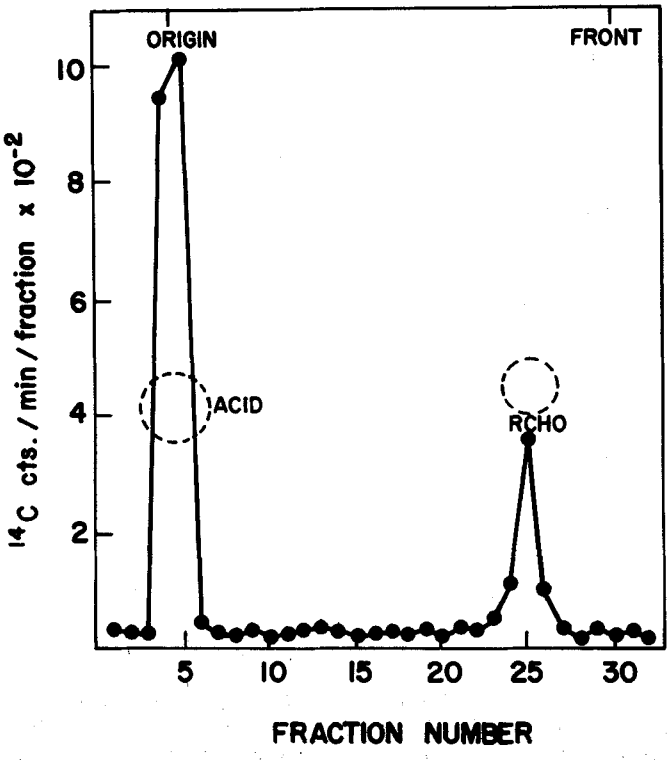

FIG. 2. Separation of dodecanal from dodecanoic acid by thin layer chromatography. A mixture of ^{14}C-dodecanoic acid and ^{14}C-dodecanal (produced and purified as shown in Results) were spotted together and developed in benzene, and radioactivity was determined on cut strips of the chromatogram (●-●). The positions of dodecanal (RCHO) and dodecanoic acid (ACID) (10 μg each) were determined after development of the chromatogram by spray reagents.

Samples were eluted from the column at the rate of 45 ml hr^{-1} with benzene at room temperature. 5 ml fractions were collected, and an aliquot of each fraction was added to a vial of scintillation fluid (4 g PPO* and 0.5 g POPOP per liter of reagent grade toluene) for radioactivity measurements by means of a liquid scintillation spectrometer (model 6804, Nuclear-Chicago, Chicago, Ill.). In Figure 1 is shown the separation of unlabelled dodecanal from the starting material, ^{14}C-dodecanoic acid. To an air-dried aliquot of

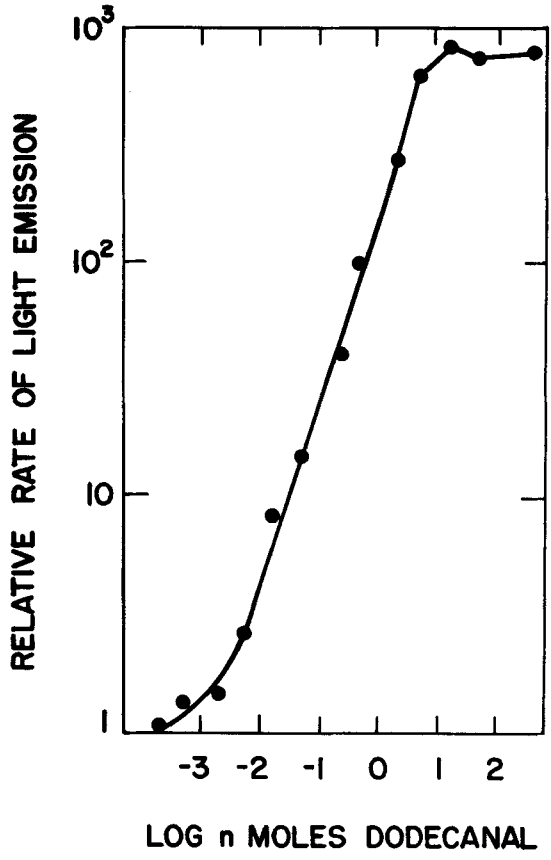

FIG. 3. Response of bacterial luminescence to dodecanal. The aldehyde was added to the reaction mixtures as an ethanolic solution (0.1 ml). The relative rate of luminescence is the measured peak of luminescence with added aldehyde compared to light emission in the absence of aldehyde.

* Abbreviations : PPO is 2,5-diphenyloxazole and POPOP is *p-bis*[2-(5-phenyl-oxazolyl)]-benzene, both obtained from New England Nuclear Corp., Boston, Mass.

FMN is flavin mononucleotide.

each fraction was added 1 ml commercial Schiff reagent (Fisher Scientific Co.) for the purpose of detecting aldehyde. After two hours the purple color was measured as optical density at 558 nm in a Beckman DU spectrophotometer (0.1 O.D. unit = 0.15 μmole dodecanal).

2.3 *Assays.*

Samples for thin layer chromatography were spotted by means of 10-μl micropipettes on Eastman Chromagram silica gel sheets (Type K301R2, Distillation Products Industries, Rochester, N. Y.). After the spots had

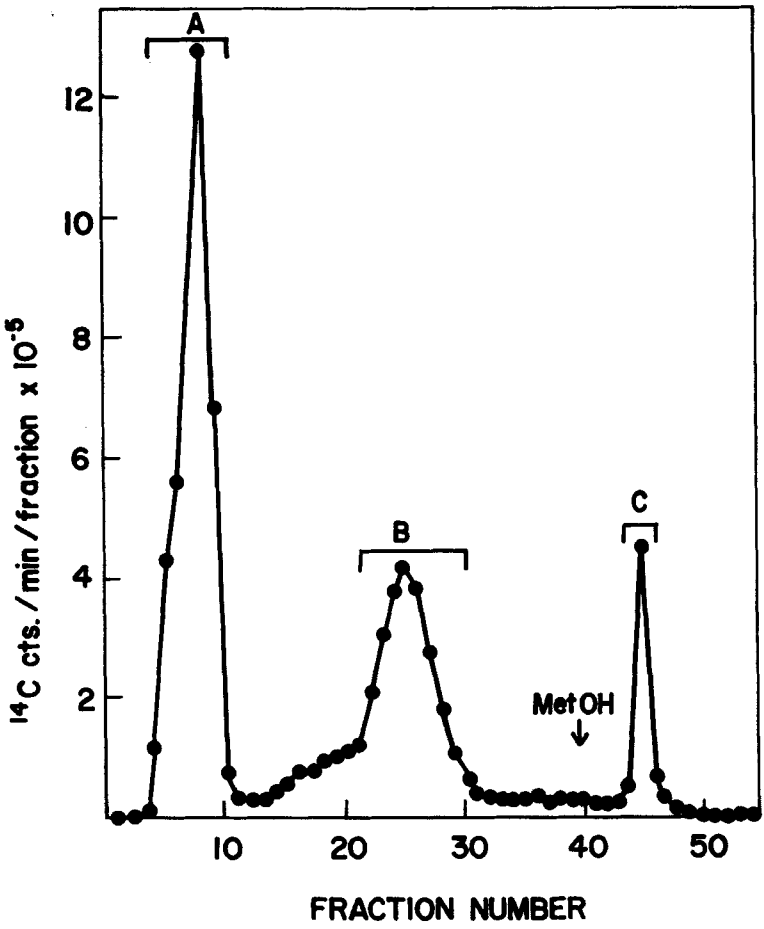

FIG. 4. Separation of the products of synthesis of ^{14}C-dodecanal from ^{14}C-dodecanoic acid by silicic acid column chromatography. Elution with benzene was followed with methanol after 40 fractions of 5 ml each had been collected.

air-dried, the sheets were developed in benzene for 90 min at room temperature in an Eastman Chromagram plate set.

After development, the chromatogram sheet was cut into 1/4 × 1 inch strips. The strips were then transferred to counting vials containing 10 ml scintillation fluid for radioactivity measurements. Figure 2 shows the separation of dodecanoic acid and dodecanal by thin layer chromatography. Unlabeled aldehyde standard was located on the chromatogram by spraying with the Schiff reagent (dodecanal appeared blue on a red background) or with 0.4 % 2,4-dinitrophenylhydrazine in 2N HCl (aldehyde appeared yellow on a white background). The unlabeled acid standard was located by spraying the sheet lightly with water. Because of its insolubility, the acid appeared white on a grey background.

Biological activity of the synthesized aldehyde was assayed using purified luciferase prepared according to the method of Kuwabara *et al.* [5] from *Photobacterium fischeri* mutant DA. This mutant barely lumineseces except in the presence of exogenous long-chain aliphatic aldehyde [2]. *In vitro* luminescence catalysed by the DA luciferase may be stimulated by dodecanal up to 1000-fold. The reaction mixture consisted of the following in a total volume of 3 ml : sodium-potassium phosphate buffer, pH 6.8, 50 μmoles; 2-mercaptoethanol, 10 μmoles; crystalline bovine serum albumin, 0.5 mg; dodecanal or unknown aldehyde dissolved in ethanol; 0.01 to 100 nmoles; purified luciferase, 5-20 μg; and $FMNH_2$, 40 nmoles. The $FMNH_2$ was freshly prepared

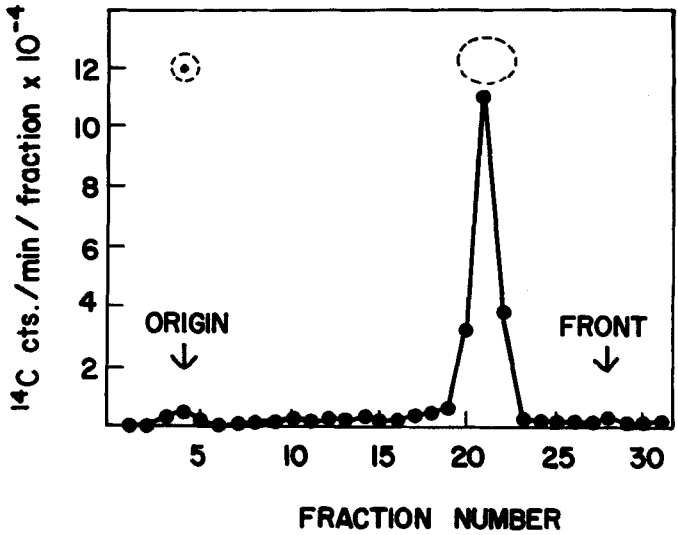

FIG. 5. Thin layer chromatogram of purified ^{14}C-dodecanal synthesised from ^{14}C dodecanoic acid. The sample was derived from fraction 8 of the silicic acid column shown in Figure 4. The spot corresponds to the position of unlabeled dodecanal chromatographed alongside the radioactive sample and identified by Schiff reagent.

by catalytic H_2 reduction of FMN. The reaction mixtures without $FMNH_2$ were prepared in glass cuvettes (1.0 cm light path) and positioned in the chamber of a Beckman DU spectrophotometer with a photomultiplier attachment. The reaction was started by injecting 0.2 ml of the reduced FMN into the cuvette by means of a tuberculin syringe.

The burst of light was recorded on a 5 in. strip chart of a potentiometric recorder moving at 1 in./min. The peak height was taken as the reaction velocity or rate of light emission in response to a given amount of aldehyde. Figure 3 shows that less than 0.1 nmole of dodecanal can be detected by this method.

3. Results and Discussion.

Figure 4 shows the radioactivity profile of the reaction products eluted from the silicic acid column. Samples of the different column fractions from Peaks A, B, and C (Fig. 4) were spotted on thin layer chromatograms. The radioactivity from peak A chromatographed with dodecanal, whereas peaks B and C contained largely radioactive acid together with a small amount of an unidentified product. In Figure 5 is shown that a high degree of radiochemical purity was achieved in fraction 8. This fraction was concentrated by evaporating off benzene, and the resultant crystals were then dissolved in ethanol. The remaining fractions in peak A were chromatographed on thin layer sheets and the aldehyde was eluted with ethanol. The final product was stored as ethanolic solutions at -20° C and appeared to be stable for at least 6 months.

Table 1. Synthesis and separation of ^{14}C-dodecanal from ^{14}C-dodecanoic acid.

	Starting acid	Fractions 5-10	Fractions 11-55
Total radioactivity (cts./min)	1.2×10^8	5.6×10^7	6.6×10^7
μmoles calculated from radioactivity	4.5	2.1	2.5
Per cent starting acid before T.L.C.	100	46	55
after T.L.C.	—	42	—
μmoles dodecanal; Schiff reagent assay	0	1.8	0
μmoles dodecanal; bioluminescence assay	0	1.9	0
Reaction with 2,4-dinitrophenyl-hydrazine	—	+	—

A reaction mixture resolved on a silicic acid column (Fig. 4) was analyzed. After T.L.C. purification only fractions 5-10 contained aldehyde with a final yield of 42 %.

The chromatographic data indicated that indeed ^{14}C-dodecanal was synthesized from ^{14}C-dodecanoic acid. The final product gave positive colour reactions with 2,4-dinitrophenyl-hydrazine and the Schiff reagent (Table 1). But more important, it was biologically active as measured by its ability to stimulate luminescence catalysed by bacterial luciferase *in vitro*. Furthermore, it had the characteristic odour of dodecanal. The observed over-all yield after purification of the final product was 42 %.

The preparation of dodecanal and decanal from their corresponding acids by catalytic reduction under pressure by formic acid [6] was attempted a number of times but failed to yield any detectable amount of aldehyde. In contrast, our rapid and simple method of synthesis followed by a one-step purification yielded a fraction containing less than 0.5 % radiotracer contaminants in the form of acid. Since the corresponding alcohol would have been located in between the acid and the aldehyde, the absence of a radioactive peak at such a position on the chromatograms indicates that no significant reduction from the aldehyde to the alcohol took place.

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