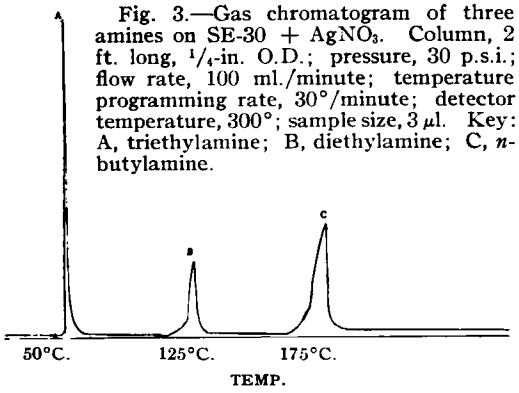

Fig. 3.—Gas chromatogram of three amines on SE-30 + $AgNO_3$. Column, 2 ft. long, 1/4-in. O.D.; pressure, 30 p.s.i.; flow rate, 100 ml./minute; temperature programming rate, 30°/minute; detector temperature, 300°; sample size, 3 μl. Key: A, triethylamine; B, diethylamine; C, *n*-butylamine.

DISCUSSION

It is evident from the chromatograms that the three amines pass through the various types of chromatographic columns at substantially different rates which are not governed by their boiling points. Silver nitrate seems to exhibit dramatic selectivity for these amines. It is difficult to state the exact nature of forces involved in the separation. However, in view of the fact that silver ion is capable of forming chelates, the strong retention behavior observed is indicative of chelate formation between the metals and the amines. It is of interest that a significant rise in temperature had to be employed to elute the 2° and 1° amines. This is consistent with the tendency for coordination increases with decreasing substitution $RNH_2 > R_2NH > R_3N$. As further evidence supporting complex formation, we observed that ethylenediamine, which theoretically is capable of forming a stronger chelate with silver, is so strongly retained that it could not be eluted even at 200°. Elution at higher temperatures was not attempted because of possible thermal decomposition of the silver nitrate. However, the use of silver bromide in place of the silver nitrate did permit use of higher temperature. In this silver bromide column ethylenediamine did emerge as a peak at a temperature of 250°.

It is of interest that the column length for this study was only 2 ft. Increasing the column length was not advantageous, and shortening it did not give good results. The method appears to be highly promising for separation of related compounds. Further studies are in progress; a complete report will be published at a later date.

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Synthesis of 5,5-Dimethyl-2,4-oxazolidinedione-2-C^{14} (DMO-2-C^{14})

By THOMAS C. BUTLER and JACK D. DAVIDSON

DMO-2-C^{14} was synthesized for use in measurements of the pH of intracellular water. It was prepared by condensing urea-C^{14} with n-butyl 2-methyllactate in the presence of sodium butylate in butanol solution. The yield of DMO-2-C^{14} from 1 mmole of urea-C^{14} was 58 per cent, and the product was radiochemically homogeneous as demonstrated by paper chromatography. The ionization exponent was determined by a system of solvent partitioning. A method is described for determination of DMO-2-C^{14} in biological samples.

THE MEASUREMENT of the pH of the water inside living cells is a difficult problem that has been approached in many ways (1). Of the various methods used, that based on the measurement of the intracellular and extracellular concentrations of a weak organic acid or base is probably most widely applicable and least subject to theoretical objections. Until recently, nearly all of the work based on this principle has employed carbon dioxide as the indicator compound. In 1959, Waddell and Butler (2) suggested that the weak acid, 5,5-dimethyl-2,4-oxazolidinedione (DMO), has the attributes desirable in a compound for the measurement of intracellular pH and should have advantages over carbon dioxide for that purpose. Using ultraviolet spectrophotometric methods for the analytical determinations of DMO in plasma and tissue, they employed the distribution of DMO for the calculation of the intracellular pH of dog muscle. Subsequently, DMO has been used by other workers in a number of different investigations of intracellular pH.

For an *in vitro* study of the intracellular pH of tumor cells, it became evident that the relatively large amount of DMO required in a sample for spectrophotometric measurement would impose undesirable restrictions on the design and scope of the experiments. A more sensitive method of measurement, as would be provided by the use of radioactive DMO, would avoid these restrictions. The synthesis of DMO-2-C^{14} was accordingly carried out. In the course of the subsequent study of tumor cells, experience with the use of DMO-2-C^{14} (in conjunction with inulin-carboxyl-C^{14} for measurement of extracellular water) has confirmed the expected advantages. Very small samples suffice, and the concentration of DMO added can be made low enough to cause no significant derangement of cellular function. Measurements of radioactivity

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can be performed with greater rapidity and probably with greater accuracy than can the corresponding spectrophotometric analyses.

EXPERIMENTAL

DMO was synthesized by a modification of the method of Stoughton (3). Urea-C^{14} was condensed with an ester of 2-methyllactic acid in the presence of sodium butylate with the production of DMO and ammonia.

The *n*-butanol was dried over anhydrous potassium carbonate. The urea was desiccated. All glassware used prior to the addition of water was oven dried shortly before use.

A lump of clean sodium weighing somewhat more than 500 mg. was dissolved in 20 ml. of *n*-butanol. After titration, the solution was adjusted to contain 1 mmole of sodium per ml. In a 15 × 150-mm. glass-stoppered test tube 60 mg. (1 mmole) of urea-C^{14} of specific activity 1 mc./mmole was dissolved in 1.5 ml. of *n*-butanol with the use of heat. To this solution were added 1.0 ml. of the sodium butylate solution (1 mmole) and 0.17 ml. of *n*-butyl 2-methyllactate (1 mmole). The loosely stoppered tube was heated in an oven at 100° for 1 hour. It was then cooled in an ice bath, and 2.5 ml. of water was added. The tube was shaken and the butanol layer was transferred by pipet to another glass-stoppered tube, in which it was shaken with 2.5 ml. of 1 *N* sodium hydroxide. The butanol was discarded. The two aqueous extracts were combined in one tube and washed with two 5-ml. portions of ethyl ether, which were discarded. The aqueous solution was acidified with 1 ml. of concentrated hydrochloric acid and washed with 5 ml. of 2,2,4-trimethylpentane, which was discarded. The aqueous solution was then extracted with three 5-ml. portions of ethyl acetate, which were combined in a glass-stoppered tube. The ethyl acetate was washed with 2 ml. of water, which was discarded. It was then evaporated in the tube by drawing over a stream of air with an impinger (4). The residue, which at first was a colorless liquid, gradually became an almost completely crystalline white mass. After desiccation, the crude product consisted of 80% DMO by weight. It contained no radioactive constituent other than DMO and no nonradioactive impurity that interferes with its use. Further purification, with attendant losses, has accordingly not been carried out. The product has been kept in methanol solution, and portions of this solution have been evaporated for preparation of aqueous solutions for use in the biological experiments.

Radiochemical homogeneity was demonstrated by paper chromatography. A small volume of the methanol solution was spotted on Whatman No. 2 paper. A descending system was used in which 1,2-dichloroethane was the moving phase and water the stationary phase. The chromatogram was scanned with a Scanogram II automatic windowless paper chromatogram scanner with associated ratemeter and recorder. No radioactivity could be detected outside of a single spot with R_f 0.63. In one chromatogram 20 mcg. of authentic unlabeled DMO was spotted on a track adjacent to DMO-2-C^{14}. The nonradioactive track was cut into strips 2.5 cm. long, which were eluted with a buffer of pH 9 for study of ultraviolet absorption. The ultraviolet absorbing material was located at a position on the chromatogram identical to that of the radioactivity. DMO-2-C^{14} that has been in methanol solution for 3 years has been shown by paper chromatography still to be radiochemically homogeneous.

An aliquot of 1/100 of the total methanol solution was evaporated for preparation of an aqueous solution that was used to provide still smaller fractions of the total for measurement of the yield in terms of ultraviolet absorbing material and in terms of radioactivity. A buffer of pH 9 containing 1/10,000 of the total yield per ml. had an ultraviolet absorption spectrum identical to that of the ionic species of authentic DMO, with an absorption maximum at 208 mμ. From comparison with standards this solution was calculated to have a concentration of 7.5 mcg./ml. The total yield is thus calculated to be 75 mg. (58%). The radioactivity of a sample of 1/10,000 of the total yield was measured by liquid scintillation counting in comparison with a benzoic acid-C^{14} standard. The activity of the sample was 0.054 μc. The total C^{14} yield would be 540 μc., corresponding to a specific activity of 0.93 mc./mmole. Owing to uncertainties in the accuracy of the C^{14} assays, the spectrophotometric estimate of yield is the more accurate, but the agreement between ultraviolet absorption and radioactivity measurements is within the expected experimental error.

The ionization exponent of DMO-2-C^{14} was determined by partitioning a measured amount of the compound between 50% ethyl acetate–50% toluene (v/v) and several buffers of different pH values and of ionic strength of 0.16. The partitioning was carried out at 37°. Radioactivity in the upper phase was measured by liquid scintillation counting by the procedure described below for the determination of DMO-2-C^{14}. The partition coefficients were calculated from these measurements. The value of pK′ was calculated from the theoretical relationship between partition coefficient and pH of the aqueous phase (5). The value so calculated did not differ by as much as the experimental error from that of 6.13 as previously measured spectrophotometrically for the unlabeled compound (2). The value of 6.13 has accordingly been used in the calculation of intracellular pH.

The following method has been found suitable for the determination of DMO-2-C^{14} in biological materials. About 100 mg. of a liquid sample or of a preparation of homogenized cells is weighed in a tared glass-stoppered test tube. One milliliter of 5 *M* monobasic sodium phosphate plus 5 ml. of 50% ethyl acetate–50% toluene (v/v) are then added to the tube. After the tube is shaken and centrifuged, 4 ml. of the upper phase is transferred to a counting vial. To the vial is then added 14 ml. of a solution of the following composition: toluene 93% (v/v), methanol 7% (v/v), and 2,5-diphenyloxazole 0.4% (w/v). Radioactivity is measured by scintillation counting. This method is applicable to samples containing inulin-carboxyl-C^{14} and DMO-2-C^{14}, since the former compound is not extracted into the organic solvent.

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