DEVELOPMENT OF SPECIFICATIONS FOR LABELLED NUCLEOTIDES AND RELATED COMPOUNDS. SPECIFIC ACTIVITY MEASUREMENTS BY MASS SPECTROMETRY.

Gerald Goldstein and W.T. Rainey.
Analytical Chemistry Division, Oak Ridge National Laboratory*, Oak Ridge, Tennesse 37830 U.S.A.
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SUMMARY

The National Academy of Science-National Research Council Committee on Specifications and Criteria for Biochemical Compounds is considering the problems involved in preparing specifications for labelled biochemicals. The Subcommittee on Nucleotides and Related Compounds has examined methods for specific activity measurement. We find that the specific activity of ^{14}C-labelled purines and pyrimidines can be determined accurately and precisely by mass spectrometry. Moreover, from the known fragmentation patterns and the isotopic abundance of carbon-14 in the fragments, the position of the labelled atoms can often be established and the labelling pattern in "uniformly labelled" compounds described. In two of the five compounds tested, the specific activity determined by mass spectrometry was 20% higher than the quoted value.

INTRODUCTION

A substantial fraction of chemical, biological, and medical research is today carried out with the aid of radioisotopes and labelled compounds. Unfortunately, along with this boon have come problems associated with quality control in their preparation, purity, and the stability of these

* Operated by Union Carbide Corporation for the U.S. Atomic Energy Commission

compounds, which have been commented on from time to time by unhappy users (1-5). A study of 500 radiochemical shipments received at the Warner-Lambert Research Institute uncovered 10 cases of clear error (6), and in a survey of workers actively engaged in the use of labelled molecules made by the IUPAC Commission on Analytical Radiochemistry and Nuclear Materials (7) 70% of the respondents indicated that they had at some time found the purity insufficient for their intended use. The conclusion frequently arrived at is that the user ought to practice defensive quality control and from his own tests decide whether the material is suitable or not. While some laboratories may be able to commit time and facilities to a testing program, the IUPAC survey showed that only about 25% of the laboratories reporting made it a practice to always analyze their shipments, so it appears that for most users defensive quality control is impractical, nor should it really be necessary. For these reasons the National Academy of Sciences-National Research Council Committee on Specifications and Criteria for Biochemical Compounds, which has been active for some years in preparing specifications for unlabelled compounds, has become interested in labelled compounds, and a general discussion of some of the problems is included in the Committee's most recent publication (8). The Subcommittee on Nucleotides and Related Compounds has been considering such information as is available concerning the preparation, purity, and applications of labelled nucleic acid derivatives (9), and the problems encountered by both users and manufacturers. We have focussed on the specific activity measurement of ^{14}C-labelled bases and nucleosides because the quoted specific activity is used frequently to quantitatively calculate the number of moles of the compound which have undergone reaction. The conventional methods of measuring specific activity from the absolute disintegration rate of a known weight of compound are not very accurate, probably not better than ± 10%, and both labelled and unlabelled impurities can cause errors. It seemed to

us that one should be able to evaluate specific activity with great precision and accuracy by mass spectrometry.

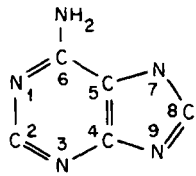
EXPERIMENTAL

The labelled compounds were selected from manufacturer's catalogs and purchased in the normal way from major suppliers.

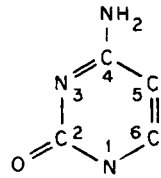
The low resolution organic mass spectrometry facility at Oak Ridge National Laboratory has been described in detail elsewhere (10). Basically, the instrument has a single-stage, 12-inch radius, 90° magnetic analyzer; 4000-volt accelerating potential; electron multiplier detector; and heated direct-inlet probe. Both 15 and 70 electron volt ionizing energies were used. Approximately 5 μg of the solid compound were introduced into the spectrometer with the direct-inlet probe and heated to 100-150° C.

RESULTS AND DISCUSSION

The numbering scheme for purines and pyrimidines which will be referred to is as follows.



Adenine



Cytosine

Figure 1 is the mass spectrum, with 70 electron volt ionizing energy, for cytosine labelled at carbon 2 with ^{14}C. The molecular ion of unlabelled cytosine is at m/e 111 and that of cytosine with one carbon-14 atom is at m/e 113. There are no molecular ions at m/e 115 or greater that cannot be accounted for by ^{13}C contribution to the m/e 113 ion and hence no molecules

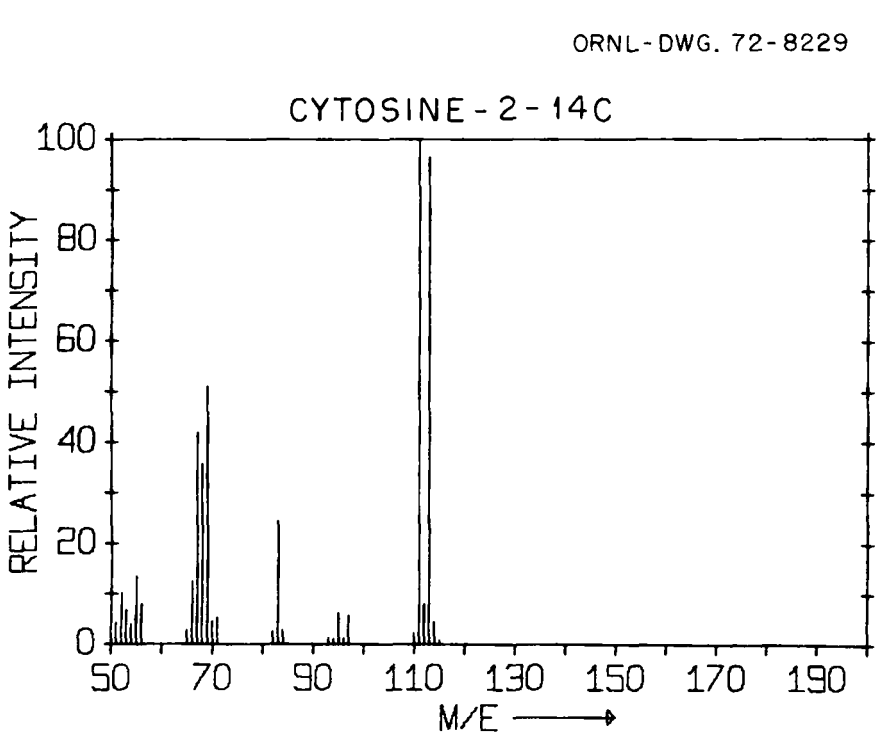


FIG. 1. MASS SPECTRUM OF CYTOSINE-2-^{14}C (ORNL-DWG. 72-8229)

with more than one labelled carbon. From the relative intensities at m/e 111 and 113, 48.9% of the molecules are labelled, and since the specific activity would be 62.4 mCi/mmole if 100% of the molecules were labelled, the specific activity of this preparation is 30.5 mCi/mmole (Table 1). By running replicate samples we obtained a relative standard deviation of about 1%. Furthermore we can learn something about the position of the labelled carbon because the fragmentation pattern of cytosine and most other purines and pyrimidines has been worked out in detail (11,12). Ions at m/e 97 and 97 result from loss of -NH_2 from the molecular ions. All the

Table 1. Specific Activity of Purines and Pyrimidines by Mass Spectrometry

Compound	Percent of Molecules Labelled	Specific Activity, mCi/mmole Found	Quoted
Adenine-2-^{14}C	37.3 (±0.5)	23.3	19.8
Adenine-8-^{14}C	89.9 (±0.6)	56.1	56
Cytosine-2-^{14}C	48.9 (±0.4)	30.5	25
Thymine-2-^{14}C	86.1 (±0.7)	53.7	53
Adenine-^{14}C(U)	100 96.8 (±1.0) [a]	302	287

[a] 96.8% of carbon atoms labelled

other ions represent fragments from which carbon-2 of cytosine has been lost. By comparison with the mass spectrum of unlabelled cytosine none of these fragments contain carbon-14 and consequently this preparation was indeed labelled only at carbon 2.

Mass spectra for two adenine preparations, adenine-2-^{14}C and adenine-8-^{14}C, are shown in Figure 2. The molecular ions are at m/e 135 (unlabelled) and 137 (having one ^{14}C atom) and the specific activity was calculated from their intensity ratio and is shown in Table 1. There are no doubly labelled molecules. The fragmentation patterns of these compounds also provide useful information about the labelling pattern. Adenine fragments by successive losses of HCN and, for the unlabelled compounds, has a fragment at m/e 108 resulting from loss of carbon 2 as HCN. The adenine-2-^{14}C preparation shows the m/e 108 ion and no ion at m/e 110, demonstrating that all the carbon-14 had been lost as HCN and was originally present only at carbon 2. The adenine-8-^{14}C preparation, on the other hand,

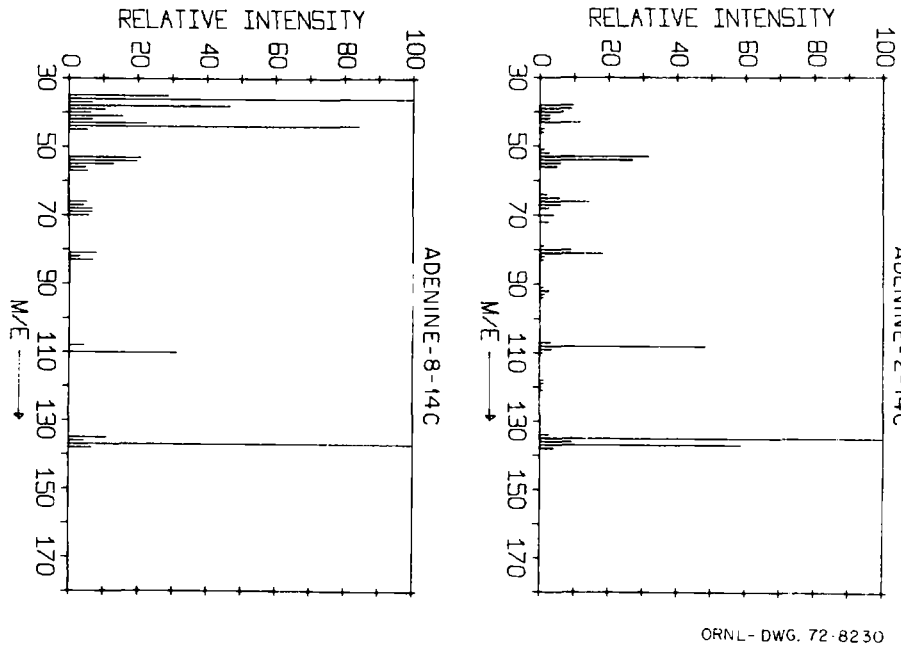


FIG. 2. MASS SPECTRA OF ADENINE-2-^{14}C AND ADENINE-8-^{14}C (72-8230)

shows an ion at m/e 110 and furthermore the fraction of the molecules labelled from the m/e 110/108 ratio (0.89) is essentially the same as that calculated from the m/e 137/135 ratio (0.90), demonstrating no loss of carbon-14 and thus no label at carbon 2. Unfortunately further fragmentation proceeds by two paths, one of which involves loss of carbon 8 and the other does not, so that it isn't possible to prove that the label was at carbon 8.

Figure 3 shows the mass spectrum of "uniformly labelled" adenine which is particularly interesting. Firstly, all molecular ions are at m/e 141, 140, and 139, representing molecules with 3, 4, and 5 carbon-14 atoms per molecule. There are no molecules with 0, 1, or 2 carbon-14 atoms. We calculated that just under 97% of the carbon atoms are labelled and the specific activity is shown in Table 1. Looking at the fragments, we find that

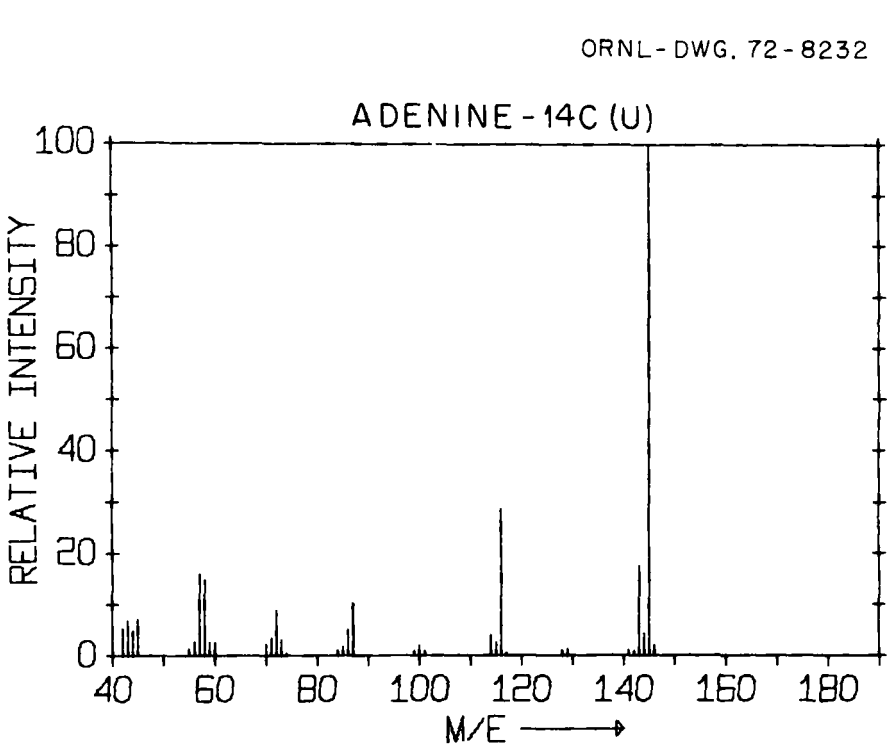


FIG. 5. MASS SPECTRUM OF "UNIFORMLY LABELLED" ADENINE (72-8232)

those representing loss of one carbon atom (m/e 112-116), two carbon atoms (m/e 83-87), and three carbon atoms (m/e 54-58) still have 97% of their carbons labelled, so that in this preparation carbon-14 was randomly distributed among the five carbon atoms in adenine. By convention, randomly labelled compounds are referred to as uniformly labelled. The fact is, however, that this preparation and many others which are called "uniformly labelled" in the accepted use of the term, is actually a mixture of molecules of different isotopic abundances of carbon-14 rather than the single molecular species that the term "uniform" suggests. This problem has been dis-

cussed by Catch (13) but we suspect is not sufficiently appreciated, and this adenine preparation is a good case in point. The adenine was prepared synthetically by condensation of five molecules of carbon-14 labelled formamide. If one assumes random combination of the formamide molecules it is possible to calculate the intermolecular distribution of carbon-14 in the adenine as a function of the isotopic abundance in the formamide. The problem is the same as taking five balls from a box containing both black and white balls. What is the probability of selecting five black balls, or four black and one white, etc., if there are equal numbers of black and white in the box, twice as many black, and so forth? This calculation was made for three cases; 50% isotopic abundance of carbon-14 in the formamide, 92% (the quoted value for the isotopic abundance in the adenine), and 97%, which is closest to what we calculate from the mass spectrum; and is shown in Table 2. The distribution that we found is also included in Table 2 and appears very reasonable for 96.8% labelling, so we expect this calculation correctly represents the distribution. If the

Table 2. Labelling Pattern in "Uniformly Labelled" Adenine

Number of Labelled Carbons	Percent of Total Molecules			
	50%[a]	92%[a]	97%[a]	Found
0	3.12	0	0	0
1	15.62	0.02	0	0
2	31.25	0.43	0.02	0
3	31.25	4.98	0.82	1.0
4	15.62	28.66	13.28	14.7
5	3.12	65.91	85.87	84.3

[a]Isotopic abundance of ^{14}C in formamide

formamide starting material were 50% labelled then the adenine synthesized would also be 50% labelled and could be called "uniformly labelled" by current definition. But 3.12% of the molecules would have no carbon-14, 15.62% would have one carbon-14 atom, 31.25% would have two carbon-14 atoms, and so forth. It is very unlikely that the purchaser of such a compound would understand that this distribution of label was implied by the term "uniformly labelled, 50% isotopic abundance", particularly since the same nomenclature could also be applied to many other distributions, for instance a 1:1 mixture of fully labelled and unlabelled adenine, It is possible of course that the labelling pattern is not of any importance to the user - only he can say - but one should be aware that different preparations may have different patterns of labelling and that the term "uniformly labelled" does not guarantee any particular pattern.

Comparing the specific activities measured by mass spectrometry with the quoted values in Table 1, the results are in reasonable agreement for three compounds but our results are about 20% higher for adenine-2-^{14}C and cytosine-2-^{14}C. We think it likely that the quoted values are incorrect because of unlabelled impurities in the preparations. The mass spectrometric value should be accurate for several reasons. Since the specific activity is calculated from the internal ratio of molecular ions in a single spectrum changes in the experimental conditions should have no effect, and the good precision of replicate measurements bears this out. We only measure $\frac{\text{intensity}}{m/e}$ ratios for the molecular ions of the particular compound of interest, so it is not likely that either labelled or unlabelled impurities would interfere because their molecular ions would almost certainly have different m/e values. A substantial impurity inthe sample that happened to yield a fragment with the proper m/e would interfere, but one would also expect to see other extraneous fragments and we have not observed any.

For those with access to mass spectrometer facilities, the cost of

obtaining a mass spectrum in terms of material consumed is small. A few micrograms of material is sufficient for replicate determinations, and for many nucleosides and bases this cost is under $10. The added cost to a manufacturer to check each lot would be insignificant. Specific activity measurement by mass spectrometry is probably limited to ^{14}C-labelled purines, pyrimidines, and their nucleosides. Nucleotides, having ionic phosphate groups, do not usually give a good mass spectrum, and tritiated compounds may undergo internal rearrangement reactions within the mass spectrometer.

ACKNOWLEDGEMENT

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