INCORPORATION OF L-METHIONINE-METHYL-^{14}C INTO GENTAMICINS

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When L-methionine-methyl-^{14}C was added to a growing culture of *Micromonospora purpurea*, a high incorporation of radioactivity into gentamicins was observed. As expected, radioactivity incorporated into gentamicins was in an increasing order of C_{1a}, C_2, and C_1.

Gentamicin C complex, produced by *Micromonospora purpurea*[1,2], is composed of three components, C_{1a}, C_2 and C_1 (Fig. 1). These components are methylaminoglycosides, which are distinct from other related antibiotics such as the kanamycins, neomycins and paromomycins.

As seen in Fig. 1, gentamicins C_{1a}, C_2, and C_1 possess one C-methyl and one N-methyl group, two C-methyl and one N-methyl groups and two C-methyl and two N-methyl groups, respectively.

Presuming that L-methionine is the donor of methyl groups of gentamicins, it may be assumed that most of the radioactivity from L-methionine-methyl-^{14}C would be incorporated into the C_1 component and least into the C_{1a} component. Experimentally, incorporation of L-methionine-methyl-^{14}C into the gentamicins was found to be highest as compared to other radioactive presumed precursors. A striking correlation between the incorporation rate of radioactivity from L-methionine-methyl-^{14}C and the number of methyl groups of each gentamicin component, was also observed.

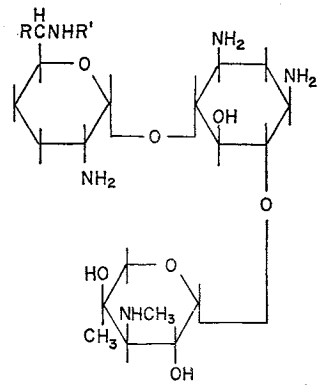
Materials and Methods

Fermentation. A four-ml portion of frozen cells of *Micromonospora purpurea* (ATCC 15835) was inoculated into a 300-ml flask containing 80 ml of inoculum medium (3 g beef extract, 5 g tryptose, 1 g dextrose, 24 g potato starch, 5 g yeast extract, and 2 g $CaCO_3$ in 1 liter tap water). The inoculum was developed in two stages of 72 hours each by shaking the flask at 37°C on a rotary shaker at 280 rpm. Four ml of the developed inoculum were added into a 300-ml flask containing 80 ml of fermentation medium (50 g potato dextrin, 5 g dextrose, 35 g soybean meal, 7 g $CaCO_3$, and 0.0013 g cobalt chloride in 1 liter tap water). The flask was shaken at 28°C at 280 rpm in the presence of added radioactive isotope.

Radioactive isotopes. L-Methionine-methyl-^{14}C was purchased from Schwarz/Mann Labs, and other radioactive isotopes (discussed under "Results") from New England Nuclear, Inc.

Addition of isotopes. Radioactive isotopes dissolved in distilled water or in 95% ethanol were added in one portion at the time of inoculation, or equivolumes of each isotope added successively at 0,

Fig. 1. Structures of gentamicin components



		R	R'
Gentamicin	C_{1a}	H	H
"	C_2	CH_3	H
"	C_1	CH_3	CH_3

24 and 48 hours after inoculation.

Recovery and purification of radioactive gentamicins. To the whole fermentation broth (65 ml), harvested from the initial volume of 80 ml, was added 0.5 g oxalic acid and the pH of the broth brought to 2.0 with 12 N H_2SO_4. Acidified fermentation broth was shaken for 1/2 hour, 1.5 g IRC-50 in NH_4^+ form added to neutralized supernatant, and the mixture (resin + supernatant) shaken for 1 hour. The solution was decanted, and the resin washed three times with distilled water. The washed resin was eluted twice with 5-ml portions of 2 N NH_4OH, and washed once with 5 ml H_2O. The eluates combined with the aqueous washings were then lyophilized.

Paper chromatography. Paper chromatograms were developed in a descending system of the lower phase of chloroform-methanol-17% ammonia (2 : 1 : 1 by volume), or of 2-butanone - tertiary butanol-methanol-conc. ammonia (16 : 3 : 1 : 6).

Assay of radioactivity. Radiochromatograms were scanned with a Nuclear Chicago scanner (Model 1002) for determination of percent radioactivity distributed into each gentamicin component, and the total radioactivity of each sample was measured employing a liquid scintillation counter (Intertech. Inst. Inc., SL 30).

Fig. 2. Radioactivity scanning of gentamicin components separated on radiochromatograms ((a) Single addition; (b) multiple addition)

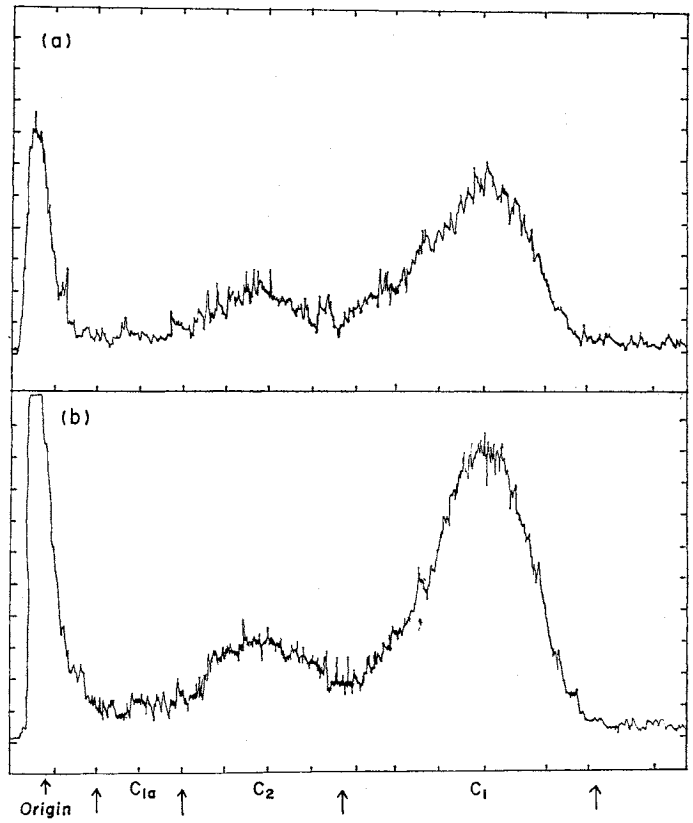


Table 1. Incorporation of ^{14}C-labeled precursors into gentamicin complex*

Precursor	Gentamicin titer		Efficiency (% Incorporation)
	μg/ml	mg/flask	
D-Glucose (UL)-^{14}C	425	28.5	0.9
D-Glucosamine-1-^{14}C	270	18.1	2.6
Glycine-2-^{14}C	545	36.5	3.5
DL-Serine-3-^{14}C	465	31.1	2.8
Betaine-methyl-^{14}C	350	23.4	0.0
Choline-methyl-^{14}C	482	32.3	0.0
L-Methionine-methyl-^{14}C	325	26.0	17.3

* Experiments not performed at the same time.

Differential ninhydrin chromatographic assay. Radiochromatograms were also scanned with an ultraviolet-visible spectrophotometer (Beckman), for determination of ninhydrin color density developed with each gentamicin component[8].

Results

Lyophilized resin eluates showed the presence of radioactive impurities and minor components which remained at the origin of the paper chromatograms (Fig. 2). No attempt was made to purify

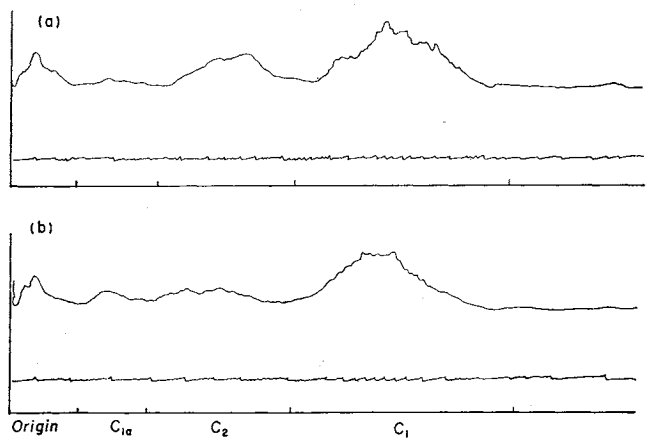
Table 2. Incorporation of L-methionione-methyl-^{14}C into gentamicin components

Addition of precursor	Radioactivity (μCi)						% Radioactivity distributed					% Incorporation into C complex
	Eluate	Origin	C_{1a}	C_2	C_1	C complex	Eluate	Origin	C_{1a}	C_2	C_1	
One portion*	6.46	1.27	0.26	0.99	4.16	5.41	100	19.59	3.99	15.35	62.17	10.8
Three portions**	10.66	2.08	0.37	1.92	6.35	8.64	100	19.50	2.88	18.05	59.55	17.3

* Added, in one portion, at 0 hour.
** Added, in three portions, at 0, 24 and 48 hours after inoculation.

the eluates further, and follow-up studies were conducted with the whole eluates which contained the total antibiotic complex.

Fig. 3. Ninhydrin color scanning of gentamicin components separated on radiochromatograms ((a) Single addition; (b) multiple addition)



Lyophilized resin eluates, each derived from 80 ml fermentation broth, weighed 18.1~36.5 mg/flask, and showed bioactivity in the range of 270 to 545 μg/ml against *Staphylococcus aureus* ATCC 6538P. Since experiments were carried out at different times with different inocula, some variations in gentamicin titers were encountered. Compared to L-methionine-methyl-^{14}C, the incorporation of glucose (UL)-^{14}C, glucosamine-1-^{14}C, glycine-2-^{14}C, betaine-methyl-^{14}C, choline-methyl-^{14}C and DL-serine-3-^{14}C into gentamicins was low (Table 1).

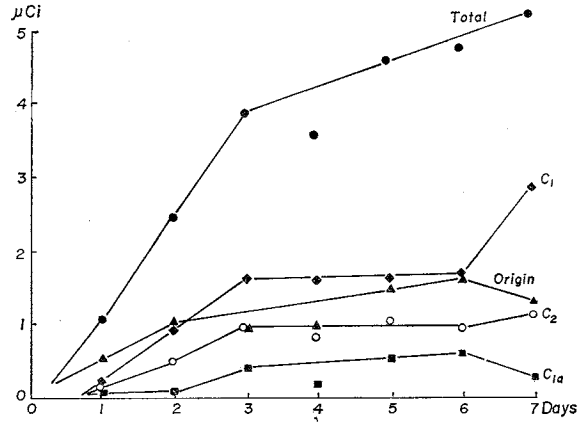
The mode of addition of L-methionine-methyl-^{14}C was studied by two methods: (1) addition of 50 μCi of the radioactive precursor, in one portion, at 0 hour, and (2) one third of 50 μCi each, in three portions, at 0, 24 and 48 hours after inoculation. As expected, better incorporation was obtained by the latter mode of addition due to the possible effect of sparing the radioactive precursor from its consumption by the cellular primary metabolism. The percentages of radioactivity, distributed into the bioactive components from the first and second modes of addition were similar and were approximately 16 : 4 : 15 : 63 (origin materials : gentamicin C_{1a} : C_2 : C_1) and 20 : 3 : 18 : 60, respectively (Table 2, Fig. 2). Percent ninhydrin densities of the four components were more or less in agreement with those of the respective radioactivities, being 10 : 4 : 28 : 68 in the first mode of addition and 11 : 6 : 25 : 70 in the second mode of addition (Fig. 3).

A time-course study showed that radioactivity incorporated into the origin and all three components increased proportionally with time up to 6 days. Further increase of label incorporated into C_2 and C_1 components was observed in samples taken after 6 days, but the same samples showed a decrease in radioactivity incorporated into the origin materials and C_{1a} (Fig. 4).

Discussion

The N-methyl groups of desosamine of both erythromycin and methymycin have been shown to be derived from the methyl group of L-methionine[4,5]. CANTONI[6,7,8] and GREENBERG[9] showed that biologically methylated compounds are formed *via* transmethylation, and that transmethylation involves conversion of L-methionine to S-adenosylmethionine, which then serves as the donor of the methyl groups.

Fig. 4. Time-course study of L-methionine-methyl-^{14}C incorporation into gentamicin components



The high efficiency of L-methionine incorporation into gentamicins (17.3 %) as compared to other radioactive isotopes, suggests that such transmethylation mechanism(s) may be operational in the biosynthesis of gentamicins. Whether L-methionine serves as a donor of N-methyl groups, C-methyl groups, or both of the groups of the antibiotics is not entirely clear. The reason why the methyl groups of choline-^{14}C and betaine-^{14}C were not incorporated into the antibiotics is not also clear. It may be that these compounds are not taken up by the organism or that they are not used as methyl donors for the gentamicin biosynthesis. From labelling experiments using L-methionine-methyl-^{13}C, DANIELS and his co-workers have just shown however that all the methyl carbon atoms of gentamicins C_{1a}, C_2 and C_1 can be derived from methionine[10]. Additional fermentations will be carried out on a larger scale using the ^{14}C precursor in order to isolate individual components with the aim of performing degradation studies to determine where the radioactivity was incorporated.

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