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

II. LARGE-SCALE PREPARATION OF METHYL-^{14}C-GENTAMICINS

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Methyl-^{14}C-gentamicins were prepared from incubation of *Micromonospora purpurea* in the presence of added methyl-^{14}C-L-methionine, and products with high specific radioactivity were isolated in satisfactory yields.

In the previous paper[1], we have shown in shake flask fermentation a high and selective incorporation of radioactivity from methyl-^{14}C-L-methionine into gentamicins. Consequently, we chose this isotope for the larger scale fermentative preparation and isolation of methyl-^{14}C-gentamicins.

Two batches of fermentation, in the presence of the isotope added at different levels, were run in a 30-liter fermenter. Each fermentation broth was worked up independently, and radioactive products were isolated and purified through a Dowex column. The resulting methyl-^{14}C-gentamicin complex showed a high radiochemical purity.

Materials and Methods

Radioactive Isotope. Methyl-^{14}C-L-methionine of specific activity 0.307 mCi/mg was purchased from New England Nuclear, Inc.

Resins. IRC-50, Amberlite IRA-401S and Dowex 1×2 (50~100 mesh) were purchased respectively from Rohm and Hass Co., Mallinckrodt Chemical Co., and J. T. Baker Chemical Co.

Paper Chromatography. Paper chromatograms were developed on Whatman No. 1 paper in a descending system of the lower phase of chloroform - methanol - 17% ammonia (2:1:1 by volume).

Measurement of Radioactivity and Bioassay. A liquid scintillation counter (Intertech. Inst. Inc., SL 30) was used for the measurement of radioactivity, a Nuclear Chicago scanner (Model 1002) for the determination of percent radioactivity incorporated into gentamicin components, and *Staphylococcus aureus* ATCC 6538 P for bioassay.

Fermentation. In batch 1, 3.5 ml portion of frozen cells of *Micromonospora purpurea* (ATCC 15835) was inoculated into a 300-ml flask containing 70 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 flask was shaken for 48 hours at 35°C on a rotary shaker at 300 rpm. Twenty-five ml each of the culture broth was inoculated into two 2-liter flasks containing 500 ml of the same inoculum medium, and the flasks were shaken for 48 hours at 28°C at 300 rpm. The inoculum (750 ml), developed in two stages, were added into a 30-liter New Brunswick fermenter (Model MF-128 S) containing 15 liters of fermentation medium (50 g potato dextrin, 5 g dextrose, 35 g soybean meal, 7 g $CaCO_3$, 0.0013 g Cobalt chloride, and 0.5 ml antifoam (Dow

Corning B-Emulsion) in 1 liter tap water). The fermentation broth was stirred with aeration of 3.5~5 liters/minute at 34°C at 350~500 rpm for 90 hours in the presence of methyl-^{14}C-L-methionine, added in three portions of 8.33 mCi each at 0, 24 and 48 hours after inoculation. During the fermentation process, additional antifoam was added automatically as required. Procedures followed in batch 2 were identical with those of batch 1, except starting of fermetation with 20-liter broth and addition of methyl-^{14}C-L-methionine in one portion of 75 mCi at 24 hours after inoculation.

Isolation and Chromatographic Purification of Products. In batch 1, 100 g oxalic acid was dissolved in 500 ml water, on a hot plate, and added to the fermentation broth. The pH of the fermentation broth was adjusted to 2 with 2 N H_2SO_4, and the acidified broth was stirred for 1 hour and filtered by gravity. The pH of the filtrate (13 liters) was adjusted to 7 with conc.NH_4OH, and the neutralized filtrate was added to 300 g of IRC-50 (NH_4^+ form) in a 5.5 cm×100 cm column. The fermentation broth was passed through the column at a rate of 3.0 liters/hour. The resin was washed with 3.0 liters of water, eluted with 3.0 liters of 2 N NH_4OH and eluate air-dried. The resulting dark brown crude (7.68 g) was passed through a column (0.75 cm×65 cm) packed with 150 g IRA-401-S (OH^- form), and eluted with water. Eluates collected between pH 8 and 12 were combined and air-dried. The decolorized crude was adsorbed on a column packed with 200 g Dowex 1×2 resin in OH^- form, and gentamicin C components were selectively eluted with water at a flow rate of 10 ml/15 minutes[2]. Eluates were collected, and tested with ninhydrin and against *Staphylococcus aureus* ATCC 6538 P. The ninhydrin-positive and bioactive fractions (tubes No. 124~168) were combined, pH of the combined eluate was brought to 4.0 with 12 N H_2SO_4, decolorized with Darco G 60, filtered, and air-dried. Similar approaches were taken for isolation and purification of products in batch 2.

Results

Scale-up of fermentation preparation of methlyl-^{14}C-gentamicins was successfully achieved from a shake flask[1] to a tank fermentation (Table 1).

Ratios of radioactivity incorporated into gentamicin C components, as well as yield of the antibiotic complex, vary depending upon fermentation conditions. In our 30-liter scale fermentation under the conditions we used, much higher percentage (54~57%) than expected (44%) incorporated into the C_1 component (Table 2).

Table 1. Fermentative preparation, isolation and purification of radioactive gentamicins

Batch. No.	Initial ferment. broth (liters)	Input of L-methionine-methyl-^{14}C (mCi)	Fermentation broth filtrate		
			Volume (liters)	Bioassay (mcg/ml)	Total radioactivity (mCi)
1	15	25	13	355	11.47
2	20	75	18	398	44.89

Batch No.	Crude product (IRC-50 Eluates)				Final product (Sulfate)			
	Wt. (g)	Bioassay (mg/g)	Total radio-activity (μCi)	Specific radio-activity (μCi/g)	Wt. (g)	Bioassay (μmg/g)	Total radio-activity (μCi)	Specific radio-activity (μCi/g)
1	7.68	608	3,370	439	2.02	580	749	371
2	11.88	462	11,467	965	5.24	476	3,210	612

Table 2. Radioactivity incorporated into gentamicin components in crude and final products

Batch No.	Radioactivity (μCi)					% Radioactivity incorporated				
	Origin	C_{1a}	C_2	C_1	C complex	Origin	C_{1a}	C_2	C_1	C complex
1-C*	1,036	329	769	1,236	2,334	30.74	9.77	22.82	36.67	69.26
1-F**	38	61	248	402	711	5.10	8.17	33.15	53.58	94.90
2-C	4,101	1,229	2,128	4,009	7,366	35.76	10.72	18.50	34.96	64.24
2-F	123	356	906	1,825	3,087	3.83	11.08	28.24	56.85	96.17

1-C* : crude product of batch 1
1-F**: final product of batch 1

The specific radioactivity of the products isolated was directly related to the level of the radioactive precursor added into the fermentation broth (Table 1). That is, as the level of the precursor added was increased, the higher was the specific radioactivity of the products isolated; 371 μCi/g products from the input of 25 mCi in batch 1, 612 μCi/g from 75 mCi input in batch 2.

Crude products, which were adsorbed on IRC 50 resin and eluted, contained substantial amounts of origin components carrying about one third of the total radioactivity. The crude

Fig. 1. Radioactivity scan of Batch I (Gentamicin complex sulfate)

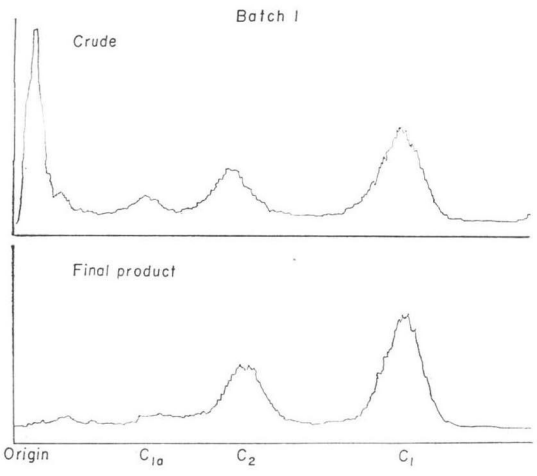


Fig. 2. Bioautogram of Batch I (Gentamicin complex sulfate)

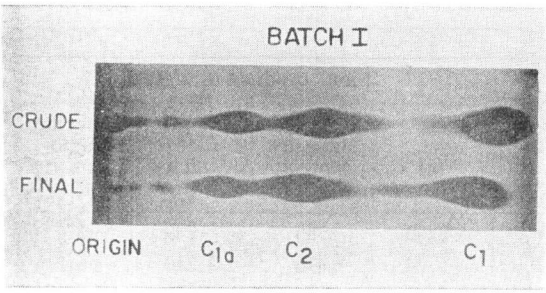


Fig. 3. Radioactivity scan of Batch II (Gentamicin complex sulfate)

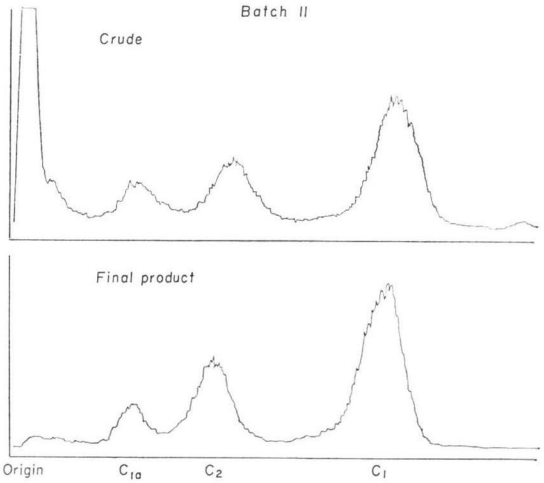
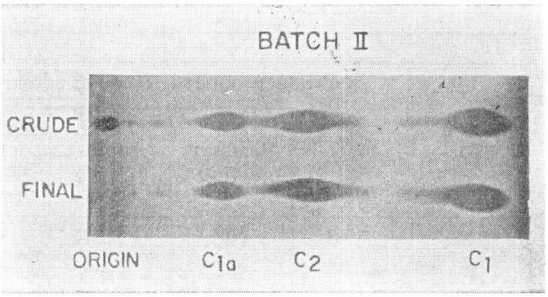


Fig. 4. Bioautogram of Batch II (Gentamicin complex sulfate)



products were purified on a Dowex 1×2 column. The chromatographic purification resulted in raising radioactivity of the gentamicin C complex content, in the final products, to 95 % and 96 % respectively in batches 1 and 2 (Table 2, Fig. 1, Fig. 3). The radiochemical purity of the final products were also in agreement with bioactivity of the products (Fig. 1 *vs.* Fig. 2 and Fig. 3 *vs.* Fig. 4).

Discussion

Methionine is reportedly converted to S-adenosylmethionine by conjugating with the adenosine moiety of ATP in the presence of Mg^{++}, then S-adenosylmethionine serves as a methyl donor in the biological transmethylation reactions[3,4,5].

Gentamicins are methylated aminoglycosides; gentamicin C_{1a} possessing one C-methyl and one N-methyl groups, C_2 with two C-methyl and one N-methyl groups, and C_1 with two C-methyl and two N-methyl groups. As previously reported[1], we have again shown a close correlation between the rate of radioactivity incorporation from methyl-^{14}C-L-methionine and the number of methyl groups of each gentamicin component. Random incorporation of methyl carbon atoms into the three gentamicin components was also shown from labelling experiments using methyl-^{13}C-L-methionine[6].

We have successfully prepared, in a tank scale, methyl-^{14}C-gentamicins using methyl-^{14}C-L-methionine. The radioactive gentamicin complex was chromatographed on a silica gel column and the individual components isolated. Studies are being carried out on the individual components for determination of radioactivity incorporated into the subunits of each component. These data will be the subject of a separate paper (Part III).

Acknowledgement

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