Inhibition by *o*-Aminoazotoluene of the Incorporation of Certain Radioactive Amino Acids into Lipids of *Mycobacterium smegmatis*

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SUMMARY

A carcinogenic substance, *o*-aminoazotoluene (AAT), inhibited the incorporation of [$1\text{-}^{14}\text{C}$]glycine and [$1\text{-}^{14}\text{C}$]leucine into the lipid fraction of *Mycobacterium smegmatis*. It inhibited transiently the incorporation of [$1\text{-}^{14}\text{C}$]glutamic acid into lipids, but it did not inhibit the incorporation of [$1\text{-}^{14}\text{C}$]acetate into lipids. AAT did not inhibit the incorporation into the protein and nucleic acid fractions of [$1\text{-}^{14}\text{C}$]glycine, [$1\text{-}^{14}\text{C}$]leucine, [$1\text{-}^{14}\text{C}$]glutamic acid, and [$1\text{-}^{14}\text{C}$]acetate.

INTRODUCTION

Yoshida (1932, 1934*a, b*) demonstrated that *o*-aminoazotoluene (AAT) produced proliferation of hepatic tissue and hepatomas in mice. Biochemical observations on carcinogenesis, including reaction of the azo dyes with proteins, have been carried out by many investigators (reviews by Miller & Miller, 1953; Rondoni, 1955; Busch, 1962; Reid, 1962). Recently we observed that the growth of mycobacteria was inhibited by low concentrations of AAT in chemically defined media, but that this inhibition was abolished in egg media. As the action of AAT on mycobacterial cells is of interest in relation to its action on the cells of higher organisms, we have studied biochemical changes produced in mycobacteria by AAT.

METHODS

Mycobacterium smegmatis strain Jucho was used throughout. The following radioactive compounds were employed for the study: [$1\text{-}^{14}\text{C}$]glycine (specific radioactivity, $57\cdot3\text{ }\mu\text{c./mg.}$), DL-[$1\text{-}^{14}\text{C}$]leucine (specific radioactivity, $44\cdot0\text{ }\mu\text{c./mg.}$), DL-[$1\text{-}^{14}\text{C}$]glutamic acid (specific radioactivity, $21\cdot0\text{ }\mu\text{c./mg.}$), and sodium [$1\text{-}^{14}\text{C}$]acetate (specific radioactivity, $148\text{ }\mu\text{c./mg.}$); all supplied by the Radiochemical Centre, Amersham, Buckinghamshire.

The test organism was cultivated at 37° for 5 days in a modified Sauton medium, in which sodium glutamate was substituted for asparagine. The bacteria were harvested, washed three times in saline, and the washed bacteria incubated in reaction mixtures containing a radioactive compound. At different times, the reaction mixtures were cooled in ice and centrifuged. The bacteria were washed once with a solution of 1% (w/v) glycine, 1% leucine, 1% sodium glutamate, or 1% sodium acetate. They were then washed three times in distilled water and fractionated according to the procedure of Schneider (1945). The method was described previously (Tsukamura, Tsukamura & Mizuno, 1964).

Samples (0.2 ml.) of each fraction were placed in stainless steel planchets and heated to dryness. The radioactivity was measured by a 2π gas flow counter and expressed as 'counts/minute/mg. dry weight organism' (the radioactivity in the sample was multiplied according to the amount of the fraction and divided by the dry weight). The radioactivity was recorded after subtracting the radioactivity at time 0.

Portions of the trichloroacetic acid (TCA)-soluble fractions were extracted five times with ethyl ether to remove the TCA and concentrated by heating under reduced pressure. Portions of the protein fractions were hydrolysed by heating in 6 N-HCl at 100° for 24 hr and concentrated by heating. These concentrates were subjected to ascending paper chromatography, using Toyo filter paper no. 50 and solvents consisting of tertiary butanol + formic acid + water (70 + 15 + 15 by vol.) and isobutanol + formic acid + water (70 + 15 + 15 by vol.). The R_f values given in this paper were obtained with the former solvent system unless it is specifically stated otherwise. The radioactivity in paper chromatograms was recorded on an automatic paper chromatogram scanner (Nihon Musen, Tokyo; running: 300 mm./hr; recording: 150 mm./hr; slit, 3 mm.; range: 300 or 1000 counts/5 sec.).

AAT (Takeda Chemical Co., Osaka) was dissolved in propyleneglycol at a concentration of 2 mg./ml. and 0.1 ml. samples of this solution were added to 4.0 ml. of the reaction mixtures. Thus, the final concentration in the reaction mixtures was 50 μ g. AAT/ml., which was two to four times higher than the minimal inhibitory concentration. Control reaction mixtures contained 0.1 ml. propyleneglycol without AAT.

RESULTS

Effect of AAT on the incorporation of [1-¹⁴C]glutamic acid

AAT did not inhibit the incorporation of [1-¹⁴C]glutamic acid into the protein fraction, but it inhibited incorporation into the lipid and nucleic acid fractions during the first 6 hr. The effects of the inhibition had disappeared after 24 hr of incubation (Table 1).

Table 1. *Effect of o-aminoazotoluene on the incorporation of [1-¹⁴C]glutamic acid into the cellular fractions of Mycobacterium smegmatis strain Jucho*

Reaction mixture	Time (hr)	Radioactivity as counts/min./mg. dry wt. bacteria			
		Fraction			
		TCA-soluble	Lipid	Nucleic acid	Protein
Control	3	1070	73	77	131
AAT	3	502	49	40	65
Control	6	740	159	102	152
AAT	6	532	49	56	170
Control	24	606	275	193	686
AAT	24	302	226	207	805

Reaction mixtures consisted of 4.0 ml. of 0.067 M-phosphate buffer (pH 7.1) containing 35 mg. wet wt. bacteria/ml., 2.5 μ c. DL-[1-¹⁴C]glutamic acid/ml., 5 μ g., sodium glutamate/ml., 125 μ g. glucose/ml. and 0 or 50 μ g. o-aminoazotoluene/ml.

Effect of AAT on the incorporation of DL-[1-¹⁴C]leucine

Incorporation of [1-¹⁴C]leucine into the lipid fraction was depressed in the presence of AAT. On the other hand, its incorporation into the protein fraction was not inhibited, but rather accelerated, by AAT (Table 2).

The only radioactive peak found in paper chromatograms of the TCA-soluble and protein fractions had an R_f (0.80) identical to that of leucine, whether AAT was present or not.

Table 2. *Effect of o-aminoazotoluene on the incorporation of DL [1-¹⁴C]-leucine into the cellular fractions of Mycobacterium smegmatis strain Jucho*

Reaction mixture	Time (hr)	Radioactivity as counts/min./mg., dry wt. bacteria			
		Fraction			
		TCA-soluble	Lipid	Nucleic acid	Protein
Control	6	614	110	171	6,280
AAT	6	450	76	109	5,710
Control	24	545	520	348	10,800
AAT	24	588	325	337	17,900

Reactions mixture consisted of 4.0 ml. of 0.067 M-phosphate buffer (pH 7.1) containing 30 mg. wet wt. bacteria/ml., 2.0 μ c. DL-[1-¹⁴C]leucine/ml., 5 μ g. L-leucine/ml., 125 μ g. glucose/ml., and 0 or 50 μ g. *o*-aminoazotoluene/ml.

Table 3. *Effect of o-aminoazotoluene on the incorporation of [1-¹⁴C]glycine into the cellular fractions of Mycobacterium smegmatis strain Jucho*

Reaction mixture	Time (hr)	Radioactivity as counts/min./mg. dry wt. bacteria			
		Fraction			
		TCA-soluble	Lipid	Nucleic acid	Protein
Control	3	6,110	653	2,140	3,040
AAT	3	6,380	736	3,090	7,070
Control	6	3,700	920	4,680	6,970
AAT	6	4,360	870	5,270	10,000
Control	24	6,840	4,600	17,300	37,000
AAT	24	4,900	1,680	15,900	44,000

Reaction mixtures consisted of 4.0 ml. of 0.067 M-phosphate buffer (pH 7.1) containing 14 mg. wet wt. bacteria/ml., 2.5 μ c. [1-¹⁴C]glycine/ml., 5 μ g. glycine/ml., 125 μ g. glucose/ml., and 0 or 50 μ g. *o*-aminoazotoluene/ml.

Effect of AAT on the incorporation of [1-¹⁴C]glycine

[1-¹⁴C]Glycine was incorporated into the lipid, nucleic acid and protein fractions. The incorporations into the nucleic acid and protein fractions were not inhibited by AAT. On the other hand, the incorporation into the lipid fraction was inhibited by AAT, and showed most clearly after 24 hr of incubation (Table 3).

A radioactive peak, identified as serine (R_f 0.36–0.37) by comparison with authentic serine, was found in paper chromatograms of the TCA-soluble and protein fractions. Since other radioactive peaks were not found in the paper chromatograms, presumably glycine was converted into serine and incorporated into protein (Fig. 1). However, in what form glycine is incorporated into lipids remains unknown.

Fig. 1. Radioactivity in paper chromatograms of the acid hydrolysates of the protein fraction obtained after incubation with [^{14}C]glycine for 24 hr in the presence and absence of AAT.

Table 4. *Effect of o-aminoazotoluene on the incorporation of sodium [^{14}C]acetate into the cellular fractions of Mycobacterium smegmatis strain Jucho*

Reaction mixture	Time (hr)	Radioactivity as counts/min./mg., dry wt. bacteria			
		Fraction			
		TCA-soluble	Lipid	Nucleic acid	Protein
Control	6	300	697	146	830
AAT	6	505	705	100	725
Control	24	492	958	188	1130
AAT	24	474	888	171	1070

Reaction mixtures consisted of 4.0 ml. of 0.067 M-phosphate buffer (pH 7.1) containing 70 mg. wet wt. bacteria/ml., 1.0 μC . sodium [^{14}C]acetate/ml., 100 μg . ammonium chloride/ml., and 0 or 50 μg . *o*-aminoazotoluene/ml.

Effect of AAT on the incorporation of sodium [1-¹⁴C]acetate

[1-¹⁴C]Acetate was incorporated into the lipid, nucleic acid and protein fractions. Almost no inhibitory effect of AAT on incorporation into these fractions was found (Table 4).

DISCUSSION

Incorporation of [1-¹⁴C]glutamic acid, [1-¹⁴C]leucine, [1-¹⁴C]glycine, and [1-¹⁴C]-acetate into the cellular fractions was observed both in the presence and absence of AAT. The concentration of AAT used was 2 to 4 times higher than the minimal growth-inhibitory concentration. Since the generation time of this organism was more than ten times longer than that of *Escherichia coli*, cultures were observed for a period of 24 hr.

The results showed that AAT inhibited the incorporation of [1-¹⁴C]glutamic acid, [1-¹⁴C]leucine and [1-¹⁴C]glycine into the lipid fraction, the incorporation of the latter two amino acids being inhibited more markedly. On the other hand, incorporation of [1-¹⁴C]acetate into the same fraction was not inhibited significantly by this agent.

It has been reported recently that serine is incorporated into lipids of *Escherichia coli* in the form of phosphatidylserine (Kanfer & Kennedy, 1964). Although it has been demonstrated in the present study that glycine is converted to serine, it remains to be tested in future whether or not glycine is incorporated into lipids via serine.

It was noted that AAT did not inhibit the incorporation of glutamic acid, leucine, glycine, or acetate into the protein and nucleic acid fractions.

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