Since β-sclerotization is associated with the presence of covalently bound catechols, the cuticular hydrolyzates were examined for the presence of catecholamine derivatives. Quantitative and specific isolation of catecholamine derivatives from cuticular hydrolyzate was achieved by alumina adsorption technique[12]. HPLC analysis of catechols thus isolated from cuticular hydrolysates revealed the presence of a major component eluting at about 12 min and a minor component eluting at about 13 min as a shoulder to the major peak (fig. 3). Comparison of retention time with related catecholamine derivatives led to the tentative characterization of the major peak to be arterenone and the minor to be dopamine. Cochromatography of the isolated peaks with authentic compound further confirmed this contention. The positive response of the major compound to Arnow's test for o-dihydroxyphenols[19], 2,4-dinitrophenyl-hydrazine and fluorodinitrobenzene indicated the presence of catecholic, carbonyl and amine groups on the molecule, respectively. UV absorption spectrum of the major peak in 0.2 M acetic acid (fig. 3, inset) corresponded well with that of the authentic compound, confirming its identity as arterenone. Similar studies led to the characterization of the minor peak to be dopamine.

Andersen and Roepstroff[20] have identified as many as eleven catechols from cuticular hydrolyzates. However, these authors failed to take precautions to avoid the decomposition of catechol during hydrolysis. For instance, hydrolysis was carried out in an oxygen rich atmosphere which causes decomposition of catechols[21]. Since our procedure involves hydrolysis in vacuum and protection of catechols with alumina during isolation, artificial product formation is highly minimized. This accounts for the isolation of a single ketocatechol. i.e. arterenone, from cuticle. The mechanism by which β-sclerotization is initiated was not clarified until recently. Andersen's group isolated 1,2-dehydro-N-acetyldopamine from β-sclerotized cuticle and suggested it to be the reagent initiating β-sclerotization[22]. As the condition used to extract this compound from cuticle certainly is drastic, involving hot alkali treatment, it is questionable whether this compound is present freely in the cuticle. Moreover, generation of colorless cuticle by the quinone of this compound as suggested by Andersen[22] is also doubtful. Alkyl substituted quinones with conjugated double bonds absorb more strongly in the visible region as compared to unconjugated quinones. As a result, the quinone from 1,2-dehydro-N-acetyldopamine is expected to give dark colored cuticle rather than light colored cuticle. On the other hand, quinone methide intermediate not only accounts for the presence of catechols in cuticle, but also for the formation of colorless cuticle[2,23]. Isolation and characterization of arterenone as a single ketocatechol from mosquito cuticle is also consistent with quinone methide formation and is inconsistent with the involvement of 1,2-dehydro-N-acetyldopamine in β-sclerotization. Recently, we synthesized 1,2-dehydro-N-acetyldopamine and tested for its participation in sclerotization of sarcophagid cuticle. Studies with radioactive trapping experiments reveal that this compound is not involved in the tanning of sarcophagid cuticle and that quinone methides are the reactive species for tanning[24].

Further studies on the characterization of aryl-amino acid adducts and the nature of enzyme system(s) involved in sclerotization reaction(s) are in progress in our laboratory.

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Ornithine decarboxylase, S-adenosyl-L-methionine decarboxylase and arginine decarboxylase from *Mycobacterium bovis* (BCG)

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Summary. Ornithine decarboxylase (ODC), S-adenosyl-L-methionine decarboxylase (AMDC) and arginine decarboxylase (ADC) activities were detected for the first time in extracts of *Mycobacterium bovis* (BCG). All the decarboxylases differed from corresponding known bacterial decarboxylases in that: a) ODC did not require GTP for activity; b) ODC was not inhibited by any known inhibitor of bacterial ODCs; c) AMDC and ADC did not require Mg^{2+}-ion for activity and were not markedly inhibited by any known inhibitor of the decarboxylases of other bacteria.

Key words. Polyamine biosynthesis; *Mycobacterium bovis* (BCG); inhibition of polyamine biosynthesis.

Numerous studies have shown that polyamines (putrescine and spermidine) are necessary for the growth of bacteria[1,2]. We have shown earlier that ethambutol, which is an effective antituberculosis drug[3], specifically inhibited spermidine synthesis in *M. bovis*, but not in non-mycobacteria[4,5]. To study the early steps of polyamine biosynthesis in *Mycobacteria* we measured the activity in *M. bovis* of the different decarboxylases needed for the biosynthesis of putrescine and tested the effect of several

inhibitors against them. Our results show that the decarboxylases from *Mycobacteria* differ from those from other bacteria in several respects including sensitivity to known inhibitors.

Materials and methods. To obtain cell extracts for the assays of different decarboxylases, *M. bovis* (BCG) was grown in Löwenstein-Jensen medium at 37 °C for 3 weeks and then treated exactly as by Pösö et al.[4,5]. The homogenate was centrifuged at 42000 × g for 45 min and the crude supernatant obtained (dialyzed overnight) was used as the source of the enzymes. ODC and AMDC from *E. coli* were obtained as described[6]. The activities of ODC[7], AMDC[8] and ADC[9] were measured as described earlier. All fluorinated derivatives of amino acids and α-methylarginine were generous gifts from the Merrel-Dow Research Center (Cincinnati, Ohio, USA). 1-Aminooxy-3-aminopropane (APA) and S-(5'-deoxy-5'-adenosyl)methylthioethylhydroxylamine (AMA) were synthesized as described earlier[10]. All derivatives of putrescine (NNNN-tetramethyl-1,4-butanediamine, NNNN-tetramethyl-2-butene-1,4-diamine, NNNN-tetramethyl-2-butyne-1,4-diamine, 1,4-diamine-2-butanone and methylacetyleneputrescine) were generous gifts from Dr Algranati (Buenos Aires, Argentina). L-(1-^{14}C)ornithine (46.7 mCi/mmol), S-adenosyl-L-(^{14}COOH)methionine (49 mCi/mmol) and DL-(1-^{14}C)arginine (46 mCi/mmol) were purchased from CEA (Gif-sur-Yvette, France). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA).

Results and discussion. GTP, which is normally needed for the full activity of bacterial ODC[2,7,11], did not stimulate the activity of mycobacterial ODC, and magic spots I (ppGpp) and II (pppGpp)[12] did not inhibit the activity of ODC from *M. bovis* as they do in the case of other bacterial ODCs[6,11] (results not shown).

Table 1 shows the effect of various common inhibitors of bacterial ODCs on mycobacterial ODC. α-Methylornithine, difluoromethylornithine (DFMO), monofluoromethylornithine (MFMO), all very potent inhibitors of common bacterial ODC[2], did not have any effect on mycobacterial ODC (table 1). Table 1 also shows that APA, a recently discovered potent inhibitor of mammalian ODC[13], did not inhibit mycobacterial ODC, but inhibited ODC from *E. coli* (table 1). Since putrescine is a competitive inhibitor of ODC from *E. coli*[2] we tested both putrescine and 5 of its derivatives, but they did not inhibit mycobacterial ODC (results not shown). These results suggest that mycobacterial ODC could be a very specific enzyme as regards its inhibition by different inhibitors.

Table 1. Effect of various inhibitors of bacterial ODCs on ODC from *M. bovis* and *E. coli*

Compound	Concentration (mM)	Activity left (%) *E. coli*	*M. bovis*
Control	–	100	100
α-Methylornithine	1.0	– [a]	87
MFMO	1.0	13.5	104
DFMO	1.0	– [a]	103
APA	1.0	0.2	90

The specific activity of the control for *M. bovis* was 15 pmoles of CO_2 per 60 min per mg of protein and for *E. coli* 1.81 μmoles of CO_2 per 60 min per mg of protein. The results are means of duplicate measurements. The results obtained from different sets of cultivation of the bacteria *M. bovis* gave essentially the same results. MFMO, monofluoromethylornithine; DFMO, difluoromethylornithine; APA, 1-aminooxy-3-aminopropane. [a] Not measured.

Table 2. Effect of various divalent cations on AMDC activity from *M. bovis* and *E. coli*

Compound	Concentration (mM)	Activity left (%) *E. coli*	*M. bovis*
Control	–	100	100
Mg^{2+}	1.0	57184	73
Mn^{2+}	1.0	56452	68
Ca^{2+}	1.0	62888	42
Co^{2+}	1.0	860	83
Zn^{2+}	1.0	96	10
Zn^{2+}	0.1	– [a]	88
Zn^{2+}	0.01	– [a]	96

The specific activity of the control for *M. bovis* was 29 pmoles of CO_2 per 60 min per mg of protein and for *E. coli* 56.3 pmol of CO_2 per 60 min per mg of protein. Other details as in table 1. [a] Not measured.

Table 3. Effect of various inhibitors of bacterial AMDCs on AMDC from *M. bovis* and *E. coli*

Compound	Concentration (mM)	Activity left (%) *E. coli*	*M. bovis*
Control	–	100	100
MGBG	1.0	5.0	87
MGBG	10.0	– [a]	42
Berenil	1.0	1.0	87
Ethambutol	1.0	– [a]	86
AMA	0.001	1.0	– [a]
AMA	0.1	– [a]	59
AMA	0.5	– [a]	31

The specific activity of the control for *M. bovis* as in table 2 and for *E. coli* 32.2 nmol of CO_2 per 60 min per mg of protein. Other details as in table 1. MGBG, methylglyoxal bis(guanylhydrazone); AMA, S-(5'-deoxy-5'-adenosyl)methylthioethylhydroxylamine. [a] Not measured.

We next studied the characteristics of AMDC from *M. bovis* (BCG). Table 2 shows that it did not need Mg^{2+} or Mn^{2+} for its full activity like all other AMDCs from bacteria studied so far[2,8,14]. However, it was interesting to see that at 1 mM Ca^{2+} and especially Zn^{2+} clearly inhibited AMDC activity in vitro (table 2). The inhibition caused by Zn^{2+} was concentration-dependent (table 2). Table 3 shows the effect of various known inhibitors of AMDC on mycobacterial AMDC. AMA (a derivative of APA[13]) caused considerable inhibition at concentrations between 0.1 and 0.5 mM and totally inhibited AMDC from *E. coli* at a concentration of 1 μM (table 3). However, MGBG and Berenil, which are known to inhibit mammalian and bacterial AMDC very strongly at micromolar concentrations[2,15] (table 3), caused only 20–30% inhibition at concentrations of 1 mM.

M. bovis has an ADC which does not need Mg^{2+}-ion for activity as do other bacterial ADCs[2,16]. Difluoromethylarginine (DFMA), which is a potent irreversible inhibitor of other ADCs[2,17], did not inhibit ADC from *M. bovis* at concentration of 1 mM, and α-methylarginine (a competitive inhibitor of ADC[17]) also did not inhibit the mycobacterial ADC (results not shown).

Our results indicate that the three decarboxylases (ODC, AMDC and ADC) involved in the biosynthesis of spermidine in *M. bovis* (BCG) are very different from the enzymes from other bacteria (and mammals). Since we have shown earlier that ethambutol, which is a drug widely used against human tuberculosis[3], inhibited specifically spermidine synthase from *Mycobacteria*[4,5], it is probably possible to find a specific drug against decarboxylases from *Mycobacteria*.

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A practicable variant of the ion exchange method for the radiometric estimation of ornithine decarboxylase activity

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Summary. A known ornithine decarboxylase assay working with ion exchange separation of [^{3}H]ornithine and [^{3}H]putrescine has been revised. The assay can be performed in disposable 1.5 ml vessels with a total of four pipetting steps. The separation of enzyme substrate and product, respectively, requires 3 h per 50 samples. The detection limit is about 50 pmoles [^{3}H]putrescine formed.
Key words. Ornithine decarboxylase; radiometric enzyme assay; ion exchange separation; Michaelis constants; pyridoxal phosphate.

Ornithine decarboxylase activity (L-ornithine carboxy-lyase, EC 4.1.1.17) is usually estimated with the $^{14}CO_2$-trapping method proposed by Morris and Pardee[1]. The preparation of this enzyme assay requires considerable experimental expenditure and equipment for the release and quantitative absorption of $^{14}CO_2$ in one incubation vessel. Numerous variants of the procedure have been published. Alternative methods are based on the separation of ornithine and putrescine by ion exchange procedures. They have the advantage of measuring exclusively the product formed by the action of ornithine decarboxylase. One method, originally published by Clark[2] and improved by Djurhuus[3], works with cation exchange paper and is suitable for small volume samples. Maderdrut and Oppenheim[4] presented another radiometric method in which non-reacted enzyme substrate, [^{3}H]ornithine, is separated from the reaction product, [^{3}H]putrescine, by an acidic ion exchange resin. Although this method works reliably with simple laboratory equipment and little experimental expenditure for the enzyme assay, it has not come into use. This may be due to some shortcomings of the procedure which can easily be overcome, as will be detailed in the following.

Materials and methods. Brains of young Wistar rats (typically 10 days old, weighing 850–1000 mg) were homogenized with 10 strokes of a Potter-Elvehjem homogenizer in 10 vols of 50 mM sodium phosphate buffer pH 7.4, 2 mM dithiothreitol. Homogenates were centrifuged for 30 min at 45,000 xg or for 12 min at 10,000 xg, resulting in protein concentrations of about 2.5 mg/ml and 3.5 mg/ml, respectively. 0.4-ml aliquots of the supernatants were combined in 1.5-ml reaction vessels with 0.1 ml of an assay medium to result in final concentrations of 0.1 mM ornithine hydrochloride, 0.1 mM pyridoxal phosphate, and 2 mM dithiotreitol in 50 mM sodium phosphate buffer pH 7.4. Blanks additionally contained 5 mM semicarbazide hydrochloride (to destroy the cofactor, pyridoxal phosphate). The samples were preincubated at 37 °C for 15 min (Thermostat 5320, Eppendorf Gerätebau, Hamburg/FRG). 1 µCi of purified[4] L-[2,3-^{3}H]-ornithine (specific activity: 15–30 Ci/mmol, New England Nuclear, Dreieich/FRG) was added in 20 µl of 50 mM sodium phosphate buffer pH 7.4 (final specific activity: 20 µCi/µmole ornithine). The reaction was terminated by addition of 0.5 ml 10 mM semicarbazide hydrochloride in 0.4 M ammonium acetate buffer pH 6.5 (2 °C). In experiments where ion exchange separation was tested homogenate, blank medium, stopping mixture, 1 µCi L-[2,3-^{3}H]ornithine, and 0.1 µCi [1,4-^{14}C]putrescine (specific activity: 80–120 mCi/mmol, New England Nuclear) were mixed and used without incubation at 37 °C.

A slurry of 30 g Amberlite CG-50 ion exchange resin (H^+-form, 100–200 mesh, Sigma Chemie GmbH, München/FRG) was prepared for use by 4 washings each with 400 ml 0.2 M ammonium acetate pH 6.5, 1 mg/ml EDTA[4] and filled into glass-wool-plugged pasteur pipettes to give columns of 3 cm height. Each column was washed with 2 ml of 0.2 M ammonium acetate buffer pH 6.5 (without EDTA). The complete enzyme assay mixture was applied to a column. To remove unreacted [^{3}H]ornithine the resin was washed with 12 ml 0.2 M ammonium acetate buffer pH 6.5 (degassed by suction) followed by 2 ml 0.2 M acetic acid. [^{3}H]Putrescine was eluted with 3 ml 8 M formic acid directly into scintillation vials.

Each eluate was mixed with 15 ml of a scintillation cocktail (Aquasol-II, New England Nuclear) and counted in a β-scintillation spectrometer, ^{3}H-counting efficiency 29.5%. Using freshly purified [^{3}H]ornithine blank counting rates were below 500 cpm, i.e., 0.1% of the added ^{3}H-label. The rates increased

Table 1. Influence of pyridoxal phosphate concentrations on enzyme activity

Ornithine [mM]	0.1	0.1	2	2
Pyridoxal phosphate [mM]	0.1	2	2	0.1
Putrescine formed* [nmoles/h/mg protein]	0.96 ± 0.04	0.26 ± 0.02	2.08 ± 0.08	1.86 ± 0.08

* Mean ± SD of 3 experiments. Assay conditions: 45,000 xg supernatant, 2.1 mg protein per ml. 2 mM dithiothreitol, 50 mM sodium phosphate buffer pH 7.4. Incubation time 4 h.

Table 2. Ornithine decarboxylase activity in postnatal rat brain

Postnatal age (days)	2	4	8	16
Brain weight (mg)	307 ± 6	499 ± 19	778 ± 42	1241 ± 45
Putrescine formed* (nmoles/h/mg protein)	1.16 ± 0.11	0.73 ± 0.07	0.37 ± 0.02	0.033 ± 0.004

* Mean ± SD, double estimations from 3 brains. Assay conditions: 10,000 xg supernatants, 3.1–3.6 mg per ml. 0.1 mM ornithine, 0.1 mM pyridoxal phosphate, 2 mM dithiothreitol, 50 mM sodium phosphate buffer pH 7.4. Incubation time 3–4 h.