Specific activity of methanol dehydrogenase in cell-free extracts of methanol-utilizing yeast

Culture	System	Type of electron acceptor	Cells grown	
			Methanol	Glucose
Pichia pinus	pH 7.0	NAD (at 340 nm)	32[a]	0
	pH 8.5	NAD (at 340 nm)	72	0
	pH 7.0	DCPIP (at 600 nm)	621	0
	pH 8.5	DCPIP (at 600 nm)	870	0
Kloeckera sp. 2201	pH 7.0	NAD (340 nm)	80	0
	pH 8.5	NAD (340 nm)	64	0
	pH 7.0	DCPIP	50	0
	pH 8.5	DCPIP	435	0

[a] Specific activity in nmoles/min/mg protein.

et al. may be due to differences in procedures employed in the preparation of the cell-free extract. OGATA et al. prepared the cell-extract by grinding cells in a mortar with alumina for 5 h at 4 °C, while in the present report, cell-extracts were prepared by sonication.

The results reveal that methanol dehydrogenase activity was inducible since dehydrogenase activity was not found in cultures propagated on glucose. In summary, an inducible methanol dehydrogenase has been detected in cellular extracts of 2 methanol assimilating yeasts, *Pichia pinus* and *Kloeckera sp.* 2201. They were found to be linked to either NAD or DCPIP. Nicotinamide adenine dinucleotide phosphate did not serve as an electron acceptor. This is the first report describing NAD-linked methanol dehydrogenase activity in yeast[5].

Zusammenfassung. Eine induzierbare Methanoldehydrogenase wurde in Zellextrakten von 2 Methanol assimilierenden Hefen, *Pichia pinus* und *Kloeckera sp.* 2201, festgestellt, die entweder von NAD oder DCPIP abhängig waren. Nicotinamid Adenin-Dinukleotidphosphat diente jedoch nicht als Elektronen-Akzeptor.

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M. G. Science Institute, Ahmedabad Gujarat (India), 21 October 1974.

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Metabolism in Porifera IV. Biosynthesis of the 3β-Hydroxymethyl-A-nor-5α-Steranes from Cholesterol by *Axinella verrucosa*

Sponges contain a great variety of sterols, which vary from one species to another[1]; knowledge of this subject has increased rapidly in recent years[2,3]. Besides conventional sterols, unusual sterols have been found. For example, aplysterol and 24(28)-didehydroaplysterol, having the cholesterol nucleus and side-chains in which an 'extra' carbon atom is attached at C-26[4], are the major sterol components in the family Verongidae[3]. Modifications of the sterol nucleus have also been found[5]. The total sterol content of *Axinella polypoides* is a mixture of 19-nor-stanols, which combine the unusual 19-nor-cholestanol nucleus with conventional saturated and Δ^{22}-unsaturated C7 (24-nor), C_8, C_9 and C_{10} side-chains[6], while *Axinella verrucosa*, in which the usual sterols are also absent, contains stanols with a 3β-hydroxymethyl-A-nor-5α-cholestane nucleus carrying C_8, C_9 and C_{10} side-chains (**1–6**)[7].

On the other hand, there is little information about the origin of sterols in sponges. We have recently shown by tracer experiments that *Verongia aerophoba* does not incorporate mevalonate into aplysterol[8]. We now report the conversion of [4-^{14}C]-cholesterol into 3β-hydroxymethyl-A-nor-5α-steranes by *A. verrucosa*. The sponge was also fed with [1-^{14}C]-acetate, and the radioactivity in the fatty acids and stanols was measured.

The labelled substrates were fed to the sponge maintained in well-aerated sea water at 14 °C by addition of aqueous (acetate) and ethanolic (cholesterol) solutions to the aquaria. Sterols were recovered from the light petroleum extract of the lyophilized tissues, while fatty acids were obtained from the subsequent chloroform-methanol extract by saponification, and then purified, after conversion into methyl esters, by chromatography on silica followed by distillation at 250 °C (experimental details are given in reference[8]). The light petroleum extract, after addition of carrier cholesterol in the case of the cholesterol incubations, was chromatographed on silica[9].

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[9] The 3β-hydroxymethyl-A-nor-5α-steranes are less polar than cholesterol (Rf on silica gel tlc in chloroform 0.45 as against Rf 0.4).

The cholesterol fractions were crystallized to constant specific activity, while the radiochemical purity of the A-nor-steranes fraction was established by crystallization and formation of derivatives. Portions of the A-nor-steranes fraction, after crystallization, were hydrogenated on palladium-charcoal and then oxidized with chromic acid to yield the carboxylic acids (part structure **7**). The latter, after addition of carrier 5α-cholestan-3-one in the case of cholesterol incubations, were chromatographed on silica and the acid fraction recovered was crystallized and methylated with diazomethane to yield the corresponding esters (part structure **8**). After each conversion (experimental details in reference [7]) the compounds were purified by chromatography and crystallization and measured for ^{14}C-radioactivity in a Beckmann LS-250 liquid scintillation system. The results are given in Tables I–III.

Acetate is incorporated efficiently into fatty acids, but utilized only to a very small extent for the biosynthesis of 3β-hydroxymethyl-A-nor-5α-steranes (Table I), suggesting that there is little or no de novo sterol biosynthesis. On the other hand, when cholesterol-4-^{14}C was administered to the sponge, the hydroxymethyl-A-nor-5α-steranes were very significantly labelled (Tables II and III). The distribution of radioactivity in the 3β-hydroxymethyl-A-nor-5α-sterane sample (290 h incubation), determined by radiogaschromatography on the acetates, revealed that all of this radioactivity was associated with 3β-acetoxymethyl-A-nor-5α-cholestane (**1**)[10].

These results indicate that *A. verrucosa* readily transforms the cholesterol nucleus into the 3β-hydroxymethyl-

[10] Carlo Erba gaschromatograph, model GV, equipped with a flame ionization detector and connected with a Nuclear Chicago flow counter, model 4998, was utilized and the analyses were performed using a 2 m × 8 mm column packed with 2% OV-17 on gaschromo programmed temperature from 220° to 260°C at 1.50/min. We thank Dr. G. Galli (Istituto di Farmacologia e Farmacognosia, Facoltà di Farmacia, Università di Milano) for radiogaschromatographic measurements.

Table I. Incorporation of label from [1-^{14}C]-acetate into fatty acids and 3β-hydroxymethyl-A-nor-5α-steranes by *A. verrucosa* [a]

	Weight (g)		dpm/mg	
	48 h	290 h	48 h	290 h
Lyophilized animals	12.2	20.4	—	—
Fatty acid methyl esters	0.20	0.21	30,170	28,700
Crude stanol fraction	0.11	0.17	—	—
After recrystallization (**1–6**)	0.06	0.11	43	120
After conversion to carboxylic acids (**7**)	0.04	0.07	36	86
After conversion to carboxylic acid methyl esters (**8**)	0.030	0.05	35	81

[a] [1-^{14}C]-acetate(62 mCi/mmole; 0.1 mCi) was fed to the animals by addition of 5 ml aqueous solution to the aquarium (10 l). 48 h after the administration, ca. half of the animals were taken, washed and frozen at −20 °C and the remaining animals were killed after 290 h incubation.

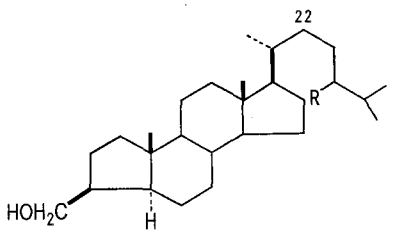
Table II. Incorporation of [4-^{14}C]-Cholesterol into 3β-Hydroxymethyl-A-nor-5α-steranes by *A. verrucosa* [a]

Period of incubation (h)	Lyophilized animals (g)	Total fed (dpm)	Total sterol recovered (mg)	(dpm)	Radioactivity recovered (%)	Radioactivity in precursor (%)	in 3β-Hydroxymethyl-A-nor-5α-steranes (%)
48	22	5.55×10^8	180	3.34×10^7	6.0	63	37
290	54.3	5.55×10^8	440	1.13×10^8	20.4	34	66

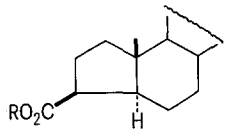
[a] [4-^{14}C]-cholesterol (61 mCi/mmole) was fed to the animals by addition of 2 ml ethanolic solution to the aquarium (50 l): 48 h after the administration, ca. half of the animals were taken, washed and frozen at −20 °C, and the remaining animals were killed after 290 h incubation.

Table III. Purification of 3β-hydroxymethyl-A-nor-5α-steranes from [4-^{14}C]-cholesterol incubations to constant specific activity

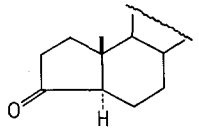
	dpm/mg		
	Free stanols (**1–6**)	Carboxylic acids (**7**)	Methyl Esters (**8**)
1st Feedings (48 h)	5.5×10^4	5.7×10^4	5.8×10^4
2nd Feeding (290 h)	1.6×10^5	1.7×10^5	1.7×10^5



1, R=H
2, R=H, Δ^{22}
3, R=Me
4, R=Me, Δ^{22}
5, R=Et
6, R=Et, Δ^{22}



7, R=H
8, R=Me



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A-nor-5α-cholestane nucleus, and suggest that in the sponge these unique A-nor-stanols (**1–6**) arise mainly by modification (ring-A contraction) of dietary sterols.

To obtain some information on the nature of this ring contraction, portions of the labelled stanols deriving from 290 h incubation with [4-^{14}C]-cholesterol (ca. 10 mg) were added to a mixture of carrier A-nor-stanols (ca. 300 mg), hydrogenated on palladium-charcoal and converted to the nor-ketones (part structure **9**). The conversion was accomplished (see reference [7]) by oxidation with dicyclohexylcarbodiimide/dimethylsulphoxide of the free stanols to the corresponding aldehydes, which were then treated with isopropenyl acetate and sulphuric acid, and the resulting enolacetates oxidized with ozone to yield the nor-ketones. The stanol mixture had a specific radioactivity of 4.22×10^4 dpm/mg and all of this was recovered in the nor-ketone mixture (specific activity 4.31×10^4 dpm/mg), showing that in the ring-contraction carbon-4 of the cholesterol nucleus is not lost, nor does it furnish the 3β-hydroxymethyl carbon of the A-nor-cholestane skeleton.

Riassunto. La spugna *Axinella verrucosa* trasforma il [4-^{14}C]-colesterolo nel 3β-idrossimetil-A-nor-5α-colestano (**1**), mentre utilizza 1′[1-^{14}C]-acetato per la sintesi dei 3β-idrossimetil-A-nor-sterani (**1–6**) in misura trascurabile. Si suggerisce che questi unici stanoli si originino principalmente per modificazione di steroli dietarici. Il carbonio-4 del nucleo del colesterolo non è nè perso nè dà origine al carbonio 3β-idrossimetilico dello scheletro A-nor-colestanico.

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Synthesis and Activity of Nonapeptide Fragments of Soybean Bowman-Birk Inhibitor

Bowman-Birk inhibitor (BBI)[1] has been known as a double-headed proteinase inhibitor which inhibits trypsin and chymotrypsin at two non-overlapping reactive sites[2]. The primary structure of BBI has been determined by ODANI and IKENAKA[3], Lys-Ser (16–17) and Leu-Ser (43–44) bonds having been estimated as antitryptic and antichymotryptic sites respectively[3,4]. The same authors have further divided the inhibitor molecule, consisting of 71 amino acid residues, into 2 fragments: one consists of 38 residues and the other 29 residues, each of which retains the inhibitory activity on trypsin or chymotrypsin, respectively[5].

In order to find out a smaller active fragment, if any, and to investigate structure-activity relationship of natural proteinase inhibitor, we attempted to synthesize heterodetic cyclic peptides with a disulfide bond. The present communication reports the syntheses and inhibitory properties of cyclic nonapeptides, X-Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys-Y (Ia: X=Ac, Y=NH_2. Ib: X=H, Y=OH), which correspond to the nonapeptide sequence of -Cys-Thr-Lys-Ser-Asn-Pro-Pro-Gln-Cys- (14–22) of BBI containing the antitryptic site.

Material and method[6]. The protected nonapeptide resin, Boc-Cys(4-OMe-Bzl)-Thr(Bzl)-Lys(2,4-Cl_2Z)-Ser(Bzl)-Asn-Pro-Pro-Gln-Cys(4-OMe-Bzl)-resin (II) was synthesized by Merrifield's solidphase method in a stepwise fashion starting with 2.19 g of Boc-Cys(4-OMe-Bzl)-resin containing 1 mmole of S-4-OMe-Bzl-cysteine. The Boc-amino acids with protected side chains were: Lys(2,4-Cl_2Z)[7], Ser(Bzl), Thr(Bzl) and Cys(4-OMe-Bzl). The coupling reactions to form peptide bonds were mediated by DCC in CH_2Cl_2 for 4 h, except in the case of Boc-Thr (Bzl) and Boc-Cys(4-OMe-Bzl), which were allowed to react for 12 h. Introduction of Boc-Gln and Boc-Asn was carried out over 12 h with the corresponding *p*-nitrophenyl esters in addition to hydroxybenzotriazole[8]. Boc groups were removed with 1 *N* HCl-AcOH, exceptionally with 50% trifluoroacetic acid in CH_2Cl_2 for Boc-Gln and Boc-Asn residues. The weight of finally obtained II was 3.24 g. The weight gain of 1.05 g (0.77 mmol), at this stage, indicated a 77% incorporation of protected peptide based on the initial Boc-Cys (4-OMe-Bzl) content in the resin.

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