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Influence of Cobalt on Fermentative Methylation

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ABSTRACT

CLARIDGE, C. A. (Bristol Laboratories, Syracuse, N.Y.), V. Z. ROSSOMANO, N. S. BUONO, A. GOUREVITCH, AND J. LEIN. Influence of cobalt on fermentative methylation. Appl. Microbiol. **14**:280–283. 1966.—*Streptomyces rishiriensis* produces at least five closely related antibiotics. Strain selection yielded a culture producing only the most active component, coumermycin A. Hydrolysis of this antibiotic by barium hydroxide yielded both 5-methyl-pyrrole-2-carboxylic acid and pyrrole-2-carboxylic acid, which could be separated by paper chromatography. Coumermycin A was thus shown to be two fractions, designated A_1 and A_2 depending upon the nature of the pyrrole carboxylic acid portion. The addition of cobalt to the fermentation medium at a level as low as 0.01 μg/ml shifted the fermentation exclusively to the production of coumermycin A_1. Other ions were ineffective, except nickel, whose activity could be explained by the presence of contaminating cobalt.

A new antibiotic, coumermycin, produced by *Streptomyces rishiriensis*, was recently described by Kawaguchi et al. (5). It is active against a wide variety of gram-positive and gram-negative organisms, with *Staphylococcus aureus* being particularly sensitive to it.

During fermentation studies with coumermycin, it became evident that other closely related antibiotics were being formed. These antibiotics have been separated by paper chromatography and have been designated A, B, C, and D in order of movement from the origin. Studies by Kawaguchi et al. (5) have shown that coumermycin A, the most active of the four components, consists of two fractions, A_1 and A_2 (3, 4), having the structure shown in Fig. 1.

Coumermycin A_1, containing 5-methyl-pyrrole-2-carboxylic acid, is found to be considerably more active against *S. aureus* than coumermycin A_2, which contains pyrrole-2-carboxylic acid. Fermentation studies were therefore directed toward obtaining coumermycin A_1, in the absence of coumermycin A_2. The introduction of the methyl group on the pyrrole-2-carboxylic acid to form coumermycin A_1 was found to be highly dependent upon the presence of cobalt; this paper gives the results of some of these fermentation studies.

MATERIALS AND METHODS

Assay of total coumermycins. The fermentations were carried out in 125-ml Erlenmeyer flasks containing 25 ml of medium incubated on a New Brunswick rotary shaker at 27 C for 6 to 8 days. Because the antibiotic is intimately associated with cellular material, it is necessary to extract the whole broth with a solvent such as methyl ethyl ketone (MEK) to detect all of the activity. The coumarin fraction will absorb in the ultraviolet region with a maximum at 340 mμ, and this fact was employed to establish a rapid spectrophotometric assay. Suitable dilutions of the MEK extract were prepared in ethyl alcohol containing 0.5% HCl, and the optical densities obtained were compared with appropriate standards. A typical standard curve is shown in Fig. 2.

Paper chromatography. The various coumermycins could be separated by paper chromatography. Schleicher and Schuell 589 Blue Ribbon paper (12-mm wide strips) was spotted with the MEK extract of the broth and then developed descendingly for 18 hr at 22 to 23 C in a system composed of acetone and 0.1 M triethanolamine previously adjusted to *p*H 7 with glacial acetic acid (2:3). After the solvent had evaporated, the strips were placed on the surface of large trays (41 by 33 cm) containing an agar medium seeded with *S. aureus* ATCC 6538P. These trays were placed at 4 C for 24 hr to permit diffusion of the antibiotic, and then were incubated at 37 C for 18 hr. A typical bioautograph is shown in Fig. 3.

These various coumermycin fractions could also be located by scanning chromatographed 25-mm wide paper strips with a Photovolt Densicord fitted with a continuous feed device. An ultraviolet lamp and a detector fitted with a 340-mμ filter allowed measurement of the coumarin fraction of the antibiotics. A typical chromatogram scan of this type is shown in Fig. 4.

5-Methyl-pyrrole-2-carboxylic and pyrrole-2-carboxylic acid determinations. Coumermycin A can be

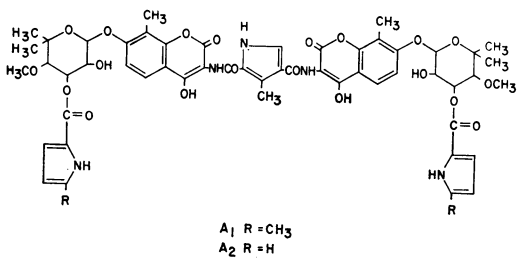

FIG. 1. *Structure of coumermycin.*

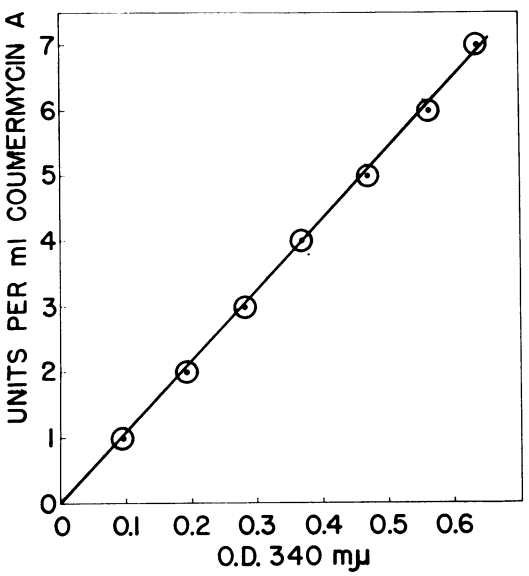

FIG. 2. *Standard curve for coumermycin A assay. Samples read on a Bausch & Lomb Spectronic-20 colorimeter.*

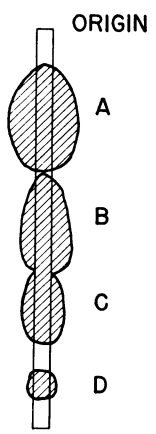

FIG. 3. *Bioautograph of coumermycin fermentation broth containing the active components. Test organism, Staphylococcus aureus ATCC 6538P.*

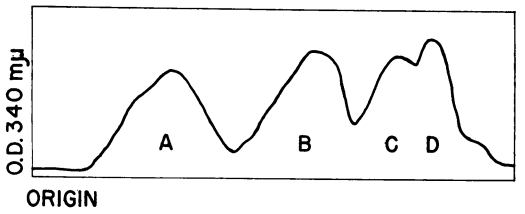

FIG. 4. *Scan of paper chromatogram on Photovolt Densicord at 340 mμ of coumermycin fermentation broth.*

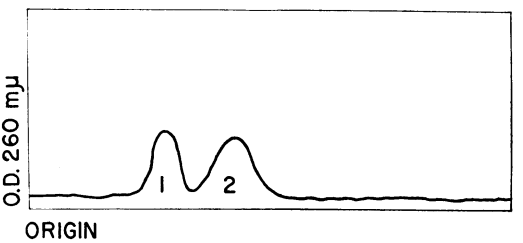

FIG. 5. *Scan of paper chromatogram on Photovolt Densicord at 260 mμ of hydrolyzed coumermycin A. (1) Pyrrole-2-carboxylic acid; (2) 5-methyl-pyrrole-2-carboxylic acid.*

hydrolyzed with $Ba(OH)_2$ to yield the 5-methyl-pyrrole-2-carboxylic acid and pyrrole-2-carboxylic acid fractions. A 10-ml amount of the MEK extract used for total assay was mixed with 1 ml of a saturated $Ba(OH)_2$ solution and heated in an autoclave with free flowing steam for 30 min. To the residue was added 1 ml of distilled water, and this solution was then adjusted to *p*H 6 to 6.5 with solid carbon dioxide. Approximately 100 μliters of this solution was spotted on 25-mm wide strips of Whatman no. 1 paper, and then developed descendingly at 22 to 23 C for 18 hr in a *n*-butanol–water–diethylamine (100:15:1) system.

The two pyrrole carboxylic acids could be located by spraying the strips with Ehrlich's reagent (*p*-dimethylaminobenzaldehyde in HCl). However, the colors formed are not stable, and thus are not suitable for quantitation. The pyrrole nucleus, however, will absorb in the ultraviolet in the region of 260 mμ. A Photovolt Densicord fitted with a shortwave ultraviolet lamp and 260-mμ filter will measure these two acids. A scan of a hydrolyzed sample showing both acids is given in Fig. 5. The pyrrole-2-carboxylic acid absorbs proportionately more strongly at this wavelength than does the 5-methyl derivative, so it is necessary to apply a correction factor when determining the amounts of these two acids in fermentation broth.

The metallic salts and vitamin B_{12} used were commercially available compounds.

RESULTS

Our earlier fermentation studies were done with cultures producing all of the coumermycin fractions. A mutant was obtained through ultra-

violet irradiation which produced essentially only coumermycin A. Chemical degradation of this fraction revealed both pyrrole-2-carboxylic acid and 5-methyl-pyrrole-2-carboxylic acid. A typical medium supporting high yields of this antibiotic consisted of 4% starch, 4% lard oil, 2% cottonseed endosperm meal, 1.5% Anheuser-Busch Yeast K-2 chip 0.25%, K_2HPO_4, and 0.5% $CaCO_3$.

Since the desired coumermycin A_1 differed from coumermycin A_2 by being methylated, attempts were made to enhance the methylation in the fermentation by the addition of methionine, betaine, and lecithin at levels from 0.05 to 0.5%, as well as by addition of small amounts of cobaltous chloride. The organic adjuvants were ineffective, but cobalt alone shifted the fermentation almost exclusively to the formation of coumermycin A_1 (Table 1). In a medium without added cobalt, the coumermycin A_1 varied from 40 to 75% of the total coumermycin A. However, the addition of as little as 0.01 μg/ml of cobalt resulted in greater than 93% coumermycin A_1, the limit of sensitivity of the assay. Various other metallic ions, Fe^{++}, Fe^{+++}, Mn^{++}, Al^{+++}, Cr^{+++}, Cd^{++}, Cu^{++}, Mg^{++}, and Pb^{++}, at levels varying from 0.04 to 80 μg/ml, were tested for their ability to shift the fermentation to the production of coumermycin A_1, but were without effect.

Of the metallic ions tested, the only other ion producing an effect similar to cobalt was nickel, which required the addition of 20 μg/ml to the medium. However, the nickel chloride used contained 0.4% cobalt, and the observed effect could be explained by this contaminant.

Vitamin B_{12} was also added to the fermentation medium to determine possible stimulation of coumermycin A_1 formation (Table 1). The high level of this vitamin required to produce the same effect as cobalt could again be explained by the cobalt content (4.34%) in the molecule. The effect of cobalt in stimulating coumermycin A_1 formation may bear no relation to the role of vitamin B_{12} in *S. rishiriensis*. The level of vitamin B_{12} producing the effect is extremely high compared with the levels used either for growth stimulation in certain bacteria (9) or as a coenzyme in enzymatic reactions (2). However, the degree of permeability of the *S. rishiriensis* cells to vitamin B_{12} is not known, and this may be an important factor.

TABLE 1. *Effect of Co^{++}, Ni^{++}, and vitamin B_{12} on the 5-methyl-pyrrole-2-carboxylic acid (MP) content of coumermycin A**

Addition	Amt	Medium N1-72		Medium N6-113	
		Coumermycin A	MP	Coumermycin A	MP
	μg/ml	*units/ml*	%	*units/ml*	%
	None	212	76	460	60
Co^{++}	0.001	196	75	436	90
	0.005	216	90	436	91
	0.01	204	>93	440	>93
Ni^{++}	1	176	89	476	89
	5	160	92	444	89
	10	160	89	528	91
	20	304	>93	548	>93
Vitamin B_{12}	0.4	212	67	288	82
	2	264	84	468	70
	4	356	>93	420	88

* Medium N1-72 contains corn syrup, cottonseed meal, soybean meal, yeast, ammonium phosphate, and calcium carbonate. Medium N6-113 contains starch, lard oil, cottonseed meal, yeast, potassium phosphate, and calcium carbonate.

DISCUSSION

The extremely low levels of cobalt required to shift the fermentation almost exclusively to coumermycin A_1 suggests its action as an activating ion of a specific enzyme. Many enzymes require metal ions for activation, such as glycylglycine dipeptidase (11), malate dehydrogenase (10), pyruvate decarboxylase (1), dialykylfluorophosphatase (7), and trimetaphosphatase (6), all of which are activated by cobalt among other divalent cations. Acetylornithine deacetylase (12) from *Escherichia coli* is specifically stimulated by cobalt. Nicholas et al. (8) reported that cobalt at a level as low as 0.1 μg per liter in the culture medium of *Clostridium pasteurianum* allows nitrogen fixation by this organism. It was recently reported that cobalt at a level of 0.01 μg/ml will enhance several-fold the production of the water-soluble basic antibiotic, gentamicin, by *Micromonospora* species (Charney, U.S. Patent 3,136,704). Cobalt is not a requisite for growth of these organisms as it is with *C. pasteurianum*, but is believed to act by stimulating a specific enzyme necessary for the antibiotic production.

Buddhari (Ph.D. Thesis, Univ. California, Davis, 1960) established that algal species of *Anabena* and *Nostoc* needed traces of cobalt for growth. The cobalt requirement was highly specific, since no other element in a series tested would replace it. Cobalt added in the form of cyanocobalamin, however, was more effective at low concentrations than an equal amount of the element in the form of inorganic cobalt salts. Such is not the case with *S. rishiriensis*, for more

cobalt in the form of B_{12} is required to produce >93% coumermycin A_1 than inorganic cobalt (Table 1).

Vitamin B_{12} acts as a coenzyme of methyl group transfer (2), and the possibility of the added cobalt being required for formation of this coenzyme cannot be ruled out on the basis of these results. The formation of coumermycin A_1 from coumermycin A_2 by a methyl group transfer would be a convenient explanation for the origin of the coumermycin A_1, although this methyl transfer may occur at stages earlier during the formation of the molecule than the last step. It is also possible that the formation of the methylpyrrole derivative arises by a different pathway than the pyrrole, a pathway which is mediated by an enzyme requiring cobalt ion as an activator.

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