JOURNAL OF BACTERIOLOGY, Mar. 1969, p. 1155-1159

Vol. 97, No. 3
Printed in U.S.A.

Role of Glycols and Tweens in the Production of Ergot Alkaloids by *Claviceps paspali*

A. MIZRAHI AND G. MILLER
Israel Institute for Biological Research, Ness-Ziona, Israel

Received for publication 17 December 1968

Several glycols and Tweens markedly stimulated the production of ergot alkaloids in submerged cultures of *Claviceps paspali*. The role of these compounds was investigated in shake flasks and bench-scale fermentors. 2,3-Butanediol was not utilized by the fungus, and *1,2*-propanediol-*1*-^{14}C was not incorporated into the alkaloids. Glycols and Tweens lowered the surface tension of the basal medium and promoted the utilization of metabolites. In the presence of glycols and Tweens, an increased uptake of labeled sorbitol and succinic acid took place, whereas the specific radioactivity of the alkaloids was not affected. These results indicated that glycols and Tweens are not involved directly in the biosynthetic process; they apparently acted as surface-active agents, facilitating transport of metabolites into the cells.

Gröger and Tyler (5) found that the addition of 1,2-propanediol to the medium of Arcamone et al. (1) promoted the production of alkaloids by *Claviceps paspali*. In a previous communication, it was reported that several normal and cyclic glycols markedly increased the production of alkaloids (11). The purpose of this investigation was to study the role of the glycols in the biosynthesis of ergot alkaloids. Two possibilities were investigated: (i) that the glycols are metabolites or precursors; and (ii) that they act as surfactants, affecting the uptake of nutrients by the mycelium. Since the second hypothesis appeared more promising, the effect of Tweens, which are typical surfactants, on the production of alkaloids was studied as well.

MATERIALS AND METHODS

Chemicals. We acquired chemicals from the following sources: Antifoam M8-J from Hodag Chemical Corp. (Chicago, Ill.); Silicone Emulsion RD from Midland Silicones Ltd. (London, England); Polyglycol 2000 from Dow Chemical Company (Midland, Mich.); *1,2*-propanediol-*1*-^{14}C (2.6 mc/mmole) from Philips-Duphar (Petten, Holland); D-sorbitol-*1*-^{14}C (3 mc/mmole) and succinic acid-*2*,3-^{14}C (16.7 mc/mmole) from Radiochemical Centre (Amersham, England); and aliphatic glycols from Fluka A. G. (Buchs, Switzerland). Cyclic glycols were a gift from Robinson Brothers Ltd. (West Bromwich, England). Tweens were purchased from Koch-Light Laboratories Ltd. (Colnbrook, England), and ergometrine maleate was from British Drug Houses Ltd. (Poole, England).

Organism. The *C. paspali* strain used was isolated from a sclerotium found in Israel on *Paspalum distichum*.

Media. The fungus was kept on slants of Potato Dextrose Agar (Difco). Seeds for submerged cultures were grown in medium A of Arcamone et al. (1). The basal medium for production of alkaloids was medium D of Kobel et al. (8), but it was prepared in tap water. Alternative production media are described in Results and Discussion; these contained additions of single glycols or Tweens, and the concentrations are indicated by percentage (v/v). For experiments in fermentors, 100 μliters/liter of Antifoam M8-J was added to the medium before sterilization.

Propagation methods. All cultures were incubated in darkness at 24 C. Most experiments were carried out in duplicate in 500-ml Erlenmeyer shake flasks. Each flask contained 50 ml of medium, unless otherwise specified. Cultures were agitated on a rotary shaker (12), with a radius of eccentricity of 44 mm, at 180 rev/min. Some production experiments were undertaken in bench-scale fermentors (Model FS-307, New Brunswick Scientific Co., New Brunswick, N.J.). In this case, 4-liter cultures were agitated at 300 rev/min, and 8 liters of air per min was supplied. Seeds for shake flasks and fermentors were prepared as described by Arcamone et al. (1). Production cultures were incubated for 11 to 12 days.

Alkaloids. Total alkaloids in the culture filtrate were determined colorimetrically with the *p*-dimethylaminobenzaldehyde reagent of Ehrlich-van Urk (17). A calibration curve was prepared with ergometrine maleate. Results are given in micrograms of ergometrine (free base) per milliliter of culture filtrate.

Thin-layer chromatography (TLC) of alkaloids. The alkaloids were extracted from the culture filtrate by a chloroform-methanol mixture (4:1, v/v) at *p*H 7 to 8. The extract was concentrated in vacuum (Rota-

vapor, Büchi Scientific Apparatus, Flavil, Switzerland), and the crude concentrate was applied to TLC plates. TLC was carried out by the method of McLaughlin et al. (9) on Silica Gel G (E. Merck, Darmstadt, Germany). The solvent system was chloroform-benzene-methanol (3:1:1; N. Castagnoli, *personal communication*). The alkaloid spots were located under ultraviolet (UV) light, collected by scraping, and then pooled.

Radioactivity experiments. The uptake of labeled sorbitol and succinic acid by the mycelium was studied in shake flasks. The additions to the basal medium (glycols, Tween 80, or labeled compounds) were made before sterilization. Labeled sorbitol or succinic acid were added at the rate of 1 μc/100 ml of medium. For each experimental condition, several flasks were run in parallel. At the time periods indicated (Fig. 2) for each condition, duplicate flasks were removed from the shaker for investigation. The mycelium was separated on an HA membrane filter (Millipore Corp., Bedford, Mass.) and washed with distilled water until no counts were detected in the wash water. Then the radioactivity of the mycelium was measured.

For radioactivity measurements, the samples (alkaloids from TLC or washed mycelium) were dispersed in 10 ml of Bray's scintillation solution (2) and counted in a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.).

Ammonium nitrogen. Residual ammonia in the culture filtrate was determined by the method of Conway (3).

2,3-Butanediol. The determinations of this glycol were carried out by Y. Klibansky who used a gas-liquid chromatographic technique (GLC). At the end of propagation in the basal medium with 0.5% of 2,3-butanediol, the mycelium was separated and washed with distilled water, and the wash water was collected. The glycol contents of the culture filtrate and wash water were determined by saturating a known volume (300 to 500 ml) with K_2CO_3, extracting with 300 ml of ether, drying the combined extracts over anhydrous Na_2SO_4, and removing the ether on the water bath. A precise quantity (6 to 16%, w/w) of xylene, as an internal standard, was added to the weighed residue, and 1- to 2-μliter samples were injected into the gas chromatograph. The value of the ratio, glycol peak area/xylene peak area, was taken as the mean from six separate injections. The corresponding value of weight of glycol/weight of xylene was obtained from a previously constructed calibration curve. Since the weight of xylene is known, the weight of glycol in the dried extract is readily computed.

The gas-chromatograph employed was the F & M model 500 (F & M Scientific Corp., Avondale, Pa.) equipped with a thermal conductivity detector. The column was a 2-m length of copper tubing (6.35 mm, outside diameter) loaded with 25% Carbowax (20 M) on alkali-treated Chromosorb W (60 to 80 mesh). The oven temperature was 180 C; the injection port temperature, 230 C; the detector block temperature, 250 C; and the carrier was helium at 40 ml/min (inlet pressure, 40 lb/in^2). A 1-mv strip-chart recorder (Minneapolis-Honeywell, Philadelphia, Pa.) was used. Peak areas were measured with a Ball and Disc integrator (Disc Instruments Ltd., Santa Ana, Calif.).

D-Sorbitol. Residual sorbitol in the culture filtrate was determined by the polarimetric method of Richtmyer and Hudson (13). The phosphomolybdate formed was removed as described by Arcamone et al. (1). A standard curve in the range of 0.2 to 4.0% (w/v) sorbitol was prepared.

Succinic acid. Residual succinic acid in the culture filtrate was assayed by the method of Slater (14), by the use of a succinic oxidase enzyme complex. This enzyme complex was freshly prepared from pig heart muscle (6). A standard curve was prepared by the use of known ammonium succinate solutions.

Surface tension. The surface tension of culture media at 20 C was determined by the method of Traube and Blumenthal (16) by means of a stalagmometer (Baird and Tatlock Ltd., Chadwell Heath, England).

RESULTS AND DISCUSSION

Metabolism and incorporation of glycols. Experiments were undertaken to investigate whether glycols are metabolized by the fungus. The fungus was grown in the basal medium with 0.5% 2,3-butanediol. This glycol was chosen because in a previous investigation (11) it produced the highest increase of alkaloids. At the end of propagation, the glycol was determined in the culture filtrate and wash water by GLC.

GLC has been employed in many cases for the identification and determination of glycols as free alcohols (15) and in aqueous solutions, sometimes down to parts-per-million range (4). Since the instrument available was equipped with a thermal conductivity detector, direct injection of dilute aqueous solutions seemed an unpromising approach. Therefore, recourse was taken to extraction of the glycol as described in Materials and Methods. In preliminary experiments with water and basal medium, both containing 0.5% glycol, virtually complete recovery was obtained by saturation with K_2CO_3 and extraction with ether. The accuracy of the GLC method was within 2 to 3% (relative) of the weight added.

After propagation, practically all of the added glycol (about 99%) was found in the culture filtrate and wash water. This indicates that the organism did not utilize the glycol.

However, one could argue that the 1% of glycol not accounted for by GLC may still have been utilized for synthesis of alkaloids. To test this possibility, the fungus was grown in the basal medium supplemented by 1,2-propanediol-*1*-^{14}C (2 μc/100 ml of medium). At the end of the fermentation process, the alkaloids were extracted and isolated by TLC. Radioactivity measurements showed that there was no incorporation of *1*-^{14}C of the glycol into the alkaloids.

These results enabled us to conclude that glycols are not directly involved in the biosynthesis of alkaloids.

Effect of Tweens on production of alkaloids. The second hypothesis was that glycols may act in this process as surface-active agents. In preliminary experiments, we found that the antifoams Silicone Emulsion RD and Polyglycol 2000, added in the range of 0.5 to 1.0% (v/v), increased the production of alkaloids. Thus, we decided to test the effect of Tweens, which are known as typical surfactants.

The results in Table 1 show that Tweens promote the production of alkaloids at a rate similar to that of glycols (11). Tweens 60 and 80 increased the production by 50 and 40%, respectively, already at a concentration of 0.02%.

The above results raised the possibility that Tweens may provide a source of fatty acids. The fatty acid components of the Tween molecules are: Tween 40, palmitic acid; Tween 60, stearic acid; and Tween 80, oleic acid. Therefore, experiments were undertaken in which the basal medium was supplemented by the sodium salts of these fatty acids (each in the range of 2 to 30 μg/ml of medium). No effect on the production of alkaloids was observed. Thus, the complete Tween molecule is required for the favorable effect.

The effect of glycols and Tweens on the surface tension of media was investigated at concentrations found to be optimal for alkaloid production.

Results in Table 2 show that all the compounds tested and found to promote alkaloid production lowered the surface tension of the basal medium.

The results described above lead to the conclusion that alkaloid production by *C. paspali* is stimulated by a wide range of surfactants which may affect several physical phenomena besides surface tension, e.g., wetting and permeability of the fungal cell wall. Kirby and Dubos (7) observed that Tween 80 increases the susceptibility of *Mycobacterium tuberculosis* to penicillin, probably by wetting of the hydrophobic bacteria.

TABLE 1. *Effect of Tweens on the production of alkaloids*

Tween	Alkaloids produced by per cent of Tween added (v/v)[a]							
	0	0.001	0.005	0.01	0.02	0.1	0.2	0.5
40	265	310	260	290	270	300	380	400
60	240	295	275	305	360	360	385	385
80	300	390	390	410	420	460	475	510

[a] Values are expressed as micrograms per milliliter. Results represent averages of duplicate shake flask cultures.

Effect of glycols and Tweens on the utilization of metabolites. The effect of glycols on the utilization of D-sorbitol, succinic acid, and ammonia was investigated in bench-scale fermentors (Fig. 1). The effect of Tween on the utilization of the same metabolites was studied in shake flasks, and the results are given in Table 3.

The results show that, in the presence of glycols and Tweens, the main metabolites were utilized by the fungus at a higher rate than in the basal medium. However, the effect on succinate utilization was less pronounced. It may be assumed that the surfactants facilitate transport of metabolites into the fungal cells, possibly by increasing the permeability of the cell wall.

Uptake and incorporation of sorbitol and succinic acid. The uptake of labeled sorbitol and succinic acid by the mycelium was studied during the lag phase (0 to 24 hr) so as to have a constant

TABLE 2. *Effect of glycols and Tweens on the surface tension of the basal medium*

Medium composition	Per cent added (v/v)	Surface tension (dynes/cm)
Tap water (control)	0	72.8
Basal medium (control)	0	96.4
Basal medium with:		
1,2-Propanediol	2.00	84.0
1,3-Butanediol	0.75	86.2
2,3-Butanediol	0.50	85.2
1,2-Cyclohexanediol	0.05	86.2
1,3-Cyclohexanediol	0.25	87.3
1,4-Cyclohexanediol	0.10	87.3
Tween 40	0.50	43.4
Tween 60	0.50	44.3
Tween 80	0.50	41.1

TABLE 3. *Effect of Tweens on alkaloid production and utilization of main metabolites*

Medium composition	Alkaloids produced[a]	Sorbitol[b]	Succinic acid[b]	Ammonia-N[b]
Basal medium (control)	230	35.0	33.0	88.2
Basal medium with:				
Tween 40 (0.5%)	290	22.4	28.3	63.4
Tween 60 (0.5%)	290	23.4	28.0	66.0
Tween 80 (0.5%)	290	24.0	26.9	68.0

[a] Values are expressed as micrograms per milliliter.

[b] Values are expressed as per cent of initial concentration found in the culture filtrate at the end of the fermentation process (residual concentration).

mycelial concentration in all samples. During the period under observation, the cultures contained about 0.08 g of dry mycelium per 100 ml (Fig. 2).

It may be seen that, in presence of glycols and Tween 80, the uptake of the metabolites by the mycelium was increased. It was shown in Table 2 that these compounds lower the surface tension of the basal medium. Tween 80, which caused the strongest depression of surface tension, also produced the highest increase in the uptake rate of the two metabolites.

To investigate whether the surfactants affect the biosynthetic process of alkaloids directly, the incorporation of labeled metabolites into the alkaloids was studied. At the end of the fermentation process (12 days), the alkaloids were isolated by TLC and their radioactivity was measured. Results (Table 4) show that the specific radio-

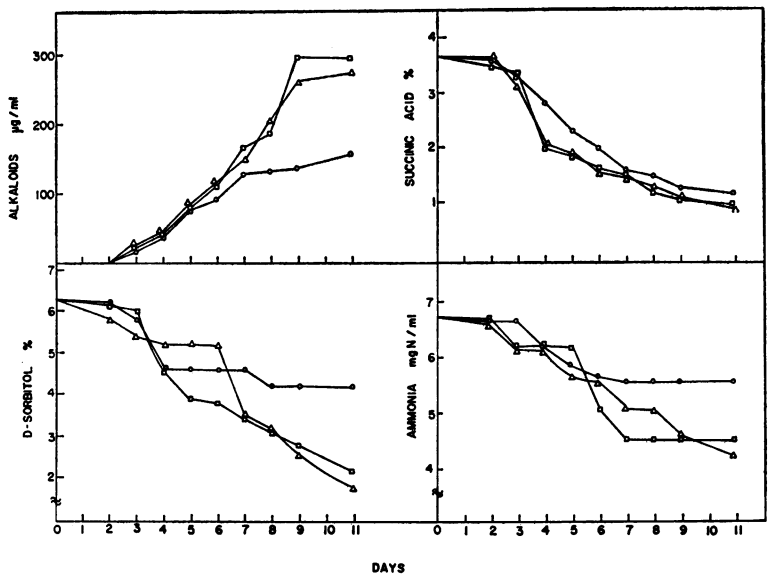

FIG. 1. *Effect of glycols on alkaloid production and utilization of main metabolites. Symbols:* ○, *basal medium;* △, *basal medium with 1.0% 1,2-propanediol; and* □, *basal medium with 0.5% 2,3-butanediol. Concentrations of metabolites found in the culture filtrate are given (sorbitol and succinic acid in per cent, w/v).*

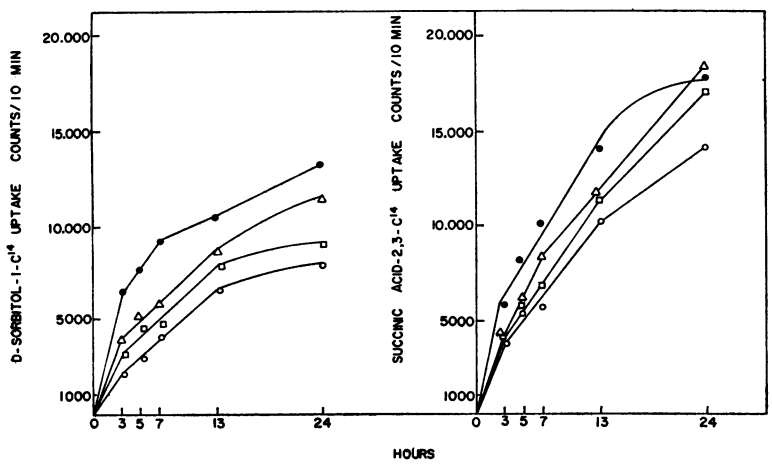

FIG. 2. *Effect of glycols and Tween 80 on the uptake by the mycelium of labeled sorbitol and succinic acid. Symbols:* ○, *basal medium;* △, *basal medium with 0.5% 2,3-butanediol;* □, *basal medium with 0.25% 1,3-cyclohexanediol; and* ●, *basal medium with 0.5% Tween 80. Radioactivity of washed mycelium from 50-ml culture is given as counts per 10 min.*

TABLE 4. *Effect of glycols and Tween 80 on the incorporation of labeled sorbitol and succinic acid into the alkaloids*[a]

Labeled metabolite	Medium composition	Alkaloids produced[b]	Counts/min of alkaloids	
			Per ml of filtrate	Per μg[c]
D-Sorbitol-*1*-^{14}C (1 μc/flask)	Basal medium (control)	287	17.0×10^3	60
	Basal medium with:			
	2,3-Butanediol (0.5%)	405	23.0×10^3	57
	1,3-Cyclohexanediol (0.25%)	360	21.0×10^3	58
	Tween 80 (0.5%)	410	24.0×10^3	58
Succinic acid-2,3-^{14}C (1 μc/flask)	Basal medium (control)	282	4.4×10^3	15
	Basal medium with:			
	2,3-Butanediol (0.5%)	420	6.8×10^3	16
	1,3-Cyclohexanediol (0.25%)	355	6.0×10^3	17
	Tween 80 (0.5%)	427	7.0×10^3	16

[a] Shake flask experiments, 100 ml of medium per flask.
[b] Values are expressed as micrograms per milliliter.
[c] Expressed as specific radioactivity of alkaloids.

activity of the alkaloids was not affected by the addition of surfactants to the medium, in spite of the marked increase in alkaloid production. One may conclude that the surfactants do not change the biosynthetic pathway.

In conclusion, the results presented in this paper suggest that glycols, Tweens, and antifoams operate as surface-active agents and facilitate the transport of metabolites into the fungal cell. The increased intracellular concentration of nutrients leads, in turn, to an improved alkaloid yield. Thus, the surfactants investigated may be classified as secondary factors in this fermentation process (10).

ACKNOWLEDGMENTS

We are indebted to S. Kindler for advice and criticism on the radioactivity experiments, and Y. Klibansky for the gas-liquid chromatographic determinations. We also thank M. Manai for his skillfull technical assistance.

LITERATURE CITED

1. Arcamone, F., E. B. Chain, A. Ferretti, A. Minghetti, P. Pennella, A. Tonolo, and L. Vero. 1961. Production of a new lysergic acid derivative in submerged culture by a strain of *Claviceps paspali Stevens & Hall*. Proc. Roy. Soc. (London) Ser. B. 155:26–54.
2. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279–285.
3. Conway, E. J. 1962. Microdiffusion analysis and volumetric error. Crosley Lockwood and Sons, London.
4. Davis, A., A. Roaldi, and L. E. Tufts. 1965. Determination of traces of glycols by gas chromatography. Develop. Appl. Spectro. 4: 289–298.
5. Gröger, D., and V. E. Tyler. 1963. Alkaloid production by *Claviceps paspali* in submerged culture. Lloydia 26:174–191.
6. Keilin, D., and E. F. Hartee. 1947. Activity of the cytochrome system in heart muscle preparation. Biochem. J. 41:500–502.
7. Kirby, W. M. M., and R. J. Dubos. 1947. Effect of penicillin on the tubercle bacillus in vitro. Proc. Soc. Exptl. Biol. Med. 66:120–123.
8. Kobel, H., E. Schreier, and J. Rutschmann. 1964. 6-Methyl $\Delta^{8,9}$-ergolen-8-carbonsäure, ein neues Ergolinderivat aus Kulturen eines Stammes von *Claviceps paspali Stevens et Hall*. Helv. Chim. Acta 47:1052–1064.
9. McLaughlin, J. L., J. E. Goyan, and A. G. Paul. 1964. Thin layer chromatography of ergot alkaloids. J. Pharm. Sci. 53:306–310.
10. Margalith, P. 1964. Secondary factors in fermentation processes. Advan. Appl. Microbiol. 6:69–90.
11. Mizrahi, A., and G. Miller. 1968. Effect of glycols on the production of ergot alkaloids by *Claviceps paspali*. Biotech. Bioeng. 10:102–103.
12. Paladino, S. 1954. A simple rotary shaker. Rend. Ist. Super. Sanità (Engl. Transl.) 17:145–148.
13. Rishtmyer, N. K., and C. S. Hudson. 1951. The rotation of polyols in ammonium molybdate solutions. J. Am. Chem. Soc. 73:2249–2250.
14. Slater, E. C. 1950. The dihydrocoenzymase-cytochrome C reductase activity of heart-muscle preparation. Biochem. J. 46:499–503.
15. Swinehart, B. A. 1968. Gas chromatographic determination of 2,3-butanediol in 1,2-propanediol using tetrahydroxyethylethylenediamine as stationary phase. Anal. Chem. 40:427–428.
16. Traube, J., and F. Blumenthal. 1905. Der Oberflächendruck und seine Bedeutung in der klinischen Medizin. Z. Exptl. Pathol. Therap. 2:117–132.
17. Voigt, R. 1959. Zur Bestimmung der Mutterkornalkaloide mit p-dimethylaminobenzaldehyd. Mikrochim. Acta, p. 619–630.