The Effects of Two Quaternary Ammonium Compounds on Citric Acid and Sterol Synthesis in *Aspergillus niger*

BY T. S. RAMAN AND E. R. B. SHANMUGASUNDARAM

University Biochemical Laboratory, Madras-25, India

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SUMMARY

A mono-quaternary amine, cetyltrimethylammonium bromide (Cetrimide), and a bisquaternary diamine, triclobisonium chloride (Triburon), were examined for their action on *Aspergillus niger*. Cetrimide, at 30 µg./ml. inhibited growth, but the organism was not inhibited by as much as 1.28 mg. Triburon/ml. Cetrimide at 20 µg./ml. inhibited citric acid accumulation. Triburon, at concentrations up to 160 µg./ml., increased the citric acid concentration, the maximum yield being at 40 µg. Triburon/ml.; at higher concentrations of Triburon there was an inhibition of citric acid production. The effect of Triburon was still perceptible in the presence of ferric ions. Cetrimide and Triburon brought about an initial increase in sterol concentration, followed by a decrease at higher concentrations of these compounds. The effects of these compounds on citric acid production by preformed mycelia were similar to the effect on cultures growing from spores.

INTRODUCTION

The use of quaternary ammonium compounds as topical antibacterial substances has become increasingly popular in recent times. A new class of these compounds, belonging to the bisquaternary group, has shown promise as systemic therapeutic agents as well (Schnitzer, Grunberg & DeLorenzo, 1960). Quaternary ammonium compounds are known to be comparatively innocuous to spore-forming organisms. A preliminary study in our laboratory showed a monoquaternary ammonium compound (cetyltrimethylammonium bromide, Cetrimide) to be highly inhibitory to the growth of *Aspergillus niger*, whereas a bisquaternary diamine (triclobisonium chloride, Triburon) was completely ineffective. The present paper deals with the effects of the two compounds on the growth and some aspects of the metabolism of *A. niger*.

METHODS

Triclobisonium chloride, *N,N'*-bis[1-methyl-3-(2,2,6-trimethylcyclohexyl)propyl]-*N,N'*-dimethyl-1,6-hexanediamine bis(methochloride), (Triburon), was a gift from Hoffmann-La Roche, Inc., Basle (see Schnitzer, Grunberg, DeLorenzo & Bagdon, 1959). Cetrimide (cetyltrimethylammonium bromide) was the gift of Imperial Chemical Industries, Ltd.

Organism. The citric acid-accumulating strain of *Aspergillus niger* (Wisc. 72-4) was used, and was maintained by monthly subculture on potato glucose agar slopes.

Growth experiments. The organism was grown in 100 ml. conical flasks containing 10 ml. of the basal medium, which contained (g./l.); glucose, 150; NH_4NO_3 , 2.5;

KH_2PO_4 , 2.5; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.25; $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$, 0.1; and $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 1.25 mg./l.; dissolved in triple glass-distilled water, and adjusted to pH 2.5 with HCl. The flasks were plugged with cottonwool and sterilized by autoclaving at 115° for 15 min. After cooling they were inoculated with a spore suspension from a 7-day slope culture. The flasks were incubated at 30° without shaking for the specified periods. After killing the organism by autoclaving, the mycelial mats were taken out, washed under the tap to remove spores, squeezed between filter-paper sheets and dried at 100°. The dry weight of the mycelium was used as a measure of growth.

Addition of the inhibitors. Cetrinide and Triburon were used as aqueous solutions and were either autoclaved in the basal medium, or sterilized separately by Seitz filtration and then added aseptically to previously autoclaved basal medium. Both methods gave identical results.

Experiments with resting mycelia of Aspergillus niger. The replacement technique used by Chughtai & Walker (1954) was adopted with minor modifications. The organism was initially grown for 4 days in 10 ml. lots of basal medium in 100 ml. conical flasks. On the 4th day, the culture medium was withdrawn and replaced aseptically with an equal volume of sterile basal medium without glucose. The flasks were then incubated overnight to make the mycelia use up any adsorbed glucose. The liquid in the flasks was again withdrawn and replaced with 10 ml. of 5% glucose solution, with or without the addition of the inhibitors as desired, and the flasks incubated at 30° for 24 hr.

Estimation of total acid and citric acid. Total acid was determined by titration of the culture medium against 0.1N-NaOH to pH 8.0. Citric acid was estimated by the colorimetric method of Saffran & Denstedt (1948).

Estimation of sterol. The dry mycelium was repeatedly extracted by grinding with acetone+ethanol (1+1 by vol.) mixture. Sterols were estimated in the extracts by the method of Vahouny, Mayer, Roe & Treadwell (1960). Pure ergosterol, freshly recrystallized from ethyl acetate and dried *in vacuo* was used as a standard.

Estimation of glucose. Glucose consumption was measured by estimating the glucose remaining in the medium by the colour reaction with anthrone (Seifter, Dayton, Novic & Muntwyler, 1950).

Oxygen consumption of Aspergillus niger mycelium in presence of Cetrinide or Triburon. The organism was grown on the usual basal medium for 4 days, by which time uniformly thick mycelia formed. The mats were taken out, washed with distilled water, and pieces of equal weight (250 mg. fresh weight) cut out and placed in Warburg flasks of 16 ml. capacity, containing 3.0 ml. fresh basal medium, and different concentrations of the inhibitors. The gas phase was air, and oxygen consumption was measured by conventional methods (Umbreit, Burris & Stauffer, 1951) during 6 hr. at 30°. Oxygen consumption of mycelia grown in presence of the inhibitors was similarly measured.

RESULTS

The effect of Cetrinide and Triburon on growth, citric acid production and sterol concentration in Aspergillus niger

The incubation periods were 5 and 8 days. Under the conditions used, maximum acid production and growth was obtained at the end of 5 days; and when the

incubation was continued longer, the yield of citric acid and the mycelial weight both decreased. The results in Table 1 show that Cetrimide was inhibitory to the organism. Triburon, on the other hand, seemed to increase the mycelial weight. Sub-inhibitory concentrations of Cetrimide increased the sterol content two- to threefold, but had practically no effect on the formation of citric acid. Triburon produced similar, but less pronounced increases in sterol concentration. Intermediate concentrations of Triburon more than double citric acid formation. At higher concentrations, Triburon inhibited citric acid formation.

Table 1. *The effect of Cetrimide and Triburon on growth, total acid, citric acid and sterol of Aspergillus niger*

The average of ten separate experiments, each in triplicate, is presented below.

Additions to medium ($\mu\text{g./ml.}$)	Dry weight mycelium (mg.)		Total acid (ml. 0.1 N-NaOH)		Citric acid (mg./flask)		Sterol content of mycelium ($\mu\text{g./100 mg.}$ dry mycelium)	
	5 days	8 days	5 days	8 days	5 days	8 days	5 days	8 days
	None	222	169	39.6	33.5	282	239	292
Cetrimide								
0.8	223	172	38.9	32.8	282	244	342	406
1.6	220	172	37.8	34.0	276	236	618	687
3.2	215	175	38.8	33.3	280	239	698	776
6.4	226	169	38.7	32.8	285	242	788	877
10.0	209	170	35.6	30.1	269	249	705	784
12.8	198	168	34.0	29.8	260	235	458	510
20.0	179	174	2.8	23.3	20	165	179	198
30.0	No growth		—	—	—	—	—	—
Triburon								
0.8	223	174	53.0	44.8	377	320	343	382
1.6	225	169	57.9	48.9	410	349	389	435
3.2	223	172	75.1	64.9	544	465	551	606
6.4	223	175	90.5	76.6	646	550	455	507
12.8	225	170	106.8	88.7	758	634	316	352
20.0	229	171	110.4	90.6	789	646	297	324
40.0	233	176	120.0	95.9	855	682	292	325
80.0	259	198	102.1	86.1	726	613	279	311
160.0	282	222	89.6	82.4	631	586	265	307
320.0	288	254	39.2	31.2	275	216	247	309
640.0	285	280	31.6	31.5	225	225	241	303
1280.0	283	282	3.7	31.4	26	220	219	309

Glucose consumption was also studied in the same flasks by estimating the residual glucose; these results are presented in Table 2, including the yields of citric acid on the basis of glucose consumed. No significant effect of either substance on glucose consumption was observed, except for the inhibition produced by the highest concentrations of Cetrimide; in this case only the fermentation period of 5 days was studied.

Effect of Triburon on citric acid production in the presence of ferric ion

Since the effect of Triburon on citric acid production (Table 1) somewhat resembles the effect of ferric ion (see Perlman, Dorrell & Johnson, 1946; Shu & Johnson,

Table 2. *The effect of Cetrimide and Triburon on glucose consumption and yield of citric acid by Aspergillus niger, growing from a spore inoculum*

The results presented are the average of ten separate experiments. The fermentation period was 5 days.

Additions to basal medium ($\mu\text{g./ml.}$)	Glucose consumed (%)	Yield of citric acid (g./100 g. glucose consumed)
None	97.1	19.4
Cetrimide		
0.8	96.8	19.5
1.6	97.2	18.9
3.2	98.4	19.0
6.4	97.9	19.4
10.0	97.5	18.4
12.8	88.3	19.6
20.0	60.1	22.2
Triburon		
0.8	98.2	25.6
1.6	98.0	27.9
3.2	97.4	37.2
6.4	97.8	44.0
12.8	98.4	51.4
20.0	97.9	53.7
40.0	98.2	58.1
80.0	98.3	49.2
160.0	97.9	43.0
320.0	98.4	18.6
640.0	98.1	15.3
1280.0	98.1	1.8

Table 3. *The effect of Triburon on citric acid production by Aspergillus niger, in the presence of ferric ion*

The results presented are the average of eight separate experiments. The fermentation period was 5 days.

Triburon added to medium ($\mu\text{g./ml.}$)	Dry weight of mycelium (mg.)		Citric acid produced (mg./flask)		Sterol content of mycelium ($\mu\text{g./100 mg. dry weight}$)	
	Fe^{3+}		Fe^{3+}		Fe^{3+}	
	1 mg./l.	15 mg./l.	1 mg./l.	15 mg./l.	1 mg./l.	15 mg./l.
0.0	225	223	542	72	305	294
0.8	223	223	694	92	347	339
1.6	226	226	760	100	407	401
3.2	222	224	822	109	575	553
6.4	219	225	859	114	462	450
12.8	224	220	889	118	316	322
20.0	230	225	952	127	290	296
40.0	234	237	1066	144	289	286
80.0	260	255	862	117	270	272
160.0	279	283	848	109	268	259
320.0	284	282	528	69	255	247
640.0	284	289	506	65	248	247
1280.0	289	280	34	5	224	210

1948) in so far as the yield of citric acid increased at first with increasing concentrations of Triburon, and then sharply decreased almost to zero, we examined whether this effect would persist in the presence of ferric ion. Two concentrations of iron were used, one being optimum for citric acid accumulation, and the other causing about 75 % inhibition of citric acid accumulation. These concentrations were determined in a separate experiment, and agreed with those reported by Shu & Johnson (1948). It was found that the effects of Triburon did persist in the two concentrations of iron, as shown in Table 3. The consumption of glucose was the same in the presence of iron as in its absence, and hence the highest amount of citric acid produced under the combined action of Triburon and iron represents a yield of about 72 % on the basis of glucose consumed.

Table 4. *The effect of Cetrimide and Triburon on glucose consumption and citric acid synthesis by preformed mycelia of Aspergillus niger*

The mycelia were incubated at 30° for 24 hr. in 5 % glucose solution. The results presented are the average of ten separate experiments.

Additions to medium (μ g./ml.)	Glucose consumed (% initial concentration)	Citric acid produced (mg./flask)
None	53	45
Cetrimide		
4.0	54	47
8.0	52	45
12.0	55	44
16.0	50	46
20.0	27	22
40.0	1	0
Triburon		
4.0	54	45
8.0	53	57
12.0	54	74
16.0	50	102
20.0	51	154
40.0	54	98
80.0	52	46
160.0	50	28
320.0	52	7
640.0	14	0

Effect of Cetrimide and Triburon on citric acid synthesis and glucose utilization by preformed mycelium of Aspergillus niger

Mycelia were incubated at 30° for 24 hr. after the addition of the replacement medium and different concentrations of the inhibitors. No measurable growth took place during this period. Citric acid and glucose were estimated in the culture fluid; the results are shown in Table 4. Intermediate concentrations of Triburon were found to stimulate citric acid production, whereas Cetrimide had no such effect.

Oxygen consumption of Aspergillus niger mycelium under the influence of Cetrimide and Triburon

The inhibitors, at the concentrations used (0.4 mg. Cetrimide/ml.; 2.0 mg. Triburon/ml.), did not significantly affect oxygen consumption of *Aspergillus niger* mycelium. Oxygen consumption of mycelia grown in the presence of the two substances also did not differ significantly from that of normal mycelium.

DISCUSSION

The wide difference in the potencies of the two quaternary amines as inhibitors of *Aspergillus niger* is surprising. We have found (unpublished) that Cetrimide and Triburon are almost equally efficient (on a weight basis) in inhibiting the growth of such diverse organisms as *Saccharomyces cerevisiae*, *Torula utilis*, several lactic acid bacteria, *Escherichia coli*, *Staphylococcus aureus* and *Streptomyces griseus*. Further, no alteration in the sterol concentration was caused by the two inhibitors in such of the above organisms as contain sterol. The toxicity of both the quaternary amines for micro-organisms, including *A. niger*, increases with increasing pH value of the medium (unpublished work), whereas their detergent properties would decrease. Besides, the minimum concentration of Cetrimide required for micelle-formation, which is considered to be essential for detergent action, appears to be several times higher than the concentration at which inhibitory action becomes manifest. Hence, it seems reasonable to assume that the differences in the properties of the two quaternary amines cannot be attributed to the lack of detergent properties of the diamine Triburon.

Yields of citric acid corresponding to 70 % of glucose consumed have been reported in submerged as well as surface fermentation (Shu & Johnson, 1948; Perlman, Dorrell & Johnson, 1946). However, we did not obtain such high yields in our usual basal medium. The high yields obtained in the presence of suitable concentrations of Triburon, taken in conjunction with the lack of effect of the compound on glucose consumption, makes it look as though Triburon specifically alters glucose metabolism in favour of citric acid accumulation. Triburon causes alterations in the amounts of citric acid and of sterol, the effects being qualitatively similar. This would suggest that Triburon affects the synthesis or utilization of a common precursor, such as acetate, if it be assumed that most of the citric acid in *Aspergillus niger* is formed via the tricarboxylic acid cycle (see Ramakrishnan, Steel & Lentz, 1955; Bomstein & Johnson, 1952). However, it may be noted that Cetrimide, while greatly increasing the concentration of sterol, had no significant effect on citric acid synthesis. The data on oxygen consumption and glucose utilization suggest that effects of the inhibitors on these functions would not account for the observed effects on citric acid accumulation. The persistence of the influence of Triburon on citric acid production in the presence of ferric ion shows that the effects of this bisquaternary diamine and of the metal are independent of each other.

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