The Keto Acid Metabolism of *Corynebacterium diphtheriae* growing in Submerged Culture

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SUMMARY

The keto acid production of *Corynebacterium diphtheriae* (strain CN2000) growing in submerged culture in complex medium was investigated. Pyruvic acid production followed a pattern in which there was a very rapid production of acid which reached a peak about 12 hr. after inoculation. The acid concentration decreased rapidly after 12 hr. and the final pyruvic acid concentration appeared to be very variable. The source of the pyruvic acid is believed to be lactic acid, the concentration of which decreased rapidly as pyruvic acid increased. The presence in the medium of sufficient iron to abolish toxin production completely did not affect the pattern of pyruvic acid production. α -Ketoglutaric acid production in the cultures appeared to vary in a random manner. *C. diphtheriae* strain G12/6 produced a maximum yield of pyruvic acid after only 6 hr. growth but was otherwise similar to strain CN2000. *C. diphtheriae* strain SM1 gave a maximum production of pyruvic acid after 24 hr. but in the presence of 3 μ g. Fe/ml. production was more rapid and was like that found for strain CN2000. No correlation was found between toxin production and the production of pyruvic acid or α -keto glutaric acid.

INTRODUCTION

In 1958 Yoneda & Ishihara showed that when they grew the SM1 strain of *Corynebacterium diphtheriae* in static culture for 4 days at 32–33° the addition of increasing amounts of iron to the culture inhibited the formation of toxin and of pyruvic acid. This seemed to be one of the few if not the only instance of a direct relationship between toxin production and a metabolite which had been reported for this organism, and it was decided to investigate the keto acid metabolism of various strains of *C. diphtheriae* growing in a stirred and aerated culture of the type described by Linggood, Matthews, Pinfield, Pope & Sharland (1955).

METHODS

Organisms. Three strains of *Corynebacterium diphtheriae* were used: CN2000 (the Wellcome Research Laboratories Culture Collection); G12/6 supplied by Dr G. D. Clarke; SM1 supplied by Dr M. Yoneda. In each case a 24 hr. growth on a Loeffler slope was used to inoculate 200 ml. medium in a 1000 ml. Erlenmeyer flask.

The flask was placed on a rotary shaker in an incubator at 35° and shaken for 24 hr. after which time the culture was used to inoculate the tank.

Culture vessels and medium. Tanks (20 l.) were used as described by Edwards & Seamer (1960) and contained papain digest of beef (11 l.; Ramon, Pochon & Amoureux, 1941) which had been treated with yeast (Pope & Smith, 1932) and to which 0.5 % (v/v) glacial acetic acid and 2.4 % (w/v) maltose were added. Antifoam A (Midlands Silicone Ltd., Upper Brook Street, London) dispersed in butyl acetate was added (15 ml. 25 %, w/v); air was passed through the tanks (at 500 ml./min.); the temperature was maintained at 35° and the cultures incubated for 48 hr.

Keto acid estimations. Estimations of pyruvic acid and of α -ketoglutaric acid were carried out by a chromatographic method (Cavallini & Frontali, 1954; Cavallini & Mandovi, 1957). The solvent used was a mixture of *n*-butanol, ethanol and 0.5N-ammonia (70 + 10 + 20, El Hawary & Thomson, 1953). Besides pyruvic acid and α -ketoglutaric acid two other keto acid hydrazones were normally found on the chromatograms (see Jännes, 1954). Since these were unidentified and the pattern of their production was in no way noteworthy they are not further discussed in this paper. On a few occasions a spot corresponding to oxaloacetic acid was also found.

Lactic acid estimations. These were carried out by the method of Barker & Summerson (1941). Since the samples were known to contain large amounts of keto acids, the deproteinized material was treated with 2:4-dinitrophenyl hydrazine and then extracted with benzene before the usual copper sulphate and calcium hydroxide treatment.

Toxin. This was estimated by the flocculation method (Ramon, 1922; Glenny & Okell, 1924). Results are expressed as Lf units/ml. (Lf/ml.).

RESULTS

Experiments with strain CN2000. Since strain CN2000 of *Corynebacterium diphtheriae* is the one in use for the routine production of high titre diphtheria toxin at these laboratories it was the one most studied. In all, nine complete analyses were performed in which samples were analysed every 6 hr. during the whole 48 hr. of the incubation period; three of the cultures contained sufficient iron to give complete inhibition of toxin production. The results were subject to variation but in most cases curves of the type shown in Fig. 1 were obtained. Pyruvic acid was produced with great rapidity and by the 12th hr. concentrations higher than 600 μ g./ml. were common. This period of production was usually followed by a period of rapid disappearance of the acid. The variations in production were of two types. In the first the height of the peak was much lower than usual (c. 300 μ g./ml.) and in the second the disappearance of pyruvic acid was relatively slow. Figure 1 also shows the production of α -ketoglutaric acid. It was very variable in the cultures studied and in the examples in Fig. 1 the final α -keto glutaric acid concentrations after 48 hr. growth were 30 and 304 μ g./ml. and the corresponding toxin titres were 345 and 305 Lf/ml.

It seemed possible that the source of the pyruvic acid in the cultures might be lactic acid. Tank contents were analysed and the results obtained during the first 24 hr. are shown in Fig. 2. The interpretation of this seems clear. Lactic acid,

present in the medium and presumably derived from the beef used in the preparation of the medium, was rapidly converted to pyruvic acid which was then further metabolized and perhaps partially converted back to lactic acid. The disappearance of the pyruvic acid is complicated by the fact that metabolism, presumably via acetate and the tricarboxylic acid cycle, will also be occurring and thus the sum of lactic and pyruvic acids, which in the very early stages of growth is sensibly constant, showed a marked decline.

Since Yoneda & Ishihara (1958) had shown an effect of iron on pyruvate formation it might have been anticipated that the presence of enough iron to inhibit toxin production completely would also exert a marked effect on pyruvic acid metabolism. The results shown in Fig. 3 show that this was not the case. The two curves differ only in the amount of pyruvic acid found after 12 hr., the pattern

Fig. 1

Fig. 2

Fig. 1. Keto acid production in two cultures of the CN2000 strain of *Corynebacterium diphtheriae*. The 0 hr. samples were uninoculated medium. SC1272 ○—○, pyruvic acid; ○—○, α -ketoglutaric acid. SC1257 ●—●, pyruvic acid; ●—●, α -ketoglutaric acid.

Fig. 2. Lactic acid and pyruvic acid in a culture (SC1597) of the CN2000 strain of *Corynebacterium diphtheriae*. The 0 hr. sample was uninoculated medium. ○—○, lactic acid; ●—●, pyruvic acid.

of metabolism remaining unchanged. Since the toxin yields produced by the two cultures were 340 and 0 Lf/ml., it is clear that pyruvic acid metabolism and toxin production were not closely linked, at least with the CN2000 strain.

This is further borne out by the results in Table 1 in which analyses of 48 hr. cultures are shown arranged in order of descending Lf values. It is clear from Table 1 that neither the α -ketoglutaric acid values nor the pyruvic acid values bear any relationship direct or indirect to the toxin titres. The extreme variability of the amounts of α -ketoglutaric acid that were found is an indication of the lack

Fig. 3

Fig. 4

Fig. 3. Pyruvic acid production by the CN2000 strain of *Corynebacterium diphtheriae* with and without added iron. The 0 hr. sample was uninoculated medium. ○—○, no added iron; ●—●, 4 µg. Fe/ml. added.

Fig. 4. Pyruvic acid production by the G12/6 strain of *Corynebacterium diphtheriae* without added iron and by the SM1 strain with and without added iron. The 0 hr. samples were uninoculated medium. ○—○, G12/6 strain; ○—○, SM1 strain without added iron; ●—●, SM1 strain with 3 µg. Fe/ml. added.

Table 1. *Analyses of 48 hr. cultures of Corynebacterium diphtheriae for toxin, pyruvic acid and α-ketoglutaric acid*

Culture number	Toxin (Lf/ml.)	Pyruvic acid (µg./ml.)	α-ketoglutaric acid (µg./ml.)
SC 1257	345	90	30
1346	340	96	0
1440	328	408	8
1394	316	142	0
1391	312	98	0
1272	305	242	304
1438	296	258	0
1444	280	49	0
1442	275	78	0
1280	263	442	518
1331	262	12	0
1441	244	188	0
1393	236	187	65
1439	216	122	100
1249	202	—	646
1443	175	0	0
1389	170	158	0

of control over the organism that existed in this work although the conditions of cultivation were kept as uniform as possible. Even when the peak values for pyruvic acid (occurring after 12 hr. growth) are used there is still no apparent relationship to the final toxin yield, for in five cultures in which final (48 hr.) toxin titres of 202, 263, 305, 340, and 345 Lf/ml. were found, the 12 hr. values for pyruvic acid were 300, 710, 637, 536 and 654 $\mu\text{g./ml.}$, respectively.

Experiments with the G12/6 and SM1 strains. Figure 4 shows the production of pyruvic acid by the G12/6 and SM1 strains. In the case of strain G12/6 the results were very similar to those found with strain CN2000 although the peak production occurred after only 6 hr. instead of 12 hr. as is usual with strain CN2000. The SM1 strain when grown without any additional iron gave a relatively slow production of pyruvic acid which continued until 24 hr. after inoculation. In the presence of iron (3 $\mu\text{g./ml.}$) the toxin production was nil (compared with 100 Lf/ml. in the control) and the production of pyruvic acid rapid, reaching a peak after 12 hr. Disappearance of pyruvic acid started after 18 hr. and was rapid. Thus in the presence of iron, pyruvic acid production by strain SM1 was more like that found for strain CN2000.

DISCUSSION

In 1958 Yoneda & Ishihara concluded that iron exerted a 'distinct influence not only on the liberation of toxin but also on the pyruvate metabolism of the organism'. From the results presented in the present paper it seems clear that no such influence was exerted on pyruvate metabolism when strain CN2000 of *Corynebacterium diphtheriae* was grown in aerated submerged culture. The SM1 strain (the one used by the Japanese workers) did appear to give a change in pyruvic acid production in the presence of iron but when the toxin titres were 100 Lf/ml. for the control culture and 0 Lf/ml. for the one containing iron, the final pyruvic acid concentrations were 94 and 271 $\mu\text{g./ml.}$ respectively. Yoneda & Ishihara on the other hand found that as the toxin titre fell from 60 to 0 Lf/ml. the pyruvic acid concentration fell from 220 to 11 $\mu\text{g./ml.}$; thus a serious difference exists between the two sets of results. The differences in the two methods of cultivation must be borne in mind, but regardless of this, the present results with the SM1 strain, together with the fact that the pyruvic acid metabolism of strain CN2000 was virtually unaffected by the presence of 3 $\mu\text{g. Fe/ml.}$, seem to rule out the possibility that toxin and pyruvic acid production are necessarily closely linked.

The rapid decrease in lactic acid concentration and corresponding increase in pyruvic acid shown in Fig. 2 suggest the presence in *C. diphtheriae* strain CN2000 of a powerful lactic acid dehydrogenase. After incubation for 12 hr. the cultures contain *c.* 10^8 – 10^9 viable organisms/ml. compared with *c.* 10^{10} viable organisms/ml. after 24 hr., so that the production of the high yields of pyruvic acid has been brought about by relatively few organisms. The cause of the change from production to disappearance of pyruvic acid is obscure, but it may be that shortage of oxygen, which has already been suggested as the factor which limits the growth rate (Edwards, 1960), is responsible for the 12 hr. culture beginning to reduce some pyruvic acid to lactic acid.

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