The Mechanism of the Formation of Organic Acids by Mould Fungi

6. THE INFLUENCE OF CYANIDE ON *ASPERGILLUS NIGER*

By I. D. CHUGHTAI AND T. K. WALKER

College of Technology, University of Manchester

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In continuation of previous studies of acid formation in *Aspergillus niger* (Walker, 1949; Chughtai, Pearce & Walker, 1950), the effects of cyanide on the utilization of glucose in cultures of this mould have been subjected to quantitative examination.

EXPERIMENTAL

The strain (N1) of *A. niger* used in our previous work was again employed and was grown at 30° on a sterilized medium made up with distilled water and containing (% w/v) NH_4NO_3 , 0.2; KH_2PO_4 , 0.1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.025; and glucose, 10. The pH was adjusted to 4.4 with HCl. This medium was dispensed in quantities of 140 ml. in conical flasks of 600 ml. capacity and having necks 35 mm. in diameter. Felts were grown from spores and when, after about 4 days, strong mycelial mats had been formed, the culture medium was withdrawn and replaced aseptically by an equal volume of the salt solution without glucose. The flasks were then left overnight in the incubator in order to allow time for assimilation of absorbed sugar.

After 12 hr. the salt solution was withdrawn and replaced by an equal volume of glucose solution (10%, w/v) without nutrient salts, but containing NaCN at concentrations of 0.006, 0.010, 0.020 or 0.040 M. Since the felts which had been standing over the inorganic-salt solution retained some of

the latter in their under-surface convolutions, this diluted the 10% solution of glucose which was used as replacement. A correction for the dilution was therefore applied in all cases. Normally, five flasks were employed for each concentration of cyanide, and controls without cyanide were also set up. The initial pH of the contents of the cyanide-containing flasks was approximately 9.5, but when incubation was resumed the activity of the mould speedily lowered it into the acid range. Samples were withdrawn at intervals for estimation of glucose utilized and acids formed. In other cases the replacement media consisted, respectively, of a solution of glucose, together with NaNO_3 , and of a solution of citric acid. The analyses were performed by the methods indicated by Chughtai & Walker (1951).

RESULTS

The range of concentration of cyanide was adopted after preliminary tests had shown that development of the mould from spores was suppressed by cyanide at 0.015 M. However, Table 1 shows that formation of acid by 'ready-formed' mycelial felts was stimulated by cyanide at all the concentrations which were adopted, the optimum effect being observed at a concentration of 0.020 M. Formation of felt also was stimulated by cyanide (Table 2). According to

Table 1. *Consumption of glucose and formation of citric, oxalic and gluconic acids by Aspergillus niger in presence and in absence of sodium cyanide*

(Glucose present initially at a concn. of 8.87 g./100 ml., equivalent to 3.55 g. carbon/100 ml.)

Cyanide concn. (M)	Time of incubation (days)	Glucose utilized (g. carbon/100 ml.)	Acid formation					
			Citric		Oxalic		Gluconic	
			(g. carbon/100 ml.)	(% yield)*	(g. carbon/100 ml.)	(% yield)	(g. carbon/100 ml.)	(% yield)
0	6	0.812	0.174	21.5	0.047	5.8	0.028	3.6
	8	1.356	0.229	16.9	0.048	3.5	0.072	5.6
	11	1.521	0.265	17.4	0.053	3.5	0.188	12.3
0.010	6	1.428	0.337	23.6	0.097	6.8	0.433	30.3
	8	1.935	0.449	23.2	0.092	4.8	0.622	32.0
	11	2.381	0.378	15.9	0.096	4.0	0.577	24.2
0.020	6	2.037	0.469	23.0	0.099	4.9	0.536	26.2
	8	2.537	0.489	19.3	0.119	4.7	0.758	29.9
	11	3.317	0.394	11.9	0.123	3.7	0.548	16.5
0.040	6	1.902	0.338	17.8	0.084	4.4	0.570	30.0
	8	2.493	0.468	18.8	0.113	4.5	0.744	29.8
	11	3.295	0.378	11.5	0.113	3.2	0.614	18.6

* Percentage yield based on carbon content of glucose utilized.

Table 2. *Influence of sodium cyanide on the weight of mycelium developed by Aspergillus niger in a glucose medium*

(Glucose present initially at a concn. of 8.85 g./100 ml., equivalent to 3.54 g. carbon/100 ml. Carbon content of felt at 3 days, before introduction of cyanide = 0.657 ± 0.112 g./flask.)

Cyanide concn. (M)	Glucose utilized (g. carbon/157 ml.)	Carbon in felt at 8 days (g./157 ml.)	Gain in carbon in felt in last 5 days		Sum of citric, oxalic and gluconic acids plus gain in felt (g. carbon/157 ml.)
			(g./157 ml.)	(% yield)*	
0	4.600	1.313	0.656	14.3	2.218
0.006	4.458	1.387	0.730	16.4	2.429
0.020	5.154	1.731	1.074	20.1	3.292

* Percentage yield on carbon content of glucose utilized.

Table 3. *Comparison of the respective effects of sodium cyanide and sodium nitrate on glucose consumption and acid formation by Aspergillus niger*

(Glucose present initially at a concn. of 8.68 g./100 ml., equivalent to 3.47 g. carbon/100 ml.)

Concn. of cyanide (M)	Concn. of nitrate (M)	Time of incubation (days)	Glucose utilized (g. carbon/100 ml.)	Gain in carbon in felt in 7 days (g./100 ml.)	Acid formation					
					Citric		Oxalic		Gluconic	
					(g. carbon/100 ml.)	(% yield)*	(g. carbon/100 ml.)	(% yield)	(g. carbon/100 ml.)	(% yield)
0	0	3	1.567	—	0.528	33.9	0.041	2.6	0.071	4.5
		5	1.743	—	0.643	36.9	0.056	3.2	0.181	10.4
		7	1.911	0.433	0.745	39.0	0.065	3.4	0.314	16.4
		10	2.348	—	0.951	40.5	0.072	3.1	0.274	11.6
		12	2.768	—	1.093	39.5	0.099	3.6	0.213	7.6
0.020	0	3	2.062	—	0.944	45.8	0.092	4.5	0.301	14.6
		5	2.700	—	1.172	43.4	0.125	4.6	0.607	22.4
		7	3.255	1.493	1.355	41.6	0.161	4.9	0.485	14.9
		10	3.359	—	1.331	39.6	0.106	3.2	0.514	15.3
		12	3.409	—	1.105	32.4	0.118	3.5	0.253	7.4
0	0.020	3	1.709	—	0.642	37.5	0.068	4.0	0.213	12.4
		5	2.348	—	0.804	34.2	0.116	4.9	0.215	9.2
		7	2.852	1.232	0.916	32.2	0.156	5.5	0.303	10.5
		10	3.174	—	1.066	33.6	0.144	4.5	0.241	7.6
		12	3.241	—	0.892	27.5	0.122	3.8	0.263	8.1

* Percentage yield based on carbon content of glucose utilized.

Table 4. *Consumption of citric acid by Aspergillus niger in presence and in absence of sodium cyanide*

(Citric acid present initially at a concn. of 2.708 g. of anhydrous acid/100 ml., equivalent to 1.015 g. carbon/100 ml.)

Cyanide concn. (M)	Time of incubation (days)	Citric acid utilized		Oxalic acid formed	
		(g. carbon/100 ml.)	(% of initial quantity)	(g. carbon/100 ml.)	(As % of citric acid consumed)
0	3	0.676	66.9	—*	—
	5	0.888	87.9	—	—
	14	1.015	100.0	—	—
0.020	3	0.044	4.4	—	—
	5	0.055	5.4	—	—
	14	0.632	62.6	0.062	9.8

* Denotes failure to detect oxalic acid by the diphenylamine test (Vogel, 1947) which is sensitive to 5 µg. and has a concentration limit of 1:10,000.

Ivanov & Osnizkaja (1934) and to Ivanov, Ivanova & Larinova (1936), *A. niger*, when in conditions of nitrogen starvation, can utilize the nitrogen of cyanide. Hence, it seemed possible that nitrogen from this source might have contributed to the increase in weight of mycelial felt so the effects of equivalent amounts of sodium cyanide and sodium nitrate were compared (Table 3).

Finally, the utilization of citric acid by *A. niger* in presence of cyanide was studied (Table 4) since it was desired to ascertain whether the higher yields of citrate which were obtained in the media containing cyanide could be ascribed wholly or in part to suppression by this compound of the mould's ability to metabolize citric acid, for aconitase is inhibited by hydrogen cyanide at relatively low concentration (Krebs & Eggleston, 1944). The utilization of free citric acid by *A. niger* in cultures in which this acid was the sole source of energy was greatly restricted in presence of cyanide. During incubation of these cultures the presence of free hydrocyanic acid could be detected by its smell (the flasks were plugged with cotton wool) when the door of the incubator was opened. In the experiments with cyanide in a glucose solution no smell of free hydrocyanic acid was ever noted, although the activity of the mould caused the pH of these solutions (initially 9.5) to fall rapidly to values usually below 3.

The highest intensity of metabolic activity as evidenced by sugar consumption, by formation of mycelial felt and by production of acid, was attained by preformed felts of the mould in presence of cyanide at concentration 0.020M. At this level (Table 1) sugar consumption at 6, 8 and 11 days was approximately double that in the control flasks, while at the same stages the yields of citric acid were respectively 2.7, 2.1 and 1.5 times those found in the controls. At these same stages the yields of oxalic acid were 2, 2.5 and 2.3 times those produced in absence of cyanide. Similarly, formation of gluconic acid was greatly enhanced by the effect of the cyanide, the amount estimated at 11 days being 2.9 times the quantity detected in the cyanide-free cultures. Moreover, the weights of felt synthesized and the total weights of acids formed in presence of cyanide are greater, calculated as percentages on the sugar consumed, than the corresponding yields of felt and acids afforded by the control flasks (Table 2), an indication that one of the effects of cyanide is to spare loss of carbon dioxide from the cultures.

DISCUSSION

The fact that addition of extra assimilable nitrogen, in the form of sodium nitrate to preformed felts of *A. niger* on a glucose solution (Table 3), served to diminish somewhat the percentage yield of citric

acid renders it improbable that the stimulation of citrate formation by an equivalent quantity of sodium cyanide was due to the nutrient value of the nitrogen of the cyano group. The effects of cyanide, as manifested in considerable increases in sugar consumed, felt produced and gluconic, citric and oxalic acids formed, indicate general stimulation of physiological function in the mould rather than activation of any one specific process. Similar effects produced by cyanide were observed by Dammann, Rotini & Nord (1938), who reported that cyanide stimulated considerably the metabolic activity of *Fusarium graminearum* Schwabe, causing an increase in the weight of felt and at the same time a higher level of activity in the fermentation of pentoses, notably of L-arabinose, whereby also the yield of alcohol was markedly increased.

Sodium arsenite, an inhibitor of oxidative decarboxylation (Krebs, 1933*a, b*; Krebs & Johnson 1937), when used at a certain concentration in glucose cultures of *A. niger* (Chughtai & Walker, 1951) produces results similar in every respect to those now noted in presence of sodium cyanide. The occurrence of pyruvic acid in glucose cultures of *A. niger* after treatment with arsenite was reported by Chughtai *et al.* (1950) and more recently α -oxoglutaric acid was detected in similarly treated glucose cultures of this mould (Walker, Hall & Hopton, 1951). These facts would seem to indicate that one of the activities of cyanide when introduced into glucose cultures of *A. niger* is to exert a stabilizing influence on the α -keto acids of the tricarboxylic acid cycle. Cyanhydrin formation no doubt occurs, since Gale & Stephenson (1939) found cyanide (0.060M) to be the only effective agent for stabilizing the oxaloacetic acid produced by the action of malic dehydrogenase.

Since the data given in Table 4 shows that hydrocyanic acid interferes powerfully with the action of those enzymes by which *A. niger* degrades citric acid, it might be expected to exercise a similar sparing action on the citric acid produced in glucose cultures of the mould. The fact that citric acid does not accumulate extensively in such cultures after the introduction of cyanide is due most probably to the speedy removal of the latter by combination with ketonic metabolites. Such a removal of hydrocyanic acid would also account for the fact that no smell of this acid has been detected coming from glucose cultures after addition of sodium cyanide, although such cultures rapidly produce sufficient acid to bring the pH to about 3. Summing up, it would seem reasonable to conclude that in glucose cultures prepared with preformed felts of *A. niger*, sodium cyanide promotes the tricarboxylic acid cycle by restricting more highly oxidative side-reactions.

SUMMARY

1. Preformed mycelial felts of *A. niger* treated with cyanide at four concentrations between 0.006 and 0.040M, utilized more sugar, synthesized more mycelial substance and formed a greater total of acids (citric, oxalic and gluconic) in a given period than did controls. The most effective concentration used was 0.020M.

2. In presence of 0.010 or 0.020M cyanide the yields of all products were not only actually greater than with the controls but were also greater when expressed as percentages on the weight of sugar consumed.

3. The breakdown of citric acid by *A. niger* is improved by cyanide.

4. Possible modes of action of the cyanide are discussed.

REFERENCES

- Chughtai, I. D., Pearce, A. A. & Walker, T. K. (1950). *Biochem. J.* **47**, 135.
 Chughtai, I. D. & Walker, T. K. (1951). *Biochem. J.* **48**, 524.
 Dammann, E., Rotini, O. T. & Nord, F. F. (1938). *Biochem. Z.* **297**, 184.
 Gale, E. F. & Stephenson, M. (1939). *Biochem. J.* **33**, 1254.
 Ivanov, N. N., Ivanova, V. E. & Larinova, N. N. (1936). *Proc. Congr. int. Microbiol.* p. 449.
 Ivanov, N. N. & Osnizkaja, L. K. (1934). *Biochem. Z.* **271**, 32.
 Krebs, H. A. (1933a). *Hoppe-Seyl. Z.* **217**, 191.
 Krebs, H. A. (1933b). *Hoppe-Seyl. Z.* **218**, 157.
 Krebs, H. A. & Eggleston, L. V. (1944). *Biochem. J.* **38**, 426.
 Krebs, H. A. & Johnson, W. A. (1937). *Enzymologia*, **4**, 148.
 Vogel, A. I. (1947). *Text-book of Qualitative Chemical Analysis*, 3rd ed. London: Longmans Green and Co.
 Walker, T. K. (1949). *Advanc. Enzymol.* **9**, 537.
 Walker, T. K., Hall, A. N. & Hopton, J. W. (1951). *Nature, Lond.*, **168**, 1042.

Heat Transformation Products of Cottonseed Oil

By J. G. CHALMERS

Cancer Research Department, Royal Beatson Memorial Hospital, Glasgow

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The high incidence of cancer of the stomach has indicated the possibility that heated foodstuffs contain a carcinogen. However, in this laboratory examination of fats heated to relatively high temperatures in cooking has not revealed the presence of agents of the aromatic hydrocarbon type, which show characteristic fluorescence. Biological tests of the carcinogenicity of heated cottonseed-oil products by feeding experiments on mice have been described by Peacock, Beck & Chalmers (1953) and additional tests made by subcutaneous injection into mice will be described later in this communication.

In the feeding experiments to which reference has been made, the following cottonseed-oil products have been used: (1) Cottonseed oil heated at the b.p. (350°) for 4 hr. (2) Cottonseed oil heated at 320° for 2.5 hr. in the presence of iron, which favours ketonization of the oil.

Considering all tests carried out in this biological investigation, in 268 animals surviving 400 days and over, there were eighteen papillomata and six carcinomata of the forestomach. Although this is a smaller percentage than that which might be expected to follow the administration of a carcinogenic hydrocarbon, it is similar to the percentage of deaths in the human species due to gastric cancer. The present work was undertaken in an attempt to determine the nature of the heated products used in

these biological tests. For a preliminary communication of this work see Chalmers (1952).

EXPERIMENTAL AND RESULTS

Apparatus. In the earlier biological experiments (Peacock *et al.* 1953), the oil was heated in a stream of N₂ in a flask fitted with an electrically heated column as described for use in the distillation of fatty esters (Hilditch, 1947). In the present experiments, owing to difficulties due to frothing, the heated column was replaced by a vacuum-jacketed column (3 ft. × 2 in. diam.). A small receiver was inserted immediately above the vacuum jacket, and water formed during the reaction together with a small quantity of liquor smelling strongly of acrolein were run off. The still-head carried a jet for the admission of N₂ in case of frothing: the main receiver was attached directly to the still-head. The path to the receiver and exhaust was about 1 in. wide.

Injection experiments. In addition to the feeding experiments to which reference has been made, several of the cottonseed-oil products were tested by subcutaneous injection, either alone or in solution in tricaprilyn, into mice which were 3 months old at the start of the experiment (Table 1). Some of these experiments are still in progress. A number of the animals died within the first year of the experiment, owing to an intercurrent infection. In Expts. 3 and 4 few of the mice lived much longer than 12 months and the results of the tests are therefore inconclusive. There has been no obvious reaction at the site of injection in any of these experiments, although in some of the older animals remote tumours were observed.