CCIV. STUDIES ON YEAST GROWN IN CYANIDE. II.

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IN the previous communication [Pett, 1935, 3] the observation was reported that yeast could be grown in media containing cyanide. The resulting yeast was found to have a high fermentative capacity, and a respiration largely insensitive to cyanide. It contained several times the normal content of flavin, in the form of flavoprotein.

The present communication gives a comparison of cyanide-yeast with yeast experimentally grown aerobically (like baker's yeast), and anaerobically (like bottom yeast), together with observations on the effect of prolonged subculture in cyanide. The results show definite differences in the three classes of yeast; prolonged subculturing in cyanide resulted in the disappearance of flavin from extracts of the yeast, with the appearance of a blue fluorescence. Some of the properties of this yeast are given.

EXPERIMENTAL.

Medium. This contained, in g. per litre, KH_2PO_4 1; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.5; NaCl 1; $(\text{NH}_4)_2\text{HPO}_4$ 4; marmite 0.5; glucose 20. The pH was adjusted to 5 before sterilizing and determined after growth, being usually 3.0–3.5.

Apparatus. 500 ml. of medium were used in a 1 litre Erlenmeyer flask, vigorously aerated through a Jena sintered glass device, or by one constructed from a Gooch crucible. The air was filtered through cotton wool in an S-shaped tube of 1 cm. bore and 50–60 cm. length. The stopper sometimes carried a separating funnel for removing samples at intervals. The stopper, complete, was wrapped and autoclaved separately, being aseptically inserted after inoculation of the flask, in a special room. The first absorption tube contained 40% NaOH , the subsequent ones N NaOH . The incoming gas, either air or nitrogen largely freed of oxygen by pyrogallol and of CO by a copper solution, was drawn through a gas wash-bottle containing either water or slightly acidified $M/750$ NaCN solution replaced every 12 hours. This procedure maintained the medium at $M/1000$ to $M/1400$ NaCN . Concentrations higher or lower can be produced by suitable variations of the solution, and frequent changes of the solution permit control of the cyanide in the medium within fairly narrow limits.

Under our conditions suction maintained a steadier flow of gas than pressure and was always used. All connexions were made with pressure tubing. The whole apparatus stood in an incubator at 26° , and the yeast was grown for 48 hours unless specified. Erlenmeyer flasks are not ideal for good aeration but

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were the most suitable of the standard glassware in possessing a large neck to admit the apparatus, and surplus room for frothing.

Procedures. Cyanide determinations were made at frequent intervals on the various solutions of the system using the sodium mercuric chloride titration to methyl yellow of Kolthoff & Furman [1929].

Fig. 1.

Respiration and fermentation were determined in the Warburg micromanometric apparatus and are expressed as Q_{O_2} and Q_{CO_2} (μ l. gas change in 1 hour for 1 mg. dry weight of yeast). The bath was set at 30° , which is the upper limit of the optimum temperature range for growing yeast. The general reproducibility of the results at different times, using different lots of yeast etc., was never better than 5%. The yeast was suspended in 0.05 M KH_2PO_4 . Fresh, neutralized KCN solution was used to give an ultimate concentration of M/600. The ultimate concentrations of the various substrates used were: glucose 2%; lactate 1.66%; alcohol 1.66%. Nitrogen was freed from oxygen by passing over glowing copper. Fluted filter-paper (Whatman No. 40) was used in the alkali, and when cyanide was used the alkali also contained cyanide [see Van Heyningen, 1935]. This precaution is actually not important with yeast at 30° for a short time, since repeated parallel experiments without cyanide in the alkali still gave the same Q_{O_2} . The pH of the mixture in each vessel was checked after the experiment.

Flavin was determined in a concentrated beam of light as described by Pett [1935, 3].

RESULTS.

Preparation of the yeast. The effect of aeration.

The yeast was centrifuged and washed 5 or 6 times with distilled water. The custom of impoverishing the yeast by vigorous aeration for 4–5 hours in buffer at pH 7, followed by additional washings, was sometimes followed. This procedure, however, is open to serious criticism. The metabolic capabilities of starved yeast are not the same as of the original yeast in several respects, though it has been assumed that the only change is a depletion of the glycogen reserves. This leads to a low Q_{O_2} without added substrate and makes various experimental effects more striking. The fact is that other changes occur. Of importance to the present discussion is the disappearance of flavin during the treatment as shown in Table I, taken from Pett [1935, 2]. Even more rapid disappearance is often observed.

Table I. *Changes in flavin content of baker's yeast during aeration in buffer.*

Time in hours	0	4	8	12	24	36
Flavin γ /g.	24	16	12	10	7	6

Clearly glycogen is not the only constituent affected by aerating the yeast, and the subsequent reactions of the yeast may thus not be "normal". This idea receives further support from observations on the respiration of yeast before and after aeration. Table II shows typical results of the effect of cyanide on the respiration before and after aeration of some commercial baker's yeast and on some similar yeast experimentally grown under aerobic conditions.

Table II. *The increased sensitivity to cyanide of the respiration of baker's yeast and similar experimentally-grown yeast, after aeration in buffer.*

	Baker's yeast		Experimental yeast	
	Before aeration	After aeration	Before aeration	After aeration
No substrate Q_{O_2}	- 6	- 2	-31	- 4
$Q_{O_2}^{CN}$	- 6	- 3	-27	- 5
Glucose Q_{O_2}	-92	-89	-32	-25
$Q_{O_2}^{CN}$	-21	-11	-19	- 5

Before aeration, especially without substrate, when the yeast is using its natural reserves, the respiration is less sensitive to cyanide. This is clearer in the fresher, experimentally grown yeast. Ogston & Green [1935] have also pointed out the CN-stability of the low natural respiration of yeast. Percentage inhibitions are not given because they give various results depending on the method of calculation. Methods in the literature could show either 40 or 100 % inhibition in Table II.

Effect of the growing time on respiration, fermentation, glutathione and flavin of various yeasts.

Large flasks were used (see Fig. 1) and samples were aseptically removed from the flasks at stated intervals and examined. The results are given in Table III.

Table III. *The effect of the growing time on the respiration, fermentation, glutathione and flavin content of yeasts grown aerobically, anaerobically and aerobically with cyanide.*

		2 days			3 days			5 days		
		Aerob.	Anaerob.	CN	Aerob.	Anaerob.	CN	Aerob.	Anaerob.	CN
No substrate	Q_{O_2}	- 6	- 5	- 2	- 5	-15	- 2	- 2	-11	- 2
Glucose	Q_{O_2}	-44	- 41	-10	-24	—	-15	-13	-17	-15
	$Q_{O_2}^{CN}$	- 3	-18	- 9	- 6	- 7	-11	- 2	- 5	-11
Lactate	Q_{O_2}	-26	- 22	- 5	-37	- 5	- 2	-25	-14	—
	$Q_{O_2}^{CN}$	-22	- 2	- 9	-14	- 2	- 6	-20	- 2	—
Alcohol	Q_{O_2}	-38	- 24	- 7	-18	- 8	- 2	-47	- 8	—
	$Q_{O_2}^{CN}$	- 3	- 2	- 2	- 2	- 1	- 2	-22	- 1	—
Glucose	Q_{CO_2}	+25	+120	+76	+18	+35	+39	+16	+52	+15
	$Q_{CO_2}^{N_2}$	+47	+148	+78	+37	+42	+30	+24	+58	+10
Flavin γ /g.		17	13	51	12	12	48	15	15	50
Glutathione	SH	—	—	—	120	47	46	170	151	115
	Total	—	—	—	121	49	153	175	156	250

In glucose the highest respiration and fermentation were found in the yeast grown the shortest time (2 days). With lactate or alcohol as substrate no general trend was noticed. Similarly the relative flavin concentrations did not change significantly, though a change in the glutathione (only twice determined) was found.

The Meyerhof quotient has been calculated but varied in a very irregular manner depending on purely fortuitous variations in the figures. Its calculation implies in such figures an absolute accuracy and significance which they do not possess.

General properties of cyanide-yeast.

The medium was always inoculated with 1 ml. of a 2-day culture of *S. cerevisiae*, grown on the same medium. Several different strains have been successfully tried, but only one was used throughout the present work. To produce cyanide-yeast, HCN-laden air was passed through the system previously described. Aseptically adding cyanide to the medium was difficult and unnecessary since the medium took up cyanide from the air in a few hours. Three controls were grown simultaneously: (1) aerobic, by passing ordinary air, (2) anaerobic, by passing nitrogen, (3) anaerobic-cyanide (called N.C.N.) by passing nitrogen through cyanide solution. It is desirable to pass gas through the flasks in this way in order to remove CO_2 and to keep the medium in motion, thus making a truer comparison with the aerobic and aerobic-cyanide yeasts. The $p\text{H}$ at the end was never less than 3.0 unless bacterial contamination had occurred. Microscopical examination both by staining and on a dark ground was a routine; only rarely were bacteria found, in which case the duplicate flask only was used.

The properties of yeasts cultured in this way may be seen in Tables III and IV (1st series). A high content of flavin must be present for typical CN-yeast as here discussed, though subculturing in cyanide may produce a flavin-free yeast which might also be called a cyanide-yeast. If a high flavin content was not found the observed properties were like those of aerobic yeast. The Q_{O_2} in glucose and lactate was about $1/4$ that of aerobic yeast and was scarcely affected by cyanide. The Q_{O_2} in alcohol was also small, but might show marked CN-sensitivity. The fermentation was usually higher than that of aerobic yeast, however determined. Cyanide-yeast has a high aerobic fermentation and this is not increased by determination in nitrogen. In other words the usual increase of anaerobic fermentation over aerobic (the Pasteur effect or reaction), which is observed in the control yeasts, was not found in CN-yeast.

The Q_{O_2} of all these yeasts may appear small to those accustomed only to commercial yeast, but is really quite high for experimentally grown yeast, especially on synthetic medium.

The glutathione content of these yeasts was twice determined but the results are only tentative. CN-yeast contained significantly more glutathione than the controls, but whereas the controls had it all in the $-\text{SH}$ form, the CN-yeast had only about $1/3$ as $-\text{SH}$. The high proportion of the oxidized form is the more remarkable since it has been suggested that oxidation of glutathione in animal tissues requires a CN-sensitive iron catalyst, and also since *in vitro* cyanide reduces GSSG. Possibly some other substance was produced and was determined by the unspecific method used.

Unfortunately quantitative measurements of cytochrome have not yet been possible, owing to the small amount of yeast available and the need for about 1 g. to make the estimation. The yield from 500 ml. medium has been from 0.5 to 1.5 g. fresh yeast, which was almost all required for the duplicate deter-

minations of flavin, respiration etc. The question is therefore open whether cytochrome still functions in CN-yeast by making use of some CN-insensitive enzyme, but it is generally held that cytochrome is exclusively and specifically oxidized by a CN-sensitive enzyme.

The fate of the cyanide is still largely unexplored, but in all cases 1/3 or more of the amount introduced disappears from the flask containing yeast, though this does not represent more than perhaps 50 mg. NaCN. Evidence of disappearance of cyanide in Warburg vessels has also been observed, often with increased O_2 uptake over controls. This suggests production of cyanates. It is possible that other reactions take place, such as formation of cyanohydrins. In this case further reaction must take place since no increased glycerol production was found, as would be expected from the familiar "trapping methods" used in yeast fermentation studies.

The effect of subculturing in cyanide.

Marked changes have been found as a result of this treatment. A series of 4 flasks, in which yeast was to be grown aerobically, anaerobically, anaerobically with cyanide and aerobically with cyanide, called the 1st cyanide series, was inoculated from the 2-day cyanide flask of an ordinary series. After 2 days' growth a new series, called the 2nd cyanide series, was inoculated from the CN-yeast of the 1st series. The 1st series was then centrifuged, washed and examined as usual. After 2 days a 3rd cyanide series was inoculated with the CN-yeast of the 2nd, etc.

The questions involved are whether yeast once grown as CN-yeast reverts on subsequent culturing aerobically or anaerobically, and whether culturing in

Table IV. *Effect of subculturing on certain properties of yeasts inoculated with cyanide-yeast, then grown aerobically, anaerobically, anaerobically with cyanide and again aerobically with cyanide.*

The 1st series was inoculated from the cyanide-yeast of an ordinary series, the 2nd series from the CN-yeast of the 1st, the 3rd series from the 2nd, etc.

Series		No substrate		Glucose		Lactate		Alcohol		Glucose		Flavin
		Q_{O_2}	$Q_{CO_2}^{CN}$	Q_{O_2}	$Q_{CO_2}^{CN}$	Q_{O_2}	$Q_{C_2}^{CN}$	Q_{O_2}	$Q_{CO_2}^{CN}$	Q_{CO_2}	$Q_{CO_2}^{N_2}$	
1st	Aerobic	- 4	- 5	-14	- 1	- 6	- 3	-13	- 1	+ 82	+104	39
	Anaerobic	- 7	-10	- 8	- 5	- 9	- 4	- 5	- 5	+130	+118	36
	N.C.N.	- 4	- 4	-12	- 1	-13	- 1	-11	- 1	+ 95	+104	40
	CN	-12	-10	-12	-11	- 3	- 3	- 6	- 6	+ 81	+ 76	60
3rd	Aerobic	- 3	- 4	-18	- 7	- 7	- 1	-14	- 4	+ 31	+ 51	40
	Anaerobic	-11	- 6	- 7	- 7	-12	- 6	-10	- 6	+104	+ 46	39
	N.C.N.	- 7	- 8	-19	-14	-12	-15	-16	- 3	+214	+120	32
	CN	-49	-40	-21	-21	-48	-21	-10	- 5	+170	+152	52
5th	Aerobic	-21	-25	-42	-31	—	—	—	—	+ 73	+ 90	11
	Anaerobic	-17	-11	-10	- 2	—	—	—	—	+119	+120	12
	N.C.N.	-21	-17	-25	-23	—	—	—	—	+112	+ 93	24
	CN	-23	-24	-21	-23	—	—	—	—	+ 43	+ 43	49
15th	Aerobic	-16	-23	-51	-57	-19	-19	-48	-16	+ 54	+106	0
	Anaerobic	-27	-11	-24	-16	- 9	-10	-25	-12	+124	+102	0
	N.C.N.	-20	-12	-26	-14	-24	-11	-32	- 8	+174	+172	0
	CN	-11	-14	-34	-54	-33	-31	-53	-25	+ 93	+ 72	0
20th	Aerobic	-18	-20	-37	-42	-20	-24	-40	-22	+ 57	+ 86	0
	Anaerobic	- 8	- 4	- 9	- 4	- 9	- 4	-12	- 4	+136	+122	0
	N.C.N.	- 7	- 4	- 9	- 7	- 9	- 6	-10	- 6	+118	+166	0
	CN	-12	-18	-25	-34	-11	-16	-29	-14	+134	+165	0

cyanide would cause changes. The results of some of these series are shown in Table IV. The intervening series (not shown) carry out the indicated trends in a regular manner.

Several things are to be noticed in Table IV. The flavin, which was high in the inoculum, was slightly lower in the aerobic, anaerobic and N.C.N.-yeasts, but still high in the CN-yeast. This suggests a disturbed mechanism not yet returned to normal. At about the 5th series the flavin was decreasing, and the same alcohol extract began to show a blue fluorescence. Estimation of the flavin green was made through a blue filter. The flavin had disappeared from all yeasts by the 10th series, and a strong blue fluorescence was found. In view of the apparent ability of some bacteria to transform flavin into a blue-fluorescing substance [Pett, 1935, 1, 3] it seemed possible that contamination had occurred. Owing to the complicated handling involved this might easily have happened, but careful microscopic examination of every flask revealed no bacteria, until the 23rd series (not shown).

The Pasteur effect continued to be absent from CN-yeast but reappeared immediately (1st series) in weakened form in the aerobic yeast, but not in the anaerobic, which shows only a slight effect in ordinary conditions. Fermentation in nitrogen was less than in air (3rd and 5th series).

In all cases respiration without substrate was not greatly inhibited by $M/600$ cyanide and in most cases was not increased with substrate. Addition of cyanide with substrate was sometimes inhibitory, though the opposite, namely a protective substrate action, is usually expected in enzyme systems. Probably other effects of cyanide are concerned than direct enzyme poisoning. From the 8th series definite activation by cyanide of the respiration in glucose was always observed, with aerobic and CN-yeasts; occasional activation in lactate was also found.

Nothing can yet be said on the nature and significance of the blue-fluorescing substance, except that it can be oxidized and reduced, but work is proceeding on this point.

SPECIFIC DISCUSSION.

These results are interesting for several reasons. Strangely enough no clear study of the effects of cyanide on growing yeast has been found in the literature. Neuberg & Perlman [1925] came close to such a study, Patterson [1931] investigated cyanide effects on different yeast preparations, and Meissel [1933] is referred to as having studied the toxic action of cyanide salts on yeast development, chiefly using heavy metal salts, however. Thus the present work opens up a new field for investigation, both with yeast and other organisms.

Points of special interest are (1) the increase in flavin in CN-yeast, (2) the relative insensitivity to CN of the respiration in CN-yeast, (3) the abolition of the Pasteur effect and (4) the development of a new type of yeast with perhaps a new respiratory pigment. In any case it is clear that cyanide-yeast represents a type distinct from either aerobic (baker's) or anaerobic (bottom) yeast.

The suggestion [Pett, 1935, 3] that the respiration of CN-yeast might be carried on by the increased flavin has been criticized by Torres [1935] on the basis of Warburg's "Wechselzahl" calculations. The fact remains that CN-yeast has 2 to 6 times more flavin than ordinary yeast and a respiration largely insensitive to cyanide.

Krah [1930] and Eichholtz [1930] had suggested that the Pasteur effect is connected with the poisoning of a ferro-catalyst so it was interesting to find that the effect has disappeared from CN-yeast. Since it reappears, even weakly, on

seeding into medium followed by aerobic growth, it must be concluded that the mechanism was not destroyed. It would be interesting to see the effect of growing yeast in ethyl carbylamine which Warburg [1926] showed to inhibit the Pasteur effect without affecting respiration or fermentation. More dilute cyanide concentrations might accomplish the same thing, as preliminary experiments have indicated.

More work is necessary on the effect of prolonged subculturing in cyanide before the significance of the observations is clear, but the indication is that some profound disturbance of the yeast has been effected and may result in a distinct type.

GENERAL DISCUSSION.

The researches of Warburg have led to the conception of cyanide as a poison of an iron-containing haem-like enzyme which is essential for the transfer of oxygen in all cells. The early work even referred to "das Atmungsferment" as though only one enzyme was responsible, though this idea has been relaxed.

It has been generally insisted that a cell's respiration must be largely inhibited by cyanide. Other results have appeared from time to time. Dixon & Elliott [1929] pointed out that while yeast respiration in general is inhibited by CN to an extent of 80 or 90 %, sometimes this did not result, and the respirations of various animal tissues were by no means totally, or even largely, inhibited by cyanide. Again Rosenthal [1931], Banga *et al.* [1931] and Shoup & Boykin [1931] found various cells and tissues partly or largely insensitive to cyanide. Of special interest here is the work of Elvehjem [1931] on cultured yeast. The studies of Frei *et al.* [1934] and of Yamaguchi [1934] have made it clear that the respiration of many, if not most, bacterial species is not very sensitive to cyanide, whether they contain cytochrome and its oxidase or not. Other possible effects of cyanide were, as usual, not studied. Occasional observations of activation by cyanide have been made. Thus Reynolds [1924] showed an increase in growth of *Fusarium*; Hanes & Barker [1931] observed an increase in the respiration of potato tubers; Tomkins [1932] observed an ultimate increased growth of moulds due to cyanide; and the respiration of algae [Watanabe, 1932] and of mammalian tissues [Kisch, 1933] has been increased by cyanide. Recently Lindahl & Örström [1936] reported an activation of top yeast respiration, which has always been the best subject on which to show inhibition. As to other effects of cyanide on cells few results have been reported. Resnichenko [1928] pointed out the importance of pH and permeability in cyanide effects, and Brinley [1928] suggested that one effect of cyanide may be to alter cell membranes and protoplasmic viscosity.

The present results, in which yeast has been actually grown in media containing cyanide, thereby effecting changes in the yeast, coupled with the above observations of others, make it imperative that the effects of cyanide on cells be considered from a broader view than heretofore. Especially must be revised the narrow view that respiration is invariably CN-sensitive and that this sensitivity is a result of specific action on certain enzyme systems, remote from all other possible effects. The importance of different effects from different cyanide concentrations must be recognized—indicating different actions, and above all the possibility of effects on permeability and metabolic processes which may or may not be reflected in the respiration, because the action is not solely, if at all, on the respiratory enzyme systems.

SUMMARY.

The observation that yeast can be grown in cyanide has been extended. Controls aerated by pure air (like baker's yeast), by nitrogen (corresponding to bottom yeast), and by nitrogen plus cyanide have been studied simultaneously. The respiration, fermentation, etc., of the different yeasts have been determined, and cyanide-yeast is shown to be a distinct type.

Cyanide-yeast showed the following properties, among others:

1. The flavin, present as flavoprotein, was consistently 2 to 6 times higher than in controls.
2. The respiration with or without substrate was largely insensitive to $M/600$ cyanide.
3. The aerobic fermentation was higher than the fermentation of aerobic yeast whether determined aerobically or in nitrogen.
4. The Pasteur effect was not demonstrable—the anaerobic fermentation was no higher than the aerobic.

The effect of prolonged subculturing in cyanide indicates the possibility of developing a new type of yeast, containing no flavin but rather a blue-fluorescing pigment.

In the general discussion it is pointed out that the effects of cyanide on cells must now be considered from a broader view, recognizing that cyanide does not always inhibit, and that several effects probably exist.

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