ON THE PRODUCTION AND USE OF PERMANENTLY ALTERED STRAINS OF YEAST FOR STUDIES OF IN VIVO METABOLIC ORGANIZATION

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I

The nature of the dynamic organization of the various chemical units found within the living cell by use of *in vitro* methods is obviously one of the more important steps in the investigation of the metabolic machinery of the cell. In the attack on this aspect of cellular biochemistry it is obviously desirable that the cell remain alive (intact) during the course of experiments designed to reveal the *in vivo* interrelationships of the components of its various metabolic systems. Unfortunately few experimental procedures are available which are definitely known to fulfill this condition.

A most valuable tool for the *in vivo* attack on mechanisms of cellular metabolism has been, thus far, the socalled "specific" inhibitor. The use of such compounds as KCN, CO, NaF, and iodoacetic acid to block certain enzymic activities within the living cell has provided data which initially guided the isolation and purification of various cellular enzymes and the *in vitro* reconstruction of our present models of cellular metabolic mechanisms. Some of these inhibitors, however, were found to be non-specific in their action, thus limiting their usefulness for the *in vivo* attack on metabolic mechanisms. Cyanide, for example, in a range of moderate concentrations reversibly blocks the cytochrome—cytochrome-oxidase complex and inhibits oxygen consumption. In a range of low concentrations, however, cyanide may stimulate oxygen consumption and bring about a marked increase in the rate of respiration. (See Commoner, 1940, for details and for other examples.)

The following properties of specific inhibitors have also limited their general usefulness:

(*a*) The inhibitor may form an inactive complex with certain extracellular substrates employed in the experiments (Green and Williamson, 1936).

(*b*) An inhibitor may simultaneously affect more than one metabolic system within the cell even in experiments made under conditions which are believed to "isolate" single metabolic activities. Cyanide, for instance, has been shown to produce a distinct inhibition of fermentation as well as respiration in yeast held under non-

proliferating conditions in sugar-containing phosphate buffer solutions (Warburg, 1925 *b*).

(*c*) The possible reversibility of the inhibitory effect in the course of an experiment may be an added complicating factor. For cyanide it has been shown that the permanence of the inhibitory effect depends on the concentration of the inhibitor and the time it remains in contact with the cells. (See Warburg 1925 *a*; Buchanan, 1926.)

(*d*) The slow penetration of the inhibitor into the cell may produce an effect which is a function of time (for cases involving iodoacetate inhibition see Saslow, 1937; for fluoride inhibition, Runnström and Sperber, 1938).

(*e*) It is technically difficult to maintain a constant concentration of such inhibitors as HCN during the course of experiments. With cyanide, compensation must be made for the tendency of HCN to distill out of experimental cell suspensions into the alkaline CO_2 absorber used in measuring the rate of O_2 uptake (Krebs, 1935; Robbie, Boell, and Bodine, 1938).

II

A method of circumventing some of the difficulties encountered in working with inhibitors has been on trial in this laboratory for several years. The method consists of treating a strain of yeast during cell proliferation with a specific inhibitor until a new stable strain of yeast possessing characteristics different from those of the original (parent) strain, and capable of maintaining the altered characteristics in the absence of the inhibitor, is obtained. Our technique was adapted from the earlier work of Meissel (1933). For other contributions on the effect of inhibitors on proliferating yeast see Pett (1936), and Yoshikawa (1938).

Our first attempt with KCN as the modifying agent has yielded a pure substrain which exhibits metabolic properties generally characteristic of yeast cells poisoned with KCN under non-proliferating conditions. The particular characteristics of this strain have remained constant for a period of 5 years during cultivation on media free from cyanide.

Our cyanide substrain does not have cytochrome activity, according to the tests employed, and is apparently entirely lacking in certain components of the enzyme complex believed to be responsible for the major portion of the oxygen consumption of its parent strain. A final pronouncement on this point depends, however, on the development of a satisfactory extraction and assay method for the cytochrome-oxidase component of yeast cells.

The disappearance of cytochrome-oxidase activity upon proliferation of the cells in contact with cyanide indicates a non-reversibility of the action of cyanide. This has not previously been definitely established by experiments under the usual non-proliferating conditions. It thus appears from our results that cyanide can bring about a temporary (reversible) inhibition of oxidase activity under non-proliferating conditions whereas, under proliferating conditions it acts irreversibly destroying the ability of growing cells to synthesize cytochrome-oxidase in an active form or at all.

The exact nature of the action of cyanide in bringing about the permanent (genetic) alteration has not been investigated as yet; neither have we studied the possibility of replacing the altered, or lost, enzyme units by (*a*) culture procedures (see Lwoff, 1933), (*b*) the addition of various isolated cell compo-

TABLE I

Comparison of the Metabolic Characteristics of the Parent Strain with Those of the Cyanide Substrain of Yeast

	Cyanide substrain	Parent strain
Respiration		
Q_{O_2} in 5 per cent dextrose	4.7	31.8
Fermentation		
Aerobic ($Q^{air}_{CO_2} - Q^{air}_{O_2}$)	238.5	156.5
Anaerobic ($Q^{N_2}_{CO_2}$)	248.8	192.8
Cytochrome-oxidase tests*	Negative	Positive
Cytochrome c absorption bands	Do not disappear in oxygen	Disappear in oxygen
Catalase test	Positive	Positive
Glucose dehydrogenase test†	Positive	Positive
Effect of inhibitors on respiration		
KCN	No effect	Complete inhibition
NaN_3	No effect	Complete inhibition

* Procedures employed were those of Keilin (1925): (*a*) Nadi reagent; (*b*) manometric method employing *p*-phenylenediamine hydrochloride.

† Anaerobic methylene blue procedure carried out in Thunberg tubes. The dehydrogenase activity was the same in both strains.

Addendum.—

Production of the Cyanide-Substrain

The procedure employed during 1935–36 in obtaining our cyanide substrain was briefly as follows: Cells of a pure strain of *Saccharomyces cerevisiae* Hansen (American Type Culture Collection No. 4360), originally derived from a single cell, were seeded into 1.5 per cent Difco malt extract broth containing an initial KCN concentration of M/1000. The culture was incubated for 48 hours at 25°C., then washed on the centrifuge with sterile M/15 KH_2PO_4, and subcultured in a modified Williams' medium (see Castor, 1939). After a series of liquid subcultures 3 single cell isolations were made and each one maintained on agar slants. Since the respiratory metabolism of the yeast from each isolation was found to be identical we finally selected one of the pure lines and have subsequently carried it on agar slants. Pure culture conditions were rigorously maintained throughout all the procedures.

The results on the cyanide substrain reported in Table I were recently obtained on yeast grown in a liquid subculture.

nents during non-proliferating conditions (Ogston and Green, 1935), or (*c*) the genetic methods developed for yeast by Winge and Laustsen (1938). These aspects of the problem will be the subject of future investigations.

The absence of cytochrome-oxidase and cytochrome c activity in cells of the cyanide substrain, together with the presence of a small cyanide- and azide-stable respiration (about 15 per cent. of the respiration of the parent strain)

makes this new strain of yeast of considerable value for direct tests of the rôle of flavoprotein in cyanide-insensitive respiration. It should also be useful for investigations of the *in vivo* activities of the cytochrome components a and b. Other examples of its advantages in investigations of intracellular metabolic mechanisms will readily suggest themselves.

III

The inhibitor-produced substrain of yeast thus makes available an unlimited supply of cells which possess a range of metabolic characteristics, and presumably an *in vivo* chemical organization, markedly different from that of its parent strain of yeast. More important, however, is the possibility of analyzing these cells, either directly by chemical methods, or indirectly by physiological methods, for their component enzyme systems without destroying the altered strain of yeast, and especially, without interference from the chemical agent originally employed in the modification.

It is our plan, as time permits, to attempt the production of other strains of *Saccharomyces cerevisiae* and other yeasts by use of various specific enzyme poisons and various physical agents which modify cellular metabolism. It is hoped that by this method we will be able to delete from, or modify in, the cell its various constitutive enzymes or enzyme systems and ascertain their probable rôle in the chemical organization of cells of the parent strain. It should be noted, of course, that the characteristic metabolic activities of the "normal" parent cells depend upon the usual functional organization of the *full* complement of the component enzyme systems of the cell. Changes in metabolic characteristics produced by the elimination or alteration of any cellular component may be not solely the expression of the loss of a particular enzyme unit, but rather the expression of a fundamentally new dynamic organization resulting from the deletion. In the latter case the results obtained by this program of research would be at variance with the usual *in vitro* reconstructions. So far, however, the results obtained with our cyanide substrain are in accord in most essentials with the *in vitro* reconstructions of Keilin and Warburg.

CITATIONS

Buchanan, J. W., 1926, Depression of oxidative metabolism and recovery from dilute potassium cyanide, *J. Exp. Zool.*, **44**, 285.

Castor, J. G. B., 1939, Metabolic characteristics of a cyanide saltant strain of yeast, Ph.D. Thesis, Harvard University.

Commoner, B., 1940, Cyanide inhibition as a means of elucidating the mechanisms of cellular respiration, *Biol. Rev. Cambridge Phil. Soc.*, **15**, 168.

Green, D. E., and Williamson, S., 1936, Pyruvic and oxaloacetic cyanohydrins, *Biochem. J.*, London, **31**, 617.

Keilin, D., 1925, Cytochrome, a respiratory pigment, common to animals, yeast, and higher plants, *Proc. Roy. Soc. London, Series B*, **98**, 312.

Krebs, H. A., 1935, Metabolism of amino-acids. III. Deamination of amino-acids, *Biochem. J.*, London, **29,** 1620.

Lwoff, A., 1933, Die Bedeutung des Blutfarbstoff für die parasitischen Flagellaten, *Zentr. Bakt., I Abt., Orig.*, **130,** 498.

Meissel, M. N., 1933, Wirkung der Cyansalze auf die Entwicklung der Hefe, *Zentr. Bakt., II Abt.*, **88,** 449.

Ogston, F., and Green, D. E., 1935, The mechanism of the reaction of substrates with oxygen. II, *Biochem. J.*, London, **29,** 2005.

Pett, L. B., 1936, Studies on yeast grown in cyanide, *Biochem. J.*, London, **30,** 1438.

Robbie, W. A., Boell, E. J., and Bodine, J. H., 1938, A study of the mechanism of cyanide inhibition. I. Effect of concentration on the egg of *Melanoplus differentialis*, *Physiol. Zool.*, **11,** 54.

Runnström, J., and Sperber, E., 1938, Zur. Kenntniss zur Beziehungen zwischen Permeabilität und Stoffwechsel der Hefezelle, *Biochem. Z.*, Berlin, **298,** 340.

Saslow, G., 1937, Oxygen consumption and respiratory quotients of caffeinized frog muscles, *J. Cell. and Compt. Physiol.*, **10,** 385.

Warburg, O., 1925 *a*, Uber Eisen, der sauerstoffübertragenden Bestandteil des Atmungsferment, *Ber. Chem. Ges.*, **58**B, 1001.

Warburg, O., 1925 *b*, Uber die Wirkung der Blausaure auf die alkoholische Gärung, *Biochem. Z.*, Berlin, **165,** 196.

Winge, Ö., and Laustsen, O., 1938, Artificial species hybridization in yeast, *Compt.-rend. trav. Lab. Carlsberg, série physiol.*, **22,** 235.

Yoshikawa, H., 1938, Studien über die Bedeutung des Eisenporphyrins im Zellstoffwechsel. II. Atmungsfähigkeit und die katalytischen Eigenschaften der Anaerob gezuchteten und der unter Cyanzusatz gezuchteten Hefen, *J. Biochem.*, Japan, **28,** 69.