Birkenshaw, J. H., Morgan, E. N., *Biochem. J.* **47**, 55 (1950).
Buttery, R. G., Ling, L. C., Guadagni, D. G., *J. Agr. Food Chem.* **17**, 385 (1969).
Di Giacomo, A., *Riv. Ital. Essenze Profumii* **44**, 610 (1965).
French, R. C., *Bot. Gaz. (Chicago)* **122**, 194 (1961).
French, R. C., *Bot. Gaz. (Chicago)* **124**, 121 (1962).
French, R. C., Gallimore, M. D., *J. Agr. Food Chem.* **19**, 912 (1971a).
French, R. C., Gallimore, M. D., *Phytopathology* **48**, 116 (1971b).
French, R. C., Gallimore, M. D., *J. Agr. Food Chem.* **20**, 421 (1972).
French, R. C., Weintraub, R. L., *Arch. Biochem. Biophys.* **72**, 235 (1957).
Guenther, E., "The Essential Oils," D. Van Nostrand, New York, N. Y., 1949.
Hartmann, G. R., Frear, D. S., *Biochem. Biophys. Res. Commun.* **10**, 366 (1963).
Macko, V., Staples, R. C., Renwick, A. A., Pirone, J., *Physiol. Plant Pathol.* **2**, 347 (1972).
Moshonas, M. G., *J. Food Sci.* **32**, 206 (1967).
Pouchert, C. J., "The Aldrich Library of Infrared Spectra," Aldrich Chemical Co., 1970, pp 191, 215, 216.
Searles, R. B., French, R. C., *Bot. Gaz. (Chicago)* **125**, 146 (1964).
Sprecher, E., *Ber. Deut. Bot. Ges.*, **72**, 150 (1964).
Staples, R. C., Wynn, W. K., *Bot. Rev.* **31**, 537 (1965).
Swern, D., *J. Amer. Chem. Soc.* **70**, 1235 (1948).
Swern, D., Findley, T. W., Scanlan, J. T., *J. Amer. Chem. Soc.* **66**, 1925 (1944).
Tulloch, A. P., *Can. J. Biochem. Physiol.* **41**, 1115 (1963).
Tulloch, A. P., Craig, B. M., Ledingham, G. A., *Can. J. Microbiol.* **5**, 485 (1959).
Tulloch, A. P., Ledingham, G. A., *Can. J. Microbiol.* **6**, 425 (1960).
Tulloch, A. P., Ledingham, G. A., *Can. J. Microbiol.* **8**, 379 (1962).

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Saccharomyces carlsbergensis: Microbiological Assay for Unidentified Factor Related to Glucose Tolerance

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A microbiological assay for an unidentified yeast growth factor from *Saccharomyces carlsbergensis*, related to the glucose tolerance factor, has been developed by using a strain of *Flavobacterium rhenanum*. Among the various media tested, one containing casein hydrolysate, glucose, and vitamins, gave best growth of the assay organism. Differences in the utilization of glucose, fructose, lactose, and acetate were detected. Increased amounts of acetate were inhibitory to the growth of the *Flavobacterium*. A linear relationship was found between yeast fractions assayed for glucose tolerance factor activity by the mammalian bioassay system and the microbiological assay regression values. Various food sources were assayed for their glucose tolerance factor activity, and their factor contents were compared.

The need for a microbiological assay for an unidentified factor obtained from *Saccharomyces carlsbergensis*, related to the chromium-containing glucose tolerance factor (GTF) (Mertz, 1969), prompted a search for microorganisms that might give a growth response to the unidentified factor. Availability of partially purified yeast fractions that had been tested for glucose tolerance factor activity in food samples made possible the screening of different organisms in order to develop a suitable assay for the factor (Toepfer *et al.*, 1973). Glucose tolerance factor has been defined as the dietary component necessary for normal glucose tolerance in rats (Mertz *et al.*, 1965). The yeast fraction has been partially purified by solvent extraction and column chromatography, but has not yet been identified. Among microorganisms examined for possible assay organisms, an isolate of *Flavobacterium rhenanum* obtained as a contaminant of a wild yeast culture gave an increased growth response when small amounts of the yeast factor were added to the growth medium. This initial observation allowed us to develop a microbiological assay for the factor that was rapid and dependable. Although impure yeast fractions containing the glucose tolerance factor were used in obtaining the increased growth response of the *Flavobacterium*, a linear relationship was found between the activity of the fractions assayed by the rat epididymal fat bioassay and the microbiological assay. Until further purification procedures are developed for the factor, the current results are reported in order that the assay system may serve in the isolation and identification of the active principle.

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METHODS

Stock cultures of the *Flavobacterium* strain were grown in Sabouraud's dextrose agar and transferred 24 hr before use as inoculum for the microbiological assay. The initial assay medium was casein hydrolysate broth (Casman, 1958) plus 0.05% glucose and an added supplement of known vitamins. The growth response of the *Flavobacterium* to the glucose tolerance factor samples was variable in Casman's medium, and better results were obtained with vitamin-free casein hydrolysate, glucose, and vitamins. Although the bacteria will grow in casein hydrolysate plus glucose, a more complete medium including the known vitamins and inorganic salts was used in the routine assay basal medium (Table I). Growth of the organism was also tested in a medium with and without nucleotides, casamino acids, and minerals plus vitamins. The composition of the nucleotide medium included the following in mg/l.: adenosine-3′-phosphoric acid, 80; guanosine-2′-phosphoric acid, 80; cytidine-3′-phosphoric acid, 80; casamino acids (vitamin-free), 5000; tryptophane, 10; K_2HPO_4, 25; KH_2PO_4, 25; $MgCl{\cdot}6H_2O$, 50; $MgSO_4$, 50;

Table I. *Flavobacterium* Basal Assay Medium

Component	g/l.	Component	mg/l.
Casein hydrolysate (vitamin-free)	30.0	Pantothenic acid	0.5
		Nicotinic acid	1.2
Glucose	0.40	Thiamine	0.4
l-Tryptophane	0.075	Riboflavin	1.0
l-Cystine	0.25	Pyridoxine	2.0
$CaCl_2$	0.10	Paraaminobenzoic acid	0.60
$MgSO_4$	0.20	Biotin	0.0015
K_2HPO_4	2.0	Folic acid	0.0015
		Vitamin B_{12}	0.0005
		Inositol	0.003
		Vitamin A acetate	0.003
		Ascorbic acid	0.005
		Choline chloride	0.002

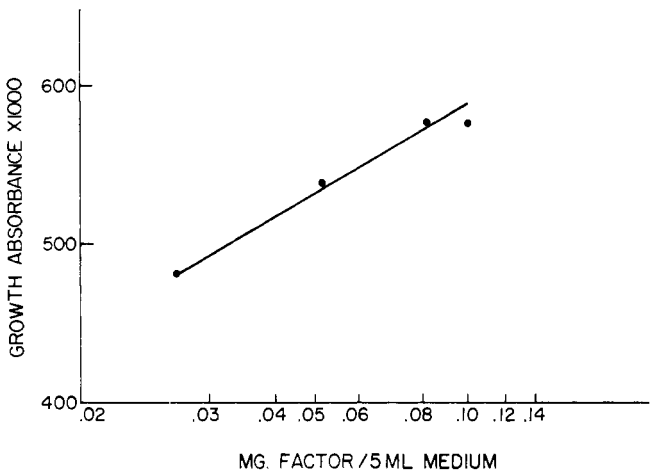

Figure 1. Growth response curve for *Flavobacterium rhenanum* and added glucose tolerance factor sample, QAE26. The dry weight values are plotted on a semi-log scale. The turbidity values are averages of triplicate tubes.

ferric citrate, 20; and the vitamin supplement at the concentration listed in Table I.

Growth of the bacteria in the assay of the yeast factor was determined by measuring the turbidity (absorbance) after incubation for 21 hr at 26°. Triplicate sets of tubes containing 5 ml of medium were prepared for each assay trial along with inoculated and uninoculated blanks. For the assay of factor activity, a 24-hr-old suspension of the organism grown at 26° in Sabouraud's dextrose agar was first decimally diluted in tubes containing 4.5 ml of casein hydrolysate broth. One-tenth milliliter of the 10^{-5} dilution of the bacterial suspension per 5 ml of assay medium was found to give a suitable amount of inocula for the assay when the first tube of the decimal dilution series gave an absorbance reading between 0.30 and 0.40 (usually corresponding to 3 mg of cell dry weight) at 650 nm. The use of heavier or lighter inocula did not result in a successful assay of the yeast factor activity. A similar phenomenon of inhibition of growth response with heavy inocula also was reported by Lichtenstein and Reynolds (1957) for the assay of vitamin B_{12} with *Lactobacillus leichmannii*. Inhibition that was due to carryover of inhibitory material with the inoculum was eliminated by repeated washing of the cells. Microbiological assay regression values, instead of absorbance at a given value of substrate (mg factor), allowed easier handling of the data. Regression value is defined as the slope of the line.

Various energy sources at different concentrations were tested in the basal assay medium with the *Flavobacterium* in order to detect and compare differences in metabolism that might exist between carbon substrates. Glucose, lactose, fructose, and acetate at levels of 1.6 to 6.4 mg in 5

Table II. Absorbance Readings for an Assay of Glucose Tolerance Factor Sample QAE26[a]

Sample added		Readings, o.d.		
mg	ml	1	2	3
		Trial no. 1		
	Blank	0.39	0.38	0.38
0.026	0.02	0.50	0.48	0.49
0.052	0.04	0.55	0.53	0.54
0.078	0.06	0.58	0.56	0.57
0.104	0.08	0.58	0.59	0.58
		Trial no. 2		
	Blank	0.40	0.41	0.39
0.026	0.02	0.50	0.48	0.48
0.052	0.04	0.54	0.55	0.52
0.078	0.06	0.56	0.57	0.56
0.104	0.08	0.58	0.60	0.60

[a] The sample regression coefficient b was calculated and used to show a linear function for the data in Trial no. 1. The latter was represented by the regression equation $y = a + b \log x$ or $y = 0.465 + 1.55 \log x$.

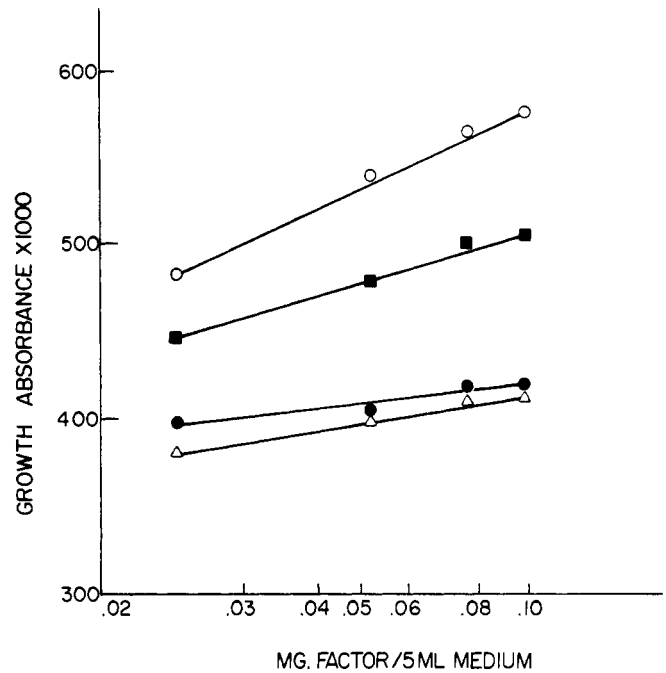

Figure 2. Growth response curves for *Flavobacterium rhenanum* measured at different time intervals; △, ●, ■, ○ = 15, 17, 19, and 21 hr, respectively. Growth was in 5 ml of the basal medium described in Table I.

ml of basal medium were used. The pH of the medium was not altered by addition of the sodium acetate.

Glucose tolerance factor samples were prepared as described by Toepfer *et al.* (1973). Vitamins and nucleotides were purchased from Calbiochem. Vitamin-free casein hydrolysate and carbohydrates were obtained from Nutrition Biochemicals Corporation.

RESULTS

The *Flavobacterium* strain used as the assay organism was proved to be a motile gram negative rod, 0.75 × 0.5–3.5 μ long. Colonies were cream to light tan. Small amounts of yellow pigment were produced only in old cultures. The carbohydrates degraded were glucose, sucrose, maltose, galactose, and fructose. Lactose and starch were not degraded. Litmus milk was reduced; indol and H_2S were not produced. The organism grew under aerobic and facultatively anaerobic conditions. The cultural properties of the isolate most closely resembled those described for *Flavobacterium rhenanum* (Breed *et al.*, 1957).

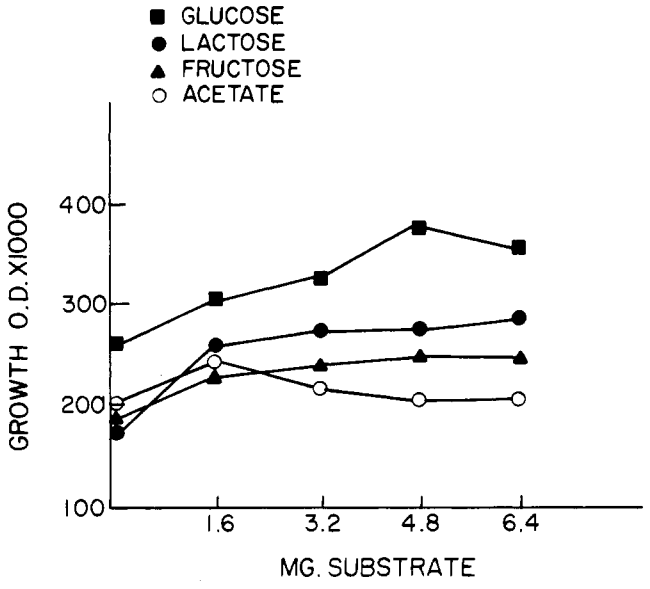

Figure 3. The influence of different carbon sources on the growth of *Flavobacterium rhenanum* in the basal medium without glucose.

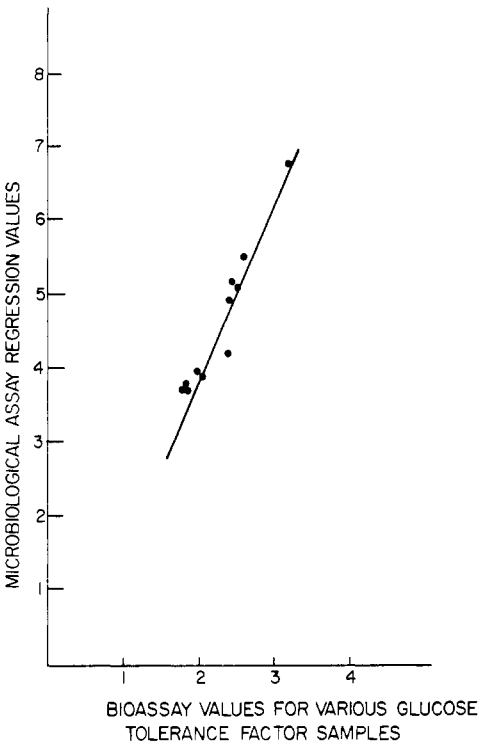

Figure 4. The relationship between rat epididymal fat bioassay values and the *Flavobacterium* microbiological assay linear regression values for glucose tolerance fractions. The slope of the line is represented by the regression equation $y = a + bx$ or $y = -1.0498 + 2.4710x$.

The absorbance readings obtained in two assay trials for a GTF sample are shown in Table II. Figure 1 illustrates the type of *Flavobacterium* growth response curve obtained by plotting the mean absorbance readings of triplicate tubes of the same sample that were provided increasing amounts of glucose tolerance factor sample (trial 1, Table II). Figure 2 illustrates the change in magnitude of the growth response to the glucose tolerance factor after various periods of incubation. Most of the growth occurred between 17 to 19 hr, with maximum yield at 21 hr. The medium used was the basal assay medium described in Table I.

The reliability of different media for the growth of the *Flavobacterium* was determined in assay trials, using samples of the yeast factor. One of the first media tested, Casman's casein hydrolysate broth plus 13 vitamins and glucose, permitted a satisfactory growth response to the glucose tolerance factor samples in initial trials, but it was later found to yield erratic results. The best medium developed included vitamin-free casein hydrolysate, glucose, inorganic salts, and vitamins, and is shown in Table I.

Since nucleotides have been reported to promote growth in marine flavobacteria (MacLeod *et al.*, 1958), a medium incorporating nucleotides plus casamino acids, minerals, and vitamins was also tested with the *Flavobacterium*. There was no stimulation of growth by a combination of the three nucleotides when compared to an identical medium that omitted the nucleotides. The addition of small amounts of unidentified factor to this medium resulted in stimulation of *Flavobacterium* growth similar to that obtained with the basal assay medium and the yeast factor. This was the medium used routinely in assaying the glucose tolerance factor (Table I).

The total growth of the organism at different levels of lactose, glucose, fructose, and sodium acetate as the substrates is shown in Figure 3. Glucose and lactose stimulated growth most, while fructose gave the least growth and acetate was only slightly utilized at the lower substrate level, with growth decreasing at the higher amounts (Figure 3). Growth response by the glucose tolerance factor was not elicited when lactose or fructose was added to the basal assay medium. Incorporation of sodium acetate in the basal medium did not interfere with the increases in growth of the *Flavobacterium* when the factor was added.

A linear relationship between various samples of the glucose tolerance factor assayed by *Flavobacterium rhenanum* and the rat bioassay was established by obtaining microbiological assay linear regression values for several glucose tolerance factor fractions and plotting these against the rat epididymal fat bioassay values obtained for the same series of fractions. The linear regressions represent the slope of the line of each trial. These results are shown in Figure 4.

Distribution of the unidentified factor in some food sources was determined by the use of the *Flavobacterium* assay, establishing their linear regression values and plotting these against the amounts of glucose tolerance factor chromium in the different foods. Extracts of food samples were prepared for the microbiological assay as described by Toepfer *et al.* (1973). Samples of shrimp, mushroom, lobster, beef, chicken, and haddock were high in glucose tolerance factor activity as evidenced by their microbiological assay growth response, while margarine and cheese were low in activity (Figure 5).

DISCUSSION

Organisms of the genus *Flavobacterium* that required growth factors were reported by MacLeod *et al.* (1954). One strain isolated would not grow in a medium with amino acids and nine vitamins unless yeast extract was also included. The requirement for yeast extract could not be replaced by vitamin B_{12}, pantethine, lipoic acid, or leucovorin. Of six strains isolated, only one could grow on acetate (MacLeod *et al.*, 1954). The *Flavobacterium* used as the assay organism in the current studies grew only on low levels of acetate; higher amounts were inhibitory. As in the *Flavobacterium* microbiological assay for the glucose tolerance factor, the factor described by MacLeod *et al.* (1958) was stimulatory rather than essential for the growth of the organism.

Preliminary isolation of the chromium-containing factor from *Saccharomyces carlsbergensis* was described in the review by Mertz (1969). The techniques developed allowed the separation by ion exchange chromatography of

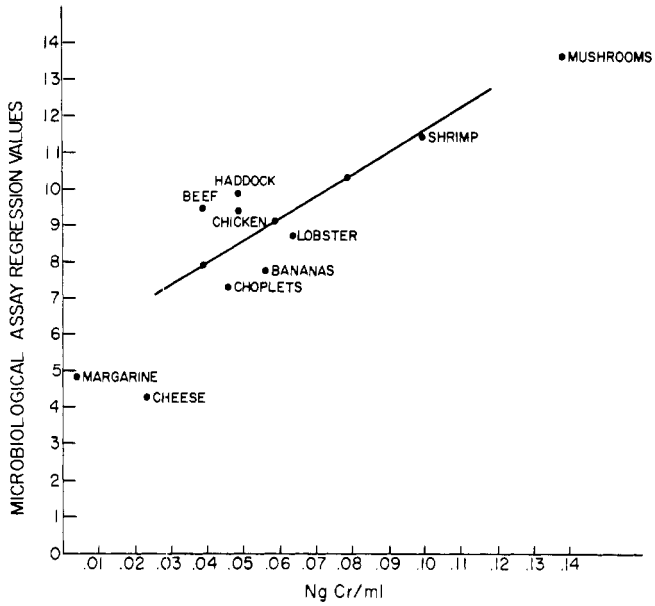

Figure 5. Biologically active glucose tolerance factor chromium distribution in different foods, with the relationship between microbiological assay regression values and the chromium in food samples. The slope of the line is given by the regression equation $y = a + bx$ or $y = 5.7273 + 54.7848x$.

the purified yeast fractions used to obtain the microbiological assay values. Using these fractions, a parallel relationship was established in this study between glucose tolerance factor bioassay values and the *Flavobacterium* microbiological assay. Thus, it appears that the growth response elicited in the bacteria by the yeast fraction is related to the biologically active chromium complex assayed by the rat epididymal fat pad assay (Mertz, 1969).

After the assay organism for the glucose tolerance factor was initially isolated, the microbiological assay was patterned after the vitamin B_{12} assay (Ford, 1953; Lichtenstein *et al.*, 1959) and the microbiological assay of vitamin B_6 (Toepfer and Polansky, 1970). The assay was used in a preliminary trial testing the content of the chromium factor in several foods, and good agreement was found between the results obtained with the rat bioassay and the microbiological assay. The microbiological assay can be applied directly to the testing of isolated yeast fractions to purify the active principle and to test for the content of glucose tolerance factor in natural sources.

LITERATURE CITED

Breed, R. S., Murray, E. G. D., Smith, N. R., "Bergey's Manual of Determination Bacteriology," 7th ed., Williams and Wilkins Co., Baltimore, Md., 1957.
Casman, E. P., *U. S. Pub. Health Rep.* **73,** 599 (1958).
Ford, J. E., *Brit. J. Nutr.* **7,** 299 (1953).
Lichtenstein, H., Beloian, A., Reynolds, H., *J. Agr. Food Chem.* **7,** 771 (1959).
Lichtenstein, H., Reynolds, H., *J. Ass. Offic. Agr. Chem.* **40,** 993 (1957).
MacLeod, R. A., Hogenkamp, H., Onofrey, E., *J. Bacteriol.* **75,** 460 (1958).
MacLeod, R. A., Onofrey, E., Norris, M. E., *J. Bacteriol.* **68,** 680 (1954).
Mertz, W., *Physiol. Rev.* **49,** 163 (1969).
Mertz, W., Roginski, E. E., Schroeder, H. A., *J. Nutr.* **86,** 107 (1965).
Toepfer, E. W., Mertz, W., Roginski, E., Polansky, M. M., *J. Agr. Food Chem.* **21,** 69 (1973).
Toepfer, E. W., Polansky, M. M., *J. Ass. Offic. Agr. Chem.* **53,** 546 (1970).

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Preparation of Solutions for Atomic Absorption Analyses of Fe, Mn, Zn, and Cu in Plant Tissue

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Dry ashing results in decreases in the apparent levels of Cu, Zn, and Fe in many types of plant tissue. The high concentrations of extraneous ions often present in plant ash solutions interfere with the determination of Fe, Mn, Zn, and Cu by atomic absorption. A procedure is proposed involving wet ashing with HNO_3, $HClO_4$, and H_2SO_4 and complete extraction of the trace elements with pyrrolidine dithiocarbamic acid in $CHCl_3$. The $CHCl_3$ is evaporated and the residue containing the trace elements is solubilized by refluxing with HNO_3. Using the proposed procedure, good recovery of added trace elements was obtained and the relative standard deviation was below 2% for the four trace elements.

There are two difficult problem areas that affect the precision and accuracy of atomic absorption (AA) analysis of trace elements in plant tissue, elemental losses that occur during organic matter destruction and extraneous inorganic ion interferences with AA.

Dry ashing of tissues in open vessels at high temperatures in a muffle furnace is a common means of organic matter destruction primarily because of the economy in terms of equipment and technician time. Elemental losses due to volatilization and incorporation in some solid material, either in the ash residue or the ashing vessel, can be quite serious with dry ashing. Since the chlorides of many metals have relatively low melting points, Gorsuch (1970) warns of the danger of volatilization of metals from tissues that are high in Cl at the temperatures used for muffling. Kometani *et al.* (1972) found it helpful to treat filter papers on which air-borne particulate matter was collected with H_2SO_4 before muffling at 500° in order to drive off Cl. At 600° they found greater volatilization losses of Cu from the $CuSO_4$ than $CuCl_2$. This also can be predicted from the melting points of the two salts. Generally temperatures must exceed 500° in ordinary muffle

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