Nonanal and 6-Methyl-5-hepten-2-one: Endogenous Germination Stimulators of Uredospores of *Puccinia graminis* var. *tritici* and Other Rusts

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Nonanal, previously isolated from distillates of uredospores of wheat stem rust *Puccinia graminis* var. *tritici* and demonstrated to be an effective stimulator of germination of these spores, was found by gas chromatography to be the chief volatile component in spore distillates and in condensates of airstreams drawn through live wheat stem rust spores. A second volatile component, 6-methyl-5-hepten-2-one, was identified in the spore extracts by gas chromatography, infrared spectrophotometry, and mass spectrometry. Both compounds stimulated germination of uredospores of crown rust of oats, leaf rust of wheat, and stem rust of wheat. Nonanal (2 to 3 μmol/g of spores) was recovered from uredospores of several rust types, including wheat stem rust (two races), wheat stripe rust, wheat leaf rust, oat crown rust, sunflower rust, corn rust, and bean rust. No nonanal was detected in similarly prepared distillates of conidia of the unrelated fungus *Pyricularia oryzae*.

Nonanal, an effective stimulator of uredospore germination in the wheat stem rust *Puccinia graminis* var. *tritici*, was identified previously in distillates of water suspensions of rust uredospores (French and Weintraub, 1957). Since that report, many compounds in addition to nonanal have been found active in stimulating spore germination (French, 1961; French and Gallimore, 1971a). The purpose of the current research was to study fresh, highly viable uredospores of wheat stem rust and to examine other rusts for the qualitative presence of nonanal and any other active compounds that might occur in significant quantity. Such a study might give clues to the metabolic generation or destruction of this potent stimulator of spore germination, as well as to its physiological mode of action.

MATERIALS AND METHODS

Viable uredospores of various species were produced in the greenhouse on appropriate hosts and stored in sealed jars at 4° or in liquid nitrogen. Uredospores used were as follows: stem rust of wheat *P. graminis* Pers. var. *tritici* (Eriks. & E. Henn.) Guyot, race 56 and race 15B; stripe rust of wheat *P. striiformis* West; leaf rust of wheat *P. recondita* Rob. ex Desm. F. sp. *tritici Eriks.;* crown rust of oats *Puccinia coronata* Cda. f. sp. *avenae* Fraser & Led.; bean rust *Uromyces phaseoli* (Reben.) Wint. var. *typica* Arth.; corn rust *Puccinia sorghi* Schw.; and sunflower rust *Puccinia helianthi* Schw. For comparison with the uredospores of rust fungi, dried conidia of the rice-blast pathogen, *Pyricularia oryzae* Cav., were included in the analyses. These spores had been produced on steeped corn grain and stored at 4°. Spores of stem rust of wheat used for airstream experiments were freshly harvested from plants and dried overnight over anhydrous calcium sulfate. Portions were refrigerated several days at 4° until quantities great enough for airstream analysis were obtained. Stripe rust spores for airstream analysis had been stored at 4° a year or more under a nitrogen atmosphere.

Chemicals. Authentic samples of nonanal and 6-methyl-5-hepten-2-one were obtained from Aldrich Chemical Co.

cis-9,10-Epoxyoctadecanoic acid and threo-9,10-dihydroxyoctadecanoic acid were synthesized according to the procedures of Swern *et al.* (1944) and Swern (1948).

Bioassay. The effectiveness of the compounds under study in stimulating uredospore germination was determined by floating spores on water suspensions of compounds, using Conway diffusion cells by methods previously described (French and Gallimore, 1971a).

Distillate Extracts of Spores. Spores (25 g) were suspended in 20 vol of distilled water in a 1-l. round-bottomed flask and distilled until half of the water with accompanying volatile materials had passed into the chilled collection flask. Further distillation yielded little additional material. About 50% w/v of salt was added to the condensates in the collection flask, and then they were extracted with 0.5 vol of redistilled isopentane. The isopentane extract of the spore distillate was concentrated to about 1 ml under a gentle airstream. Small quantities of this extract or a further concentration of it were injected into the gas chromatograph (gc) for analysis of extracted volatiles.

Water Extract of Spores. Spores were suspended in 5 vol of distilled water, allowed to soak for 2 hr with occasional shaking, and removed by filtration through Whatman no. 1 filter paper. Extraction was repeated; the extracts were combined, extracted with 0.25 vol of redistilled isopentane, and concentrated for analysis as before.

Airstream Extracts of Spores. Air was dried by passage through drying tubes containing $CaSO_4$ granules or humidified by bubbling through distilled water, and then drawn by gentle suction through spores (150 g) contained in a gas-washing bottle fitted with a fritted disk and a side inlet. This air then passed through 20 ml of isopentane in a finger-type condenser cooled by ethanol–dry ice in order to trap volatiles released by the spores. At various time intervals, the solvent plus spore volatiles was removed from the trap, concentrated in an airstream, and analyzed by gc. As a control, room air was drawn through the system for a period of several hours with no spores present. Amounts of volatiles recovered from room air were negligible compared to the amounts of volatiles from spores being studied.

Analytical Procedures. Routine separation of the components of the spore extracts was made with a Perkin-Elmer model 800 dual-column gas chromatograph with flame ionization detectors, equipped with a 4-to-1 effluent splitter to permit sample collection. The principal gc analyses reported here were made using a 6-ft × ⅛-in. o.d. stainless steel column packed with 15% DC-550 (phenyl-type silicone oil) on 80–100 mesh GC 22 (Perkin-Elmer Co.). Nitrogen carrier flow was 35 cm^3/min. Injection port and detector temperatures were 180° and 210°, respectively. For identification and measurement of extract compo-

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nents, a temperature program of 50° to 200° at 12.5°/min was employed. When effluent fractions were collected for further analyses, the program rate was 5°/min. Gc effluent fractions were collected using a Haak heated-line collection apparatus attached to a 0.01-mm AgCl infrared microcell cooled in an ice bath. Infrared spectra of the collected fractions and of gc purified authentic samples of various compounds were determined, using a Beckman Model IR 8 infrared spectrophotometer. Mass spectrometric analyses of components of spore extracts were made, using a Perkin-Elmer Hitachi RMU-6E single-focusing, magnetic-scan instrument, electron voltage, 70 V. The gas chromatograph was attached to the mass spectrometer through a Biemann-Watson separator, with all connecting lines held at 200°. The gas chromatograph was a Hewlett-Packard Model 5750 with thermal conductivity detector. The effluent stream was split between the mass spectrometer and gas chromatograph, so that both gc and ms peaks could be recorded. In addition, the gc peaks were observed on the total ion monitor of the mass spectrometer.

RESULTS

Distillates of Uredospores of Stem Rust of Wheat. French and Weintraub (1957) identified nonanal as the principal biologically active component in the distillates of wheat stem rust uredospores through the formation and subsequent identification of semicarbazone, thiosemicarbazone, and 2,4-dinitrophenylhydrazone derivatives. Gas chromatography, a technique more sensitive than derivatization, was used in the present studies. Analyses of isopentane extracts of spore distillates, using DC-550, Carbowax 1500, diethylene glycol succinate (DEGS), and Apiezon L-type columns, all indicated, by peak amplification, that nonanal was the principal component of spore distillates. The gc pattern shown in Figure 1A shows the predominance of nonanal over a background of numerous other volatile components. Treatment of similar distillate samples with sodium bisulfite before isopentane extraction indicated removal of carbonyl compounds by elimination of many of the component peaks, including that for nonanal.

The identity of nonanal as the chief component of stem rust spore distillate was further confirmed by infrared analysis of the gc effluent corresponding to the major peak.

Final confirmation of nonanal as the major distillate component was made using a gc–mass spectrograph (gc–ms) combination, in which the spectrum of the largest gc peak corresponded to that of an authentic sample of nonanal.

Water Extracts of Wheat Stem Rust Spores. Previous studies of nonanal in rust spores were based on products resulting from distillation of spore suspensions. Spores floated on water release a water-soluble germination inhibitor (Allen, 1955; Staples and Wynn, 1965), recently identified as methyl-*cis*-ferulate (Macko *et al.*, 1972); hence it was of interest to determine if nonanal were also released. Since French (1962) reported that high humidity in an airstream passing through spores greatly increased the amount of volatile aldehyde released into the airstream, attempts were made to recover nonanal from live spores by soaking them in large volumes of water. Water extracts of spores were made as described. The principal component from gc analysis of the water extract appeared to be a compound that eluted a few degrees before the expected peak for nonanal (Figure 1B). This component was absent from a bisulfite-treated sample, indicating the presence of a carbonyl group. No significant amounts of nonanal appeared to be present in the water extract of live spores, yet a distillate of the water extract proved to be quite effective in stimulating germination. This finding was not surprising because numerous volatile compounds had been shown to be effective in stimulating uredospore germination (French, 1961; French and Gallimore, 1971a). The compound observed to be the chief volatile component of water extracts was found by peak amplification also to be present in distillates as one of the more prominent secondary peaks. However, it is present in distillates in much smaller quantities than is nonanal. The infrared spectrum of a sample collected from gc effluent had a major peak at 1712 cm^{-1}, indicating the presence of a carbonyl group, as did the bisulfite results, but the spectrum did not match that of numerous 7-, 8-, 9-, and 10-carbon aldehydes and ketones (Pouchert, 1970).

The main volatile component of water extracts of the spores was identified as 6-methyl-5-hepten-2-one by use of a gc–ms combination. Comparison of the gc, ir, and ms properties of an authentic sample of this compound con-

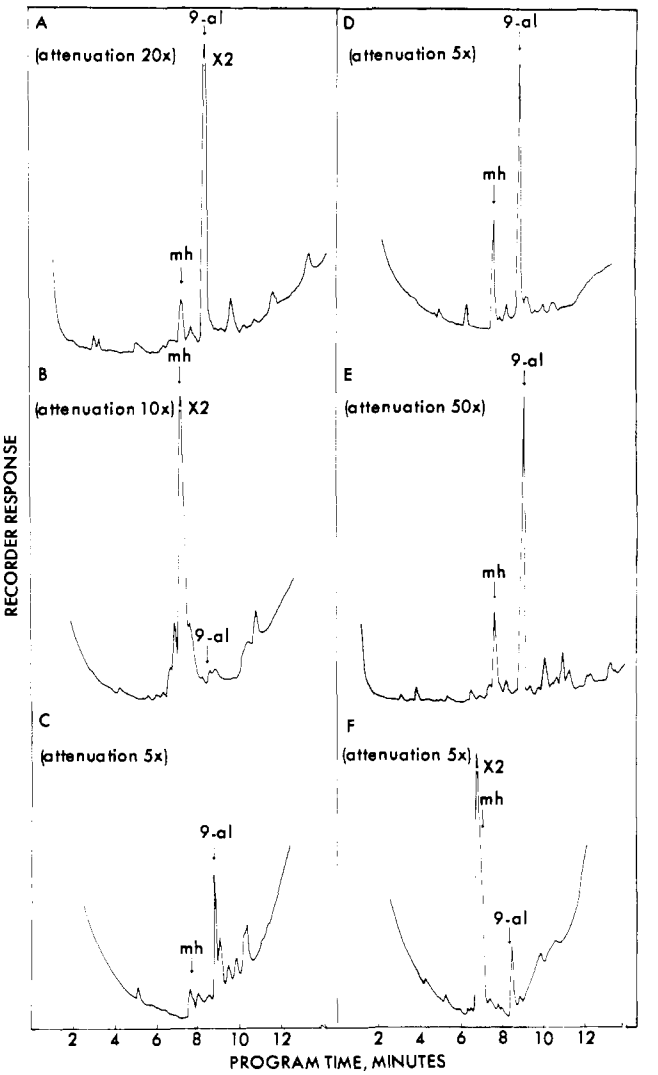

Figure 1. Gas chromatograms of isopentane extracts of spores. Samples run on a 6-ft × ⅛-in. o.d. stainless steel column of 15% DC-550 (phenyl-type silicone oil) packed on 80–100 mesh GC 22, with a program of 50–200°, run at 12.5°/min. Peak positions, as determined from duplicate amplification runs, are indicated for nonanal (9-al) and 6-methyl-5-hepten-2-one (mh). A. 10-μl extract of a distillate of wheat stem rust uredospores. B. 20-μl extract of a cold water extract of wheat stem rust uredospores. C. 25-μl extract of condensate from moist airstream drawn through wheat stem rust uredospores for 24–48 hr. D. 10-μl extract of a distillate of wheat stripe rust uredospores. E. 30-μl extract of condensate from moist airstream drawn through wheat stripe rust uredospores for 0–24 hr. F. 25-μl extract of a distillate of conidia of *Pyricularia oryzae*. Note that for *Pyricularia* neither peak aligns with the peak positions of nonanal (9-al) and 6-methyl-5-hepten-2-one (mh) determined in peak amplification runs.

Table I. Effect of 6-Methyl-5-hepten-2-one on Germination of Stem Rust Spores. Spores Were Floated on a 2.0-ml Suspension of Compound at 22° for 90 min

Concentration, μl/2.0 ml	ppm	% germination	
		Experiment 1	Experiment 2
10	5,000	0	0
5	2,500	0	0
2	1,000		14
1	500	66	47
0.8	400	77	88
0.6	300	86	91
0.4	200	88	94
0.2	100	93	95
0.1	50	88	95
0.01	5	34	
0.001	0.5	10	
H_2O	0	4, 6	6, 7

firmed the proposed identity. The ir and ms absorption patterns of this rather unusual compound are shown in a recent paper by Moshonas (1967), in which he reports its presence in orange oil.

The authentic sample of 6-methyl-5-hepten-2-one, like many related compounds, was quite stimulatory to uredospore germination (Table I). Also, like these compounds, it was active in stimulating germination of spores still contained within the rust pustule (French and Gallimore, 1972).

In the gc pattern (Figure 1A) of the spore distillate, the smaller peak corresponding to 6-methyl-5-hepten-2-one is marked. However, analysis of this peak in the gc–ms combination revealed that what appears to be separating as a single component on the DC-550 column is actually composed of one or two additional compounds of unknown identity, as well as the methylheptenone. Some of the minor components in the rust spore distillates have been tentatively identified from gc–ms analyses as hexenal, 2,4-dimethylhexenal, and crotonaldehyde. Other components had mass spectra whose fragmentation patterns suggested they were aldehydes, ketones, and unsaturated hydrocarbon molecules, but more specific characterizations were not made. *n*-Nonanol, the possible reduction product of uredospore action on nonanal (Searles and French, 1964), was observed by French and Gallimore (1971a) to be even more effective than nonanal in stimulating spore germination. However, this compound was not detected in either distillates or water extracts of spores.

Airstream Extracts of Wheat Stem Rust Uredospores. French (1961) had reported that airstreams drawn through uredospores contained volatile substances that stimulated uredospore germination. Aldehydes, as measured by reaction with *p*-hydroxybiphenyl, were found to be present in condensates of these airstreams and, like the stimulatory capacity of the airstream condensate, to be present in much greater amounts in humidified airstreams than in dry ones. It was proposed that the observed increase in germination among rust spores exposed to high humidities for several hours (spore hydration) might be caused by the release of endogenously produced nonanal by the spores. For this reason, attempts were made to identify and measure nonanal in condensates of dry and moist airstreams drawn through spore masses. In an experiment where first dry air for 4 hr and then humidified air was drawn through 150 g of uredospores of wheat stem rust (freshly collected and dried over anhydrous $CaSO_4$ at 4°), with samples of the air condensate taken at various time intervals, no apparent increase in amount of volatiles was observed after the shift to the moist airstream. However, the amount of volatiles released into either type of airstream was so low during the first few hours that neither nonanal nor any other component could be positively identified and measured. After 24 hr, enough volatile material from the moist airstream was recovered to allow identification of nonanal as the chief component.

The change in percent moisture of uredospores was not followed during these experiments, and perhaps water saturation of spores is required before rapid release of volatiles is observed. Similar samples were taken and analyzed at various times over the next 120 hr. The gc pattern of these samples changed little during the course of collection. Nonanal appeared, by gc peak amplification and comparison with authentic nonanal, to be the major volatile component released. Figure 1C shows the gc pattern of the airstream condensate sample collected during the period 24 to 48 hr. When all the airstream condensate samples were pooled and injected into the gc–ms combination, the presence of nonanal and also 6-methyl-5-hepten-2-one was firmly established. Thus, nonanal is either present in or can be produced by live stem rust uredospores and is not generated solely during distillation as a degradation product.

The failure to observe a rapid release of nonanal into the air by spores exposed to moist airstreams does not rule out the possibility that water vapor (hydration) stimulates spore germination by triggering an intracellular buildup of nonanal. However, neither soaking a sample of live spores overnight in water nor exposing a sample to high humidity for several days gave a significant increase in nonanal recovered in distillates of the spores. Again, such an intracellular conversion of precursor to nonanal (germination stimulator) would not be detected if this same conversion occurs in all samples by heat during distillation. Attempts to isolate nonanal directly from solvent extracts of ground uredospores through the formation of bisulfite and other derivatives were unsuccessful because of interference by the large quantities of fats and oils in spores.

9,10-Epoxy- and 9,10-dihydroxyoctadecanoic acids, which comprise a significant portion of the fatty acids of wheat stem rust uredospores, have been suggested as possible precursors of the nonanal in these spores (Tulloch, 1963; Tulloch *et al.*, 1959). When these two acids were subjected to the normal spore distillation procedure, both in the presence and in the absence of spores, there was no evidence that nonanal was being generated from the acids by the heating process.

Endogenous Stimulators in Uredospores of Other Rusts. Stripe rust of wheat (*Puccinia striiformis*) uredospores was studied using both distillation and airstream procedures. By either method, the gc patterns of the volatiles derived from the stripe rust spores were similar to those obtained from stem rust spores except that 6-methyl-5-hepten-2-one was present in two to three times greater amounts (Figures 1D and E) than in stem rust spores. The gc identification of nonanal and 6-methyl-5-hepten-2-one in this sample was confirmed by mass spectrometry.

Because fewer spores are needed for the distillation extraction than for the airstream process, the volatile components of the other spore types studied were analyzed only by the distillation method. Table II shows the amount of nonanal recovered from each of the rusts analyzed by distillation. For each, the nonanal was identified and amounts were estimated by comparison of gc patterns with those of known quantities of authentic nonanal. No attempt is made to present data for methylheptenone levels because this compound, if present in the spore sample, was usually present in such small amounts that it could not be positively identified and measured. Also, mass spectrometry had shown that the gc peak corresponding to methylheptenone in many of these samples contained other components that eluted from the GC-550 column at about the same time. Although we have not been able to determine how accurately the amount of nonanal recov-

Table II. Nonanal Recovered in Distillates of Uredospores of Various Rusts and From Conidia of *Pyricularia oryzae*

Rust	μl of nonanal/g of spores
Wheat stem rust, race 56	0.046
Wheat stem rust, race 15B	0.050
Wheat stripe rust	0.048
Wheat leaf rust	0.055
Bean rust	0.034
Sunflower rust	0.026
Oat crown rust	+[a]
Corn rust	+[a]
Pyricularia	None[b]

[a] Nonanal present as predominant distillate component, but amount not determined. [b] In this sample, amounts as low as 0.0005 μl should have been detectable if present.

ered by our distillation technique reflects the level of the compound in live ungerminated spores, nonanal in all tests seemed to be the predominant volatile present, and its level was amazingly consistent among the various spore types studied. As may be noted in Table II, the distillate of the conidia of one unrelated fungus *Pyricularia oryzae* was also analyzed. Nonanal was not detected in this sample, although it should have been if it were present (and recoverable by these techniques) at a level of less than 1% of that found in the rust spores. A comparison of the gc pattern of a 100-fold more concentrated distillate of these *Pyricularia* conidia (Figure 1F) with that of rust spore distillates (Figures 1A and D) reveals a much lower level of volatile components in the conidia. Furthermore, the volatile peaks that were present were not reduced by prior treatment of the extract with bisulfite, providing further evidence for a lack of nonanal and methylheptenone in these spores.

When uredospores of the different rusts in which nonanal had been detected in their distillate extracts were, in turn, tested for germination responses to nonanal, 6-methyl-5-hepten-2-one, and similar compounds, three of them—stem rust of wheat, leaf rust of wheat, and crown rust of oats—showed a positive response. In the other types, no stimulatory response was observed.

DISCUSSION

Nonanal and a second compound, 6-methyl-5-heptene-2-one, were identified in distillates of spores and in condensates of airstreams drawn through live wheat stem rust spores. Methylheptenone is present in both distillates and airstream extracts in much smaller amounts than is nonanal and appears to be slightly less effective as a stimulator of rust spore germination. However, the presence of this compound is of interest because, as far as we could determine, this is the first report of 6-methyl-5-hepten-2-one occurring in a fungus and also having a potential physiological function. This compound has been found in wood-rotting fungi, *Ceratocystis* sp. (Endoconidiophora) (Birkenshaw and Morgan, 1950; Sprecher, 1964) and in many of the essential oils such as oil of ginger and oil of lemongrass (Guenther, 1949) and has more recently been identified in lemon oil (Di Giacomo, 1965) and orange oil (Moshonas, 1967).

The presence of nonanal and methylheptenone in the volatile substances from live uredospores indicate that these stimulators are available to affect spore germination if other environmental conditions are favorable. The release of these compounds from spores is enhanced by the presence of water vapor. Recently it was shown (French and Gallimore, 1971b) that spores could be pretreated with a low concentration of nonanal vapor and saturated water vapor for several hours and then show a stimulation in germination when transferred to water. All of these observations further implicate a role for endogenous stimulators in the stimulation observed in some spore lots when pretreated with water vapor.

Still another interesting water–water vapor–nonanal interaction may have been discovered in the apparent absence of nonanal in water extracts of stem rust spores. This compound perhaps is released from spores only in the presence of water vapor or by heat of distillation. Its preferential absorption on filtration equipment is possible (Buttery *et al.*, 1969), but one might expect methylheptenone to behave similarly; hence, this probably does not explain the absence of nonanal in water extracts of stem rust spores. Although the presence of nonanal and methylheptenone may have a pronounced effect on germination, the inhibitor, methyl-*cis*-ferulate, and the presence of and exposure to water vapor and liquid water must also be kept in mind when a control mechanism for germination is considered.

The effects of nonanal on germination have been studied in detail only with uredospores of wheat stem rust. Uredospores of crown rust of oats and leaf rust of wheat also respond to nonanal, but the others studied apparently do not.

The demonstrated presence of nonanal in distillates of spores of various types of rusts, including some for which a positive germination response has not been observed, raises many questions about its occurrence among other fungal spore types. *cis*-9,10-Epoxyoctadecanoic acid, which has been postulated to be a possible precursor of nonanal, has been reported to be present in abundant amounts in teliospores and aeciospores, as well as uredospores, of many different rust species. The epoxy acid was absent from the fats of the smuts and mildews (Tulloch and Ledingham, 1960). If the epoxy acid is indeed a precursor of nonanal, then one might expect a correlation between the occurrence of these two compounds among various spore types. In this regard, our finding of nonanal in bean rust (*Uromyces phaseoli*) and in corn rust (*Puccinia sorghi*) uredospores in amounts almost equal to that in wheat stem rust uredospores is of special interest, since Tulloch and Ledingham (1962) report no detectable epoxy acid in uredospores of either bean or corn rust. However, the amount of nonanal recovered in spore distillates is so small (about 0.05 μl, or 0.3 μmol/g of spores) compared to the amount of epoxy acid normally observed that quantities of the epoxy acid sufficient to serve as precursor of the nonanal may not be detected. The very large discrepancies between the quantities of the two compounds in many spore types further suggest that not all the epoxy acid would normally be degraded into nonanal.

Hartmann and Frear (1963) studied the metabolism of ^{14}C-labeled *cis*-9,10-epoxyoctadecanoic acid by crude cell-free homogenates of flax rust (*Melampsora lini* (Ehrenb.) Lév.) uredospores, using autoradiographic analysis of thin-layer chromatograms of the products. They found that the epoxy acid was enzymatically hydrolyzed, first to the dihydroxy acid and then probably to a monohydroxy acid. However, they probably would not have detected the formation of a small amount of nonanal if it had occurred.

Further surveys of various fungal spore types would be of interest, not only in terms of the possible presence of nonanal and other volatiles, but also to test for any potential of these volatile compounds to stimulate germination or otherwise affect spore metabolism.

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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;