level, the decarboxylation showed a sharp decrease. The maximum activity was reached at a weaker concentration with long-chain acids. The depression of decarboxylation at concentrations exceeding the maximum is not due to aldehyde formation, but to penetration of the keto acid at a rate exceeding the decarboxylation velocity, and its consequent accumulation in the yeast cell.

The reverse behaviour of straight-chain α-keto acids with yeast preparations and with intact yeast can only be explained by assuming that the cell membrane of intact yeast cells offers a barrier particularly to the penetration of short-chain keto acids. This assumption has been confirmed experimentally by potentiometric measurement of the penetration speeds.

The only branched α-keto acid investigated, α-ketoisovaleric acid, was decarboxylated slower by yeast preparations than were the next straight-chain keto acids. With intact yeast, α-ketoisovaleric acid was decarboxylated less rapidly than α-ketobutyric acid.

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GLUCOSONE

II. INHIBITION OF YEAST METABOLISM, YEAST HEXOKINASE ACTIVITY AND TISSUE GLYCOLYSIS*

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D-Glucosone has been reported[1,2,3] to inhibit the anaerobic fermentation of glucose by baker's yeast, but the reports were not in agreement as to the amount of glucosone required to produce inhibition. There was also lack of agreement as to whether glucosone does or does not inhibit respiration of yeast cells[2,3]. These variances,

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indicated the desirability of additional studies with a purified well-characterized sample of D-glucosone. The preparation and properties of such a sample of glucosone has been described in detail in Part I[4]. The present report includes an investigation of the effect of this purified glucosone on yeast metabolism together with studies of its effect on yeast hexokinase activity and on tissue-slice glycolysis.

MATERIALS AND METHODS

The yeast was Fleischmann's starch-free pressed baker's yeast. The hexokinase was Pabst's purified yeast hexokinase which, it is stated, has an activity of approximately 28,000 K.M. units per g at 30°. The tumor tissue slices were cut from Walker 256 carcinomas which had been grown subcutaneously for 5 to 7 days in male white rats of the Wistar strain. The brain tissue was sliced from normal brains excised from rats of approximately the same size as those in which the tumors had been grown. The tissues were sliced to approximately 0.5 mm in thickness with a Stadie-Riggs microtome.

The usual Warburg manometric techniques[5] were used for the fermentation, glycolysis and respiration studies. The direct method, in which KOH is placed in the center well of a flask, was used for the respiration measurement. The reaction rates were measured in a total volume of 2 ml at 37°. The gas phase was 5% CO_2 in N_2 for anaerobic and air for aerobic studies. The yeast hexokinase reaction was carried out in a total volume of 2 ml at 30° in an atmosphere of 5% CO_2 in N_2 according to the method of COLOWICK AND KALCKAR[6].

The yeast cells suspended in water were placed in the side-arm of the flask, and the substrate or substrate plus glucosone and water were placed in the main part of the flask. In some cases, as indicated, buffers to give a final concentration of 0.04 *M* were also added to the main part of the flask. For the yeast hexokinase reaction, hexokinase and substrate, or hexokinase, substrate and glucosone were placed in the side-arm of the flask and the main part contained the following in the final concentrations as indicated: 0.01 *M* adenosine triphosphate (ATP) adjusted with NaOH to a pH about 7.5, 0.01 *M* $MgCl_2$ and 0.02 *M* $NaHCO_3$. For tissue glycolysis the tissues were suspended in Krebs-Ringer bicarbonate which contained 0.015 *M* glucose and glucosone in the amount specified for each experiment. The amount of tissue was determined as the dry weight of the tissue which was taken from the suspending medium at the conclusion of the experiment and dried in an oven at 90°.

RESULTS AND DISCUSSION

Effect on anaerobic fermentation of yeast

Glucosone inhibition of yeast fermentation has been found to vary in intensity with different yeast samples and with storage of the yeast. The data given in Table I gathered from a series of experiments with the same yeast sample within a short period of time show that the pH of the medium is an important factor in the degree of inhibition of yeast fermentation by glucosone. When an aqueous glucosone solution was made less acid with NaOH before addition to the medium, the degree of inhibition increased. This effect was most apparent when the molar ratio of glucosone to glucose was 3:1, at which level the average inhibition was 32% when the glucosone was unadjusted (pH 3.5), but 42 and 100% when the pH was raised to 4.5 and 5.5, respectively. When phosphate or succinate buffers were added to the system to maintain the pH during the course of the reaction, the inhibition was greater than when the system was unbuffered. The degree of inhibition produced by glucosone adjusted to pH 5.5 and in a molar ratio of 2:1 with glucose increased from 40% in the unbuffered to about 70% in the buffered medium.

Glucosone inhibited the anaerobic fermentation of fructose to a greater extent than it inhibited the fermentation of glucose. Unadjusted glucosone in a ratio of 1:1 with fructose gave an average of 35% inhibition in an unbuffered medium and

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TABLE I

EFFECT OF pH ON D-GLUCOSONE INHIBITION OF ANAEROBIC FERMENTATION OF GLUCOSE BY BAKER'S YEAST

Glucosone / Glucose (molar ratio)	Percentage inhibition in various media						
	Unbuffered			KH_2PO_4	Na_2HPO_4–KH_2PO_4		Succinate
	pH 3.5	pH 4.5	pH 5.5	pH 4.5	pH 5.5	pH 6.5	pH 5.5
1:1	0	0	0	7	31	24	34
2:1	0	0	40	67	69	73	71
3:1	32	42	100	100	100	—	100
5:1	100	100	—	—	—	—	—

Yeast, 2 mg; glucose concentration, 0.005 *M*; buffer concentration, 0.04 *M*. Glucosone was adjusted to the indicated pH with NaOH before addition to the medium. The unadjusted glucosone solution had a pH of 3.5.

57% when buffered with KH_2PO_4. Under similar conditions with glucose as the substrate, glucosone produced very little or no inhibition.

To determine whether the degree of inhibition was a function of the yeast concentration, the amount of yeast was varied and the amount of glucosone and sugar was held constant as required in the ACKERMAN AND POTTER[7] test for type of inhibition. When the reaction rates in the presence or absence of glucosone with either glucose or fructose as the substrate were plotted against yeast concentration (Fig. 1), the lines obtained passed through the origin. This indicates that glucosone did not combine irreversibly with any of the yeast enzymes and that the inhibition produced by glucosone was of the competitive type. This result substantiates the suggestion by MITCHELL AND BAYNE[1] that the inhibition by glucosone is competitive.

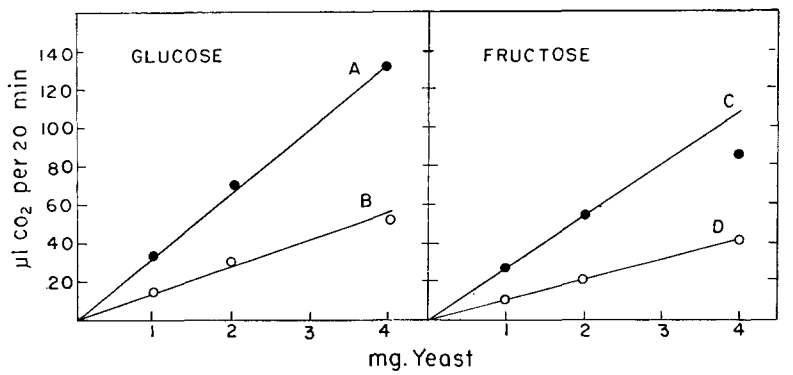

Fig. 1. Effect of amount of yeast on D-glucosone inhibition of anaerobic fermentation of glucose and of fructose. Curves: A, glucose 0.01 *M*; B, glucose 0.01 *M* and glucosone 0.01 *M*; C, fructose 0.01 *M*; D, fructose 0.01 *M* and glucosone 0.005 *M*.

The glucosone:glucose ratio necessary to inhibit anaerobic yeast fermentation had been reported by BECKER[3] and by MITCHELL AND BAYNE[1] to be greater than 2:1. Our present findings when the fermentation system is unbuffered and the glucosone solution adjusted to no higher than pH 4.5 are in agreement with their results. However, in our experiments, less glucosone was required for inhibition when adjusted

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to a higher pH or when buffer of pH 4.5 to 6.5 was added to the fermentation system. Our previous finding[2] that a 1:1 ratio of glucosone to glucose in an unbuffered system produced 56% inhibition may have been obtained because of the particular yeast sample used, or because of the glucosone sample which was obtained in syrup form and probably was not as pure as the sample used in this study.

Effect on respiration and aerobic fermentation of yeast

Glucosone, when adjusted to pH 4.5 and added in a glucosone:glucose ratio as high as 5:1, had little if any effect on the respiration of several yeast samples when the respiration was measured with 0.005 *M* glucose, 0.04 *M* KH_2PO_4 and 4 mg yeast present in the reaction mixture. Aerobic fermentation, measured under the same conditions, was inhibited when the glucosone:glucose ratio was as low as 2:1. The degree of inhibition of aerobic fermentation was about the same as that of anaerobic fermentation, which is shown in Table I, when the glucosone:glucose ratio was 3:1 or 2:1 in the presence of KH_2PO_4 buffer. However, the degree of inhibition varied somewhat with different yeast samples as had also been found to be the case in anaerobic fermentation.

The failure of glucosone to inhibit respiration is in agreement with the finding of BECKER[3]. The inhibition of respiration observed in our first studies[2] with glucosone may have been due to impurities in the original glucosone sample or to the particular yeast sample used.

Effect on yeast hexokinase activity

Glucosone has been found to inhibit the phosphorylation of glucose and of fructose by yeast hexokinase as is shown in Table II. The degree of inhibition appears to depend upon the molar ratio of glucosone to substrate. As this ratio was increased,

TABLE II

D-GLUCOSONE INHIBITION OF PHOSPHORYLATION OF GLUCOSE AND OF FRUCTOSE BY YEAST HEXOKINASE*

Glucosone / Substrate (molar ratio)	*Glucosone concentration M*	*Substrate concentration M*	*Inhibition of phosphorylation %*
	Glucose as substrate		
0.05:1	0.00025	0.005	7
0.10:1	0.0005	0.005	34
0.20:1	0.001	0.005	42
0.25:1	0.00025	0.001	43
0.50:1	0.0005	0.001	54
	Fructose as substrate		
0.05:1	0.0001	0.002	45
	0.00025	0.005	42
0.10:1	0.0005	0.005	68
	0.0001	0.001	52
0.25:1	0.0005	0.002	55
	0.00025	0.001	55
0.50:1	0.0005	0.001	62

* 0.2 mg of hexokinase used per flask.

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regardless of the absolute concentration of inhibitor and substrate, the inhibition tended to increase.

Glucosone had a greater inhibitory effect in the hexokinase reaction when fructose was the substrate than when glucose was the substrate, as had been found in the case of yeast fermentation. This increase in inhibition was more pronounced with lower proportions of inhibitor to substrate than with the higher proportions. At a molar ratio of glucosone to sugar of 0.05:1, the glucose phosphorylation by hexokinase was only slightly inhibited by glucosone, whereas the fructose phosphorylation was inhibited about 42%. At a molar ratio of 0.5:1, glucose phosphorylation was inhibited 54% and fructose phosphorylation 62%.

Indication that the inhibition was competitive in nature was obtained (Fig. 2) by the ACKERMAN AND POTTER[7] test. The competitive nature of the glucosone inhibition was further substantiated by results obtained with the LINEWEAVER AND BURK[8] test, which are shown in Fig. 3. The Michaelis enzyme–substrate dissociation constant, K_s, and the enzyme-inhibitor dissociation constant, K_i, were calculated from the data in Fig. 3. The K_s for fructose was found to be $1.0 \cdot 10^{-3}$ M and the

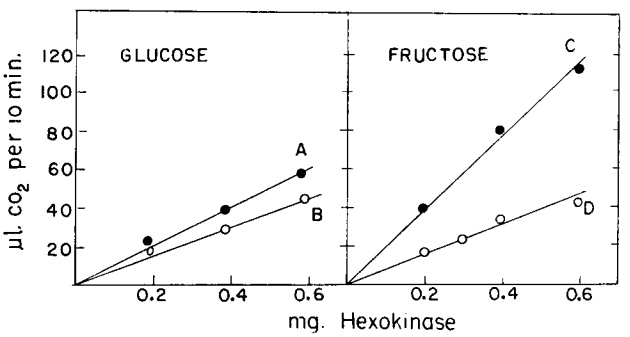

Fig. 2. Effect of amount of yeast hexokinase on D-glucosone inhibition of phosphorylation of glucose and of fructose. Curves: A, glucose 0.005 M; B, glucose 0.005 M and glucosone 0.0005 M; C, fructose 0.005 M; D, fructose 0.005 M and glucosone 0.0005 M.

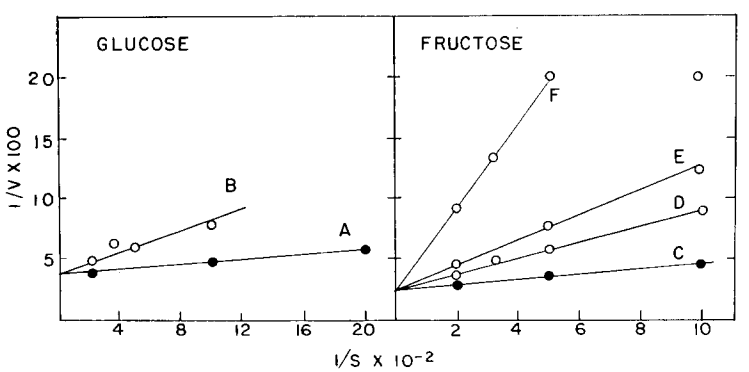

Fig. 3. Effect of increasing substrate concentration on D-glucosone inhibition of phosphorylation of glucose and of fructose by yeast hexokinase. The data were plotted according to the method of LINEWEAVER AND BURK. Curves: A, glucose (0.0005 M to 0.005 M); B, glucose plus glucosone 0.00025 M; C, fructose (0.001 M to 0.005 M); D, fructose plus glucosone 0.0001 M; E, fructose plus glucosone 0.00025 M; F, fructose plus glucosone 0.0005 M.

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K_s for glucose $2.0 \cdot 10^{-4}$ M. These constants are in fair agreement with those reported by other investigators[9] for fructose and glucose. The K_i for glucosone, averaged from the data in the three experiments with fructose as the substrate, was calculated to be $5.93 \cdot 10^{-5}$ M. The K_i for glucosone calculated from the experiment in which glucose was the substrate was $6.25 \cdot 10^{-5}$ M, which is almost identical with the K_i calculated when fructose was the substrate.

Glucosone was found by SOLS AND CRANE[10] to be very slowly phosphorylated by brain hexokinase, and they reported a dissociation constant of $1.0 \cdot 10^{-5}$ M for glucosone with brain hexokinase; however, phosphorylation of glucosone was not obtained by EEG-LARSEN AND LALAND[11] with their preparation of brain hexokinase. The phosphorylation of glucosone by a partially purified yeast hexokinase was reported by JOHNSTONE AND MITCHELL[12]. The phosphorylation of glucosone in the present experiments when 0.2 mg hexokinase was used has been found to be negligible, but when an amount of hexokinase 10 or 100 times greater was used (personally suggested by Dr. SOLS), there was a measurable amount of phosphorylation of glucosone.

Effect on glycolysis of tissue slices

Glucosone is shown by the data in Table III to be a very effective inhibitor of anaerobic glycolysis in tissue slices. With tumor-tissue slices, glucosone in a molar ratio of 0.067:1 with glucose gave 62% inhibition of glycolysis. With the Krebs ascites tumor cells, YUSHOK had found that glucosone in the same ratio with glucose produced 75% inhibition[13] and in a ratio of 0.043:1 with glucose produced 50% inhibition[14]. The effectiveness of glucosone against tumor tissue slices is therefore about the same as against ascites tumor cells.

TABLE III

D-GLUCOSONE INHIBITION OF GLYCOLYSIS OF RAT TISSUE SLICES

Glucosone concentration M	*Glucosone / Glucose (molar ratio)*	*Glycolysis rate*			*Inhibition of glycolysis* %
		Glucose + glucosone added $Q_{CO_2}^{N_2}$	*Glucose alone added* $Q_{CO_2}^{N_2}$	*No addition* $Q_{CO_2}^{N_2}$	
		Walker 256 tumor			
0.00005	0.0033:1	43.8	42.0	3.6	0
0.00025	0.017:1	28.8	39.2	4.8	26
0.0005	0.033:1	23.8	37.0	3.9	36
0.001	0.067:1	14.0	37.0	3.9	62
		Brain			
0.00001	0.00067:1	15.6	15.0	4.2	0
0.00005	0.0033:1	9.0	16.8	4.6	64
0.0001	0.0067:1	3.9	12.0	4.4	100

$Q_{CO_2}^{N_2}$ = μl CO_2/mg dry weight of tissue/h. Glucose concentration = 0.015 M.

With brain tissue, studied as an example of a normal tissue with a high anaerobic rate of glucose utilization, glucosone in a ratio of 0.0033:1 gave 64% inhibition. The glycolysis of brain tissue slices was therefore about 20 times more sensitive to glucosone than was that of tumor tissue slices.

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It is difficult to compare the effectiveness of glucosone on tissue glycolysis and on yeast fermentation since necessarily glycolysis and fermentation are carried out under different conditions of pH and since, as shown above, the potency of glucosone in yeast fermentation is somewhat pH-dependent. Under the conditions of these experiments, tissue glycolysis appears to be much more susceptible to glucosone inhibition than is yeast fermentation.

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The authors wish to express their appreciation to Dr. CHARLES E. BECKER for a generous sample of his glucosone for comparison studies.

SUMMARY

D-Glucosone inhibition of anaerobic yeast fermentation has been found to vary in degree with different yeast samples and to be somewhat dependent on the acidity of the medium. Glucosone did not affect the respiration of yeast but affected aerobic fermentation to about the same extent as it did anaerobic fermentation. The fermentation of fructose was inhibited by lower concentrations of glucosone than was the fermentation of glucose.

Glucosone was found to inhibit phosphorylation by yeast hexokinase more than fermentation by whole yeast with either glucose or fructose as substrate. The phosphorylation of fructose was inhibited to a greater extent than was the phosphorylation of glucose. The inhibition was found to be competitive in nature and the enzyme–inhibitor dissociation constant was calculated to be of the order of $6.0 \cdot 10^{-5}$ *M* with either glucose or fructose as substrate.

Glucosone has also been found to be inhibitory to the glycolysis of tumor- and brain-tissue slices. Much lower concentrations of glucosone were required for inhibition of tissue glycolysis than for inhibition of yeast fermentation. Brain tissue was more sensitive to glucosone than was tumor tissue.

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