Aerobic Metabolism of the Hepatopancreas of *Helix pomatia*

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The presence of the tricarboxylic-acid cycle (Krebs's cycle) has been demonstrated in most mammalian tissues investigated (Krebs & Johnson, 1937; Green, Loomis & Auerbach, 1948), where it appears to be the major pathway for the oxidation of carbohydrate, fat and protein intermediaries. No detailed examinations have been made in the invertebrates. However, evidence has been presented of Krebs's cycle activity in certain nematodes (Massey & Rogers, 1950), the cockroach (Barron & Tahmisiian, 1948) and *Drosophila* (Spirtes, 1951). No similar studies have been made in *Helix pomatia*. The evidence of Biedermann (1911), Baldwin (1938) and Vonk, Mighorst & de Groot (1950) suggests that the hepatopancreas plays an important role in the metabolism of the animal.

The enzyme systems examined in the present investigation were those contained in suspensions and mitochondrial preparations from the hepatopancreas of both hibernating and active *H. pomatia*, since there is evidence of changes in the enzymes of an organism during hibernation (Baldwin, 1938; Suomalainen, 1935).

METHODS

The snails used in these experiments were either kept in the refrigerator in the hibernating state, or had been active and fed on cabbage in the laboratory at 22° for at least 7 days before use. Unless otherwise specified, results reported in this paper were obtained with preparations from active *H. pomatia*.

Enzyme preparations

Suspensions. The hepatopancreas was rapidly removed and ground in an ice-cold Potter 'homogenizer' in either 0.2M-sucrose solution or 0.5% KCl solution. The suspension was diluted with the appropriate medium to give a final concentration of approximately 20% tissue.

Mitochondria. These were isolated according to the methods described by Schneider (1948) using 10% hepatopancreas suspensions made in 0.2M-sucrose. All preparations were carried out in a cold room at 2°, a Servall SS-1-a high-speed centrifuge being used for the isolation of the mitochondria.

Hexokinase. This was obtained by the method of Berger, Slein, Colowick & Cori (1946) as described by Cross, Covo, Taggart & Green (1949). The preparation was stored as a frozen solution at -10° and retained its activity for many months. The conditions for autolysis for baker's yeast were those described by Bailey & Webb (1948).

Determinations

Inorganic orthophosphate was determined by the method of Fiske & Subbarow (1925), and α -ketoglutarate and pyruvate by the method of Friedemann & Haugen (1943). Citrate was determined by the method of Natelson, Pincus & Lugovoy (1948) and sometimes by that of Weil-Malherbe & Bone (1949). Succinate was determined by the method of Krebs (1937). O_2 uptakes were determined manometrically; the gas phase was air.

Reagents

Adenosine triphosphate (ATP) was prepared by the method of Dounce, Rothstein, Beyer, Meier & Freer (1948). By chemical and spectrophotometric analysis (Kalekar, 1947) it was found to be 98% pure.

Commercial adenosine 5'-phosphate was crystallized twice from hot water. On spectrophotometric analysis it was found to be 99% or more pure.

Sodium citrate, L-glutamate, L-malate, α -ketoglutarate and oxaloacetate were commercial preparations. Succinic acid was recrystallized three times from hot water. The succinic acid was dissolved, neutralized and the sodium salt precipitated with ethanol. The salt was dissolved and crystallized from aqueous ethanol. Octanoic acid was obtained commercially and distilled *in vacuo*. Cytochrome *c* was prepared by the method of Keilin & Hartree (1937) and dialysed against distilled water. Sodium pyruvate was prepared from commercial pyruvic acid after distillation *in vacuo*, by a method described by Lardy (1949).

Diphosphopyridine nucleotide (DPN) was prepared by an unpublished method of Ochoa, and coenzyme A by the method of Lipmann, Kaplan, Novelli & Tuttle (1950). Hexose diphosphate and phosphoglyceric acid were gifts from Mr J. H. Ashby and by chemical analysis were 98% pure. Hexose monophosphate was a gift from Dr J. D. Judah.

Standard medium. Final concentrations in the Warburg flask were 0.025M-KCl; 0.0066M-Mg²⁺; 0.001M-adenylic acid (AMP); 1×10^{-5} M-cytochrome *c*; 0.014M-sodium phosphate buffer, pH 7.4; substrate as indicated in text. Final volume was made up to 3 ml. with water. The enzyme preparations were added in either KCl or sucrose solution (0.5 ml.). 0.1 ml. 20% KOH was placed in the centre well with filter papers. The equilibration was carried on for 10 min.

RESULTS

The endogenous respiration of suspensions prepared in 0.5% KCl or 0.2M-sucrose from both active and hibernating specimens of *H. pomatia* was measured. No significant difference was observed between the levels of the endogenous respiration of the active and hibernating snails as shown in Table 1.

Table 1. *Effect of Krebs's cycle intermediates on the respiration of hepatopancreas suspensions*

(System: standard medium, 30 μ moles substrate added except in the case of succinate where 100 μ moles added. Temp. 38°.)

Condition of animal	Substrate	O ₂ uptake (μ l./mg. N/hr.)		No. of observations
		Mean	Range	
(1) 0.5% KCl suspensions				
Active	Nil	11	9.3-14.1	5
Hibernating	Nil	12	9.1-15.5	7
Active	Succinate	36	25-43.5	8
Hibernating	Succinate	39	37-41.5	6
Active	α -Ketoglutarate	16	12.1-18.4	5
Hibernating	α -Ketoglutarate	17	10.8-23.0	5
Active	Citrate	19	16.6-19.9	6
Hibernating	Citrate	19	9.6-27.8	7
Active	Fumarate	15	11.6-16.9	5
Hibernating	Fumarate	21	14.7-24.4	6
Active	L-Malate	18	13.2-25.6	5
Hibernating	L-Malate	17	10.2-19.5	5
(2) 0.2M-Sucrose suspensions				
Active	Nil	14	11.9-15.4	5
Hibernating	Nil	10	8.1-13.1	6

The effect of the addition of various glycolytic intermediates on the respiration of 0.2M-sucrose suspensions was next investigated (Table 2). All substrates tested were found to increase the rate of respiration. Baldwin (1938) observed that tissue slices of the hepatopancreas of active *H. pomatia* utilize galactose, whereas preparations from hibernating animals do not. It has now been demonstrated that galactose was oxidized by 0.2M-sucrose suspensions of the hepatopancreas prepared from the active animal, but not by similar preparations from hibernating animals (Table 2).

Table 2. *Effect of added carbohydrates and glycolytic intermediates on the respiration of 0.2M-sucrose suspensions of the hepatopancreas*

(System: standard medium, 30 μ moles substrates added. Tissue added equivalent to 4 mg. N. Temp. = 38°. Duration of experiment = 30 min.)

Condition of animal	Substrate added	O ₂ uptake (μ l.)
Active	Nil	22
	Galactose	34
	Fructose 6-phosphate	34
	Hexose diphosphate	36
	Phosphoglyceric acid	42
	Glucose	35
Hibernating	Nil	16
	Phosphoglyceric acid	32
	Galactose	17

In Table 1 are also shown the results of the addition of Krebs's cycle intermediates to 0.5% potassium chloride suspensions prepared from the hepatopancreas of both active and hibernating animals. All of the Krebs's cycle intermediates

tested were found to increase the rate of respiration of the suspension. The extent of the increase due to the addition of the substrate was of the same order for the hepatopancreas preparations from both active and hibernating snails. The addition of Krebs's cycle intermediates to hepatopancreas suspensions after some 30 min. incubation in the Warburg bath was found to restore the level of the endogenous respiration, the endogenous substrate presumably being exhausted.

Table 3. *Effect of malonate on the oxidation of glucose and galactose in the presence and absence of malate*

(System: standard medium, malonate final concentration = 0.033M; glucose and galactose 30 μ moles. 0.2M-sucrose suspension added equivalent to 4 mg. N. Temp. = 38°. Duration of experiment = 30 min.)

Additions	Oxygen uptake (μ l.)
Nil	38.0
Malonate	32.0
Glucose	43.0
Glucose + malonate	37.0
Glucose + malonate + 5 μ moles malate	48.0
Galactose	46.0
Galactose + malonate	37.0

The effect of malonate on the respiration of hepatopancreas preparations

The effect of malonate on the respiration of a 0.2M-sucrose suspension in the presence of glucose, galactose, and L-malate was investigated. The results in Table 3 show that the addition of malonate causes some reduction in the oxidation of glucose, but that the original level is restored by the addition

of L-malate. Malonate was also found to inhibit the oxidation of all the Krebs's cycle intermediates tested (Table 4).

Table 4. *Effect of malonate on the oxidation of Krebs's cycle intermediates by 0.5% hepatopancreas suspensions*

(System: Standard medium, substrates 30 μ moles (succinate 100 μ moles), malonate 0.033 M. Tissue suspension added equivalent to 3 mg. N. Temp. = 38°. Duration of experiment = 1 hr.)

Substrate	O ₂ uptake (μ l.)		Inhibition (%)
	No malonate	With malonate	
Nil	30.0	25.0	16.0
Succinate	362	62.0	86.0
Fumarate	167	47.0	72.0
Citrate	60	36.0	40.0
α -Ketoglutarate	40	25.0	38.0

It is considered that these experiments indicate that the hepatopancreas of *H. pomatia* contains enzymes which will oxidize Krebs's cycle intermediates. The inhibition of the endogenous respiration which occurs on the addition of malonate, and the effect of the latter on the oxidation of all added Krebs's cycle intermediates, suggests that succinoxidase and possibly the Krebs's cycle plays a part in the endogenous respiration. The individual reactions of the Krebs's cycle were next investigated.

Reactions of the Krebs's cycle

The reaction citric acid $\rightarrow$ α -ketoglutarate was examined, further oxidation of α -ketoglutarate being prevented by the addition of arsenite. In Table 5 are shown the results of some typical experiments. The addition of citric acid to the hepatopancreas suspension was found to increase considerably the amount of α -ketoglutarate which accumulated.

Table 5. *Conversion of citric acid to α -ketoglutarate in 0.5% KCl suspension of the hepatopancreas of active Helix pomatia*

(System: standard medium and final arsenite concentration = 0.006 M. Suspension added equivalent to 4 mg. N. Duration of experiment = 1 hr.)

Additions	Oxygen utilized (μ atoms)	Citrate utilized (μ moles)	α -Keto-glutarate formed (μ moles)
Nil	13.0	1.5	2.0
30 μ moles citrate	18.2	11.0	9.0
30 μ moles citrate	13.2	10.0	7.0
30 μ moles citrate	11.0	9.0	7.5
No arsenite	5.7	7.0	4.1
+ 30 μ moles citrate			

In Table 6 are shown the results of experiments demonstrating the synthesis of citrate from a series of precursors. All the reactions were investigated in Thunberg tubes under anaerobic conditions to

Table 6. *Synthesis of citrate by hepatopancreas preparations*

(System: 0.0066 M-Mg²⁺; sodium phosphate buffer, pH 7.4, 0.014 M; ATP as indicated in the table; 20 μ moles substrates. Final volume = 3 ml. Duration of experiment = 30 min. Temp. = 28°. Results expressed as μ g. citrate synthesized/mg. N of suspension or mitochondria. Tissue suspensions or mitochondria added equivalent to 4 mg. N.)

	Oxalo-acetate	HCO ₃ '	Pyruvate	ATP concn. ($\times 10^{-3}$ M)	Citrate synthesized (μ g.)
(1) KCl suspension from active animal	+	-	+	2	13
	+	-	+	1	18
	+	-	+	2	20
	+	-	+	3	24
	-	+	+	1	19
	-	-	-	1	0
(2) Mitochondria from active animal	+	.	+	1	20
	-	+	+	1	0
(3) KCl suspension from hibernating animal	+	-	+	1	10
	-	+	+	1	0
	-	-	-	1	0

Table 7. *Accumulation of succinate by 0.5% KCl suspensions of the hepatopancreas of active Helix pomatia*

(System: standard medium; final concentration of malonate = 0.033 M. Temp. = 38°. Duration of experiment = 60 min. Suspension added equivalent to 4 mg. N.)

Additions	Oxygen utilized (μ atoms)	α -Ketoglutarate		Succinate formed (μ moles)
		Present (μ moles)	Utilized (μ moles)	
Nil	3.5	1.0	—	2.0
10 μ moles α -ketoglutarate	5.0	8.5	1.5	4.0

Table 8. *Intracellular distribution of succinoxidase, cytochrome oxidase and nitrogen in 0.2M-sucrose suspensions of hepatopancreas*

(Results expressed as percentage of original enzyme activity or nitrogen content.)

Condition of animal	Enzyme system	Whole suspension	Supernatant I	Cellular debris	Mitochondria	Supernatant II
Active	Succinoxidase	100	75	16	69	4
Active	Cytochrome oxidase	100	79	2	43	32
Active	Nitrogen	100	82	19.9	14	54.5
Hibernating	Nitrogen	100	79	17	13.5	55.0

prevent further utilization of the citrate formed. Citrate synthesis was not investigated in sucrose suspensions since sucrose interferes with the analysis for citric acid.

The reaction α -ketoglutarate $\rightarrow$ succinate was studied with the aid of malonate to prevent further oxidation of succinate. Respiring suspensions of the hepatopancreas accumulated succinate in the presence of malonate and the yield of succinate was increased by the addition of α -ketoglutarate. The results of these experiments are shown in Table 7.

The intracellular localization of Krebs's cycle enzymes in the hepatopancreas of Helix pomatia

Differential centrifugations were carried out on 0.2M-sucrose suspensions prepared from the hepatopancreas of both active and hibernating *H. pomatia*. The tissue suspension was centrifuged at 3000 g for 10 min., and the supernatant (*S*₁) centrifuged for 10 min. at 15000 g at 2° to sediment the mitochondria from the supernatant (*S*₂). The mitochondria were washed by suspension in 0.2M-sucrose and centrifugation at 15000 g. The distribution of the nitrogen, succinoxidase and cytochrome oxidase of these fractions are shown in Table 8. Succinoxidase and cytochrome oxidase were determined by the method of Schneider & Potter (1943).

Table 9. *Oxidation of Krebs's cycle intermediates and L-glutamate by mitochondria isolated from the hepatopancreas of active Helix pomatia*

(System: standard medium; 30 μ moles substrates (succinate 100 μ moles). Temp. = 38°.)

Substrate	Oxygen uptake (μ l./mg. N./hr.)
Nil	0
α -Ketoglutarate	24.0
L-Malate	44.0
Succinate	110.0
Citrate	7.0
L-Glutamate	0

The oxidation by mitochondria of α -ketoglutarate, L-malate, fumarate, succinate and citrate was investigated and the results are shown in Table 9. Additions of DPN were found to increase

the rate of oxidation of L-malate and α -ketoglutarate (Table 10).

Table 10. *Effect of DPN additions on the oxidation of L-malate and α -ketoglutarate by hepatopancreas mitochondria*

(System: standard medium; additions as described in the table. Mitochondria added equivalent to 3 mg. N. Temp. = 38°. Duration of experiment = 30 min.)

Additions	Oxygen uptake (μ l.)
30 μ moles malate	26.0
30 μ moles malate + 1 μ mole DPN	47.0
30 μ moles α -ketoglutarate	28.0
30 μ moles α -ketoglutarate + 1 μ mole DPN	37.0

The synthesis of citric acid from pyruvate and oxaloacetate by mitochondrial preparations of both active and hibernating animals was investigated. It was found that the synthesis of citrate was proportional to added ATP but that the addition of coenzyme A had no effect on the reaction. The synthesis of citric acid from added bicarbonate and pyruvate took place in the presence of mitochondrial preparations from active animals but not in that of preparations of hibernating animals (Table 6).

Acetate and octanoate oxidation with hepatopancreas mitochondria and suspensions was studied using L-malate or succinate as primer. Both in the absence and presence of primer, acetate and octanoate failed to increase the oxygen uptake above that shown by the control. Substitution of ATP and/or the addition of DPN or coenzyme A failed to elicit oxidation of acetate or octanoate.

Investigations of oxidative phosphorylation in the hepatopancreas

The esterification of inorganic phosphate was studied in sucrose suspensions of the hepatopancreas. Sodium fluoride was added to inhibit adenosine triphosphatase activity. The effect of the oxidation of Krebs's cycle intermediates on the level of inorganic phosphorus esterified was tested. The increased oxygen uptake in the presence of these intermediates was found not to be associated with phosphorylations. The phosphate that was esterified during the experiment was found to be unaffected by 2:4-dinitrophenol (DNP) (Table 11).

Experiments were carried out over a range of temperatures, 22–38°, but lowering the temperature did not initiate oxidative phosphorylation associated with added Krebs's cycle intermediates. The addition of respiratory co-factors (coenzyme A, DPN, triphosphopyridine nucleotide (TPN), thiamine pyrophosphate, or flavin-adenine dinucleotide) to the hepatopancreas suspensions did not elicit oxidative phosphorylation.

Table 11. *Effect of Krebs's cycle intermediates and dinitrophenol on phosphate esterification by tissue suspensions*

(System: standard medium; 90 μ moles glucose; 0.05 ml. hexokinase solution; dinitrophenol final concentration 10^{-5} M. Tissue added equivalent to 6 mg. N. Temp. = 25°. Duration of experiment = 90 min.)

Substrate	Dinitrophenol	P esterified (μ moles)
Nil	–	13.0
Nil	+	9.0
Succinate	–	14.0
Succinate	+	13.0
L-Glutamate	–	14.0
L-Glutamate	+	14.0
L-Malate	–	13.0
L-Malate	+	13.0

In view of the experiments of Kielley & Kielley (1951) measurements were made both in the presence and absence of fluoride. With suspensions, a greater esterification was found in the presence of sodium fluoride. Numerous experiments were made with mitochondrial preparations. These were carried out with and without sodium fluoride; with added myokinase (Kalckar, 1947); with both ADP and AMP as phosphate acceptors and with and without the respiratory co-factors listed above. In no case was any phosphorylation observed during the oxidation of Krebs's cycle intermediates, which included succinate, L-malate, fumarate and α -ketoglutarate. L-Glutamate was also tested in this system, but without effect.

The absence of any esterification of phosphate by the mitochondrial preparations and the stability of the phosphate esterified in the suspensions to DNP suggest that these phosphorylations result from anaerobic reactions, e.g. glycolytic reactions. We have found in fortified rat-liver suspensions that the phosphorylations associated with glycolysis are not interrupted by DNP.

DISCUSSION

The snail, as a poikilothermic animal, has in the laboratory a body temperature of about 20–23°. Manometric measurements of the hepatopancreas at this temperature were so small that for the determination of μ l. O_2 /mg. N/hr., a working temperature of 38° was adopted. No instability in the

respiration of preparations in the presence of substrates was noted during experiments of 40 min. duration.

The activity shown by the hepatopancreas suspensions is about one-tenth that of similar preparations of rat liver. From a study of the data of Massey & Rogers (1950) it would appear that the nematode tissue examined possessed an aerobic respiration of the same order as that of the hepatopancreas. These activities are low in contrast to those obtained by us for muscle preparations of *Locusta migratoria* (unpublished) and for cockroach muscle (Barron & Tahmisiian, 1948).

It was not possible in *H. pomatia* to investigate muscle. The consistency of the foot muscle is such that it did not permit the preparation of tissue dispersions which could be examined, while the columella muscle in this species is so small that it is not practicable for investigations.

The evidence for the presence of Krebs's cycle activity in the hepatopancreas may be summarized as follows:

(1) The addition of fumarate, α -ketoglutarate, citrate, *cis*-aconitate, succinate, malate and oxaloacetate caused an increase in the oxygen uptake.

(2) In the presence of malonate, succinate accumulated and the amount formed was increased by the addition of α -ketoglutarate.

(3) Malonate inhibited the respiration, and this inhibition was partially removed when intermediates of the Krebs's cycle were added.

(4) Added citrate was utilized by the suspension and α -ketoglutarate accumulated in the presence of arsenite. Even in the absence of inhibitor, however, α -ketoglutarate accumulated during the oxidation of citrate.

(5) Small quantities of citrate were synthesized by the hepatopancreas preparations from added pyruvate and oxaloacetate. Like mammalian liver, the hepatopancreas of active snails appears to possess a β -carboxylase (see Table 8). It was not possible, however, to detect β -carboxylase activity in the hepatopancreas of the hibernating animal. The level of citrate synthesis was dependent on the ATP concentration, but added coenzyme A was without effect, and it appears therefore that the limiting factor in the cycle probably lies in the concentration of 'condensing enzyme'.

Judah & Rees (1953) have shown that in rat-liver preparations ATP will activate fatty acid oxidation. The inability of hepatopancreas preparations to oxidize added octanoate or acetate in the presence of Krebs's cycle intermediates and ATP suggests either that the tissue does not possess this oxidase or that, under the conditions of our experiments, this enzyme is destroyed.

The distribution of nitrogen in the hepatopancreas suspension showed that about 12% of the nitrogen

of the tissue suspension was localized in the mitochondrial fraction. This may be contrasted with the 25% localized in the mitochondrial fraction of rat liver. The rate of oxidation of Krebs's cycle intermediates in the presence of hepatopancreas mitochondria was about 15% of the activities for rat-liver mitochondria. The Krebs's cycle activity of the mitochondria was of the order that would be expected from the activity of the whole suspension. The distribution of cytochrome oxidase and succinoxidase in the hepatopancreas suspension were similar to the results obtained by Hogeboom, Schneider & Pallade (1948) for rat-liver preparations.

The addition of co-factors, e.g. DPN, are without effect on the oxidation of cycle intermediates by rat-liver and rat-kidney mitochondria preparations (Judah, 1951). This is in marked contrast to the stimulatory effect of DPN on the oxidation of L-malate and α -ketoglutarate by hepatopancreas mitochondria.

In mammalian tissues the oxidation of Krebs's cycle intermediates is accompanied by the esterification of inorganic phosphate from the medium. The system used in our investigations was tested in rat-liver preparations and the P:O ratios found for succinate, L-glutamate and octanoate were of the same order as those reported in the literature. Esterification of inorganic phosphate took place at 28° in the whole 0.2M-sucrose hepatopancreas suspensions but the level of phosphate esterified was not increased by the co-oxidation of Krebs's cycle intermediates. Variation in the temperature of incubation, salt and sucrose concentrations and the addition of respiratory co-factors did not elicit oxidative phosphorylation. The observation that the phosphate esterification is not interrupted by the addition of dinitrophenol suggests that such esterification as is observed is not oxidative in nature. Under anaerobic conditions there was no decrease in the phosphate esterified. It appears likely that these phosphorylations are due to anaerobic glycolysis.

Krebs, Gurin & Eggleston (1952) have suggested that the Krebs's cycle may have a dual function. At higher levels of animal organization the cycle may function both as a mechanism for energy production and as a pathway for the synthesis of special molecules. At a lower level of organization the cycle may only possess the latter function. Such a hypothesis as proposed by Krebs may be an explanation of our failure to demonstrate phosphorylations associated with Krebs's cycle activity.

No variation in the oxidation of Krebs's cycle intermediates was observed in a comparison of preparations of the hibernating and active *H. pomatia*. All results were examined statistically and no significant differences were found. The absence of a β -carboxylase in the hibernating hepatopancreas could reduce cycle activity. With the loss of the ability to utilize galactose in the hibernating animal, there exist in the hepatopancreas during hibernation at least two mechanisms for reducing the utilization of carbohydrate.

SUMMARY

1. The oxidation of a number of Krebs's cycle intermediates by tissues suspensions and mitochondria of *H. pomatia* have been studied.
2. Reactions of the Krebs's cycle were demonstrated to occur.
3. The evidence for the presence of the cycle and its role in the hepatopancreas is discussed.
4. Differences in the metabolism of preparations from active as compared with hibernating animals are reported and discussed.

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Enzyme Formation in *Saccharomyces fragilis*

1. INVERTASE AND RAFFINASE

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The phenotype of an organism is a variable property which is dependent upon an interaction between its genotype and its environment and can be altered by changes in either of these. A necessary corollary of this is that the genotype can only be reliably deduced from the phenotype when either the environment remains constant or the influence of the environment on the phenotype is accurately known. Experimentally, it is no simple matter to maintain growing cells in a constant environment, and a detailed knowledge of the effect of the environment on the phenotype would appear to be an essential prerequisite for an understanding of the mechanism of the genic control of enzyme formation. It was with this aim in view that investigations into the factors affecting enzyme formation in *Saccharomyces fragilis* were begun. This paper will be chiefly concerned with the anaerobic utilization of sucrose and raffinose by this yeast.

Willstätter, Lowry & Schneider (1925) and De Ley & Vandamme (1951) have described increases in the invertase content of brewer's yeast following continuous addition of sucrose to the washed yeast suspended in a salt solution containing ammonium, potassium, magnesium, nitrate and phosphate ions. The former authors used as their starting material a yeast in which invertase activity had become attenuated during repeated fermentations in the brewery and they showed that the slow addition of sucrose gave a much bigger stimulation than a large single addition at the start. Glucose and maltose were almost as effective if added in the same way. The maximum increase in invertase activity which they obtained was about 14-fold. De Ley & Vandamme (1951) showed that invertase activity of the cells was greatest during the stationary phase

of growth on a beer-wort medium and that the ability to form extra invertase on slow addition of sucrose was also greatest for cells harvested during the stationary phase. Omission of ammonium ions or phosphate resulted in a marked fall in the amount of invertase formed, whereas replacement of ammonium ions by asparagine resulted in increased invertase formation.

It is possible that the glucose-fructose linkage in raffinose is hydrolysed by invertase. However, the rate of hydrolysis is only about one-tenth that for sucrose by the same enzyme preparations. Adams, Richtmeyer & Hudson (1943), among others, considerably purified invertase from brewer's yeast and tested the preparations for ability to hydrolyse a number of di-, oligo-, and poly-saccharides. Their most highly purified preparations possessed β -fructofuranosidase, α -D-galactosidase, β -D-glucosidase, β -D-mannosidase and inulase activities. The ratio (rate of sucrose hydrolysis)/(rate of raffinose hydrolysis) varied in these experiments from 4.11 to 8.59, yet despite the fact that their preparations were obviously impure, the authors were of the opinion that sucrose and raffinose were hydrolysed by the same enzyme. There is, therefore, as has been stated by Neuberg & Mandl (1950), no certainty that hydrolysis of the glucose-fructose linkage in sucrose and raffinose is due to the activity of invertase. Recently, however, Gilliland (1949) and Winge & Roberts (1952) have shown that *Saccharomyces italicus* has three polymeric genes controlling the ability to ferment raffinose, and that the ability to ferment sucrose appears to be controlled by the same three genes. A considerable number of yeast hybrids was examined, and in every case fermentation of raffinose and rapid fermentation of sucrose