2. Non-proliferating mycelia, grown at pH 2.1, convert glucose into itaconic acid with about equal yields at pH 6.0 and 2.1. Non-proliferating mycelia, grown at pH 6.0, do not accumulate itaconic acid from glucose either at pH 6.0 or 2.1. However, mycelia, grown at pH 6.0 and then transferred to a full growth medium of pH 2.1, will gradually develop the ability to produce itaconic acid.

3. The results indicate that an acid environment is necessary for the formation of an essential enzyme system operating in the conversion of glucose into itaconic acid.

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The Mechanism of Itaconic Acid Formation by *Aspergillus terreus*

2. THE EFFECT OF SUBSTRATES AND INHIBITORS

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Published work on the itaconic acid fermentation is virtually limited to investigations of the optimum conditions for obtaining high yields of itaconic acid from glucose and sucrose. Very little information is available concerning the conversion of other carbon sources into itaconic acid by mould fermentation. Calam, Oxford & Raistrick (1939) reported that glucose was the only compound, among a number tested, which gave rise to substantial amounts of itaconic acid in surface culture experiments with *Aspergillus terreus*. Citric, malic, pyruvic and acetic acids, either alone or in mixture, gave no or only very small amounts of itaconic acid.

In the present investigation the ability of *A. terreus* to form itaconic acid from a variety of carbon sources has been investigated, using the methods developed in our laboratory for the study of itaconic acid production by cultures growing on a shaker (Larsen & Hovden, in preparation) and by non-proliferating mycelial suspensions (Larsen & Eimhjellen, 1955). The results of these experiments, which have been carried out in both the presence

and absence of metabolic inhibitors, serve as a basis for a discussion of the mechanism of itaconic acid formation.

EXPERIMENTAL

The experiments were carried out with *A. terreus*, strain NRRL 1960. Growth experiments were carried out with cultures grown in acid media on a shaking machine as described in the previous paper (Larsen & Eimhjellen, 1955); using this technique various compounds were tested as carbon source both for growth and for itaconic acid production.

To measure the Q_{O_2} (μ l. O_2 consumed/mg. dry wt. mycelia/hr.) of non-proliferating mycelia the organism was grown on the acid glucose medium (Larsen & Eimhjellen, 1955) at 30° for 50-60 hr., centrifuged and washed with and suspended in either tap water acidified to pH 2.1 with H_2SO_4 or in 0.067 M sodium-potassium phosphate buffer (pH 7.0). Before use the suspensions were aerated for 2 days at 30° to reduce the endogenous respiration. Oxygen uptake was measured in conventional Warburg manometers, and the CO_2 was absorbed by 10% (w/v) KOH in the centre well.

To test for itaconic acid production from various substrates by suspensions of non-proliferating mycelia, 50-60 hr. cultures grown in the acid glucose medium were harvested by filtration as described in the previous paper

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(Larsen & Eimhjellen, 1955) and suspended in the acid tap-water solution of pH 2.1. The suspensions were incubated with substrate according to Larsen & Eimhjellen (1955). Samples were withdrawn at suitable time intervals and analysed after removal of the mycelium by centrifuging.

Reagents. *cis*-Aconitic acid was prepared according to Malachowski & Maslowski (1928) and Krebs & Eggleston (1944); DL-citramalic acid according to Michael & Tissot (1892) with the modification introduced by Marckwald & Axelrod (1899); itaconic acid according to Larsen (1954); and DL-isocitric acid according to Fittig & Miller (1889) and Ochoa (1948). Oxaloacetic acid was obtained by saponification of the diethyl ester (Krampitz & Werkman, 1941); commercial pyruvic acid was purified according to Robertson (1942). Preparations of L-sorbose, L-rhamnose, L-fucose, L-xylose and D-lyxose were kindly furnished by Professor N. A. Sørensen. Other organic reagents were commercial preparations of highest purity available and were used without further purification.

Analytical methods. Dry matter was determined as previously described (Larsen & Eimhjellen, 1955); glucose and maltose according to Hagedorn-Jensen with the modification of Fujita-Iwatake (Barrenscheen & Pany, 1941); total acid by alkali titration; itaconic acid by a semimicro modification of Friedkin's method (Larsen, 1954); citric acid according to Lardy (1949) and *cis*-aconitic acid according to Nekhorocheff & Wajzer (1953). Control experiments showed that glucose, citric and itaconic acids did not interfere with the determination of *cis*-aconitic acid. Itaconic acid did not interfere with the citric acid determination to any significant extent. Pyruvic acid was determined by the method of Friedemann (1950). Acetaldehyde formed by the mycelium in the presence of arsenite evaporated from the reaction mixture and was trapped in absorption tubes either as the bisulphite compound or as the 2:4-dinitrophenylhydrazone. The amount of acetaldehyde trapped in the bisulphite solution was determined iodometrically.

For the identification of pyruvic acid and acetaldehyde the 2:4-dinitrophenylhydrazones were prepared and recrystallized from 96% (v/v) ethanol. The m.p.'s and mixed m.p.'s were determined using authentic samples prepared from commercial pyruvic acid and acetaldehyde. The R_f values of the 2:4-dinitrophenylhydrazones were determined by paper chromatography according to Cavallini, Frontali & Toschi (1949).

When sodium arsenite was present glucose and itaconic acid were determined by the methods given above, save that determinations of blank corrections were made on aqueous solutions of sodium arsenite of the appropriate concentration instead of on water.

The various metabolic reaction mixtures were tested routinely for the presence of organic acids by paper chromatography as previously described (Larsen & Eimhjellen, 1955).

RESULTS

Growth experiments. These experiments were carried out with shake cultures. At the time of peak alkali titre the itaconic acid content was determined and the amount of growth judged by visual inspection. The results, which are presented in Table 1, show that *A. terreus*, strain NRRL 1960, can grow on a large variety of sugars and alcohols;

even the methyl pentose L-rhamnose is utilized for growth. During growth the pH fell to 2.0-2.2.

The ability of the mould to produce itaconic acid from these compounds presents a somewhat different picture. Some of the substrates gave rise to a fair production of itaconic acid, but the yields were variable (Table 1). In other cases no itaconic acid was detected in the fermentation liquor, although the substrate gave rise to good growth.

Table 1. *Growth and itaconic acid formation with sugars and alcohols as substrates*

20 ml. medium with 5% (w/v) substrate in 100 ml. flasks. (+), Barely visible growth; +, slight growth; ++, fair growth; +++, good growth; —, not tested.

Substrate	Growth	Itaconic acid formed in % (w/w) of substrate added
Inulin	+++	12
Starch	+++	0
Glycogen	+++	0
Raffinose	+++	0
Cellobiose	+++	41
Maltose	+++	0
Lactose	(+)	—
Sucrose	+++	57
D-Glucose	+++	52
D-Mannose	+++	32
D-Galactose	++	4
D-Fructose	+++	26
L-Sorbose	(+)	—
L-Rhamnose	+++	0
L-Fucose	(+)	—
D-Arabinose	+	—
L-Arabinose	+++	18
D-Xylose	+++	31
L-Xylose	+	—
D-Ribose	+	—
D-Lyxose	+	—
mesoInositol	++	0
D-Mannitol	+	—
mesoGalactitol (= dulcitol)	(+)	—
Glycerol	+++	23
Ethanol	+++	11

Growth experiments with citric and acetic acids showed that these compounds were effectively utilized as sole source of carbon provided that the pH was kept above 4. However, no itaconic acid accumulated under these conditions, although the pH of the medium, which increased during growth, was frequently readjusted during the experiment to the lower limit at which growth occurred.

Respiration experiments. The results of the Q_{O_2} measurements are presented in Table 2, and reflect the ability of *A. terreus* to attack a large number of common metabolic intermediates. The addition of members of the tricarboxylic acid cycle caused a significant increase in the rate of oxygen uptake,

although the effect of oxaloacetic acid was small. Of the C₂ compounds tested, acetic acid and ethanol gave relatively high Q_{O_2} values, whereas the addition of ethylene glycol and glycolic acid caused an almost negligible stimulation of oxygen uptake.

Table 2. *Oxidation of substrates by mycelial suspensions at pH 2.1 and pH 7.0*

Mycelia suspended in water acidified with H₂SO₄ to pH 2.1 or in 0.067 M sodium-potassium phosphate buffer of pH 7.0. To each Warburg vessel were added 2.0 ml. of mycelial suspension in the main compartment and 0.2 ml. (20 μ moles) of substrate in the side bulb. Substrate was added to the mycelial suspension at zero time. Gas phase air. Temp. 30°.

Substrate	Q_{O_2} *(μ l./mg. dry wt./hr.)	
	pH 2.1	pH 7.0
D-Glucose	-17	-22
Pyruvic acid	-6	-11
Acetic acid	-6	-17
Succinic acid	-14	-8
DL-Malic acid	-8	-11
Fumaric acid	-14	-11
Oxaloacetic acid	—	2
L-Tartaric acid	—	-1
Citric acid	-12	-11
cis-Aconitic acid	-14	-8
trans-Aconitic acid	—	-5
DL-isoCitric acid	-5	-4
Itaconic acid	—	-1
α -Oxoglutaric acid	-10	—
Ethylene glycol	—	0
Glycolic acid	—	-2
Ethanol	—	-34

* Endogenous Q_{O_2} = 10 is subtracted from all figures.

Formation of itaconic acid by non-proliferating mycelia. The extent of itaconic acid formation by non-proliferating mycelia in the presence of a variety of substrates is shown in Table 3. All attempts to reduce the considerable endogenous activity were invariably accompanied by an even stronger reduction of the capacity of the suspension to form itaconic acid from substrates. The rate of endogenous itaconic acid production was particularly rapid at the start of the experiment and usually fell to about zero after 2-4 hr. (cf. Larsen & Eimhjellen, 1955, Fig. 1). For the evaluation of such experiments it was thus made the rule to start measuring the changes taking place 2-3 hr. after the suspension had been transferred to the aerated tubes in the water bath, and to subtract the amount of endogenous itaconic acid production from that formed in the presence of the substrate.

By determining the amount of substrate utilized it was possible to calculate the 'yield' of itaconic acid from the substrate. Though the yield was identical in duplicate experiments with the same substrate, mycelia harvested at different times showed large variations. This effect has been attributed largely to the extraordinary sensitivity of the mycelium to slight variations in the working conditions during harvesting. In experiments on itaconic acid production from glucose by mycelial suspensions, yields of 0-65% (mole for mole) have been obtained; usually the molar yield was about 40% (i.e. about 29% by weight). In calculating these figures itaconic acid formed by endogenous activity was subtracted.

Table 3. *Formation of itaconic acid by non-proliferating mycelia*

Mycelia suspended in tap water solution of pH 2.1. Expt. no. 1 lasted for 10 hr., Expt. no. 2 for 9 hr. and Expt. no. 3 for 10 hr.

Expt. no.	Substrate	Substrate added (mg.)	Itaconic acid formed (mg.)	
			Total	Total minus endogenous
1	None	—	18	—
	D-Glucose	140	52	34
	Glycerol	415	33	15
	Glyceric acid	340	30	12
	Glycollic acid	400	0	-18
	Ethanol	375	33	15
	Citric acid	140	43	25
	cis-Aconitic acid	140	18	0
	DL-Malic acid	280	34	16
	None	—	14	—
2	D-Glucose	145	51	37
	Pyruvic acid	190	7	-7
	Oxaloacetic acid	140	9	-5
	{ Pyruvic acid + oxaloacetic acid	190	9	-5
	DL-Citramalic acid	140	—	—
	DL-Citramalic acid	280	13	-1
3	None	—	27	—
	D-Glucose	140	59	32
	Maltose	140	47	20
	Acetic acid	50	4	-23

Maltose, which gave rise to good growth but no itaconic acid in the growth experiments, was also readily utilized by non-proliferating mycelia grown in an acid glucose medium. Concomitantly with the utilization of maltose itaconic acid was produced at a rate well above that found in the absence of substrate (Table 3). It thus seems probable that maltose can be converted into itaconic acid by *A. terreus*.

molar yield of itaconic acid than did glucose, but occasionally the yield was as high or even higher. Table 4 shows the results of an experiment in which citric acid and glucose were tested separately and in a mixture. In this particular case the yield of itaconic acid from citric acid was higher than that from glucose. It should be noted that when citric acid and glucose were tested together, no citric acid was consumed by the mycelium, whereas glucose

Table 4. *Formation of itaconic acid in the presence of glucose and citric acid*

Non-proliferating mycelia suspended in tap-water solution of pH 2.1. Added at start: tube 1: no addition; tube 2: 705 μ moles glucose; tube 3: 775 μ moles citric acid; tube 4: 705 μ moles glucose + 775 μ moles citric acid. Figures below refer to changes taking place in the tubes between fourth and tenth hr. after addition of substrates.

Tube no.	Substrate	Glucose utilized (μ moles)	Citric acid utilized (μ moles)	Itaconic acid formed (μ moles)	Molar yield* (%)
1	None	—	—	10	—
2	Glucose	390	—	165	40
3	Citric acid	—	435	225	49
4	Glucose + citric acid	395	0	160	38

* Assuming that one mole of substrate can theoretically give rise to one mole of itaconic acid. Itaconic acid formed by endogenous activity has been subtracted.

Table 5. *Formation of itaconic acid in the presence of glucose and cis-aconitic acid*

Non-proliferating mycelia suspended in tap-water solution of pH 2.1. Added at start: tube 1: no addition; tube 2: 775 μ moles glucose; tube 3: 805 μ moles *cis*-aconitic acid; tube 4: 775 μ moles glucose + 805 μ moles *cis*-aconitic acid. Figures below refer to changes taking place in the tubes between fourth and ninth hr. after addition of substrates.

Tube no.	Substrate	Glucose utilized (μ moles)	<i>cis</i> -Aconitic acid utilized (μ moles)	Itaconic acid formed (μ moles)	Molar yield* (%)
1	None	—	—	25	—
2	Glucose	435	—	130	24
3	<i>cis</i> -Aconitic acid	—	0	25	—
4	Glucose + <i>cis</i> -aconitic acid	465	60	250	43

* Assuming that one mole of substrate can theoretically give rise to one mole of itaconic acid. Itaconic acid formed by endogenous activity has been subtracted.

Table 3 shows that the substrates tested can be divided into three classes: (1) Substrates causing a clear-cut increase in itaconic acid production, viz. glucose, maltose, glycerol, ethanol, glyceric, citric and malic acids. (2) Substrates which neither cause an increase in production of itaconic acid nor an inhibition of its endogenous formation, viz. *cis*-aconitic and citramalic acids. (3) Substrates inhibiting the endogenous formation of itaconic acid, viz. pyruvic, acetic, oxaloacetic and glycollic acids.

Formation of itaconic acid in the presence of citric acid. The addition of citric acid resulted in a substantial accumulation of itaconic acid by non-proliferating mycelia (Table 3). Citric acid was utilized at a rate about equal to that of glucose. In several experiments with citric acid as substrate yields of itaconic acid ranging from nil to about 50 % (mole for mole) have been found. When tested simultaneously citric acid generally gave a lower

was converted into itaconic acid as if no citric acid was present. Other experiments of similar type substantiated this latter finding and showed that the attack on citric acid did not start until all glucose had been consumed.

Formation of itaconic acid in the presence of cis-aconitic acid. Non-proliferating mycelia, prepared so as to test their itaconic acid-producing activity, attacked *cis*-aconitic acid either very slowly or not at all. Although in some experiments a significant amount of *cis*-aconitic acid was metabolized, no itaconic acid was produced over and above that formed by endogenous activity. But when a mixture of *cis*-aconitic acid and glucose was added, an amount of itaconic acid accumulated which was far greater than that accumulating in the presence of glucose alone (Table 5). However, the glucose was utilized at the same rate in the two cases, and only very little *cis*-aconitic acid disappeared from the mixture.

Formation of itaconic acid in the presence of pyruvic acid. Although pyruvic acid was rapidly metabolized by non-proliferating mycelia, the amount of itaconic acid formed was less than that produced by the control without substrate (Table 3). When a mixture of glucose and pyruvic acid was added, no glucose was consumed until all the pyruvic acid had been utilized. The amount of itaconic acid formed in the presence of such a mixture was not only smaller than that formed in the presence of glucose alone, but considerably smaller than that formed endogenously (Table 6).

Table 6. *Formation of itaconic acid in the presence of glucose and pyruvic acid*

Non-proliferating mycelia suspended in tap-water solution of pH 2.1. Added at start: tube 1: no addition; tube 2: 780 μ moles glucose; tube 3: 2270 μ moles pyruvic acid; tube 4: 780 μ moles glucose + 2270 μ moles pyruvic acid. Figures below refer to changes taking place in the tubes during the first 9 hr. of incubation.

Tube no.	Substrate	Glucose utilized (μ moles)	Pyruvic acid utilized (μ moles)	Itaconic acid formed (μ moles)
1	None	—	—	135
2	Glucose	725	—	265
3	Pyruvic acid	—	1170	70
4	Glucose + pyruvic acid	0	1010	42

Inhibition of metabolism by fluoroacetate. Table 7 shows the results of an experiment on the influence of sodium monofluoroacetate on itaconic acid formation by non-proliferating mycelia in the presence of glucose. In the presence of a concentration of fluoroacetate of 10^{-4} M or higher, the yield of itaconic acid was greater than that found in the absence of fluoroacetate.

The values for the yield of itaconic acid (Table 7, column 5) have been calculated on the assumption that fluoroacetate has no significant influence on the rate of endogenous itaconic acid formation. This is only true in part, since separate experiments have shown that at fluoroacetate concentrations greater than 10^{-3} M, the rate of endogenous itaconic acid formation is lowered; consequently the corresponding values for '% molar yield' in Table 7 are probably too low. On the other hand, separate experiments on the rate of endogenous itaconic acid formation in the presence of 10^{-4} M and 2×10^{-4} M fluoroacetate have occasionally shown a small increase in the rate over and above that in the absence of fluoroacetate. However, in the latter case the effect could only be shown during the first 2-3 hr. of the experiment, when the endogenous activity was greatest. At concentrations below 10^{-3} M it therefore seemed permissible to disregard

the influence of fluoroacetate on the endogenous activity in experiments of the type reported in Table 7, in which the conversions taking place between the second and the sixth hour have been used as a basis for the calculations.

Table 7. *Formation of itaconic acid from glucose in the presence of fluoroacetate*

Non-proliferating mycelia suspended in tap-water solution of pH 2.1. Added at start 460 μ moles glucose and varying amounts of sodium monofluoroacetate. Figures below refer to changes taking place between second and sixth hr. Endogenous production of itaconic acid in the absence of fluoroacetate amounted to 30 μ moles, and has been subtracted from the values for itaconic acid produced in the presence of glucose given below.

Fluoroacetate (M)	Glucose utilized (μ moles)	Inhibition of glucose utilization* (%)	Itaconic acid produced from glucose (μ moles)	Molar yield† (%)
0	230	0	105	46
2×10^{-5}	235	0	105	45
10^{-4}	205	11	125	61
2×10^{-4}	185	20	110	59
5×10^{-4}	165	28	105	64
2×10^{-3}	160	30	100	63†
5×10^{-3}	120	48	70	58†
10^{-2}	75	67	40	53†

* Inhibition of utilization rate in % of rate in the absence of fluoroacetate.

† Assuming that one mole of glucose can theoretically give rise to one mole of itaconic acid.

‡ Values possibly too low, see text.

Several experiments have been carried out to see whether citric acid accumulated when suspensions of non-proliferating mycelia metabolized glucose in the presence of fluoroacetate. The results of these experiments were equivocal. In three out of a total of five experiments, in which concentrations of fluoroacetate of 2×10^{-4} M and 5×10^{-4} M were used, no citric acid accumulated over and above the small amount formed in the absence of fluoroacetate. The amount detected accounted for about 1 % (mole for mole) of the glucose consumed. However, the presence of fluoroacetate caused a clear-cut increase in the yield of itaconic acid in each case. In two of the five experiments a significant amount of citric acid accumulated over and above that found in the absence of fluoroacetate. In the presence of 5×10^{-4} M fluoroacetate the amount of citric acid formed accounted for 3.1 % (mole for mole) of the glucose utilized; in the absence of fluoroacetate citric acid similarly accounted for 1.5 %. The corresponding figures for itaconic acid formation were 46 % (mole for mole) and 34 % (mole for mole), respectively. The fifth experiment, in which a concentration of 2×10^{-4} M fluoroacetate was used, gave similar values.

Table 8 shows the results of an experiment on the influence of fluoroacetate on itaconic acid formation with citric acid as substrate. Fluoroacetate caused a pronounced increase in the yield of itaconic acid from citrate. It should also be noted that fluoroacetate was a much more powerful inhibitor of citric acid utilization (Table 8, column 3) than of glucose utilization (Table 7, column 3).

In quantitative experiments with non-proliferating mycelia, the amounts of glucose utilized and itaconic acid, pyruvic acid and acetaldehyde produced in both the presence and absence of sodium arsenite were estimated. In the absence of sodium arsenite glucose was converted into itaconic acid with a yield of 40 % (mole for mole). No acetaldehyde and only a faint trace of pyruvic acid was

Table 8. *Formation of itaconic acid from citric acid in the presence of fluoroacetate*

Non-proliferating mycelia suspended in tap-water solution of pH 2.1. Added at start 730 μ moles citric acid and varying amounts of sodium monofluoroacetate. Figures below refer to changes taking place between fourth and eighth hr.

Fluoroacetate (M)	Citric acid utilized (μ moles)	Inhibition of citric acid utilization* (%)	Itaconic acid produced (μ moles)		Apparent itaconic acid production from citric acid, $b - a$ (μ moles)	Molar yield† (%)
			(a) In the absence of citric acid	(b) In the presence of citric acid		
0	400	0	20	90	70	18
5×10^{-5}	195	51	20	90	70	36
2×10^{-4}	95	76	20	80	60	63
10^{-3}	0	100	10	10	0	—

* Inhibition of utilization rate in % of rate in the absence of fluoroacetate.

† Assuming that one mole of citric acid can theoretically give rise to one mole of itaconic acid.

Table 9. *Metabolism of glucose in the presence of arsenite*

Non-proliferating mycelia suspended in tap-water solution of pH 2.1. Added at start 800 μ moles glucose and sodium arsenite to a final concentration of 10^{-3} M. Figures below refer to changes taking place between third and twelfth hr., during which 230 μ moles glucose were consumed.

Products	Amount products formed (μ moles)		Apparent production from glucose, $b - a$ (μ moles)	Production in % of theoretical*
	(a) In the absence of glucose	(b) In the presence of glucose		
Pyruvic acid	80	425	345	75
Acetaldehyde	30	120	90	20
Theoretical recovery	—	—	—	95

* Assuming that one mole of glucose can theoretically give rise to two moles of pyruvic acid or two moles of acetaldehyde.

Inhibition of metabolism by arsenite. Preliminary experiments on the effect of sodium arsenite on the utilization of glucose by non-proliferating mycelia showed that substantial amounts of pyruvic acid accumulated in the reaction tubes. The pyruvic acid 2:4-dinitrophenylhydrazone had m.p. 212°; the mixed m.p. with an authentic sample (m.p. 211°) was 211.5° (all m.p.'s are uncorrected). The R_F values of the new and the authentic samples of the 2:4-dinitrophenylhydrazones were identical.

It was also noticed that the air coming from the tubes to which sodium arsenite had been added smelt of acetaldehyde. On passing the air through an acid solution of 2:4-dinitrophenylhydrazine a copious precipitate was formed, which, after recrystallization, had m.p. 150°. The mixed m.p. with an authentic sample of acetaldehyde 2:4-dinitrophenylhydrazone (m.p. 152°) was 149°. The R_F values of the new and the authentic samples were identical.

detected; the latter compound accounted for less than 1 % (w/w) of the glucose consumed. Although in the presence of 10^{-3} M sodium arsenite the rate of glucose utilization was lowered to 30 % of the rate in the absence of arsenite, it remained constant during the 12 hr. of the experiment. No itaconic acid accumulated in the reaction mixture. However, practically all glucose consumed could be accounted for, on a molar basis, as pyruvic acid and acetaldehyde. The results of the experiment are summarized in Table 9. The figures show that substantial amounts of pyruvic acid and acetaldehyde also accumulated in the absence of glucose (column 2), apparently owing to an inhibition of the endogenous metabolism. On the assumption that the endogenous metabolism is equally inhibited in the presence of glucose, the figures for endogenous metabolism have been subtracted from those obtained in the presence of glucose to give apparent production of pyruvic acid and acetaldehyde from

glucose (column 4). Since one mole of glucose can, on the basis of the Embden-Meyerhof scheme, give rise to two moles of pyruvic acid in an aerobic fermentation, 75 % of the glucose utilized are accounted for as pyruvic acid. Similarly, assuming by analogy with well-known biochemical reactions that one mole of glucose can, theoretically, give rise to two moles of acetaldehyde, 20 % of the glucose utilized is accounted for in the latter product. Thus the amounts of pyruvic acid and acetaldehyde found are 95 % of that expected on a theoretical basis.

DISCUSSION

The growth experiments with various sugars and alcohols indicate that closely related compounds differ markedly in their ability to act as sources of itaconic acid (Table 1). It is especially striking that no itaconic acid was detected in the fermentation liquor in a number of cases where good growth was observed and where the pH was found to be within the range of 2.0-2.2 (Larsen & Eimhjellen, 1955). No general relation has been found between the chemical structure of the substrates and itaconic acid production, thus indicating that a cautious interpretation of the data is desirable. This is further validated by the fact that maltose, which gave rise to good growth but no itaconic acid in the growth experiments (Table 1), is readily converted into itaconic acid by non-proliferating mycelia (Table 3). Previous investigations (Larsen & Hovden, in preparation) have shown that the itaconic acid-producing mechanism is extremely sensitive to external influences, thus suggesting that the great variations in the yields of itaconic acid from the compounds listed in Table 1 might in part be due to toxic trace contaminants in the preparations. A similar argument might be used in the cases of no or only slight growth, although our experience indicates that growth is far less sensitive to external influences than itaconic acid production. On the other hand, the slight growth observed in some of the cases might be due to contamination of the substrates with utilizable carbon compounds.

The fact that citric and acetic acids are not converted into itaconic acid, although they support good growth, is in agreement with the finding of Calam *et al.* (1939). This phenomenon might be explained on the basis of the finding reported in the previous paper (Larsen & Eimhjellen, 1955) that the enzyme system producing itaconic acid is formed only at a low pH. It seemed possible, therefore, that although the pH in the present experiment was as low as could be tolerated by the organism when citric or acetic acids were substrates for growth, it was none the less not low enough to allow the formation of the itaconic acid-producing system. This hypothesis was tested by allowing non-proliferating

mycelia, from cultures grown in glucose medium at pH 2.1, to act on citric and acetic acids. Tables 3 and 4 show that under these conditions citric acid, though apparently not acetic acid, does give rise to itaconic acid.

Kinoshita (1931) suggested that itaconic acid might be formed from glucose by *A. itaconicus* via citric and aconitic acids as intermediate steps, the latter giving rise to itaconic acid by a simple decarboxylation. The evidence behind the suggestion was his observation that citric acid accumulated in calcium carbonate-buffered cultures, whereas itaconic acid was the main product in acid cultures.

Corzo & Tatum (1953) have recently reported that in experiments with [*methyl*-¹⁴C]acetate, [*carboxy*-¹⁴C]acetate and asymmetrically ¹⁴C-labelled citrate they have obtained evidence for the relationship of itaconic acid to the tricarboxylic acid cycle intermediates. These authors used the same strain of *A. terreus* as that used in the present investigation. They found that itaconic acid is formed from citric acid by a pathway which involves loss of the carbon atom derived from the acetate carboxyl, and the conversion of the carbon atom derived from the methyl group of acetate into the methylene carbon of itaconic acid. It should be noted that if *cis*-aconitic acid were an immediate precursor of itaconic acid, the findings of Corzo & Tatum are incompatible with the present knowledge of the asymmetric behaviour of citric acid (Potter & Heidelberger, 1949).

The results of the present investigation support the finding of Corzo & Tatum (1953) that itaconic acid is formed from citric acid and suggest that some activated form of citric acid is an intermediate in the conversion of glucose into itaconic acid by *A. terreus*. This conclusion is based upon the following observations with non-proliferating mycelia: (a) citric acid can be converted into itaconic acid with an efficiency which is at least as great as that in the conversion of glucose (Table 4); (b) citric acid can accumulate when glucose is the substrate, particularly in the presence of fluoroacetate; (c) in the presence of both glucose and citric acid no citric acid is utilized until all glucose is consumed (Table 4). The latter observation indicates that, when metabolized, these two substrates form the same intermediate, and that this intermediate, which is possibly an activated form of citric acid, is more readily formed from glucose than from citric acid itself.

The experiments reported above have shown that fluoroacetate stimulates the formation of itaconic acid both from glucose (Table 7) and, in particular, from citric acid (Table 8). In the latter case it was possible to raise the molar yield of itaconic acid from 18 to 63 %. According to present knowledge the toxic action of fluoroacetate is due to its conversion

into fluorocitrate, which compound is a specific inhibitor of aconitase (Peters, 1952; Morrison & Peters, 1954). There has been some confusion as to whether aconitase is one or two enzymes, but most investigators now seem to agree that only one catalytic centre of the aconitase protein is concerned in the enzymic conversion of citrate into isocitrate, possibly via *cis*-aconitate (Buchanan & Anfinsen, 1949; Martius & Lynen, 1950; Morrison, 1954). If this is the case, the consequence of the present observations would be that citric acid is a more immediate precursor of itaconic acid than is *cis*-aconitic acid. Besides the experiments with fluoroacetate further support for this viewpoint is furnished by the observation that non-proliferating mycelia are unable to convert *cis*-aconitic acid into itaconic acid (Table 3), and by the observation that when a mixture of glucose and *cis*-aconitic acid is metabolized the amount of itaconic acid formed is appreciably greater than the sum of the amounts of itaconic acid formed from each of the two substrates (Table 5).

In interpreting the latter observation the possibility was considered that the presence of glucose might stimulate the conversion of *cis*-aconitic acid into itaconic acid. However, even if this were the case, the amounts of itaconic acid formed under these conditions were so great that by far the larger part of it must have been formed from glucose, and with a greater efficiency than the corresponding conversion in the absence of *cis*-aconitic acid. It can therefore be concluded that *cis*-aconitic acid affects the metabolism of the mycelium in such a way that glucose is converted into itaconic acid with a significantly higher yield than in the absence of *cis*-aconitic acid. This conclusion suggests that *cis*-aconitic acid inhibits the tricarboxylic acid cycle at the stage of *cis*-aconitic acid itself, thus diverting a greater proportion of the metabolic intermediates to the pathway leading to the formation of itaconic acid. Integrating the observations presented it thus seems possible that the presence of *cis*-aconitic acid promotes the conversion of activated citric acid into itaconic acid.

Thus postulating activated citric acid as a close precursor of itaconic acid, one might envisage its decarboxylation to citramalic acid followed by a dehydration to form itaconic acid. The experiments showed, however, that DL-citramalic acid was not converted into itaconic acid under the conditions tested (Table 3).

No satisfactory explanation can be formulated at present for the fact that pyruvic, acetic and oxaloacetic acids, and a mixture of pyruvic and oxaloacetic acids are not, apparently, converted into itaconic acid and even inhibit its endogenous formation (Table 3). It should be noted in this connexion that in a mixture of glucose and pyruvic acid, the

utilization of glucose is completely inhibited whereas pyruvic acid is consumed as if no glucose were present (Table 6). It might therefore be that pyruvic, acetic and oxaloacetic acids completely inhibit the endogenous formation of itaconic acid, and that the small but definite production of itaconic acid observed in the presence of these compounds is due to their partial conversion into itaconic acid.

The fact that non-proliferating mycelia had very little or no ability to convert pyruvic acid into itaconic acid (Table 3), not even in the presence of oxaloacetic acid, might also be taken as an indication that pyruvic acid is not an intermediate in the conversion of glucose into itaconic acid. However, the experiments with arsenite strongly suggest that glucose is converted into a C_3 compound before the formation of itaconic acid, and that the C_3 compound is pyruvic acid or a compound closely related to it (Table 9). Arsenite is reported to inhibit oxidative decarboxylation of α -oxo acids (Walker, 1949), thus further suggesting that the C_3 compound is normally decarboxylated to form 'active' acetic acid.

Acetaldehyde, the other product which accumulated when glucose was metabolized in the presence of arsenite (Table 9), may be formed by the decarboxylation of pyruvic acid as in yeast. The latter reaction is known to be little affected by arsenite (Szent-Györgyi, 1930).

SUMMARY

1. Experiments with growing and non-proliferating mycelia of *Aspergillus terreus*, strain NRRL 1960, have shown that this organism is able to convert a number of different compounds into itaconic acid.

2. Experiments with non-proliferating mycelia, to which were added metabolic inhibitors and substrates singly or in mixtures, have led to the following postulate: glucose is converted into itaconic acid via a C_3 stage and activated citric acid. The C_3 stage is pyruvic acid or a compound closely related to pyruvic acid. The results indicate that activated citric acid is a closer precursor to itaconic acid than is *cis*-aconitic acid.

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Biosynthesis of Fatty Acids in Cell-free Preparations

2. SYNTHESIS OF FATTY ACIDS FROM ACETATE BY A SOLUBLE ENZYME SYSTEM PREPARED FROM RAT MAMMARY GLAND

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We have reported previously the synthesis of short- and long-chain fatty acids from acetate by cell-free suspensions (homogenates) of mammary gland of lactating rats and sheep (Popják & Tietz, 1954a). The following conditions were required for synthesis in the preparations of rat mammary gland: (a) aerobic incubation, (b) the co-oxidation of any of the three keto acids: pyruvate, oxaloacetate or α -oxoglutarate, and (c) the addition of adenosine triphosphate (ATP), although the last was not strictly required with pyruvate and α -oxoglutarate. The maximum synthesis of fatty acids from acetate occurred in the presence of acetate (0.02M), oxaloacetate (0.02M) and of ATP (0.01M) and when the gas phase was air instead of pure O₂.

In this article the preparation and some of the properties of a soluble enzyme system from the mammary gland of lactating rats are described. The results have already been presented in a preliminary form (Popják & Tietz, 1953, 1954b).

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MATERIAL AND METHODS

Preparation of the soluble enzyme system. Homogenates of the mammary gland of lactating rats, 10-14 days after parturition, were prepared first as described previously (Popják & Tietz, 1954a) and were then fractionated by high-speed centrifuging. The homogenate, from which whole cells, cell debris, nuclei, etc., have been removed by preliminary centrifuging at 400 g for 10 min., was centrifuged first at 25 000 g for 30 min. and at 0°. A clear, transparent pink supernatant (Sp. I) was taken off and filtered through a small pad of cotton wool to remove a thin film of fat from the top. The sediment, designated as 'mitochondria', was washed twice by dispersion in fresh, ice-cold buffer and centrifuging at 25 000 g for 15 min. A sample of Sp. I was centrifuged further at 2-4° and at 104 000 g for 30 min. in a Spinco preparative centrifuge. The supernatant (Sp. II), which in appearance was similar to Sp. I, was taken off and filtered as described for Sp. I. The sediment, a pinkish brown translucent pellet, designated as 'microsomes', was washed once with fresh buffer and sedimented at 104 000 g for 10 min.

The 'mitochondria' obtained from 5 ml. of homogenate were suspended with the aid of a small glass homogenizer in 2.5 ml. of buffer or in the same volume of Sp. I, to provide 2 ml. for incubation and 0.5 ml. for determination of dry weight. The 'microsomes' obtained from 10 ml. of Sp. I were treated in the same way with buffer or Sp. II.

After it was found that all the enzymic activity required for fatty acid synthesis was present in Sp. II, the procedure was shortened. Homogenates were prepared in either phosphate buffer (0.154M-KCl, 100 parts; 0.154M-MgCl₂,