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The Biosynthesis of Kojic Acid

2. THE OCCURRENCE OF ALDOLASE AND TRIOSEPHOSPHATE ISOMERASE IN *ASPERGILLUS* SPECIES AND THEIR RELATIONSHIP TO KOJIC ACID BIOSYNTHESIS

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The experiments with ^{14}C -labelled glucose and dihydroxyacetone described in the preceding paper (Arnstein & Bentley, 1953) indicated that dihydroxyacetone is not an intermediate in the biosynthesis of kojic acid from glucose. Moreover, the distribution of ^{14}C in the kojic acid derived from [$1\text{-}^{14}\text{C}$]glucose suggested that most of the kojic acid was formed by a direct conversion of the glucose. However, the possibility that kojic acid might be synthesized by condensation of dihydroxyacetone phosphate and a C_3 unit, e.g. glyceraldehyde phosphate, not in equilibrium with each other, could not be excluded by the isotope experiments, as the expected distribution of ^{14}C in the kojic acid would be similar in both cases. It therefore became important to determine whether the organisms producing kojic acid used in this work contained the enzymes aldolase, which is concerned with the formation of dihydroxyacetone phosphate and glyceraldehyde phosphate, and triosephosphate isomerase; the presence of triosephosphate isomerase would be particularly significant as this enzyme would bring about the equilibrium between any dihydroxyacetone phosphate formed and

glyceraldehyde phosphate. In addition to experiments demonstrating the presence of both these enzymes in the mycelium of the moulds used for kojic acid production in this work, the present paper also discusses the influence of phosphate on kojic acid formation and the evidence for the participation of phosphorylated intermediates in kojic acid biosynthesis.

The method of numbering the kojic acid carbon atoms throughout this paper corresponds with that adopted in the previous paper (Arnstein & Bentley, 1953).

EXPERIMENTAL

Enzyme experiments. In experiments to determine whether the organisms contained the enzyme aldolase, extracts were prepared by grinding mycelia with sand. These extracts were incubated with hexose diphosphate and the formation of triosephosphate was observed by measuring the amount of alkali-labile and inorganic phosphate present at definite time intervals.

Material and Methods

Fructose diphosphate. Calcium hexose diphosphate (2.2 g.) was suspended in a little water and treated at 0° with 2N-HCl until almost all was dissolved. A little insoluble material was centrifuged off and solid potassium oxalate (1.58 g.) added to the solution. After shaking, the precipi-

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tated calcium oxalate was spun down. Finally, the clear solution was adjusted to the desired pH value (7 or 10) by careful addition of dil. NaOH and diluted to 10 ml.

Preparation of extracts. The method of Lynen & Hoffman-Walbeck (1948) was used with slight modifications. Unless otherwise described, the organisms were grown as surface cultures on 200 ml. of Czapek-Dox medium (10% (w/v) glucose with the addition of 1% (w/v) yeast extract) in plugged 1 l. conical flasks at 28–29°. Before use, the culture was chilled in the refrigerator for 30 min.; the medium was decanted and the mycelium washed with portions of ice-water. It was dried as completely as possible between layers of filter paper, and the wet weight was determined. (For determination of dry weight a portion was weighed and dried to constant weight in a vacuum desiccator.) Portions of the mycelium were ground with an equal weight of acid-washed sand and ice-cold water (5 ml./g. mycelium) using a porcelain mortar and pestle. The mortar and pestle were previously chilled to 0° and the mortar was held in an ice bath during the grinding operation. The mixture of cell material and sand was transferred to a chilled measuring cylinder and made up to a given volume with ice-cold distilled water. The mixture was shaken and the supernatant liquid was transferred to a Pyrex boiling tube; various additions of buffer solutions, etc., were made and, last of all, the solution of potassium hexose diphosphate was added. After rapid mixing, 1 ml. of the supernatant solution was removed and added to 20% (w/v) trichloroacetic acid (TCA) (2 ml.). The boiling tube was placed as rapidly as possible in a bath at 30° and further 1 ml. samples were removed and deproteinized with TCA at 10 min. intervals.

Phosphate analyses. The TCA solutions were filtered, and the filtrate and washings made up to 10 ml. Portions (2 ml.) were removed for determination of inorganic phosphate (*I*), and the inorganic phosphate produced after treatment with 2N-NaOH (1 ml.) for 20 min. at room temp. (*A*). The method of Allen (1940) was used for these determinations. The value of $A - I$, the alkali-labile phosphorus, was used as a measure of triosephosphate.

In experiments* designed to show the presence or absence of triosephosphate isomerase, the triosephosphate mixture was differentiated by oxidation with iodine in slightly alkaline solution. Under these conditions only glyceraldehyde phosphate is oxidized; the decrease in alkali-labile phosphate on iodine oxidation is therefore a measure of the glyceraldehyde phosphate present (Meyerhof & Lohmann, 1934; Meyerhof & Kiessling, 1935; Herbert, Gordon, Subrahmanyam & Green, 1940; Utter & Werkman, 1941). These experiments were carried out under the following conditions, which are slightly different from those in the aldolase experiments.

Cultures of *Aspergillus flavus-oryzae* were grown on the culture medium previously described except that the glucose concentration was increased to 15% (w/v) and 100 ml. portions of medium were used in 500 ml. conical flasks. The extracts were prepared with sand and in some cases glycine buffer was used instead of water during the grinding process. During the course of the reaction, 1 ml. samples were withdrawn from the tubes and were added to

10% (w/v) TCA (2 ml.). After the addition of water (7 ml.) the solutions were mixed and filtered.

Portions (1 ml.) of the TCA filtrates were used for the estimation of inorganic and alkali-labile phosphate using the Fiske-Subbarow reagent (Fiske & Subbarow, 1925). The optical density was observed with a Klett-Summerson photoelectric colorimeter, the final volume being 10 ml.

For the reaction with iodine, 2 ml. of the TCA filtrate were carefully neutralized to pH 8 with 5% (w/v) Na_2CO_3 ; 0.02N- I_2 (1 ml.) was added and the solution incubated at 37° for 20 min. At the end of this time, the excess iodine was back titrated with 0.02N- $\text{Na}_2\text{S}_2\text{O}_3$ and the solution made up to a volume of 10 ml.; 5 ml. was removed for the determination of alkali-labile phosphate and the remainder of the solution was used for the determination of inorganic phosphate.

Influence of phosphate level on kojic acid production by *A. flavus-oryzae*. The basal medium used in this experiment had the composition described earlier (Arnstein & Bentley, 1953) but the K_2HPO_4 was omitted. A 5% (w/v) solution of K_2HPO_4 was added to individual 1 l. flasks, containing 200 ml. basal medium, to give final concentrations of K_2HPO_4 equal to 0.25, 0.1, 0.01 and 0.001% (w/v) respectively. The plugged flasks were sterilized, inoculated in the usual way and incubated at 28°. Kojic acid production was followed quantitatively on portions of medium removed under sterile conditions: 1 ml. of suitably diluted medium was mixed with 4 ml. 1% (w/v) aqueous FeCl_3 solution and 5 ml. water and the colour produced was compared with that obtained with a kojic acid solution of known strength, using a Spekker photoelectric absorptiometer (blue-green filter, no. 603).

RESULTS

Effect of pH. The results given in Table 1 indicate that these extracts contained aldolase activity and show the effect of pH on the reaction. Only in those experiments where a relatively high pH was maintained was there a steady increase in the amount of triosephosphate (alkali-labile phosphate) produced. At pH values lower than 8, the amount of triosephosphate produced appeared to increase initially but then decreased after about 20 min. It is probable that these preparations, where no attempt had been made to purify the aldolase, contained enzymes destroying triosephosphate and that this was diminished at higher pH values. Indeed, many moulds are known to contain powerful phosphatases which are almost always acid phosphatases (Roche, 1950). Earlier experiments with enzyme preparations of *A. oryzae* (Taka diastase) had shown the presence of at least two enzymes with maximum activity towards α - and β -glycerophosphate at pH 4.1, and 6.2 (Bamann & Salzer, 1936; Nguyen-van-Thoi, 1942).

The presence of phosphatases in our extracts was confirmed by the increased production of inorganic phosphate when hexose diphosphate was present compared with controls where no phosphate esters were added (Table 2) and by the liberation of H^+ during the incubation (Table 1). The majority of the

* These experiments were carried out by one of us (R. B.) at the Department of Biochemistry, Columbia University, College of Physicians & Surgeons, New York. A grant from the Nutrition Foundation Inc. which made this work possible, is gratefully acknowledged.

experiments were therefore carried out in the presence of glycine buffer, pH 9 or 10, with the solution of hexose diphosphate previously adjusted to pH 10 by the addition of sodium hydroxide. The necessity of working at an alkaline reaction to obtain optimal activity agrees with the findings of Herbert *et al.* (1940) for muscle aldolase and con-

firms the experience of Utter & Werkman (1941); these authors, using *Escherichia coli* preparations, had to work at pH values greater than 9 in order to avoid phosphatase action.

Control experiments. Table 3 presents results which show that the enzyme was destroyed on boiling and that there was no significant decomposition

Table 1. *Effect of pH on the production of triosephosphate by mycelial extracts of Aspergillus oryzae*

(In these experiments 1 g. portions of mycelium (total wet wt. = 7.2 g.) of a culture of *A. oryzae*, 4 days old, which was not producing kojic acid at that time, were used and the initial suspension was made up to 5 ml.)

Exp. no.	Additions				pH		Time (min.)	I* (mg.)	A† (mg.)	A - I‡ (mg.)
	Glycine buffer (ml.)		0.43N-Potassium hexose diphosphate (ml.)		Initial	Final				
	pH 9	pH 10	pH 7	pH 10						
1	—	—	1	—	7.5	5.6	0	1.91	2.97	1.06
							10	1.91	3.46	1.55
							20	2.56	3.24	0.68
2	3	—	1	—	8.0	6.0	0	1.77	2.88	1.11
							10	1.77	3.82	2.05
							20	3.03	3.93	0.90
3	—	3	1	—	8.5	7.8	0	2.29	2.74	0.05
							10	2.29	3.30	1.01
							20	2.25	3.60	1.35
4	3	—	—	1	9.0	—	0	2.20	2.52	0.32
							10	2.25	3.10	0.85
							20	2.56	3.71	1.15

* I = inorganic phosphate. All values for phosphates in all the tables are calculated for the original volume of incubation solution, and are given as mg. P.

† A = inorganic phosphate after alkaline hydrolysis (20 min. at room temperature).

‡ A - I = triosephosphate P.

Table 2. *Absence of triosephosphate production by endogenous metabolism*

(In each case, the mycelium (1 g.) was ground with an equal weight of sand and ice-cold distilled water (5 ml.). The hexose diphosphate solution was adjusted to pH 10 before use.)

Exp. no.	Additions		Water (ml.)	Time (min.)	<i>I</i> * (mg.)	<i>A</i> * (mg.)	<i>A - I</i> * (mg.)
	0.2M-Glycine buffer, pH 10 (ml.)	0.2M-Hexose diphosphate (ml.)					
1†	5	—	2	0	0.42	0.46	0.04
				10	0.59	0.60	0.01
				20	0.64	0.67	0.03
				30	0.87	0.90	0.03
2†	5	2	—	0	1.08	1.28	0.20
				10	1.33	1.65	0.32
				20	1.95	2.53	0.58
				30	2.34	2.95	0.61
3‡	5	—	2	0	0.23	0.27	0.04
				10	0.28	0.31	0.03
				20	0.36	0.39	0.03
				30	0.39	0.42	0.03
4‡	5	2	—	0	0.96	1.14	0.18
				10	1.20	1.43	0.23
				20	1.29	1.53	0.24
				30	1.43	1.84	0.41

* Symbols I and A have the meaning given in Table 1.

† Culture of *A. flavus-oryzae*, 4 days old, just beginning to produce kojic acid.

‡ Culture of *A. flavus-oryzae*, 8 days old, giving an intense colour when the culture medium was treated with ferric chloride.

Table 3. *Effect of heat and possible inhibitors on the production of triosephosphate by extracts of Aspergillus flavus-oryzae mycelium*

(1 g. portions of mycelium (total wet wt. = 5.5 g.) from a 4-day-old culture of *A. flavus-oryzae*. The initial suspension was made up to 10 ml.)

Exp. no.	Vol. of enzyme suspension (ml.)	Notes	Buffer + hexose diphosphate (ml.)*	Water (ml.)	Time (min.)	I† (mg.)	A† (mg.)	A - I† (mg.)
1	10	Heated in boiling-water bath for 10 min. before use	4	1	0	2.81	3.15	0.34
					10	2.58	2.25	-0.33
					20	2.58	2.18	-0.40
					30	2.44	2.58	0.14
2	10	Initial pH = 9.2. Final pH = 8.7	4	1	0	2.92	3.48	0.56
					10	2.73	3.67	0.94
					20	3.03	4.53	1.50
					30	3.22	5.81	2.59
3	0	—	4	11	0	2.43	2.58	0.15
					10	2.32	2.55	0.23
					20	2.17	2.33	0.16
					30	1.98	2.44	0.46
4	10	—	4	—‡	0	2.81	3.07	0.26
					10	3.03	—	—
					20	3.07	3.90	0.83
					30	3.18	4.72	1.54
5	10	—	4	—§	0	2.81	3.67	0.86
					10	2.78	—	—
					20	3.04	4.79	1.75
					30	3.19	5.36	2.17

* Potassium hexose diphosphate (pH 7) (5 ml.) mixed with 0.2M-glycine buffer, pH 10 (16 ml.) and adjusted to pH 10 by addition of 2N-NaOH. Each tube contained 4 ml. of this mixture.

† Symbols *I* and *A* have the meaning given in Table 1.

‡ This tube contained 1 ml. of 0.1M-NaF.

§ This tube contained 1 ml. of 0.1M-kojic acid.

Table 4. *Effect of enzyme concentration on the production of triosephosphate by extracts of Aspergillus flavus-oryzae mycelium*

(In Exps. 1 and 2 a 3-day-old culture, in Exps. 3 and 4 a 7-day-old culture of *A. flavus-oryzae* was used. In a control experiment carried out at the same time, but without addition of hexose diphosphate, only a small amount of inorganic phosphate and no alkali-labile phosphate was produced.)

Exp. no.	Mycelium (g.)	Vol. of suspension (ml.)	0.2M-Glycine, pH 9 (ml.)	Water (ml.)	Hexose diphosphate, pH 10 (ml.)	Time (min.)	I* (mg.)	A* (mg.)	A - I* (mg.)
1	0.5	10	5	4	1	0	2.00	2.00	0.00
						10	2.10	2.25	0.15
						20	2.00	2.75	0.75
						30	2.10	3.10	1.00
2	1.0	10	5	4	1	0	2.25	2.00	-0.25
						10	2.25	3.25	1.00
						20	2.30	3.61	1.31
						30	2.50	4.45	1.95
3	1.0	19	—	—	1.8	0	2.88	3.15	0.27
						10	2.83	3.51	0.68
						20	3.04	4.20	1.16
						30	3.30	4.83	1.53
4	2.0	19	—	—	1.8	0	2.99	3.35	0.36
						10	3.41	5.14	1.73
						20	3.62	5.77	2.15
						30	4.04	6.88	2.84

* Symbols *I* and *A* have the meaning given in Table 1.

Table 5. *Production of triosephosphate by a centrifuged enzyme preparation obtained from Aspergillus flavus-oryzae mycelium*

(The enzyme solution was prepared from the mycelium (total wet wt. = 5.6 g.) of a culture of *A. flavus-oryzae*, 4 days old, which was just beginning to produce kojic acid. All of this material was ground with sand (equal wt.) and water (20 ml.) and centrifuged at 2500 rev./min. for 5 min. at -5° . The supernatant was filtered and made up to a volume of 30 ml. (pH of solution = 7).)

Exp. no.	Vol. of enzyme solution (ml.)	Water (ml.)	0.1M-Glycine, pH 9 (ml.)	Hexose diphosphate, pH 10 (ml.)	Time (min.)	<i>I</i> * (mg.)	<i>A</i> * (mg.)	<i>A - I</i> * (mg.)
1	5	5	3	2	0	2.25	2.32	0.07
					10	2.25	2.40	0.15
					20	2.28	2.63	0.35
					30	2.29	2.81	0.52
2	10	—	3	2	0	2.25	2.55	0.30
					10	2.43	2.85	0.42
					20	2.55	3.57	0.82
					30	2.58	3.60	1.02
3	10	3	—	2	0	2.40	2.73	0.37
					10	2.40	2.66	0.22
					20	2.25	2.66	0.41
					30	2.40	2.66	0.26

* Symbols *I* and *A* have the meaning given in Table 1.

Table 6. *Demonstration of presence of triosephosphate isomerase in mycelial extracts of Aspergillus flavus-oryzae by iodine oxidation of the triose mixture*

Exp. no.	Preparation of extract	Buffer + HDP* (ml.)	Time (min.)	<i>I</i> † (mg.)		<i>A</i> † (mg.)		<i>A - I</i> † (mg.)	
				Before <i>I</i> ₂ treatment	After <i>I</i> ₂ treatment	Before <i>I</i> ₂ treatment	After <i>I</i> ₂ treatment	Before <i>I</i> ₂ treatment	After <i>I</i> ₂ treatment
1	Mycelium (1 g.) ground with sand (1 g.) and iced water (5 ml.)‡	7	0	0.96	—	1.07	—	0.11	—
			10	1.23	—	1.59	—	0.36	—
			20	1.58	1.58	2.23	2.21	0.65	0.63
			30	1.83	1.70	2.61	2.55	0.78	0.85
2	As in Exp. no. 1	7	0	0.89	—	1.05	—	0.16	—
			10	1.13	—	1.48	—	0.35	—
			20	1.27	1.31	1.83	1.83	0.56	0.52
			30	1.59	1.64	2.21	2.20	0.62	0.56
3	Mycelium (1 g.) ground with sand (1 g.) and iced glycine buffer; pH 10 (5 ml.)‡	7	0	0.91	—	1.13	—	0.22	—
			10	1.13	—	1.47	—	0.34	—
			20	1.47	1.47	1.91	1.91	0.44	0.44
			30	1.62	1.58	2.10	2.10	0.48	0.52
4	As in Exp. no. 1	7	0	0.80	—	1.02	—	0.22	—
			10	0.96	—	1.28	—	0.32	—
			20	1.24	1.20	1.60	1.60	0.36	0.40
			30	1.42	1.39	1.81	1.74	0.39	0.35
5	Mycelium (2 g.) ground with sand (2 g.) and iced water (10 ml.)§	7	0	0.96	—	1.07	—	0.11	—
			10	1.37	1.56	1.75	1.99	0.38	0.43
			20	1.81	1.92	2.54	2.71	0.73	0.79
			30	2.16	2.30	3.22	3.34	1.06	1.14
			40	2.55	2.58	3.66	3.74	1.11	1.16

* Mixture of 0.2M-glycine buffer, pH 10, and 0.2M-sodium hexose diphosphate, pH 10, in the proportion of 5:2.

† Symbols *I* and *A* have the meaning given in Table 1.

‡ Culture of *A. flavus-oryzae*, 4 days old, just beginning to produce kojic acid.

§ Culture of *A. flavus-oryzae*, 7 days old, giving an intense colour when the culture medium was treated with ferric chloride.

of hexose diphosphate in the absence of enzyme. Since moulds are known to have a high rate of endogenous metabolism it was possible that triosephosphate was produced by the extracts even in the absence of added hexose diphosphate. This possibility was ruled out by the results of experiments shown in Table 2.

Effect of varying quantity of enzyme. When the amount of mycelium was increased there was a corresponding increase in the amount of triosephosphate formed (Table 4). Thus, after incubation for 30 min., 0.5 and 1.0 g. mycelium gave 1.00 and 1.95 mg. of alkali-labile phosphate, respectively (Table 4, Exps. 1 and 2). Using a different culture,

1.53 and 2.84 mg. of alkali-labile phosphate were produced from 1.0 and 2.0 g. of mycelium, respectively (Table 4, Exps. 3 and 4). The experiments detailed in Table 5 were carried out with a solution obtained after centrifugation of the initial suspension. They show that the enzyme was water-soluble as was the case with the preparations obtained by Lynen & Hoffman-Walbeck (1948) from *Penicillium notatum*. The extracts were particularly sensitive to the absence of buffer. When the amount of enzyme solution was increased from 5 to 10 ml., the amount of triosephosphate present after 30 min. correspondingly increased from 0.52 to 1.02 mg.

Effect of sodium fluoride and kojic acid. In a preliminary experiment (Table 3, Exps. 4 and 5) sodium fluoride and kojic acid were added to investigate whether these compounds influenced the

formation of triosephosphate. No significant effects were noted with kojic acid. In some cases, in the presence of sodium fluoride, the amount of triosephosphate formed was slightly less than in the control experiments; in the small number of experiments that were carried out no consistent results were obtained.

Triosephosphate isomerase. The results given in Table 6 show that there was no significant change in the amount of alkali-labile phosphate after treatment with iodine. This means that the reaction mixture contained virtually no glyceraldehyde phosphate. Since the equilibrium mixture of triosephosphate formed in presence of triosephosphate isomerase contains about 96 % of dihydroxyacetone phosphate (Meyerhof & Lohmann, 1934; Herbert *et al.* 1940; Meyerhof & Junowicz-Kocholaty, 1943;

Table 7. *Hydrolysis of adenosinetriphosphate by extracts of Aspergillus flavus-oryzae mycelium*

(In each set of experiments, a 4-day-old culture of *A. flavus-oryzae* was used, giving an intense red coloration when the culture medium was treated with ferric chloride. The mycelium (wt. in each case, 3.5 g.) was ground with sand (3 g.) and the appropriate buffer (15 ml.). The mixture was centrifuged (3000 rev./min.) for 5 min. at -5° , the supernatant being decanted through a filter paper and stored at 0° until used. Exps. 1-5: buffer used in grinding mycelium and for addition as above was 0.2M-acetate, pH 4. Exps. 6-8: buffer used in grinding mycelium and for addition as above was 0.2M-trishydroxymethylaminomethane, adjusted to pH 8 with 6N-HCl.)

Exp. no.	Enzyme solution (ml.)	Buffer (ml.)	ATP (ml.)	Water (ml.)	0.25M-NaF (ml.)	Time (min.)	I* (mg.)	B† (mg.)	ATP‡ (mg.)	ATP§ less blank (mg.)
1	1	1	0.1	0.9	—	0	0.09	0.51	0.42	0.36
						10	0.22	0.55	0.33	0.27
						20	0.35	0.62	0.27	0.21
2	1	1	—	1	—	0	0.06	0.14	0.08	—
						10	0.07	0.15	0.08	—
						20	0.09	0.14	0.05	—
3	1 (boiled for 5 min. before use)	1	0.1	0.9	—	0	0.08	0.51	0.43	0.37
						10	0.09	0.54	0.45	0.39
						20	0.09	0.52	0.43	0.37
4	2	0.9	0.1	—	—	0	0.13	0.65	0.52	0.40
						10	0.46	0.72	0.26	0.14
						20	0.53	0.76	0.23	0.11
5	1	1	0.1	0.7	0.2	0	0.07	0.51	0.44	0.38
						10	0.09	0.52	0.43	0.37
						20	0.10	0.53	0.43	0.37
6	1	1	0.1	0.9	—	10	0.13	0.54	0.41	0.35
						20	0.14	0.54	0.40	0.34
						40	0.16	0.55	0.39	0.33
7	1	1	—	1	—	10	0.09	0.16	0.07	—
						20	0.09	0.15	0.06	—
						40	0.10	0.15	0.05	—
8	1 (boiled for 5 min. before use)	1	0.1	0.9	—	10	0.12	0.52	0.40	0.34
						20	0.11	0.53	0.42	0.36
						40	0.11	0.53	0.42	0.36

* I=inorganic phosphate.

† B=total phosphate present after hydrolysis for 7 min. at 100° in N-HCl.

‡ Amount of ATP=B-I.

§ Amount of ATP after subtraction of average blank of 0.06 mg./ml. enzyme solution obtained from Exps. 2 and 7 where no ATP was added.

Oesper & Meyerhof, 1950), these results indicate that the extracts contained triosephosphate isomerase.

Action of extracts on adenosinetriphosphate (ATP). In preliminary experiments to find out whether phosphate esters other than hexose diphosphate were hydrolysed, centrifuged extracts of *A. flavus-oryzae* were incubated with ATP. As shown in Table 7, inorganic phosphate was produced and the amount of 7 min. acid-hydrolysable phosphorus diminished. The enzyme was active at pH 4 but not at pH 8. In the presence of 0.016 M-sodium fluoride, inorganic phosphate was not produced and the amount of ATP did not diminish (see Table 7, Exp. 5).

experiment (Fig. 1) demonstrate that the level of phosphate had a marked influence on the rate of appearance and on the subsequent metabolism of kojic acid. High phosphate levels (0.1–0.25 %, w/v) resulted in rapid kojic acid production, reaching a maximum in about 10 days, followed by almost complete destruction of the kojic acid within 30 days. At lower phosphate levels (0.01–0.001 %, w/v) kojic acid formation still took place, but was much slower and did not quite reach the amounts produced with the high phosphate medium. A steady state was maintained for some time, but the kojic acid was then slowly destroyed. However, after 150 days appreciable amounts were still present.

Fig. 1. Influence of phosphate level on the production of kojic acid by *A. flavus-oryzae*. Each curve represents the mean of two experiments. Details of the medium and method of kojic acid estimation are given in the Experimental Section. The different phosphate levels were as follows: A, 0.25 %; B, 0.1 %; C, 0.01 %; D, 0.001 % (w/v) K_2HPO_4 .

On prolonged incubation (1–2 hr.) such solutions were shown by paper chromatography to contain a hexose and a pentose identified by comparison with control chromatograms as glucose and ribose. The 'ribose spot' was not formed in the absence of ATP nor after boiling the enzyme preparations. Glucose, however, was present under these conditions. The 'ribose spot' was formed at pH's 4 and 6, hardly at all at pH 7 and not at pH's 2 and 8.

It is concluded that in addition to dephosphorylation of ATP these extracts can also split off ribose either from ATP itself or the dephosphorylated product.

Influence of phosphate level on the production of kojic acid by A. flavus-oryzae. The results of this

DISCUSSION

The presence of aldolase and triosephosphate isomerase in mycelial extracts of Aspergillus oryzae and A. flavus-oryzae

The evidence for the existence in fungi of the enzymes and intermediates of the Embden-Meyerhof pathway is meagre (see review by Foster, 1949). Recently, Lynen & Hoffman-Walbeck (1948), using extracts of ground *P. notatum* mycelia, observed the formation of triosephosphate from fructose diphosphate. Since the mixture was not analysed separately for dihydroxyacetone phosphate and glyceraldehyde phosphate, only the presence of aldolase was conclusively shown. However, under suitable conditions phosphoglyceric acid was

isolated after the action of mycelial extracts on fructose diphosphate, and it seems likely that the preparations must have contained triosephosphate isomerase. Using similar extracts from *A. flavus-oryzae* and *A. oryzae*, we found that triosephosphate was formed from fructose diphosphate and that the aldolase activity of these preparations was of the same order as that obtained from *P. notatum*.

On treatment with iodine the amount of triosephosphate obtained with the *Aspergillus* extracts was not diminished indicating that there was virtually no glyceraldehyde phosphate present. Since preparations of triosephosphate isomerase are known to convert triosephosphates into an equilibrium mixture which contains about 95 % of dihydroxyacetone phosphate (Meyerhof & Lohmann 1934; Herbert *et al.* 1940; Meyerhof & Junowicz-Kocholaty, 1943; Oesper & Meyerhof, 1950) these results strongly suggest that triosephosphate isomerase was also present in the mycelial extracts, the alternative possibility that fructose diphosphate is split in this case to two molecules of dihydroxyacetone phosphate being much less likely.

The above interpretation is in agreement with the experiments on the formation of kojic acid from [1^{14}C]glucose (Arnstein & Bentley, 1953). In these experiments, up to 17 % of the total radioactivity was located at carbon atom 6 of the kojic acid. Moreover, the glucose re-isolated at the end of the fermentations contained a significant amount of radioactivity in carbon atom 6, indicating the presence in the mould of an enzyme system which causes the radioactivity to become distributed at random between carbon atoms 1 and 6 of the glucose as well as of the kojic acid; such a redistribution would be expected if glucose were converted to an equilibrium mixture of triosephosphates (by the action of aldolase and triosephosphate isomerase) and partly resynthesized during fermentation.

It should be emphasized, however, that there is no evidence that fructose diphosphate is a normal intermediate in the glucose metabolism of these moulds; indeed, it is possible that the reactions of the Embden-Meyerhof scheme exist and can be detected, although they may not be of major importance in the metabolism of glucose by these organisms. Such a possibility receives some support from the observations on the radioactivity of the respiratory carbon dioxide produced when [1^{14}C] or [$3,4^{14}\text{C}_2$]glucose was the substrate (Arnstein & Bentley, 1953); these results suggested that initially carbon atom 1 of glucose was converted to carbon dioxide more extensively than carbon atoms 3 and 4. We conclude, therefore, that the moulds contain both aldolase and triosephosphate isomerase and that some dihydroxyacetone phosphate is formed and is in equilibrium with glyceraldehyde phosphate, although this reaction is not of major importance.

The participation of phosphorylated intermediates in kojic acid biosynthesis

As already discussed in the previous paper (Arnstein & Bentley, 1953) there appear to be two pathways of kojic acid formation. Although the mould mycelium contains both aldolase and triosephosphate isomerase, it seems that when glucose is available the postulated condensation of triosephosphates to kojic acid is not as important as the direct conversion of glucose to kojic acid. Although phosphorylation is probably essential for the formation of reactive trioses, and hence for the synthesis of kojic acid by the secondary pathway, the evidence for the participation of phosphorylated intermediates in the major, direct conversion of glucose to kojic acid is not clear-cut. Barham & Smits (1934) found that variation of the phosphate concentration in the fermentation of xylose by *A. flavus* resulted in two maxima of kojic acid production, the lower concentration giving the higher yield. Later, Gould (1938) observed that the rate of kojic acid production in replacement cultures of *A. tamarii* was higher on a phosphorus-free glucose medium than on a phosphorus-containing control culture and he suggested that therefore phosphorylation was not involved in kojic acid biosynthesis. A similar conversion of glucose to kojic acid in replacement cultures in the absence of added phosphate had been observed earlier by Kluyver & Perquin (1933). Gould also obtained kojic acid production in phosphorus-free replacement cultures containing glycerol or xylose. Since in each case the mycelium itself must have contained phosphorus compounds this evidence is not unequivocal.

In other experiments, Gould (1938) showed that the addition of sodium fluoride or sodium iodoacetate, in amounts known to inhibit reactions involving phosphorylated intermediates in other moulds (Walker, 1936, 1949; Mann, 1944), to cultures of *A. tamarii* actually increased the rate of kojic acid production. Although Gould's experiments on the production of kojic acid are not conclusive they do suggest that phosphorylation of glucose is not concerned with kojic acid biosynthesis. Further evidence along this line may be adduced by analogy with the oxidation of glucose or gluconic acid to 2-ketogluconic acid by *Pseudomonas aeruginosa*. This oxidation is not blocked by sodium fluoride or sodium iodoacetate, nor does it require ATP nor involve phosphorylated intermediates (Campbell & Norris, 1950; Stokes & Campbell, 1951). There seems to be a similarity between this bacterial aerobic oxidation (where the carbon skeleton of the substrate is not drastically changed) and the formation of gluconic, ketogluconic and kojic acids in fungi. If the 3-keto group of kojic acid were formed in an analogous manner to

the 2-keto group of 2-ketogluconic acid (in the bacterial system), then phosphorylated compounds might be involved only in the secondary pathway of kojic acid formation, i.e. condensation of triose-phosphates.

In experiments reported in this paper, the effect of phosphate level on kojic acid production was followed over long periods of time (Fig. 1). High phosphate levels (0.25%, w/v) resulted in rapid kojic acid production, reaching a maximum in about 10 days, followed by almost complete destruction of the kojic acid within 30 days. At lower phosphate levels (0.01%, w/v) kojic acid formation still took place, but was much slower and did not quite reach the amounts produced with the high phosphate medium. A steady state was maintained for some time, but the kojic acid was then very slowly destroyed, appreciable amounts still being present after 150 days. We conclude, therefore, that low phosphate levels affect both the formation and destruction of kojic acid and probably also the general metabolic processes of the mould rather than kojic acid production in particular.

The further utilization of kojic acid has been

noted by several workers (Corbellini & Gregorini, 1930; Birkinshaw, Charles, Lilly & Raistrick, 1931; Gould, 1938) and Foster (1949) has suggested that a study of this breakdown would provide a clue to the nature of the biosynthesis. The significance of the reassimilation still remains virtually unknown but kojic acid should probably be considered as a normal metabolite rather than as an end product. It may serve as a storage material and be later transformed into more complex pyrones; in addition, its antibiotic property would prevent the growth of other organisms.

SUMMARY

1. The presence of aldolase and triosephosphate isomerase in extracts of the mycelium of *Aspergillus flavus* and *A. flavus-oryzae* is shown.

2. The participation of phosphorylated intermediates in kojic acid biosynthesis is discussed.

3. The effect of varying phosphate levels on kojic acid production has been investigated.

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