APPLIED BIOCHEMISTRY AND BIOTECHNOLOGY 8, 237–247 (1983)

Enzymatic Synthesis of Flavin Adenine Dinucleotide

SAKAYU SHIMIZU,* KIYOSHI YAMANE,† YOSHIKI TANI,‡ AND HIDEAKI YAMADA

Department of Agricultural Chemistry and ‡Research Center for Cell and Tissue Culture, Kyoto University, Sakyo-ku, Kyoto 606, Japan

Received and accepted January 12, 1983

Abstract

A new and simple enzymatic method for the synthesis of flavin adenine dinucleotide (FAD) from flavin mononucleotide by the transadenylylation reaction using microbial cells is described.

Among various microorganisms tested, *Artherobacter globiformis* IFO 12138 and two soil bacteria were selected as useful enzyme sources. Under suitable reaction conditions, the amount of FAD synthesized was 2.25 μmol/mL with cells of *A. globiformis*.

The transadenylylation reaction could be coupled with the ATP supplying system through a glycolysis process with yeast.

Index Entries: FAD, enzymatic synthesis of; ATP, supplying system for enzymatic synthesis of FAD; reactor, for FAD synthesis under coupling with ATP generation system; transadenylylation, of FAD synthesis under coupling with ATP generation system; *Arthrobacter globiformis*, useful enzyme source for FAD synthesis; yeast, as an ATP generator; enzymatic synthesis, of FAD; synthesis, enzymatic, of FAD.

Introduction

Flavin adenine dinucleotide (FAD) has been prepared by extraction from microbial cells (*1–4*), by chemical synthesis (*5, 6*) and by a fermentative process using *Brevibacterium ammoniagenes* (*7*) and *Sarcina lutea* (*8, 9*). However, there has been no published synthesis of FAD by an enzymatic process with a good yield

†On leave from the Central Research Laboratory, Toyobo Co., Otsu 520, Japan

0273–2289/83/0600–0237$2.20

except for our reporting that immobilized bacterial cells produce FAD in a significantly high yield from flavin mononucleotide (FMN) and ATP by the transadenylylation reaction (*10*). Since the transadenylylation to form FAD requires ATP as an adenosyl donor, the construction of an ATP-generating system is indispensable for economic utilization of this reaction. Several routes for generation of ATP were evaluated by Langer et al. (*11*). Among those routes, the utilization of microbial cells as the generator is more advantageous, since the process is not complicated and the yield of ATP is higher than those with other processes. Basic studies on utilization of yeast cells as an ATP generator were first described by Ogata and coworkers (*12*). Later, this process was improved and utilized for the synthesis of ATP itself and various reactions requiring ATP (*13–16*).

In this study, we applied this system in order to supply ATP for the FAD-synthesizing reaction. We first describe a survey of microorganisms having high FAD-synthesizing activity from FMN and ATP and the essential conditions for this synthesis and, second, conditions for the coupling of FAD synthesis to the ATP-generating system.

Materials and Methods

Microorganisms, Cultivation, and Preparation of Dried Cells

All microorganisms used were strains preserved in our laboratory. The media for bacteria, yeasts, and mold, and the conditions for cultivation and preparation of dried cells as an enzyme source were described previously (*17–19*), unless otherwise stated. Baker's yeast used was a commercially available pressed paste (Oriental Yeast Co., Tokyo).

Reaction Mixture for FAD Synthesis

The standard reaction mixture for the synthesis of FAD contained, in 0.5 mL, 2.5 μmol FMN, 7.5 μmol ATP, 5 μmol $MgSO_4$, 100 μmol potassium phosphate, pH 7.0, and 50 mg dried cells. The reaction was carried out at 37°C for 6 h in the dark and stopped by immersing the reaction tube in a boiling waterbath for 3 min. The supernatant after centrifugation was analyzed for FAD.

Reaction Mixture for ATP Synthesis

The standard reaction mixture for the synthesis of ATP contained, in 1 mL, 20 μmol adenosine, 400 μmol glucose, 10 μmol $MgSO_4$, 300 μmol potassium phosphate, pH 7.0, and 80 mg dried cells. The reaction was carried out at 37°C and stopped by immersing the reaction tube in a boiling waterbath for 3 min. The supernatant after centrifugation was analyzed for ATP.

Analytical Methods

FAD in the supernatant was separated by ascending paper chromatography (PPC) on Toyo Roshi No. 53 paper using solvent system I, *n*-butanol–acetic acid–water (4:1:5, by vol., top phase), and measured by application of the lumiflavin proce-

dure to eluates of the spots as described by Yagi (*20*). For analysis of isolated FAD, solvent system II, acetone–isobutyric acid–water 55:30, by vol.) was also used. Thin layer chromatography (TLC) on a Kieselgel G60 F_{245} plate (E. Merck, Darmstadt) was carried out with a solvent system of water saturated with isoamyl alcohol. Phosphorus contents of FAD samples were determined by the method of Fiske and SubbaRow (*21*). The D-amino acid oxidase test was carried out as described by Friedmann (*22*). ATP was measured enzymatically (*23*). Growth of *Arthrobacter globiformis* IFO 12138 was measured turbidimetrically using an absorbancy at 610 nm–dry cell mass calibration curve. All spectrophotometric analyses for identification of FAD were carried out in 0.1*M* potassium phosphate, pH 7.0, using a Hitachi 200-10 spectrophotometer.

Results and Discussion

Survey of FAD-Synthesizing Reaction Conditions

Selection of FAD-Synthesizing Microorganisms. FAD-synthesizing activity was assayed in 111 strains, that is, 66 bacteria, 29 yeasts, and 16 molds. Screening was also done on 50 bacteria isolated from soil samples. High activity was found to be widely distributed in the genera of *Corynebacterium, Micrococcus, Arthrobacter,* and *Brevibacterium*. No strain with high activity was found in the yeasts and molds tested. Fourteen bacterial strains that synthesized more than 0.20 μmol FAD/mL were retested under the standard conditions with sodium laurylbenzene-sulfonate (SLBS). The results are given in Table 1. These strains also showed relatively low

TABLE 1
FAD-Synthesizing Activity in Various Bacteria[a]

Strain	FAD, μmol/mL
Erwinia aroideae IFO 3830	0.41
Corynebacterium glutamicum ATCC 13032	0.76
Corynebacterium paurometabolum IFO 12160	0.35
Micrococcus sp. AKU 0511	0.59
Micrococcus luteus IFO 1099	0.70
Micrococcus luteus IFO 3232	0.96
Arthrobacter globiformis IFO 12138	1.26
Arthrobacter simplex IFO 12069	0.93
Brevibacterium ammoniagenes IFO 12071	1.16
Brevibacterium ammoniagenes IFO 12072	0.96
Isolate L	0.54
Isolate N	1.50
Isolate O	1.05
Isolate P	1.23

[a]Reactions were carried out under the standard conditions with 1 mg SLBS.

degrading activity toward FMN and ATP as substrate (data not shown). This may be a necessary feature for efficient FAD synthesis. *A. globiformis* IFO 12138 showed the highest activity. *A. globiformis* and two additional strains, namely isolates N and O that were gram-positive coryneforms, were selected as promising sources of the enzyme for the synthesis of FAD.

Culture Conditions for the Preparation of Dried Cells with High FAD-Synthesizing Activity. *A. globiformis* was grown at 28°C for 36 h in a basal medium with various carbon sources. Cells with high enzyme activity were prepared by growing cells with glucose, mannose, fructose, choline, or nicotine as a major carbon source (Table 2). Casamino acid, ammonium acetate and ammonium citrate, as well as peptone, were effective major nitrogen sources for preparation of cells with high activity. An increased cell yield without any decrease in the enzyme activity was obtained when the peptone concentration was increased to 1.5%. A further increase in the peptone concentration, however, caused a decrease in the activity of cells, though the cell yield was increased. Cells from 36 to 48 h cultures in the basal medium with 1% glucose and 1.5% peptone showed the highest activity. Prolonged cultivation in the same medium led to a decrease in the activity. Both isolates N and O also showed high enzyme activity under the same culture

TABLE 2
Effect of Carbon Source for Growth on FAD-Synthesizing Activity[a]

Carbon source	Growth, g/100 mL	FAD μmol/mL
Glucose	0.39	1.10
Fructore	0.31	0.91
Galactose	0.20	0.59
Mannose	0.37	1.03
Ribose	0.23	0.76
Sucrose	0.20	0.60
Lactose	0.16	0.30
Soluble starch	0.18	0.02
Na-acetate	0.16	0.15
Na-citrate	0.22	0.40
Na-succinate	0.18	0.37
Peptone	0.28	0.29
Casamino acid	0.21	0.51
Nicotine	0.19	1.30
Choline	0.25	1.23

[a]*A. globiformis* was grown in a basal medium with 1% of the indicated carbon source for 36 h with shaking. The composition of the basal medium was 0.5 g peptone, 0.3 g K_2HPO_4, 0.2 g NaCl, 0.02 g $MgSO_4 \cdot 7H_2O$, and 0.1 g yeast extract in 100 mL water, pH 7.0. Reaction conditions were the same as those in Table 1.

conditions as those above (data not shown). In the following experiments, cells from a 36 h culture at 28°C with the basal medium containing 1% glucose and 1.5% peptone were used.

Reaction Conditions for the Synthesis of FAD. The reaction conditions for FAD synthesis with dried cells of *A. globiformis*, and isolates N and O were studied.

(i) FMN concentration. The amount of FAD synthesized by *A. globiformis* increased with increased addition of FMN, and reached a maximum (0.81 μmol/mL) at 15 μmol FMN/mL. Maximum values of FAD synthesis with isolates N and O were 0.38 and 0.42 μmol/mL, respectively, at an FMN concentration of 5 μmol/mL (Fig. 1). None of the three strains synthesized FAD significantly from riboflavin substituted for FMN, even when a surfactant was present in the reaction mixture.

(ii) ATP concentration. As shown in Fig. 2, in the reaction mixture with FMN either at 2.5 or 10 μmol/mL, the amount of FAD synthesized increased almost linearly up to an ATP concentration of 20 μmol/mL, when *A. globiformis* was the

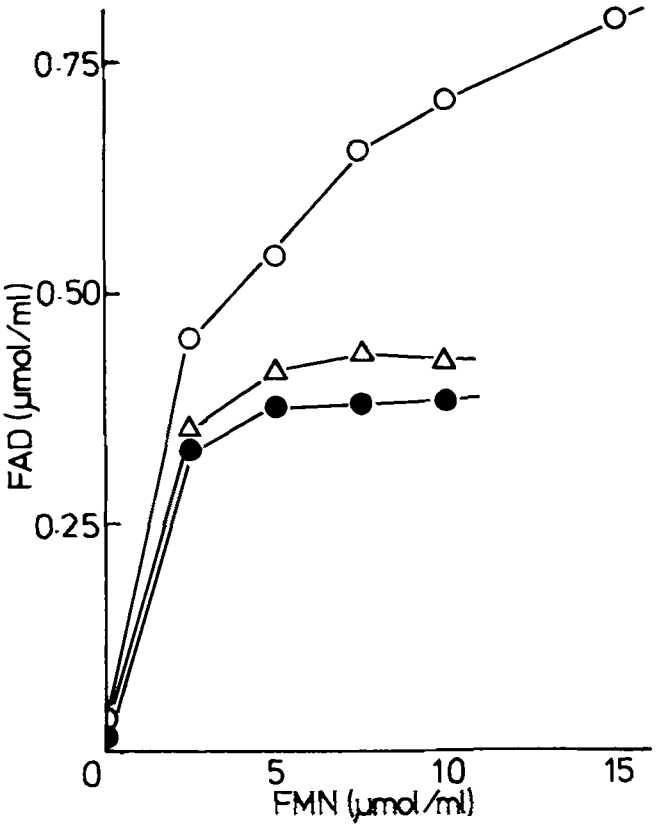

Fig. 1. Effect of FMN concentration on the synthesis of FAD. Cells of *A. globiformis* (○), isolate N (●), and isolate O (△) were incubated with various concentrations of FMN as shown. Other conditions were given in the text.

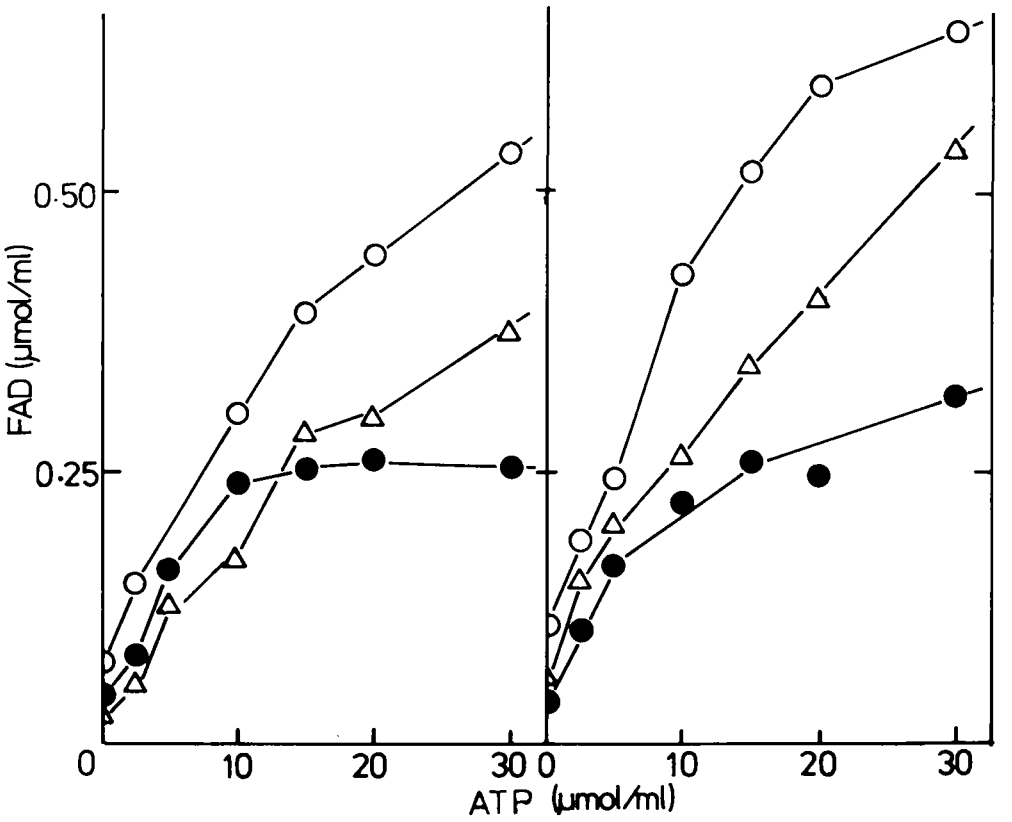

Fig. 2. Effect of ATP concentration on the synthesis of FAD. Cells of *A. globiformis* (○), isolate N (●) and isolate O (△) were incubated with various concentrations of ATP as shown. Other conditions were given in the text.

enzyme source. Isolate O showed a similar pattern of FAD synthesis to that of *A. globiformis*. On the contrary, a remarkable increase in FAD synthesis was not observed at ATP concentrations of more than 10 μmol/mL, when isolate N was the enzyme source.

(iii) Effect of buffers. Figure 3 shows the synthesis of FAD with various buffers. Of the buffers tested, imidazole-HCl was the most effective buffer for FAD synthesis. On replacing potassium phosphate with imidazole-HCl, a 25–80% increase in FAD synthesis was observed with any of the three strains as the enzyme source. Optimal pHs for the FAD synthesis were 7.5–8.0 with *A. globiformis*, 6.5–7.0 with isolate N, and 6.5 with isolate O. When potassium phosphate, pH 7.0, was the buffer, the optimal concentration of the buffer for FAD synthesis with *A. globiformis* was 100 μmol/mL and with increasing concentration of the buffer, the amount of FAD synthesized slightly decreased.

(iv) Cell concentration. The maximum yield of FAD was obtained at a cell concentration around 100 mg/mL in all three strains. A further increase in cell concentration caused a decrease in the synthesis of FAD.

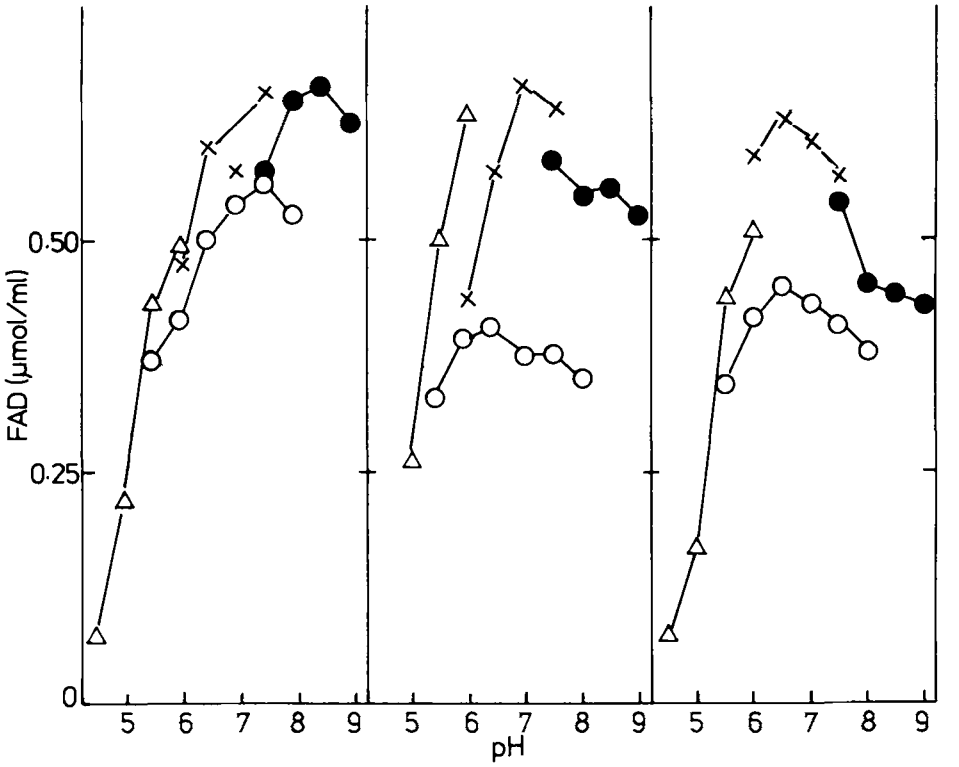

Fig. 3. Effect of buffers on the synthesis of FAD. Reactions were carried out with the following buffers: acetate (△), potassium phosphate (○), imidazole-HCl (X), Tris-HCl (●). Cells used as the enzyme source were *A. globiformis* (left), isolate N (center), and isolate O (right). Other conditions were given in the text.

(v) Effect of reaction temperature. The maximum synthesis of FAD was attained at 42°C with *A. globiformis* as the enzyme source and about a 50% decrease in the synthesis was observed at 50°C.

(vi) Effect of divalent metal ions. FAD was effectively synthesized by cells of *A. globiformis* even when $MgSO_4$ was omitted from the reaction mixture. A significant change in FAD synthesis was not observed with varying concentrations of $MgSO_4$ up to 10 μmol/mL. Addition of $MnSO_4$, $FeSO_4$, $CoSO_4$, $NiSO_4$, or $ZnSO_4$ at 10 μmol/mL instead of $MgSO_4$ did not cause any significant change in the synthesis of FAD. On the other hand, the synthesis of FAD was almost completely repressed by replacing $MgSO_4$ with $CuSO_4$.

(vii) Effect of surfactants. The stimulative effect of surfactants on the synthesis of FAD is shown in Table 3. Among the surfactants tested, SLBS showed remarkable stimulation. With this surfactant, a 1.5-fold increase in FAD synthesis was observed when *A. globiformis* was the enzyme source. The maximum synthesis of FAD was obtained at the concentration of 4 mg SLBS/mL.

(viii) Time course for FAD synthesis. Based on the results described above, the productivity of FAD was compared among the three strains. As shown in Fig.

TABLE 3
Effect of Surfactants on the Synthesis of FAD[a]

Surfactant	FAD synthesized (μmol/mL) with		
	A. globiformus	Isolate N	Isolate O
SLBS	0.78	0.48	0.49
Sodium laurylsulfate	0.65	0.40	0.45
Laurylpyridinium chloride	0.66	0.41	0.41
Cetyltrimethylammonium chloride	0.65	0.38	0.36
Tween 60	0.58	0.35	0.36
Span 60	0.58	0.34	0.38
None	0.50	0.33	0.38

[a]Reactions were carried out under the standard conditions with or without 0.5 mg of the indicated surfactant.

4, cells of *A. globiformis* synthesized more than 2.3 μmol FAD/mL with imidazole-HCl. The values for isolates N and O were 1.9 and 1.1 μmol/mL, respectively.

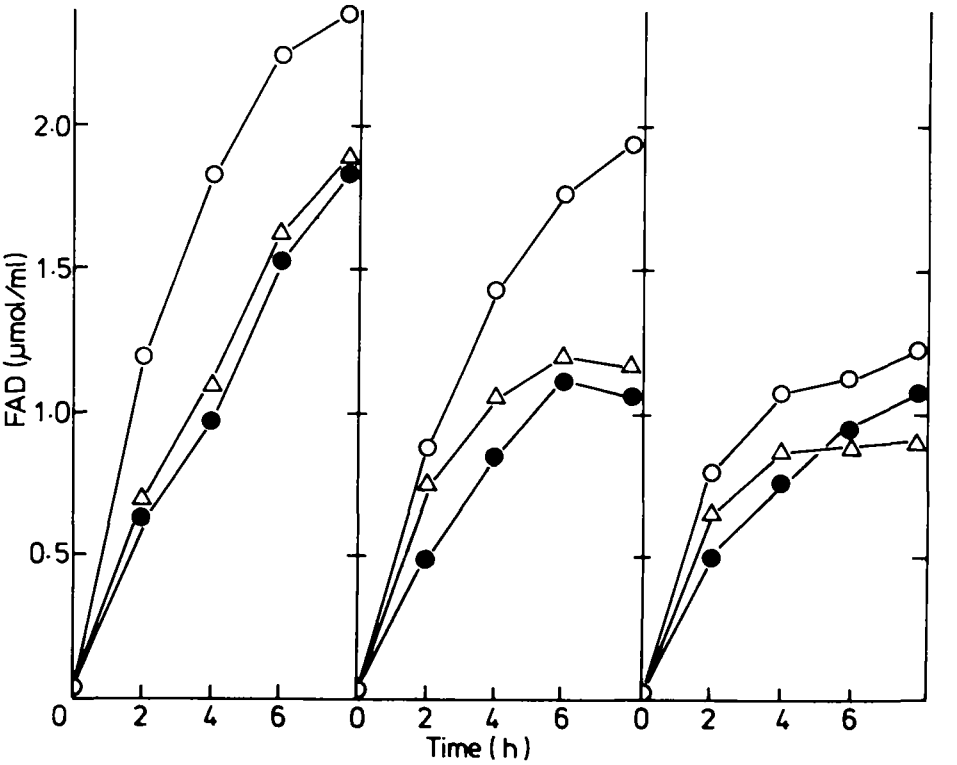

Fig. 4. Time course for FAD synthesis. Reactions were carried out with potassium phosphate, pH 7.0, 100 μmol/mL (●); imidazole-HCl, pH 7.0, 200 μmol/mL (○); or Tris-HCl, pH 8.0, 200 μmol/mL (△). Cells used as the enzyme source were *A. globiformis* (left), isolate N (center), and isolate O (right). Other conditions were given in the text.

Isolation and Identification of FAD. FAD synthesized in the reaction mixture with *A. globiformis* was easily isolated with a good recovery through procedures including charcoal treatment (*18*), DEAE-cellulose column chromatography (*5*), and Florisil treatment (*24*). Data for characterization of the isolated FAD were as follows: λ_{max} nm (ϵ): 263 (36,900), 375 (9300), 450 (111,000) [authentic FAD (Kyowa Hakko Co., Tokyo), 263 (36,900), 375 (9070), 450 (112,000); reported (*25*), 263 (38,000), 375 (9300), 450 (11,300)]; ratio of riboflavin:phosphorus, 1:1.82 (required, 1:2); D-amino acid oxidase test, O_2 uptake μL/min, 6.0 (authentic FAD, 6.5); PPC, R_f in solvent I, 0.11 (authentic FAD, 0.11), R_f in solvent II, 0.16 (authentic FAD, 0.16); TLC, R_f, 0.44 (authentic FAD, 0.44).

Synthesis of FAD Coupled with the Yeast ATP-Generating System

The results in Fig. 2 show that only a few percent of the ATP added was utilized for FAD synthesis. When ATP was added to the reaction mixture at appropriate intervals, the amount of FAD synthesized markedly increased. These results strongly suggest that increased accumulation of FAD may be expected, if a suitable ATP-generating system is coupled with the FAD-synthesizing system. As previously reported, AMP or adenosine is phosphorylated to ATP in a high yield during the fermentative process of yeasts when high concentrations of inorganic phosphate and glucose are present (*12*). We tested several yeast strains as ATP producers. First, several reaction mixtures in which ATP had been accumulated by yeast cells were tested as ATP sources. *A. globiformis* cells produced FAD well in these reaction mixtures. The amount of FAD produced was closely related to the amount of ATP in the reaction mixture used as the ATP source (Table 4). Figure 5 shows synthesis of FAD in the reaction mixture in which ATP was actively generated by yeast cells. The highest accumulation of FAD was found in the reaction mixture in which *A. globiformis* cells and FMN were added 2 h after the initiation of the reaction with yeast cells. At this phase, the ATP available for FAD synthesis was about 8 μmol/mL. Both ATP and FAD in the reaction mixture increased in parallel for several hours. On the contrary, addition of *A. globiformis* cells and FMN to the 4-h

TABLE 4
Synthesis of FAD in the Reaction Mixture in Which ATP Had Been Accumulated from Adenosine by Yeast Cells[a]

Yeast	Available ATP for FAD synthesis, μmol/mL	FAD synthesized with, μmol/mL	
		A. globiformis	Isolate N
Baker's yeast	15.3	1.02	0.98
Saccharomyces sake AKU 4110	12.0	1.01	0.75
Hansenula jadinii AKU 4324	17.3	1.15	0.83

[a]Dried yeast cells as indicated were incubated for 3.5 h as described in the text. After boiling the reaction tube, 15 μmol FMN, 2 mg SLBS, and 100 mg dried cells of *A. globiformjia* or isolate N were added to the reaction mixture, and the reaction was continued for a further 6 h. Otheer conditions were given in the text.

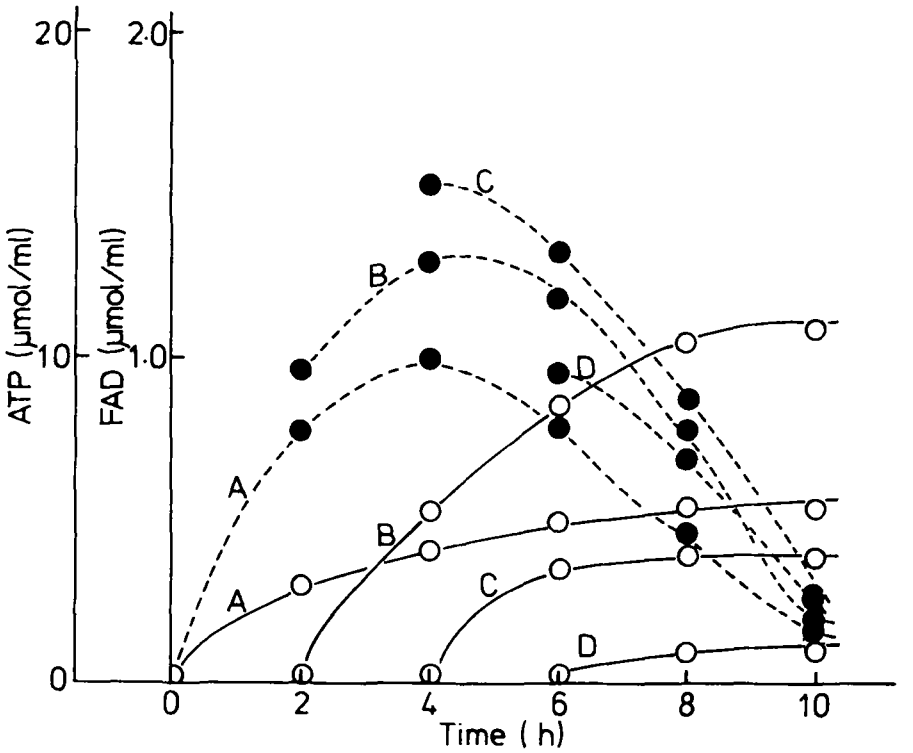

Fig. 5. Synthesis of FAD under coupling with the ATP-generating system of yeast. ATP-synthesizing reactions were carried out with Baker's yeast as the enzyme source using four sets of tubes (A, B, C, and D) as described in the text. At the indicated times, 15 μmol FMN and 100 mg cells of *A. globiformis* were put into one tube of each set as shown, and the reactions were continued for a further several hours. (○), FAD synthesized; (●), available ATP in each tube during FAD synthesis.

reaction mixture did not cause remarkable accumulation of FAD, although the ATP available for the FAD synthesis was about twofold higher than that in the former case.

The results obtained here indicate the effectiveness of the yeast glycolytic pathway as an ATP-generating system in a reactor for FAD synthesis. A schematic diagram of the FAD synthesis described here is presented in Fig. 6.

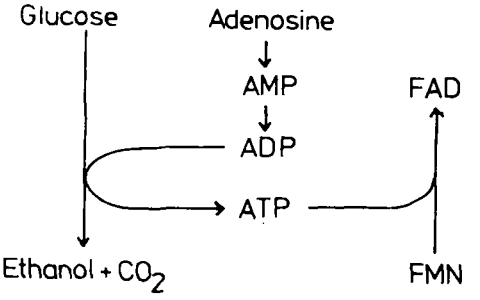

Fig. 6. Schematic representation of the reaction system for FAD synthesis under coupling with the yeast ATP-generating system.

References

1. Kuwada, S. (1955), *Vitamins* **9,** 453.
2. Izumiya, M., and Kobayashi, T. (1962), *Vitamins* **26,** 93.
3. Shimizu, S., Ishida, M., Kato, N., Tani, Y., and K. Ogata (1977), *Agric. Biol. Chem.* **41,** 2215.
4. Shimizu, S., Ishida, M., Tani, Y., and Ogata, K. (1977), *J. Ferment. Technol.* **55,** 630.
5. Moffat, J. G., and Khorana, H. G. (1958), *J. Am. Chem. Soc.* **80,** 3756.
6. Michelson, A. M. (1964), *Biochim. Biophys. Acta* **91,** 1.
7. Nakamura, N., Takasawa, S., and Tanaka, M. (1968), *Abstr. 20th Ann. Mtg. Vitamin Soc. Japan.*
8. Sakai, T. Watanabe, T., and Chibata, I. (1973), *Agric. Biol. Chem.* **37,** 849.
9. Watanabe, T., Uchida, T., Kato, J., and Chibata, I. (1974), *Appl. Microbiol.* **27,** 531.
10. Yamada, H., Shimizu, S., and Tani, Y., (1980), in *Enzyme Engineering,* Weetall, H. H., and Royer, G. P., eds., vol. 5, Plenum, New York, pp. 405–411.
11. Langer, R. S., Hamilton, B. K., Gardner,. C. R., Archer, M. C., and Colton, C. K. (1976), *AIChE. J.* **22,** 1079.
12. Tochikura, T., Kuwahara, M., Yagi, H., Okamoto, H., Tominaga, Y., Kano, T., and Ogata, K. (1967), *J. Ferment. Technol.* **45,** 511.
13. Tochkura, T., Kariya, Y., Yano, T., Tachiki, T., and Kimura, A. (1974), *J. Ferment. Technol.* **49,** 59.
14. Samejima, H., Kimura, Y., Ado, Y., Suzuki, Y., and Tadokoro, T., (1978), in *Enzyme Engineering,* Broun, G. B., Manecke, G., and Wingard, Jr., L. B., eds., vol. 4, Plenum, New York, pp. 237–244.
15. Asada, M., Nakanishi, K., Matsuno, R., and Kamikubo, T., (1982), *Agric. Biol. Chem.* **46,** 1687.
16. Kimura, A., Tatsutomi, Y., Mizushima, N., Tanaka, A., Matsuno, R., and Fukuda, H. (1978), *Eur. J. Appl. Microbiol. Biotechnol.* **5,** 13.
17. Ogata, K., Shimizu, S., and Tani, Y., (1972), *Agric. Biol. Chem.* **36,** 84.
18. Shimizu, S., Kubo, K., Morioka, H., Tani, Y., and Ogata, K., (1974), *Agric. Biol. Chem.* **38,** 1015.
19. Ogata, K., Shimizu, S., and Tani, Y. (1970), *Agric. Biol. Chem.*, **34,** 1757.
20. Yagi, K., in *Methods in Enzymology,* McCormick, D. B., and Wright, L. D., eds., vol. 18, Academic Press, New York, pp. 290–296.
21. Fiske, C. H., and SubbaRow, Y., (1925), *J. Biol. Chem.* **66,** 375.
22. Friedmann, H. C. (1974) in *Methods of Enzymatic Analysis,* Bergmeyer, H. U., ed., vol. 4, 2nd ed., Academic Press, New York, pp. 2182–2185.
23. Lamprech, W., and Trautschold, I. (1974), in *Methods of Enzymatic Analysis,* Bergmeyer, H. U., ed., vol. 4, 2nd ed., Academic Press, New York, pp. 2101–2110.
24. Peel, J. L. (1958), *Biochem. J.* **69,** 403.
25. Whitby, L. G. (1953), *Biochem. J.* **54,** 437.