*MECHANISM OF AMINOACYL RNA SYNTHESIS: STUDIES WITH ISOLATED AMINOACYL ADENYLATE COMPLEXES OF ISOLEUCYL RNA SYNTHETASE**

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Enzyme-aminoacyl adenylates function as intermediates in the synthesis of aminoacyl RNA's.[1] Each amino acid is activated and transferred to specific t-RNA molecules by an individual enzyme according to the following equations:

$$\text{Enzyme} + \text{ATP} + \text{AA} \rightleftharpoons \text{Enz-AA-AMP} + \text{PP}_i$$

$$\text{Enz-AA-AMP} + \text{t-RNA} \rightleftharpoons \text{Enzyme} + \text{AA-RNA} + \text{AMP}$$

A crucial aspect of the specificity of protein synthesis resides in the selectivity of each aminoacyl RNA synthetase for both amino acid and t-RNA. Clearly structural variations between different t-RNA molecules are important. Likewise, since the transfer apparently has considerably more stringent specificity with respect to the amino acid than does the formation of the enzyme-aminoacyl adenylate complex, the transfer reaction must be sensitive to subtle changes in the structure of this complex.

The latter point is illustrated by the behavior of the isoleucyl RNA synthetase from *Escherichia coli*.[2] It had been inferred from the amino acid-dependent ATP-pyrophosphate exchange reaction that this enzyme could form a valyl as well as an isoleucyl adenylate complex; however, it transfers only the isoleucyl moiety to the appropriate t-RNA. The t-RNA molecules specific for isoleucine thus react differently with identical protein molecules complexed to isoleucyl or valyl adenylates.

Our aim is to exploit this property to investigate the broader problem of protein-nucleic acid interactions. We have initiated studies of the factors affecting this specificity by using highly purified isoleucyl RNA synthetase in substrate quantities. The enzyme-isoleucyl and enzyme-valyl adenylate complexes have been produced and isolated from reaction mixtures by gel filtration through Sephadex. The isolated complexes have been directly compared in two reactions: their ability to transfer the adenylate portion to inorganic pyrophosphate with the formation of ATP, and their ability to transfer the aminoacyl moiety to t-RNA. Both complexes serve as precursors of ATP, but the enzyme-valyl adenylate complex, in contrast to the isoleucyl derivative, fails to function as a donor of the aminoacyl group to t-RNA. Transfer of the valyl moiety is abortive in the sense that the enzyme-valyl adenylate complex breaks down when reacted with t-RNA containing isoleucine-specific chains.

Experimental.—Materials: H^3-ATP was purchased from Schwarz Laboratories, and U-C^{14}-L-isoleucine and U-C^{14}-L-valine from New England Nuclear Corp. PP_i^{32} was prepared according to Berg.[3] β,γ-P^{32}-ATP was prepared by exchange of ATP with PP_i^{32} using purified isoleucyl-RNA synthetase.

E. coli B cells, purchased from Grain Processing Corp., had been grown on a complex medium and harvested during exponential phase. Transfer RNA from these cells was isolated by a modi-

fication of the method of Zubay.[4] The partition chromatography for separation of specific t-RNA chains has been reported.[5]

In several experiments, t-RNA lacking the terminal adenylate or the adenylate and subterminal cytidylate was used. For these preparations, t-RNA was treated with purified Crotalus venom phosphodiesterase[6] using the 20°-incubation conditions reported by Zubay[7] to remove primarily the terminal adenylate and subterminal cytidylate yielding RNA . . . pC. Incubation of RNA . . . pC with either CTP alone, or CTP plus ATP, and the enzyme described by Preiss *et al.*[8] should yield RNA . . . pCpC and RNA . . . pCpCpA, respectively, as the major products. The material digested with venom diesterase, as well as the material which had been incubated with CTP, was inactive as isoleucine acceptor, whereas the material into which both adenylate and cytidylate were incorporated had 70% of the initial acceptor activity for isoleucine.

Isoleucyl RNA synthetase was isolated from the above cells and was purified by a new procedure which will be described in another article.[9] The characteristics of the purified enzyme[9] that are relevant to this study are as follows:

(*a*) The specific activity equals 650 units per mg of protein (1 unit equals an exchange of 1 μmole of PP_i^{32} with ATP in 15 min at 37° under the conditions described earlier[10]), representing an enrichment of 325-fold over the initial extract, and a fourfold increase in specific activity over earlier preparations.[2]

(*b*) The ratio of isoleucine- and valine-dependent ATP-pyrophosphate exchange activities was constant during the latter stages of the purification.

(*c*) The enzyme appeared homogeneous by the criteria of starch gel electrophoresis, boundary sedimentation velocity (schlieren optics, $S_{20,w} = 5.8$), and zone sedimentation velocity in sucrose gradients ($S_{20,w} = 5.3$).[11]

(*d*) Equilibrium sedimentation analysis[12] yielded weight-average and z-average molecular weights of 114,000 and 111,000, respectively. The agreement between these values suggests little or no molecular weight heterogeneity and allows us to use their average (112,500) in calculating the moles of enzyme.

Isolation of enzyme-aminoacyl adenylate complexes: The complete reaction mixture (0.20 ml) for the formation of enzyme-aminoacyl adenylate complex contained 0.4 μmole of potassium phosphate buffer, pH 7.5; 1.0 μmole of $MgCl_2$; 2.0 μmoles of 2-mercaptoethanol; 0.10 μmole of H^3-ATP (specific activity 8.25×10^7 cpm/μmole); 0.10 μmole of C^{14}-L-isoleucine (specific activity 6.45×10^7 cpm/μmole) or 4.0 μmoles of C^{14}-L-valine (specific activity 9.95×10^6 cpm/μmole) and 0.1–0.4 mg of the most purified enzyme fraction. In some experiments, the C^{14}-amino acid was replaced by the C^{12}-amino acid.

After 5 min at room temperature the reaction mixture was chilled to 0° and placed onto a Sephadex G-75 column (0.75 cm by 20 cm) previously equilibrated with a solution containing 0.05 *M* sodium succinate, pH 6.0, 0.05 *M* KCl, 0.001 *M* reduced glutathione, and 0.001 *M* EDTA. Fractions eluted with the same buffer were assayed for enzyme activity and for radioactivity by drying a 0.1-ml aliquot on a Whatman glass filter (GF/C, diameter 2.4 cm). The dried filter was placed in a vial containing 10 ml of toluene scintillator solution and counted in a Packard Tricarb liquid scintillation spectrometer. Recovery of the enzyme and of radioactivity from the column was 95–100%.

Reaction of isolated enzyme-aminoacyl adenylate complexes with PP_i^{32}: Enzyme-isoleucyl or enzyme-valyl adenylate was assayed immediately after isolation for its ability to form ATP in the presence of PP_i^{32}. The reaction mixture (1.0 ml) contained 100 μmoles of Tris-HCl, pH 8.0; 5 μmoles of $MgCl_2$; 5 μmoles of 2-mercaptoethanol; 0.2 μmole of PP_i^{32} (specific activity 1.22×10^8 cpm/μmole); and 0.1–0.5 mμmole of enzyme-aminoacyl adenylate complex (as determined by the content of C^{14}-amino acid). After 15 min at 37° the reaction was stopped by the addition of 0.25 ml of 14% PCA and 0.25 ml of 14% Norit by weight. The Norit was collected by centrifugation and washed 8 times with a solution containing 1% PCA and 0.01 *M* sodium pyrophosphate, then twice with water. The Norit was suspended in 0.3 *N* ammonia in 50% ethanol, and an aliquot of the suspension was plated and counted in a Nuclear-Chicago window counter.

Reaction of isolated enzyme-aminoacyl adenylate complexes with t-RNA: Transfer of the amino acid from the isolated enzyme-aminoacyl adenylate to t-RNA was determined essentially as described by Berg *et al.*[13] The reaction mixture (0.5 ml) contained 50 μmoles of sodium cacodylate buffer, pH 7.0; 5 μmoles of 2-mercaptoethanol; 5 μmoles of KCl; sufficient t-RNA to accept 3–4

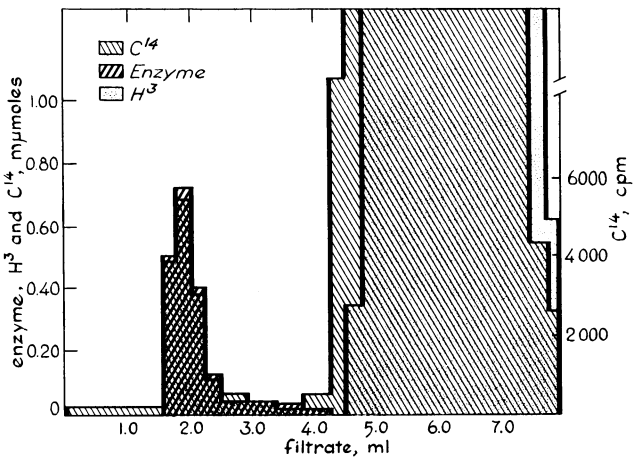

FIG. 1.—Separation of enzyme-isoleucyl adenylate from free substrates on Sephadex G-75. Details as described in *Methods*.

mμmoles of isoleucine; and 0.01–0.10 mμmole of enzyme-C^{14}-isoleucyl adenylate (or C^{14}-valyl adenylate). After 15 min at 0° the RNA was precipitated by the addition of 2 ml of cold 2 *N* HCl and filtered on a Whatman glass filter. After washing 4 times with 2 ml of 2 *N* HCl, the filter was dried and counted as described above. In other experiments the entire incubation mixture (after 15 min at 0°) was placed on a second Sephadex G-75 column, identical to the one from which the enzyme-aminoacyl adenylate had been isolated, and was eluted with succinate buffer as before.

Results.—Isolation of enzyme-aminoacyl adenylate complexes: The elution pattern obtained when substrate levels of enzyme were incubated with H^3-ATP, C^{14}-isoleucine, and Mg^{++}, and then subjected to Sephadex gel filtration, is shown in Figure 1. It may be seen that the enzyme activity appeared well before the bulk of the radioactive substrates, but that nearly one molar equivalent of H^3 and of C^{14}, with respect to the enzyme, was found in the fractions containing enzyme (Table 1). In the absence of ATP, no detectable C^{14}-isoleucine was eluted with the enzyme.

TABLE 1

COMPOSITION OF ISOLATED ENZYME-AMINOACYL ADENYLATE COMPLEXES

Substrate	Amount of enzyme (mμmoles)	Enzyme-bound (moles/mole of enzyme) H^3-AMP	C^{14}-amino acid
H^3-ATP + C^{14}-isoleucine	1.0	0.94	0.95
C^{14}-isoleucine	2.5	—	<0.01
H^3-ATP	2.8	0.2	—
H^3-ATP + C^{14}-valine	2.5	0.61	0.65

The procedure for the formation and isolation of the enzyme-bound complexes has been described under *Methods*. Amount of enzyme is based on the enzyme activity recovered from the Sephadex column (95–100% of original) and a turnover number of 4.85 units min^{-1} of ATP-pyrophosphate exchange per mμmole of enzyme (at 37°). "Enzyme-bound" H^3-AMP and C^{14}-amino acid is based on the total radioactivity associated with the enzyme fractions, as illustrated in Fig. 1. The specific activities of the substrates are given in *Methods*.

Without amino acid, variable quantities of H^3-label appeared in the enzyme fraction. This H^3-label was not in ATP but rather in AMP, since in an experiment containing β,γ-P^{32}-ATP as well as H^3-ATP, less than 10 per cent of the P^{32} relative to the amount of H^3-label was eluted with the enzyme. When the H^3-ATP and P^{32}-ATP were cochromatographed on Dowex-1-Cl, the ratio of H^3 to P^{32} in the ATP fractions, in which 90 per cent of the initial material was recovered, was identical

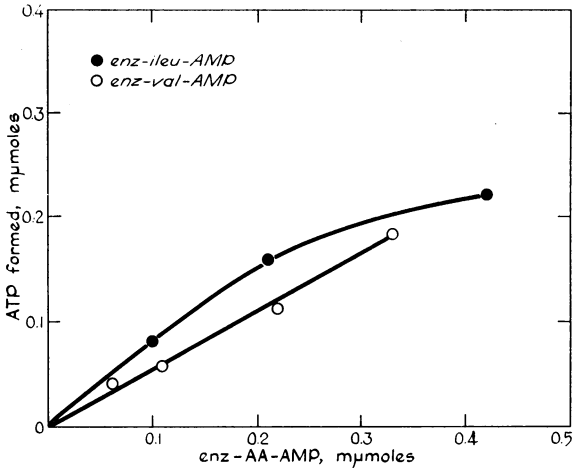

FIG. 2.—Synthesis of ATP from isolated enzyme-aminoacyl adenylates. Conditions for the reaction of isolated enzyme-aminoacyl adenylate with PP_i^{32} and the estimation of ATP^{32} formed are described in *Methods*.

to the ratio in the crude mixture. We presume that H^3 bound to the enzyme in the absence of added amino acid is due to trace quantities of amino acid contaminating the enzyme preparations. In support of this is the fact that a heat-inactivated enzyme preparation (10 min at 100°) supported a rate of pyrophosphate-ATP exchange when added in lieu of isoleucine consistent with the presence of 1 molecule of isoleucine for every 12 molecules of enzyme. Moreover, the molar ratio of H^3-AMP associated with the enzyme varied from 0.2 to 0.9 with different enzyme preparations and in one preparation was reduced from 0.6 to 0.27 after filtration of the enzyme through Sephadex G-75. Further experiments are necessary, however, to establish the nature of AMP binding to enzyme which occurs in the absence of added amino acid.

When C^{14}-valine replaced isoleucine in the incubation with H^3-ATP and enzyme, the enzyme fractions of the Sephadex gel effluent contained nearly equivalent amounts of H^3-AMP and C^{14}-valine in amounts equivalent to 60–65 per cent of the recovered enzyme (Table 1).

Reaction of isolated enzyme-aminoacyl adenylates with PP_i^{32}: Isolated enzyme-isoleucyl AMP and enzyme-valyl AMP can react with PP_i^{32} to form ATP^{32} (Fig. 2). Nearly 75 per cent of the AMP from the isoleucyl complex and 50–60 per cent of the AMP from the valyl complex was converted to ATP^{32} on the basis of adsorption to Norit. After elution of the product from Norit, 83 per cent of the P^{32} migrated with ATP on paper electrophoresis (0.02 *M* sodium citrate buffer, pH 3.5, 1 hr, 1200 volts). Failure to obtain quantitative yields of ATP is probably due to the competing spontaneous breakdown of the complex under these conditions. Added Mg^{++} stimulated the reaction by only 30 per cent, suggesting that Mg^{++} may be associated with the isolated complex.

Reaction of isolated enzyme-aminoacyl adenylates with t-RNA: The isolated enzyme-C^{14}-isoleucyl adenylate transfers the isoleucyl moiety to t-RNA (Fig. 3). Again, incomplete transfer of the aminoacyl moiety to the RNA probably results from some breakdown of the complex between the time of its isolation and its

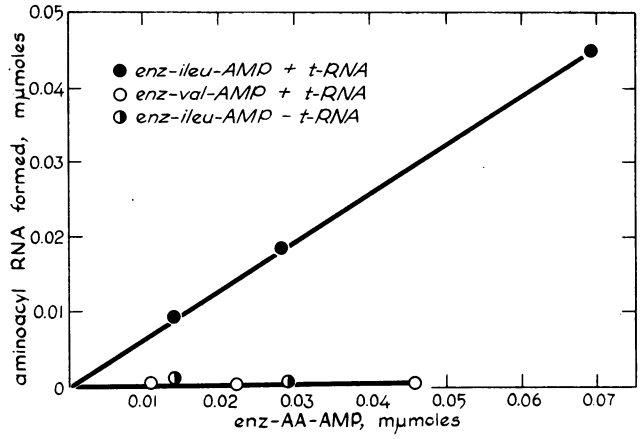

FIG. 3.—Transfer of C^{14}-isoleucine from isolated enzyme-isoleucyl adenylate to t-RNA. Assay conditions for the transfer of C^{14}-amino acid from isolated enzyme-C^{14}-aminoacyl adenylate to t-RNA and the specific activities of C^{14}-isoleucine and C^{14}-valine are given under *Methods*.

utilization as an aminoacyl donor. Additional Mg^{++} was not required for this reaction and, in fact, was inhibitory (20%). The product of the transfer to t-RNA was destroyed after treatment with 0.01 *M* KOH at room temperature for 10 min.[14] When RNA was omitted, or had previously been digested with ribonuclease, less than 1 per cent of the C^{14} was acid-precipitable. This implies that the complex is unstable under these acid conditions. That aminoacyl transfer was specific is suggested by the fact that t-RNA enriched for tyrosine acceptor chains, but lacking isoleucine-accepting activity, did not yield aminoacyl RNA (<3%).

In contrast to the enzyme-isoleucyl adenylate complex, the enzyme-C^{14}-valyl adenylate complex incubated with the same t-RNA preparation did not give rise to any detectable valyl RNA (Fig. 3).

Instability of enzyme-valyl adenylate in the presence of t-RNA: To determine why the enzyme-valyl adenylate fails to transfer the valyl moiety to t-RNA, we studied the fate of the isolated complex after exposure to t-RNA. For this purpose, H^3-AMP-labeled enzyme-valyl adenylate was incubated with an excess of t-RNA in terms of its isoleucine-accepting chains. The mixture was then filtered through Sephadex G-75 as described earlier. With intact t-RNA containing functional isoleucine acceptor chains, none of the H^3-AMP remained bound to the enzyme (Table 2). An RNase digest of t-RNA, a t-RNA lacking an intact terminal trinucleotide sequence, or a t-RNA freed of isoleucine-specific chains, did not induce the breakdown of complex beyond that found when RNA was omitted, i.e., that due to spontaneous breakdown. The acceptor activity of the t-RNA was not decreased by the reaction and was recovered from the column.

Because spontaneous breakdown of the complex is appreciable and the precise rate of breakdown has not been measured, the conclusions drawn from this experiment can only be qualitative. What is clear, however, is that some form of interaction between the enzyme-valyl adenylate complex and a class of intact t-RNA chains occurs which increases the lability of the complex. The relationship between

TABLE 2

BREAKDOWN OF ENZYME-VALYL ADENYLATE IN THE PRESENCE OF T-RNA

t-RNA preparation	Isoleucine-acceptor activity (mμmoles/μmole RNA)	Breakdown of enzyme-C^{14}-valyl-AMP (per cent)
None	—	15–25
RNA . . . pCpCpA	0.20	100
RNase digest of above	0.00	20–35
RNA . . . pCpCpA	0.39	100
RNA . . . pC	0.00	40
RNA . . . pCpC	0.00	45
RNA . . . pCpCpA (regenerated)	0.27	100
RNA enriched for tyrosine-acceptor activity	0.00	30
RNA enriched for isoleucine-acceptor activity	0.96	100

In each experiment, approximately 0.5 mμmole of isolated enzyme-valyl-H^3-adenylate (specific activity 8.23×10^4 cpm/mμmole) was incubated with 5 μmoles of t-RNA nucleotide. Each RNA preparation was assayed for isoleucine-acceptor activity as previously described.[13] After 15 min at 0° the mixture was filtered through a Sephadex G-75 column. Fractions were collected and assayed for enzyme and H^3 as described in *Methods*. The results are expressed as the percentage of H^3 retained on the Sephadex column relative to the amount of H^3 in the complex used as the substrate.

Transfer RNA enriched for tyrosine or isoleucine acceptor activities was obtained by partition chromatography.[5] The former fraction contained no detectable isoleucine or valine acceptor activity, while the latter was approximately fivefold enriched for isoleucine and accepted 0.27 mμmoles of valine per μmole of t-RNA.

this observation and the inability of the complex to transfer valine to t-RNA chains remains to be investigated.

Discussion.—Sephadex gel filtration has been used extensively to effect separations between high- and low-molecular-weight substances. The relatively mild conditions and the rapidity with which the filtration can be carried out makes it especially suitable for the isolation of somewhat stable enzyme-bound intermediates. This is illustrated in the present experiments as well as in those recently reported for the isolation of enzyme-acetyl adenylate[15] and enzyme-threonyl adenylate[16] complexes. The availability of the intermediate enzyme-aminoacyl adenylate as an isolated complex enables us to examine additional parameters involved in the specificity of aminoacyl RNA synthesis.

On the basis of the C^{14} and H^3 content of the isolated complex, one mole each of isoleucine and AMP are bound per mole of isoleucyl RNA synthetase. This suggests that there is only one site per enzyme molecule at which isoleucyl adenylate binds. The failure to achieve an equivalent amount of binding with ATP and valine is not yet clear, but differences in the stability between the isoleucyl and valyl adenylate complexes with the enzyme may be the reason. Refiltering isolated enzyme-valyl adenylate through a Sephadex column resulted in about 30–40 per cent loss of bound AMP and valine, whereas less than 10 per cent of the isoleucyl adenylate complex broke down during repeated filtration. Relevant to this point, it is known that with methionyl RNA synthetase[17] as well as tryptophanyl RNA synthetase,[18] the K_m for the homologous adenylate is of the order of 100 times lower than for nonspecific aminoacyl adenylates.

The present experiments do not clarify the nature of the binding of the amino acid and AMP to the enzyme. Our assumption is that the amino acid and AMP are linked to each other through an acyl phosphate bond; this is based on the ability of the isoleucine-containing complex to yield ATP or isoleucyl RNA in the presence of inorganic pyrophosphate or RNA, respectively, much in the same way that synthetic aminoacyl adenylates behave in the presence of enzyme and appropriate supplements. Of considerable interest is the nature of the linkage of the amino-

acyl adenylate to the protein. The aminoacyl adenylate appears to be stabilized since under the conditions of the gel filtration, free aminoacyl adenylates, by analogy with other aminoacyl adenylates, would have been completely hydrolyzed.[17,18] Assuming that the K_m's of the individual substrates (10^{-4} to 10^{-6} M) approximate the dissociation constants, then the binding between the enzyme and the adenylate must be considerably stronger. The affinity of aminoacyl adenylates to their respective enzymes has not generally been measured, although in one instance the dissociation constant between luciferyl adenylate and luciferase to form an active intermediate in light emission is of the order of 5×10^{-10}.[19]

The present finding that the enzyme-valyl adenylate complex, in contrast to the analogous isoleucyl derivative, fails to transfer its aminoacyl group to t-RNA confirms the earlier conclusion based on experiments using catalytic quantities of enzyme.[13] Several explanations could be offered for this behavior, among which are (*a*) a failure of enzyme-valyl adenylate complex to bind the t-RNA$_{ileu}$ chains, (*b*) an intrinsic inability of t-RNA$_{ileu}$ to bind an L-valyl group to its terminal adenylate residue, and (*c*) an inability of the enzyme-valyl adenylate to effect normal transfer of the aminoacyl group to the bound t-RNA. While our present findings do not identify the correct explanation, they do indicate that the enzyme-valyl adenylate complex can interact with t-RNA containing isoleucine-specific chains. The end result of this interaction is breakdown of the complex rather than aminoacyl transfer.

The mechanism of the breakdown of the valyl complex is so far unknown. It has not been shown, for instance, whether the reaction involves an attack on the acyl bond or on the point of attachment of the valyl adenylate to the enzyme. Whereas an intact t-RNA chain, including the . . . CpCpA terminal trinucleotide, is essential for the breakdown reaction, it is not clear whether the terminal sequence is necessary for binding of the RNA to the enzyme-aminoacyl adenylate complex. The terminal adenylate of the functional RNA might conceivably participate in the catalytic hydrolysis of the complex. Alternatively, the binding of t-RNA may produce a conformational change in the protein which decreases the stability of the complex.

Summary.—Highly purified isoleucyl-RNA synthetase from *E. coli* forms both enzyme-isoleucyl adenylate and enzyme-valyl adenylate complexes, which have been isolated from reaction mixtures by Sephadex gel filtration. Both isolated complexes react with inorganic pyrophosphate to form ATP. On the other hand, only the isoleucyl adenylate complex transfers its aminoacyl moiety to t-RNA. Transfer RNA containing active isoleucine-specific chains induces breakdown of the enzyme-valyl adenylate complex.

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CONTROL OF THE WAVEFORM OF OSCILLATIONS OF THE REDUCED PYRIDINE NUCLEOTIDE LEVEL IN A CELL-FREE EXTRACT

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We have reported preliminary observations on damped sinusoidal oscillations of the level of reduced pyridine nucleotide (DPNH) in suspensions of yeast cells[1-3] and have found similar oscillations to take place in cell-free extracts containing the glycolytic system of these cells.[4] Here we report biophysical observations on the control of the waveform, phase, and period of the oscillations which are of significance to the possible biological function of this interesting reaction. Biochemical aspects of the metabolic control phenomena in the extract are to be reported in detail elsewhere.[5]

Preparations and Experimental Method.—The enzyme system consists of the soluble cytoplasmic constituents of *Saccharomyces carlsbergensis*, obtained by high-speed centrifugation of ruptured cells.[4] Microscopic examination shows no visible cell fragments, and the extract exhibits no measurable respiratory activity. Enzymatic activities of the glycolytic sequence are, however, present. The substrate and nucleotide levels are appropriate for the oscillatory reaction. The extracts prepared as described previously[4] are designated Type I; however, it has recently been found that the extracts may be prepared in the same way from cells which have been stored at 0° for several days. Preparations from these stored cells are designated Type II and are described in detail in a separate communication;[5] the number of hours that the cells have been stored at 0° is noted in parentheses in the figure legends.

Experimental Results.—Waveform of the oscillations: In previous reports, we have observed the waveform of the oscillations to be approximately sinusoidal.[1, 4] However, more recent detailed studies indicate interesting variations and interconversions of waveforms, a typical example of which is shown in Figure 1. The oscillations, followed at 340 mμ in the double-beam spectrophotometer, with a