9.6 with 1.0 *N* sodium hydroxide and was extracted with three 300-ml. portions of chloroform. After drying over anhydrous sodium sulfate, the chloroform solution was concentrated under reduced pressure to about 50 ml. The antibiotic activity was transferred to aqueous solution by extracting the chloroform with four 20-ml. portions of 0.1 *M* phosphate buffer at *p*H 5.2. The combined aqueous extracts were adjusted to *p*H 9.5, and again were extracted with chloroform. Evaporation of the chloroform yielded a light brown residue that weighed 0.89 g. and contained an activity equivalent to 460 mg. of erythromycin. Further purification of such preparations was achieved by chromatography or countercurrent distribution.

Chromatography.—Three grams of cellulose powder was added to 10 ml. of an acetone solution that contained an antibiotic preparation equivalent to 176 mg. of erythromycin. The mixture was evaporated to dryness under vacuum.

The activity adsorbed on cellulose powder was added to a column previously packed with dry cellulose, and the column was developed by gravity with 0.01 *N* ammonium hydroxide saturated with methyl isobutyl ketone. Fractions (15-ml.) were collected by an automatic fraction collector. Fractions 1 to 5 contained no activity. Fractions 6–14 contained erythromycin, and fractions 15–20 contained a mixture of erythromycin and erythromycin B. The remainder of the active fractions (21–48) contained only erythromycin B. The recovery of activity from the column was almost quantitative. The erythromycin B fractions were combined, adjusted to *p*H 10.5, and extracted with two 100-ml. portions of chloroform. After drying over anhydrous sodium sulfate, the chloroform was removed under reduced pressure. The residue was extracted with three 25-ml. portions of ether which were combined, filtered, and dried. After evaporation of the ether, the residue was dissolved in 5 ml. of warm acetone. Crystalline erythromycin B was deposited as the solution cooled.

Craig Countercurrent Distribution.—The solvent system was prepared by mixing 10 l. of 0.1 *M* phosphate buffer at *p*H 6.5, 10 l. of methyl isobutyl ketone and 0.5 l. of acetone. Ten ml. of the upper phase was used to dissolve 590 mg. of the crude antibiotic. A 100-tube countercurrent distribution was performed in a Craig machine having a capacity of 10 ml. per phase in each tube. Upon completion of the distribution, samples from every fifth tube were withdrawn for assay. The results are plotted in Fig. 1. Paper chromatograms showed that tubes number 30 through 50 contained only erythromycin while tubes number 60 through 80 contained only erythromycin B. The latter fractions were combined and concentrated to about 40 ml. under reduced pressure. The *p*H was adjusted to 9.6 and the antibiotic activity was extracted into chloroform. The residue remaining after removal of the chloroform by evaporation was crystallized from 6 ml. of acetone. The first crop of rectangular plates weighed 95 mg.

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Mechanism of Thiamine-catalyzed Reactions[1]

BY SHUNZI MIZUHARA[2] AND PHILIP HANDLER

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High concentrations of thiamine in alkaline solution catalyze several reactions of carbonyl compounds known to occur in biological systems, among them the formation of acetoin and acetate from acetaldehyde and diacetyl. Acetaldehyde-C^{14} has been employed to demonstrate the mechanism of this reaction.

It is well established that in biological systems thiamine functions in the oxidative and nonoxidative decarboxylation of pyruvic and other α-keto acids which arise in metabolism.[3] Under some circumstances, however, thiamine pyrophosphate together with a specific protein such as the "carboxylase" of pig heart has been observed to catalyze the formation of CO_2 and acetoin, rather than acetaldehyde or acetate, when incubated with pyruvate. This reaction is favored by the presence of acetaldehyde.[4] Presumably related is the "diacetylmutase" reaction in which diacetyl yields acetate and acetoin with preparations from pigeon breast muscle.[5] The formation of furoin from furfural also has been observed in protein-free, model systems in the presence of various quaternary thiazole compounds including thiamine[6,7] which also catalyzes acetoin formation from pyruvate[8] and the diacetylmutase reaction.[9] Significantly these reactions proceed only if 2 equivalents of alkali are added per mole of thiamine hydrochloride,[8,9] since under these conditions there exist appreciable quantities of thiamine pseudobase.[10]

A mechanism for the mode of action of thiamine in these systems has been suggested[8,9] which considers that the lone pair of electrons on the tertiary thiazole nitrogen of thiamine pseudobase coordinates with the carbo-cation of pyruvate or diacetyl. The resulting intermediate decarboxylates (or loses a ketone-like group) leaving a complex which acetylates acetaldehyde to form acetoin. To strengthen the arguments on which this mechanism rests, the catalytic action of thiamine in alkaline solution was verified and, with the use of

(1) This work was done under contract AT-(40-1)-289 between Duke University and the Atomic Energy Commission.

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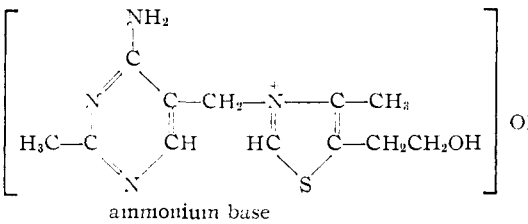
acetaldehyde-1,2-C^{14} it has been shown that 2 of the carbons of acetoin are derived from acetaldehyde under these conditions.

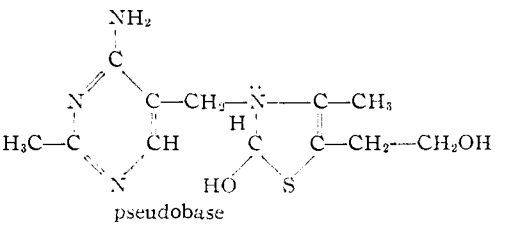
The phosphoroclastic cleavage of pyruvate to acetyl phosphate and formate and the formate–pyruvate exchange catalyzed by *E. coli* require cocarboxylase.[11,12] These reactions had not previously been attempted in model systems. When formate was substituted for acetaldehyde in the presence of pyruvate and thiamine, only acetoin was obtained, with no evidence for the formation of methylglyoxal which might have been expected. When formaldehyde was used in place of acetaldehyde, both acetoin and monohydroxyacetone were formed. These experiments, therefore, did not provide a mechanism for the pyruvate–formate exchange. Similarly, no incorporation of $C^{14}O_2$ into pyruvate was noted when these were incubated with thiamine or cocarboxylase at *p*H 8.4.

Experimental Section

Acetaldehyde-1,2-C^{14} was prepared[13] from barium carbide-C^{14} obtained on allocation from the U. S. Atomic Energy Commission. Acetoin formed during the incubations was converted to diacetyl and the nickel salt of dimethylglyoxime isolated by Langenbeck's procedure[14] and recrystallized from hot pyridine. Radioactivity was assayed in a gas flowcounter with a commercial scaler to an error of ±5%. Incubations were performed in evacuated Thunberg tubes at 38° for 330 min. in a total volume of 3.0 or 6.0 ml.

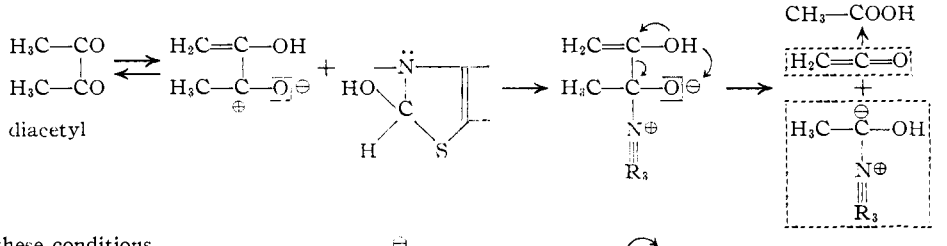


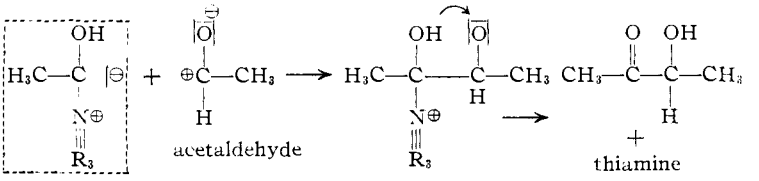
ammonium base



pseudobase

The results of studies of the diacetylmutase and carboxylase model systems are shown in Tables I and II. The recovery of acetoin (as Ni-dimethylgloxime) was maximal when two equivalents of alkali per mole of thiamine hydrochloride was added in both systems, and only in complete systems under these conditions were significant quantities of activity incorporated into the acetoin. The specific activity of the material formed in the diacetylmutase system was reduced by the unreacted diacetyl remaining.





Large scale runs were attempted with formate and formaldehyde. The incubation mixtures contained 2 mM. of thiamine hydrochloride, 2 mM. of sodium pyruvate and 4 meq. of NaOH with 4 mM. of either sodium formate or formaldehyde in a total volume of 50 ml. maintained at 38° in an evacuated Thunberg tube for 6 hr. Saturated 2,4-dinitrophenylhydrazine in 2 *N* HCl was added till no further precipitation occurred. The precipitate was removed and the filtrate boiled for 10 min. and cooled overnight and filtered. The latter precipitate was washed with hot alcohol and the hot filtrate, containing diacetyl dinitrophenylhydrazone discarded. The remaining material on the funnel was consecutively washed with cold pyridine, hot pyridine and hot nitrobenzene. The hot pyridine filtrate yielded reddish crystals, of unknown composition, melting with decomposition at 272–276°. The hot nitrobenzene filtrate yielded orange-red crystals which melted with decomposition at 311–312° and showed no depression with an authentic sample of diacetyl-2,4-dinitrophenylosazone.

A similar procedure was followed after incubation with formaldehyde. The hot pyridine fraction yielded yellow-brown crystals which melted with decomposition at 295–298° and showed no depression with an authentic sample of methylglyoxal-2,4-dinitrophenylosazone. Again, the 2,4-dinitrophenylosazone of diacetyl was obtained from the hot nitrobenzene fraction.

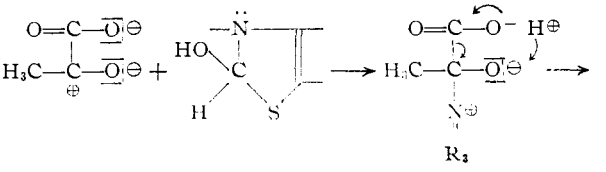
Discussion

The experiments with acetaldehyde-C^{14} leave little doubt that the reaction

$$CH_3COCOCH_3 + CH_3CHO \longrightarrow CH_3COCHOHCH_3 + CH_3COOH$$

catalyzed by thiamine is not an oxidation–reduction but involves scission of the diacetyl into two moieties one of which reacts with acetaldehyde to yield acetoin while the other appears as acetate. This reaction does not occur in neutral or acid solutions but only when the *p*H is sufficiently high to permit pseudobase formation from thiamine. In still more alkaline solutions thiamine is irreversibly destroyed and the diacetylmutase reaction does not occur. These facts are compatible with the mechanism suggested earlier.[8,9]

The formation of acetoin from pyruvate and acetaldehyde proceeds in similar fashion



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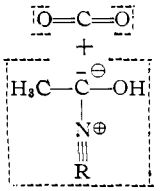


TABLE I

DIACETYLMUTASE MODEL SYSTEM

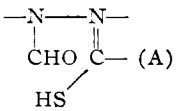
Thiamine, μM.	Diacetyl, μM.	Acetaldehyde, μM.	NaOH, μeq.	pH	Ni-Dimethylglyoxime isolated Mg.	Ni-Dimethylglyoxime isolated C.p.m.
100	100	200	300	9.2	1.0	381
100	100	200	200	8.4	3.4	3413
100	100	200	100	6.8	1.1	125
100		200	200	8.4	0	
	100	200	50	8.6	0.3	100
	100	200	25	7.0	1.6	184
	100	200		4.0	12.5	445

TABLE II

CARBOXYLASE MODEL SYSTEM

Thiamine, μM.	Pyruvate, μM.	Acetaldehyde, μM.	NaOH, μeq.	pH	Ni-Dimethylglyoxime isolated Mg.	Ni-Dimethylglyoxime isolated C.p.m.
200	200	400	600	9.2	Trace	107
200	200	400	400	8.4	1.1	15,609
200	200	400	200	6.9	Trace	280
	200	400	50	8.1	0	
	200	400		6.7	0	

Karrer[15] has suggested that the first step in pyruvate decarboxylation may involve opening of the thiazole ring to form (A) with subsequent formation



of a thioacetal of pyruvate. However, at the *p*H of these experiments there is no evidence for such a thiol form.[10] An "aldehyde–coenzyme complex" such as that shown above was also proposed recently by Littlefield and Sanadi.[16] The mechanism proposed by Langenbeck[17] and modified by Weil-Malherbe[18] involved an initial Schiff base formation between the carbonyl compound and the amine group on the pyrimidine ring. No evidence has been adduced in support of this hypothesis and it may be noted that stoichiometric addition of sufficient alkali to repress the dissociation of the pyrimidine amino group and thus permit Schiff base formation does not permit acetoin formation in these systems.

According to the recent formulation of Reed and DeBusk,[19] "lipothiamide" pyrophosphate, rather than cocarboxylase, is the coenzyme of pyruvate oxidase. These authors postulate an internal migration of the C_2 unit derived from pyruvate from the thiamine moiety to one sulfur of the lipoic acid moiety, opening the –S–S bridge with formation of CH_3CO–S–R–SH. Before attempting to reproduce such a reaction in a protein-free system, Fisher–Hirshberger models were made of a compound of the structure proposed for lipothiamide pyrophosphate[20] and of a lipothiamide pyrophosphate–acetaldehyde complex similar to that for thiamine shown above. When properly rotated, the —C—N≡ of the thiamine–aldehyde moiety came into perfect juxtaposition and contact with the –S–S– group of the lipoic moiety. Attempts to produce CH_3–CO–S– in protein-free alkaline solutions of thiamine or cocarboxylase plus pyruvate and the disulfide form of lipoic (thioctic)[21] acid were entirely unsuccessful. Under these conditions a small amount of sulfhydryl-reacting material appeared which was paralleled by equivalent thiochrome formation. This reaction was not, however, dependent upon the presence of pyruvate and represents an unrelated and apparently trivial direct reduction of lipoic acid by thiamine. These negative findings in no wise contravene the arguments of Reed and DeBusk in support of their hypothesis.

The model systems described above operate only in media considerably more alkaline than those in biological systems. It seems entirely possible, however, that cocarboxylase or lipothiamide pyrophosphate bound to a proton accepting site on an enzyme molecule may exist in the pseudobase form.

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