[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, COLUMBIA UNIVERSITY]

Studies on Model Systems for Thiamine Action. Synthesis of Reactive Intermediates, and Evidence on the Function of the Pyrimidine Ring[1]

BY RONALD BRESLOW AND EDWARD MCNELIS

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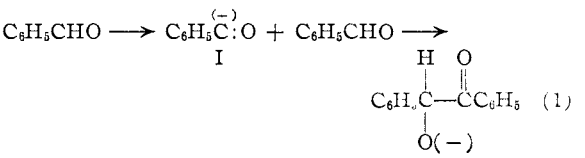
Strong support for the mechanism previously proposed[2] for model systems for thiamine action has been added by the synthesis of suggested intermediates and the finding that they are much more effective in the test system than are the simple related thiazolium salt catalysts. It has also been found that the effect of aromatic substituents in the catalysts, such as the pyrimidine ring in thiamine, can be ascribed to inductive electron withdrawal operating to accelerate at least two of the steps in the model reactions.

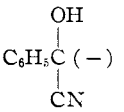
Previous studies on model systems for thiamine action have led to the proposal of a general mechanism by which the models operate, and by extension this mechanism has been proposed for the biochemical catalyses involving thiamine pyrophosphate itself.[2] The fundamental similarity of all these reactions, biochemical as well as chemical, lies in the fact that all can formally be considered to involve an intermediate acyl carbanion, R–CO(−), although the true structure of this formal species, which would of course be rather unstable, is presumably that of a derivative involving the catalyst.

Thus, cyanide ion catalyzes the benzoin condensation, which can be formally represented as in equation 1. In the cyanide-catalyzed reaction the



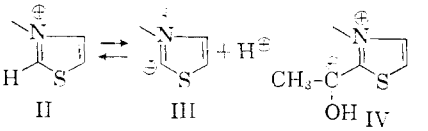
(1)

intermediate anion I is of course really the corresponding cyanohydrin which is a reasonable reso-



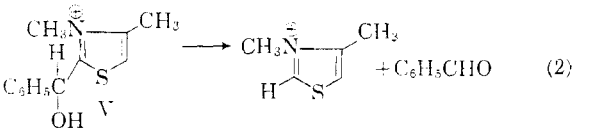
nance-stabilized anion. On this formal basis decarboxylation of pyruvate to acetaldehyde, as well as condensation of acetaldehyde to acetoin, can also be considered as involving an acetyl carbanion, $CH_3CO(-)$, and one might expect to find cyanide ion catalysis, as has been observed.[3]

The function of the cyanide is clear. Thiazolium salts (II) and in some cases imidazolium salts also catalyze these reactions,[2,4] but their structures are more complex, and hence their mode of catalysis is less obvious; we have recently observed that they are in equilibrium with zwitterions (III) under mild conditions[4b]; again an anion is available for reaction which is situated on a carbon multiply bonded to nitrogen. We have accordingly suggested[2] that thiazolium salts act by a mechanism similar to that of cyanide ion, and that the formal acyl carbanion is in this case simply (IV).



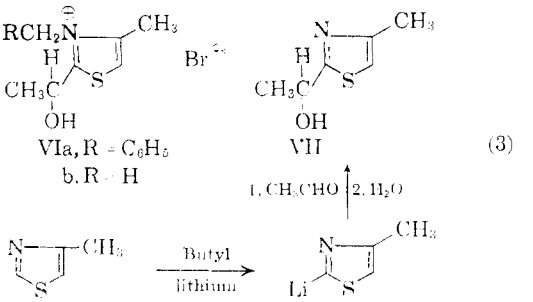
II III IV

Although the indirect evidence for this mechanism seems strong, the attempt to synthesize V, a proposed intermediate in the benzoin condensation, furnished only moderate direct support as V was rather unstable (equation 2), and could not be tested in the benzoin reaction.[5]



(2)

It is well known, however, that adducts of aromatic aldehydes dissociate more readily to the components than do adducts of aliphatic aldehydes. Accordingly it seemed possible that substances such as VI might be stable enough for testing in the acetoin-forming model reaction.[6] The synthesis of the parent thiazole VII was readily accomplished (equation 3); the metalation[7] of 4-methylthiazole should of course occur in the 2-position, reflecting in the thiazole series an acidity of the C-2 hydrogen which becomes rather marked in the thiazolium salts themselves. As proof of structure, VII has been degraded with sodium hypoiodite to iodoform and 2-carboxy-4-methylthiazole,[8] identical with an authentic sample.



VIa, R = C_6H_5 VII (3)
b, R = H

(1) A preliminary report of this material was presented at the 134th Meeting of the American Chemical Society in Chicago, Ill., September, 1958.

(2) R. Breslow, THIS JOURNAL, **80**, 3719 (1958).

(3) V. Franzen and L. F. Leutscher, *Ann.*, **613**, 1 (1958); for an earlier report, *cf.* Y. Takeuchi, K. Okuda, S. Hayakawa and S. Mizuhara, *J. Biochem. Japan*, **42**, 93 (1955).

(4) (a) T. Ugai, S. Tanaka and S. Dokawa, *J. Pharm. Soc. Japan*, **63**, 269 (1943); T. Ugai, T. Dokawa and S. Tsubokawa, *ibid.*, **64**, 7A, 3 (1944); S. Mizuhara, R. Tamura and H. Arata, *Proc. Japan Acad.*, **27**, 302 (1951); S. Mizuhara and P. Handler, THIS JOURNAL, **76**, 571 (1954); (b) R. Breslow, *ibid.*, **79**, 1762 (1957).

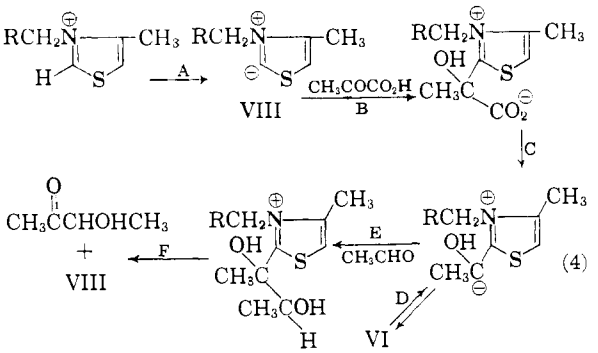
(5) R. Breslow, *Chemistry & Industry*, 893 (1957).

(6) A similar compound has recently been prepared in the thiamine series by L. O. Krampitz, *et al.*, THIS JOURNAL, **80**, 5894 (1958).

(7) *Cf.* H. Gilman and J. A. Beel, *ibid.*, **71**, 2328 (1949).

(8) H. Schenkel, E. Marbet and H. Erlenmeyer, *Helv. Chim. Acta*, **27**, 1437 (1944).

As expected, the N-alkylthiazolium salts (VI) did not eliminate acetaldehyde under mild conditions, and could accordingly be used in the acetoin test (equation 4).[2,9]



(4)

Since the yield of acetoin in the test is ordinarily only 10% or less, based on catalyst, one might well expect an intermediate in the reaction to be more effective than the simple related thiazolium salt. That this is indeed the case for VIa and VIb is revealed in Table I, where comparison of case 2 with 3, and 4 with 5, demonstrate clearly the greater effectiveness of the proposed intermediates in the model system.

TABLE I

ACETOIN FORMATION

	Compound	No. of runs	Acetoin, micrograms
1	Thiamine	6	375 ± 18
2	2-(α-Hydroxyethyl)-3-benzyl-4-methylthiazolium bromide (VIa)	8	239 ± 19
3	3-Benzyl-4-methylthiazolium bromide	5	79 ± 22
4	2-(α-Hydroxyethyl)-3,4-dimethylthiazolium bromide (VIb)	6	34 ± 4
5	3,4-Dimethylthiazolium bromide	5	17 ± 7
6	No catalyst	4	15 ± 4

It is striking, however, that even these intermediates yield only limited amount of acetoin, suggesting that, in the reaction sequence (equation 4), steps D, E and F are still important in determining the rate of acetoin formation, which is of course possible if intermediates accumulate in the reaction or if there are competing side reactions.

It is also of interest that N-benzyl substituted thiazolium salts (Table I, cases 2 and 3) are more effective than the corresponding N-methyl compounds (cases 4 and 5). This effect in the simple thiazolium salts has been discussed previously,[2] when it was pointed out that, in equation 4, an aromatic substituent R would be expected to facilitate step A by furnishing an inductive electron-withdrawing effect. We have corroborated this by measuring the rate of deuterium exchange at C-2 of 3-benzyl-4-methylthiazolium bromide and of the corresponding 3,4-dimethylthiazolium bromide. At 25° and *p*H 5.37 the benzyl compound exchanges

(9) The reaction sequence is formulated in an arbitrary way, using pyruvic acid in step B and acetaldehyde in step E. If pyruvate were used in both steps the product would be acetolactic acid instead of acetoin. Both would be oxidized to diacetyl in our test, however, so the "acetoin" reported in Table I is really acetoin *plus* acetolactate, whose sum we feel to be the best measure of catalysis.

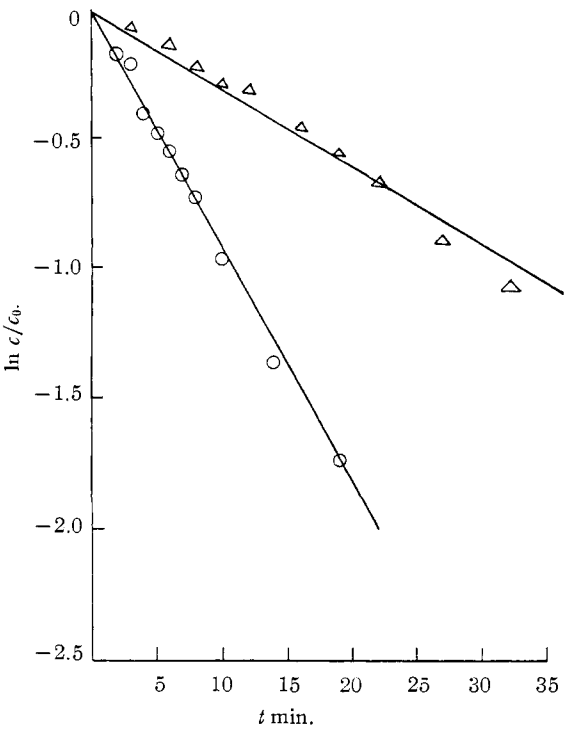


Fig. 1.—Washing out of deuterium from thiazolium salts at 25° in phthalate buffer, *p*H 5.37. Concentration of the deuterio-thiazolium salt, obtained from infrared measurements, in a standard first-order logarithmic plot: △, 3,4-dimethylthiazolium bromide (2-*d*); ○, 3-benzyl-4-methylthiazolium bromide (2-*d*).

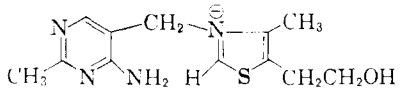
3 times as fast as the methyl derivative (Fig. 1). Thus step A is favored by the aromatic substituent. It is also clear from Table I that this effect is not large enough to explain the difference between the catalytic ability of the two compounds (cases 3 and 5) and this is supported by the observation that even in the intermediates VI a benzyl compound is more effective than a methyl (cases 2 and 4). It is thus clear that the inductive effect operates at steps other than A, and steps D and F seem likely to be affected also. Step F gives VIII, the zwitterion, and should of course be accelerated by an electron-withdrawing phenyl for the same reasons as operated in step A. The ionization step D also seems likely to be accelerated by electron withdrawal, but no direct evidence is available on this point yet.

Thiamine (IX) is still the most effective in the acetoin test (Table I). The aminopyrimidine ring would be expected to have an even greater electron-withdrawing effect than a phenyl ring, and this has recently been confirmed by some *pK* measurements.[10] It is, however, also true that the amino group of thiamine might assist steps A, D and F by internal proton removal; this factor is yet to be evaluated.

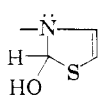
Finally it should be pointed out that, while the electron-withdrawing inductive effect furnished by aromatic rings accelerates several steps in the model reaction sequence and is thus beneficial to catalysis, it would be possible for too much electron with-

(10) Ralph G. Yount and David E. Metzler, private communication.

drawal to decrease the catalytic activity. There are other possible forms of the first conjugate base of a thiazolium salt besides the zwitterion VIII; such forms as X, a pseudobase, are also involved in the equilibrium, and while an electron-withdrawing R group would favor formation of both VIII and X it should also displace the equilibrium



IX



X

between them toward the pseudobase.[11] Metzler has observed[10] that *p*-nitrobenzylthiazolium salts are poor catalysts in the model reaction, although the inductive effect here should be quite large. It is thus possible that the inductive assistance furnished by the aminopyrimidine ring of thiamine is optimal, considering the various equilibria which occur.

Although chemical function can thus be assigned to the two major structural features of thiamine, the thiazolium ring which is the primary site of catalytic activity and the aminopyrimidine ring which bolsters it inductively, one must remember that in biochemical systems other functions, associated with binding to proteins, will also play a role. In this respect it should be remarked that an aminopyrimidine ring is part of the purine adenine, an ubiquitous "handle" for binding such biological co-factors as diphosphopyridine nucleotide and triphosphopyridine nucleotide, coenzyme A, flavin adenine dinucleotide and many others. One might well expect a similar role from the aminopyrimidine group of thiamine. It is then tempting to speculate that the methyl group at C-2 of the aminopyrimidine ring helps to differentiate thiamine from the other substances mentioned and thus contributes to the selectivity which is one of the most striking aspects of enzymatic reactions.

Experimental[12]

2-(α-Hydroxyethyl)-4-methylthiazole.—4-Methylthiazole (8.0 g.) was added to butyllithium (0.2 mole) in ether at −75° under N_2. After 10 min., freshly distilled acetaldehyde (3.2 g.) was added and the mixture was stirred for 50 min. The mixture was worked up with dilute acid; addition of base, ether extraction and distillation yielded 5.53 g. (48%) of colorless 2-(α-hydroxyethyl)-4-methylthiazole, b.p. 91–94° (2.9 mm.), $n^{25}D$ 1.5339.

Anal. Calcd. for C_6H_9NOS: C, 50.32; H, 6.33; N, 9.78. Found: C, 50.56; H, 6.47; N, 9.49.

This product with NaOI under the usual conditions gave iodoform and 2-carboxy-4-methylthiazole, whose decomposition point (95–97°) and infrared spectrum were identical with those of an authentic sample.[8]

3,4-Dimethyl-2-(α-hydroxyethyl)-thiazolium Iodide.—The methiodide was prepared by reaction of the above thiazole with methyl iodide in ethanol; m.p. 149–150°, unchanged on recrystallization from methanol–ether.

Anal. Calcd. for $C_7H_{12}INOS$: C, 29.59; H, 3.90; N, 4.93. Found: C, 29.58; H, 4.04; N, 4.77.

The methiodide (100 mg.) was dissolved in 60 ml. of freshly distilled pyridine in a nitrogen-swept flask. An exit tube led to a test solution of 2,4-dinitrophenylhydrazine. Heating the pyridine solution for 0.5 hour at 70–80° caused no precipitation in the test solution. The pyridine solution, on addition of ether, yielded 50 mg. of recovered methiodide.

3,4-Dimethyl-2-(α-hydroxyethyl)-thiazolium bromide was prepared similarly, m.p. 166–167.5°.

Anal. Calcd. for $C_7H_{12}BrNOS$: C, 35.30; H, 5.08; N, 5.88. Found: C, 35.20; H, 5.04; N, 5.59.

3-Benzyl-2-(α-hydroxyethyl)-4-methylthiazolium bromide was prepared from the corresponding thiazole with benzyl bromide in refluxing methyl ethyl ketone; after recrystallization from methanol–ether to white crystals showed m.p. 159–160°.

Anal. Calcd. for $C_{13}H_{16}BrNOS$: C, 49.68; H, 5.13; N, 4.46. Found: C, 49.81; H, 5.29; N, 4.41.

A stability test in hot pyridine as described for the methiodide gave no 2,4-dinitrophenylhydrazone precipitate.

Acetoin Test.—The activities listed in Table I were determined by a procedure previously reported.[2] As the assay involves a preliminary oxidation to diacetyl, both acetoin and acetolactic acid are detected together and reported as "acetoin." As indicated, reproducibility was good; standard deviations of the mean were considerably smaller than the reported mean deviations.

Rates of Deuterium Exchange.—The thiazolium salts were equilibrated with deuterium oxide at room temperature overnight; the solvent was then removed and the salts were dried *in vacuo*. Completeness of deuteration was established by examination of the infrared spectra (KBr) at 4.5–4.6 μ for the C–D stretching absorption and at 10.7–11.0 μ for loss of the C(2)–H bending absorption; drying was continued until no O–D absorption at 4.0 μ was present. The deuterated salt (0.27 mmole) was dissolved in 0.25 ml. of 0.02 *M* phthalate buffer (*p*H 5.37) and the solution was placed in a CaF_2 infrared cell (0.15 mm.). The appearance of the O–D band at 3.98 μ in the infrared was followed using a Perkin–Elmer Infracord model 137 with slit override at 25. Mechanical standards were used to maintain pen balance during readings and between readings, when the cell was removed from the light path to avoid heating. Temperatures were 24.5–25.5°.

We have found that under these conditions Beer's law is followed in the region 20–80% transmittance for solutions of known DOH concentration. Final values of the optical density were used to construct first-order plots in the standard fashion, as shown in Fig. 1. Duplicate runs gave close agreement, and from these data the following half-times can be calculated: 3,4-dimethylthiazolium bromide (2-*d*), 23 ± 1 min.; 3-benzyl-4-methylthiazolium bromide (2-*d*), 7.5 ± 1 min.

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(11) The equilibria between the various conjugate bases of thiazolium salts have been discussed previously (ref. 2). It is clear that an electron-withdrawing inductive effect will destabilize the thiazolium salt most, the zwitterion form of the first conjugate base less, and the other conjugate bases less yet, in order of decreasing positive charge on nitrogen.

(12) Melting points were taken on a calibrated Fisher–Johns block.