in former papers (cf. Osterhout, W. J. V., *Ergebn. Physiol.*, **35**, 967 (1933); Hill, S. E., and Osterhout, W. J. V., *Jour. Gen. Physiol.*, **21**, 541 (1937–38); Blinks, L. R., *Ibid.*, **13**, 495 (1929–30).

[3] Osterhout, W. J. V., *Ibid.*, **18**, 987, figure 2 (1934–35).

[4] Osterhout, W. J. V., *Ibid.*, **13**, 715 (1929–30).

[5] Hill, S. E., and Osterhout, W. J. V., *Proc. Nat. Acad. Sci.*, **24**, 312 (1938).

[6] We may write

$$\text{P.D.} = 58\,\frac{(uC_{Na} - vC_{Na}) - (uC_K - vC_K}{(uC_{Na} + vC_{Na}) - (uC_K + vC_K)}\log\frac{uC_{Na}+vC_{Na}}{uC_K + vC_K}$$

$$= 58\,\frac{(u - v)(C_{Na} - C_K)}{(u + v)(C_{Na} - C_K)}\log\frac{C_{Na}(u + v)}{C_K(u + v)}$$

$$= 58\,\frac{u - v}{u + v}\log\frac{C_{Na}}{C_K}.$$

[7] As $u_{Na} = u_K = u$ increases the P.D. approaches the limiting value P.D. $= 58 \log C_{Na} \div C_K$.

[8] Osterhout, W. J. V., *Jour. Gen Physiol.*, **20**, 13 (1936–37).

[9] Osterhout, W. J. V., *Ibid.*, **21**, 707 (1937–38).

[10] Unpublished.

SYNTHESES CARRIED OUT IN VIVO BY ISOLATED PEA ROOTS: I

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There has long been interest in the mechanisms by which chemical reactions take place *in vivo*, and numerous techniques have been applied to the study of this problem. A precise interpretation of purely analytical results is, however, often difficult or impossible, particularly if the reactions in question have been allowed to take place in the intact organism. It is desired to present in the present paper a few of the results obtained with the aid of a new experimental approach to the problem of physiological syntheses. A single relatively simple organ has been cultivated *in vitro* under conditions which have been closely controlled both as to external environment and as to nutrient supply, and the metabolism related to a single well defined and readily determinable substance has been investigated.

In earlier papers[1,2] it has been shown that isolated pea roots may be successfully grown *in vitro* provided only that a suitable nutrient medium is used. Such a nutrient medium must of course contain carbohydrates (in

this case sucrose), a source of nitrogen (in this case nitrate) and the correct kinds and proportions of inorganic salts. The medium must in addition contain a small amount, 0.1 mg. per liter or less, of vitamin B_1 if continued growth of the root is to occur. It is with the metabolism of vitamin B_1 and related substances that the present paper is concerned.

It has been found possible[3] to replace B_1 by various other similarly constituted substances or combinations of such materials. The question naturally arises whether or not these substances are transformed by the organism into the vitamin itself. An answer can be obtained if the rate of formation of B_1 *in vivo* is substantially greater than the rate of its destruction and if an assay method can be made available which is specific for the vitamin molecule. Both of these conditions are well enough met in the cases to be discussed so that some picture can be presented of modes and limitations of such syntheses as effected by isolated pea roots.

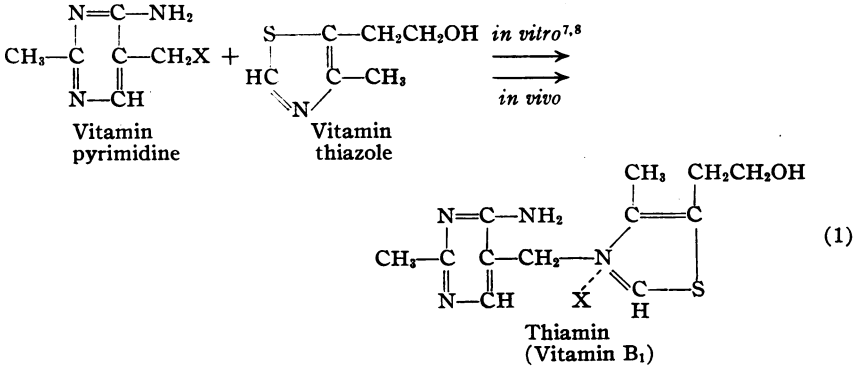
The methods used have been described in detail elsewhere[2,3,4] and need not be gone into here. Three general types of measurements have been made:

1. Ability of substances related to vitamin B_1 to replace the latter in supporting the growth of excised pea roots.

2. A quantitative assay with the fungus *Phytophthora cinnamomi*, which is used for the determination of amounts of vitamin B_1.[5]

3. A quantitative assay with the fungus *Phycomyces Blakesleeanus*, which is used for the determination of the total of vitamin B_1 and/or free vitamin pyrimidine plus vitamin thiazole.[4,6]

The vitamin B_1 molecule contains a substituted pyrimidine nucleus linked through a methylene bridge to a substituted thiazole nucleus. These two portions, which will be referred to hereafter as the "vitamin pyrimidine" and the "vitamin thiazole," may be linked *in vitro* to form the vitamin itself. In order that this condensation (Reaction (1)) take place in the test tube it is essential that the 5-methyl group of the pyrimidine be substituted with a reactive group X, such as a Br atom.



(1)

It has been shown[9] that numerous organisms which require vitamin B_1 as an accessory growth factor are able to utilize a mixture of the vitamin pyrimidine and the vitamin thiazole in place of the vitamin itself. In fact, pyrimidines which cannot *in vitro* be linked with the vitamin thiazole, such as the 5-aminomethyl pyrimidine (X = NH_2), may even be used as the pyrimidine component for the growth of certain microörganisms and for the pea root.[3] It has been generally supposed that these organisms actually combine the pyrimidine and thiazole halves *in vivo* to form the vitamin. In no case, however, has such a synthesis been actually demonstrated. Evidence that the pea root does in fact synthesize vitamin B_1 was obtained from experiments in which pea roots were supplied with a mixture of the 5-aminomethyl pyrimidine and the vitamin thiazole. After the roots had grown with this mixture as their growth factor, root tips were removed and assayed (*a*) by the *Phytophthora* test, which determines the vitamin B_1[5] but not the pyrimidine-thiazole mixture, and (*b*) by the *Phycomyces* test, which determines vitamin B_1 and/or any of the uncombined intermediates. Table 1 shows that the roots contain only vitamin B_1 and no significant amount of the uncombined intermediates.[10]

TABLE 1

VITAMIN B_1 CONTENT OF PEA ROOT TIPS AFTER CULTIVATION WITH VARIOUS GROWTH FACTORS

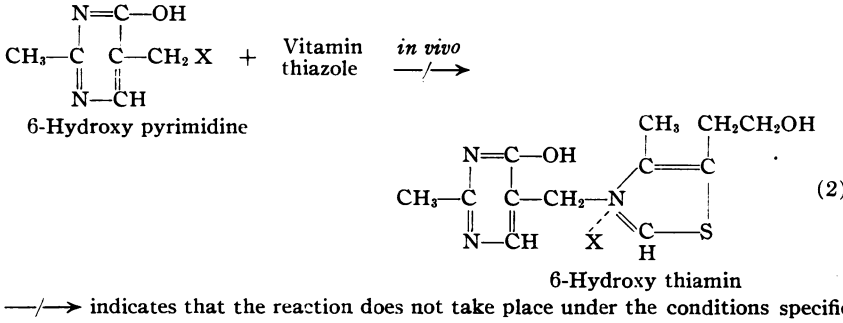
	GROWTH FACTOR SUPPLIED		
CONTENT OF	NONE	PYRIMIDINE + THIAZOLE	VITAMIN B_1
Vitamin B_1 (*Phytophthora* assay)	0	100%	100%
Vitamin B_1 and/or pyrimidine + thiazole (*Phycomyces* assay)	0	100%	100%
Pyrimidine + thiazole (difference of the two assays)	0	insignificant	insignificant

It is also of interest that the roots supplied with the mixture of intermediates contain as much of the vitamin as do roots supplied with the vitamin itself. Control roots contain no appreciable amount of the vitamin. This means, then, on the assumption made in footnote 5, that the pea root must be capable of conducting *in vivo* a *synthesis* of the vitamin which does not take place in the test tube and we postulate that this synthesis is due to the action of a specific enzyme[11] furnished by the organism.

It was further found, by experiments similar to these, that the root is also able to synthesize vitamin B_1, in the presence of the vitamin thiazole, from pyrimidines in which the 5-methyl group is substituted with either an ethoxy (X = OC_2H_5) or a thioformamido group (X = NHCSH).

By means of what we shall term "competition" experiments, it was found possible to study the limitations of this *in vivo* coupling of pyrimidine and

thiazole by roots. In this paper the application of this tool to the study of the following reaction is pointed out; it should be noted, however, that the method is capable of considerably wider application.

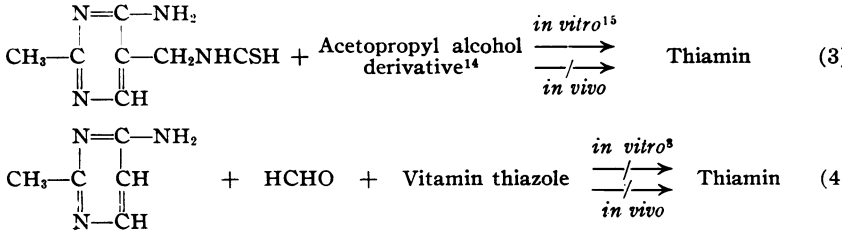


(2)

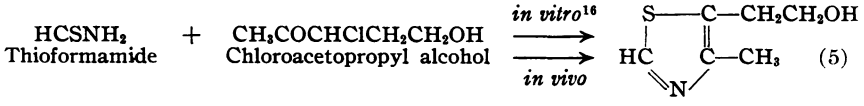
—/→ indicates that the reaction does not take place under the conditions specified.

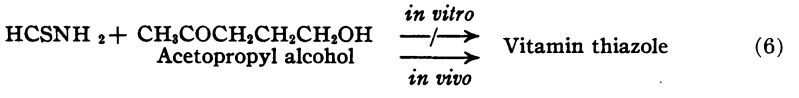
6-Hydroxy vitamin does not support the growth of pea roots[12] either alone or in combination with vitamin pyrimidine.[13] Therefore this vitamin analog does not break up appreciably *in vivo* to make the vitamin thiazole available for recombination. Moreover, when 6-OH pyrimidine (X = OC_2H_5) is added, even in large excess, to a mixture of vitamin pyrimidine (X = OC_2H_5) and vitamin thiazole, it is found to exert no effect on the activity of the mixture in supporting the growth of pea roots. Reasoning from these results, the stability of the inactive vitamin analog and the inability of the inactive pyrimidine to "compete" with the active pyrimidine for the available vitamin thiazole, we conclude that an enzymatic synthesis (Reaction (2)) of the 6-OH vitamin is not accomplished at a detectable rate *in vivo*. Hence the enzyme system which we assume to be responsible for *in vivo* synthesis of the vitamin exhibits a certain degree of specificity in its action. Numerous analogs of the vitamin thiazole are active as the thiazole component in supporting the growth of pea roots and hence are presumably synthesized to vitamin analogs *in vivo*, so that the specificity of the above enzyme system is by no means complete.

The fact that it has not been found possible to obtain evidence for an *in vivo* production of thiamin according to reactions (3) and (4) lends further weight to the view that the vitamin is actually produced in nature by a simple joining of its two halves.



The pea root is unable to synthesize either vitamin pyrimidine or vitamin thiazole from the sucrose and the inorganic constituents of the basic nutrient medium. We have found that it is, however, able to synthesize the vitamin thiazole from appropriate simpler substances. Of the two reactions given here for formation of the thiazole only the first one takes place in the test tube whereas pea roots can be shown to accomplish both of them.





When supplied with a mixture of vitamin pyrimidine, thioformamide,[17] and either chloroacetopropyl alcohol or acetopropyl alcohol, the roots grow as well and contain as much thiamin (as judged by the *Phycomyces* assay) as roots supplied with vitamin B_1 itself.

TABLE 2

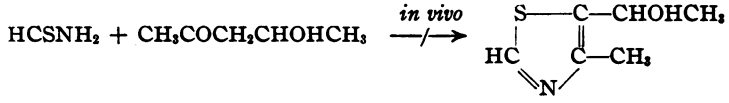
VITAMIN B_1 CONTENT OF PEA ROOT TIPS AFTER CULTIVATION WITH VITAMIN PYRIMIDINE AND ACYCLIC THIAZOLE INTERMEDIATES

	GROWTH FACTOR SUPPLIED			
	NONE	PYRIMIDINE + VITAMIN THIAZOLE	PYRIMIDINE + THIOFORMAMIDE + CHLOROACETOPROPYL ALCOHOL (REACTION (5))	PYRIMIDINE + THIOFORMAMIDE + ACETOPROPYL ALCOHOL (REACTION (6))
Vitamin B_1 content (*Phycomyces* assay)	0	100%	100%	100%

Control experiments with *Phycomyces* show that this organism cannot in either case utilize the thiazole intermediates to replace the vitamin thiazole under the present conditions. This, then, justifies the use of the *Phycomyces* assay in the experiments of table 2 and also indicates that under our conditions the synthesis of thiazole according to Reaction (5) is enzymatic in nature. It may be concluded that the pea root is able to effect a ring closure from thioformamide and either acetopropyl alcohol or its chloro derivative with the formation of the vitamin thiazole. It would seem not unlikely that the synthesis of the thiazole ring in nature is accomplished in a similar manner.

The 4-methyl, 5-α-hydroxyethyl thiazole possesses a considerable activity as the thiazole component for the growth of pea roots. The synthesis

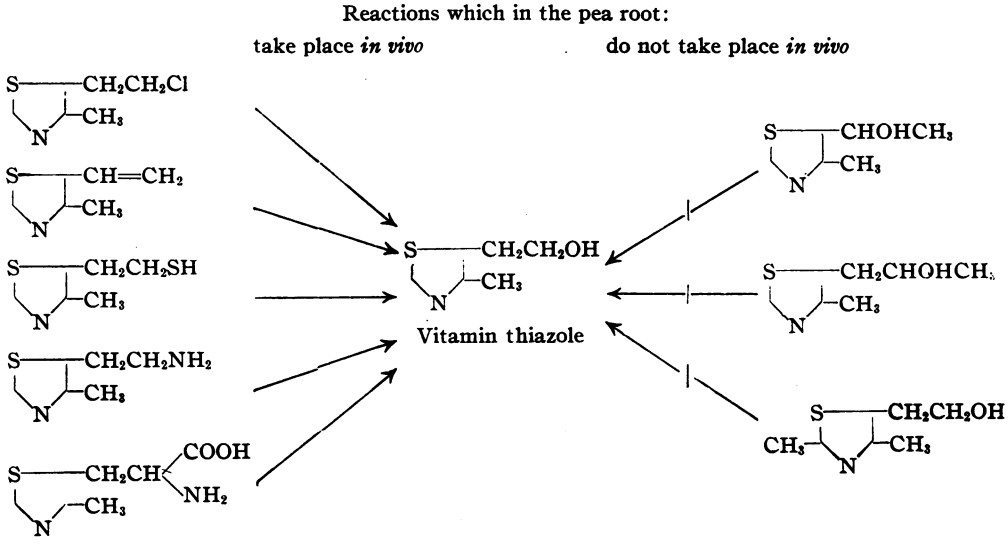
of this analog of the vitamin thiazole can be formulated as follows:



(7)

Such a synthesis is not, however, effected by pea roots, i.e., although the roots are apparently able to utilize this analog if it is supplied to them ready made, they are unable to synthesize it as they are the vitamin thiazole. This indicates that also in the case of the enzyme system responsible for thiazole synthesis there is a considerable specificity.

A number of analogs of the vitamin thiazole are highly active as the thiazole component for the growth of isolated pea roots. In the figure are



shown, at the left, five such analogs[18] each of which could conceivably be converted *in vivo* by the organism to the vitamin thiazole, shown in the center. Such a transformation actually does take place; roots supplied with vitamin pyrimidine plus any one of these five analogs of the vitamin thiazole not only grow as well, but also contain essentially the same amount of vitamin B_1 as do roots grown upon the vitamin itself. The vitamin assays were carried out in these cases by the *Phycomyces* method which is permissible[19] since the five analogs under discussion have themselves activities upon the growth of *Phycomyces* which are much smaller than that of the vitamin thiazole.[4]

The three thiazole analogs[18] which are shown at the right of the figure are also active in supporting the growth of pea roots. It would seem *a priori* unlikely from a chemical standpoint that any of these should be

metabolized to the vitamin thiazole by the root. Experiment shows that in no case do pea roots which have *grown* with one of these analogs as the thiazole component of the medium contain any significant amount of substance active in supporting the growth of Phycomyces. It must therefore be concluded that indeed no *in vivo* conversion to the vitamin thiazole takes place in these cases and consequently that each of these three thiazoles can form a part of a hormone molecule differing in structure from, but having physiological properties similar to the natural vitamin.

It has been shown in the present paper that the pea root synthesizes vitamin B_1 (or a substance indistinguishable from vitamin B_1 by the *Phytophthora* bioassay) from a mixture of the pyrimidine and thiazole components of the vitamin molecule, and that this reaction is carried out *in vivo* under conditions such that no *in vitro* reaction can occur. There is a considerable specificity as to the structures of the pyrimidine and thiazole which may take part. This must then be a synthesis in which a specific enzyme, a "thiaminase" (from "thiamin," the chemical name proposed for the vitamin[20]), takes part. A second and distinct enzyme system is able to effect closure of the thiazole ring from suitable acyclic substances to form the vitamin thiazole. Since analogs of the vitamin thiazole are apparently not formed in analogous fashion, this "thiazolase" must also be somewhat specific in its action. The suggestion is made that both "thiaminase" and "thiazolase" play a part in the natural synthesis of thiamin by the plant. It has further been shown that certain thiazole derivatives are transformed to the vitamin thiazole *in vivo* by enzymatic reactions corresponding to deamination, decarboxylation, hydrolysis and hydration; whereas certain other growth-promoting thiazoles are not so transformed. It is suggested that the methods outlined in the present paper may offer a new and more exact approach to the problem of the mechanism of biosyntheses.

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[1] Bonner, J., *Science*, **85**, 183 (1937).

[2] Bonner, J., and Addicott, F., *Bot. Gaz.*, **99**, 144 (1937).

[3] Bonner, J., *Amer. Jour. Bot.*, **25**, 543 (1938).

[4] Bonner, J., and Erickson, J., *Amer. Jour. Bot.* (in press).

[5] Robbins, W. J., *Proc. Nat. Acad. Sci.*, **24**, 53 (1938). Experiments in this laboratory have confirmed this report that *Phytophthora* responds to vitamin B_1 but not to a mixture of vitamin pyrimidine plus vitamin thiazole. The *Phytophthora* assay will be assumed here, then, to determine vitamin B_1 and not the mixture of intermediates. It may be that this assay determines combined forms of the vitamin, such as co-carboxylase, as well, but this, if true, would not alter the arguments presented here.

[6] Schopfer, W. H., and Jung, A., *Compt. rend. 5ème Congrès Intern. tech. et chim. Ind. agr., Scheveningue*, **1937**, 22.

[7] Williams, R. R., and Cline, J. K., *Jour. Amer. Chem. Soc.*, **58**, 1504 (1936)

[8] Cline, J. K., Williams, R. R., and Finkelstein, J., *Jour. Amer. Chem. Soc.*, **59**, 1052 (1937).

[9] First demonstrated for *Staphylococcus aureus:* Knight, B. C. J. G., *Biochem. Jour.*, **31**, 966 (1937); see reference 3, and review by Schopfer, W. H., *Arch. Mikrobiol.*, **9**, 116 (1938).

[10] The average amount of vitamin B_1 found in one root tip 1 cm. long, cultivated for one week in nutrient medium containing either vitamin B_1 or a vitamin pyrimidine-vitamin thiazole mixture, was $6 \times 10^{-6} \pm 0.65 \times 10^{-6}$ mgs. In tables 1 and 2, relative values are used for the sake of simplicity. "100%" indicates no experimentally significant difference from the vitamin B_1 control tips. "0%" indicates no experimentally significant amount of vitamin B_1 or its intermediates.

[11] Robbins, W. J. (see reference 5) has suggested "that the synthesis of the vitamin from its intermediates is enzymatic."

[12] The inability of 6-hydroxy vitamin and other vitamin analogs (see reference 3) to support the growth of pea roots is evidence that the organism is *unable* to convert these substances into thiamin by an *in vivo* synthesis.

[13] In place of 6-hydroxy vitamin these experiments were actually carried out with the readily available "chlorooxy vitamin" (Buchman, E. R., and Williams, R. R., *Jour. Amer. Chem. Soc.*, **57**, 1751 (1935)). From the results presented in this paper on the conversion of chloro thiazole to vitamin thiazole it may be inferred that chlorooxy vitamin and 6-hydroxy vitamin are physiologically equivalent in work with the pea root.

[14] Both acetopropyl alcohol and chloroacetopropyl alcohol were used in an attempt to demonstrate an *in vivo* synthesis.

[15] Todd, A. R., and Bergel, F., *Jour. Chem. Soc.* (London) **1937**, 364.

[16] Buchman, E. R., *Jour. Amer. Chem. Soc.*, **58**, 1803 (1936).

[17] The thioformamide is undoubtedly largely decomposed during the autoclaving of the medium (a procedure used in all of the experiments reported here). In accordance with this it was found that approximately 10 times as much of the mixture of thiazole intermediates as of vitamin thiazole must be used to support the growth of pea roots equally well. The *in vivo* synthesis of vitamin thiazole does not take place in the absence of added thioformamide, and hence either it itself or its decomposition products must enter into the reaction.

[18] Buchman, E. R., and Richardson, E. M., unpublished.

[19] The reasonable assumption is made that all substances, save only the vitamin itself, derived *in vivo* from these five thiazoles and vitamin pyrimidine, have *Phycomyces* activities comparable to those of the thiazoles when tested in a mixture with vitamin pyrimidines. See also reference 4.

[20] Williams, R. R., *Jour. Amer. Med. Assoc.*, **110**, 727 (1938).