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A Reappraisal of the Relationship Between Free and Bound Coumarin in *Melilotus*

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A Reappraisal of the Relationship Between Free and Bound Coumarin in *Melilotus*[1]

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DUNCAN and Dustman (3, 4), Clayton and Larmour (1), and Stevenson and Clayton (13) were among the first investigators to attempt the assay of coumarin in sweetclover (*Melilotus* spp.). All of these workers apparently believed that the coumarin measured by their assay methods existed in the free form in the plant. Roberts and Link (8, 9) recognized the presence of bound coumarin in sweetclover seeds and green tissues and indicated that provision must be made for the hydrolysis of this form if reliable values for total coumarin content were to be obtained. However, they stated that in succulent, green tissues the free form usually predominated over the bound form (9). Slatensek and Washburn (12) observed that Pioneer sweetclover, a variety described by Stevenson and White (14) as being low in coumarin on the basis of colorimetric analysis of alcoholic extracts, appeared to be high in coumarin when assayed by a fluorometric method which involved heating the plant tissue in alkali. The difference in values obtained by the two methods was attributed to the presence of bound coumarin which was hydrolyzed in the fluorometric procedure but not in the colorimetric assay. Although the fluorometric assay described by Slatensek and Washburn (12) did not permit distinction between free and bound coumarin, it is apparent that these investigators considered coumarin to be in the free form in all coumarin-containing varieties other than Pioneer.

In recent years, Goplen et al. (5) have reported on the influence of two pairs of alleles, *Cu/cu* and *B/b*, upon the level and form of coumarin in sweetclover. A qualitative colorimetric method was used for the detection of free coumarin in alcoholic extracts; and, for quantitative measurements of total coumarin, a fluorometric assay similar to that described by Slatensek and Washburn (12) was used. The authors concluded that the *Cu/cu* allelic pair governed presence or absence of coumarin and that the *B/b* alleles determined the type of coumarin (free or bound) present in *Cu* individuals. The assay methods did not permit determination of free coumarin levels, but it may be inferred from their discussion that the authors considered the coumarin present in *CuCuBB* plant tissues to be in the free form.

Haskins and Gorz (6) used a modification of the Slatensek and Washburn (12) procedure for the determination of free and total coumarin in aqueous extracts of plant tissues representing the four genotypes homozygous with respect to *Cu/cu* and *B/b*. Their conclusions regarding the influence of these two pairs of alleles differed somewhat from the conclusions of Goplen et al. (5). Thus, they suggested that the *Cu/cu* alleles determined whether the level of coumarin would be high or low, and the *B/b* alleles influenced the presence of free and bound coumarin as opposed to bound coumarin only. It was reported that in young leaves of the *CuB* phenotype, approximately 37% of the coumarin was in the free form. Similarly, Clopton (2) has indicated that approximately one-third of the coumarin in Hubam sweetclover seeds occurs in the free form.

Still more recently, Rudorf and Schwarze (10) have found that when proper precautions are taken to prevent glycosidase activity during extraction, extracts of "bitter" Hubam sweetclover contain little if any free coumarin. They used dilute sulfuric acid as an extracting solution, and reported that bound coumarin, which was presumed to be the glycoside of *cis*-o-hydroxycinnamic acid, was extracted without being hydrolyzed.

Although by no means a complete review of the literature on coumarin in sweetclover, the foregoing is sufficient to indicate that the point of view regarding the form in which coumarin exists in normal, coumarin-containing sweetclover plants has shifted. Thus, early workers apparently thought that the free form predominated, then it was believed that both forms were present in substantial quantity, and most recently it has been suggested that only the bound form exists in the intact plant.

The present study substantiates the findings of Rudorf and Schwarze (10), but employs a different method of extraction and assay, and utilizes various genotypes of biennial sweetclover rather than the annual variety, Hubam.

MATERIALS AND METHODS

Plants of biennial white-blossomed sweetclover (*Melilotus alba* Desr.) of the four genotypes, *CuCuBB*, *CuCubb*, *cucuBB*, and *cucubb*, were used in this study. The plants were representative

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of several F_9 lines that had been derived from a single, doubly heterozygous F_6 plant. The original cross from which this F_6 plant was derived involved a *cucuBB* plant as the female parent and a *CuCubb* plant as the male parent. The seed from which these two parent plants were grown was obtained from W. K. Smith, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, and the University of Wisconsin.

Samples for coumarin assay were obtained from the first-year growth of plants seeded in the field in rows without a companion crop in the spring of 1960. Sixteen plants of each genotype were sampled on October 4, 1960, and 16 additional plants of each genotype were sampled on October 6. For each plant sampled, the terminal 8- to 10-inch portion of one branch was removed and the cut end was immediately immersed in a test tube of tap water. Samples were then carried to the laboratory, where a young, fully expanded leaf was selected on each of the branches. This leaf was removed and the three leaflets were cut from the petiole with a sharp razor blade. Each leaflet was immediately weighed to the nearest 0.1 mg. on a direct-reading balance. The mid-leaflet was oven-dried for the determination of percentage dry matter. One side leaflet was dropped into 2 ml. of distilled water at room temperature, contained in a test tube. Two drops of 95% ethyl alcohol were applied to the leaflet to facilitate submersion, and the tube and contents were then autoclaved for 15 min. at approximately 15 psi. This method of extraction, designated procedure "A", has been described in an earlier publication (6). The other side leaflet was subjected to extraction procedure "B," which differed from procedure "A" in that the leaflet was dropped into 2 ml. of water that had been heated in a boiling water bath for 10 to 15 min. Addition of alcohol was not required for submersion of the leaflet under these conditions. The tube was left in the boiling water for approximately 10 sec. after the leaflet had been added and was then allowed to stand at room temperature until it was placed in the autoclave. Leaflets to be extracted by procedure "A" and "B" were autoclaved simultaneously. Autoclaved extracts were cooled, leaflets were removed and discarded, and the extracts were stored in a freezer for later assay. The assay method used was essentially the fluorometric procedure for free and total coumarin which was described by Haskins and Gorz (6).

In both extraction procedures, care was taken to avoid bruising the leaflets. Operations were scheduled in such a way that the maximum time elapsing between removal of a leaf from the branch and addition of the leaflets to water was approximately 40 min., and the maximum time elapsing between addition of the leaflets to water and the start of autoclaving was 8 min. In an earlier experiment it was found that leaflets could stand in dry tubes at room temperature for at least 6 hours without appreciable alteration in levels of free and total coumarin.

Extraction procedure "B" also was used in obtaining extracts for assaying 5 plants of each of 9 sweetclover varieties. Four yellow-blossomed varieties—Common Yellow, Madrid, Goldtop, and Erector (*M. officinalis* (L.) Desr.) and 5 white-blossomed varieties—Common White, Spanish, Evergreen, Arctic, and Israel (*M. alba*)—were used in this portion of the study. With the exception of the annual variety, Israel, these varieties are biennial in growth habit. Samples were collected on October 10, 1960, from field-grown plants seeded in rows without a companion crop in the spring of 1960.

RESULTS AND DISCUSSION

Mean values for free and total coumarin in "A" and "B" extracts of young leaves from 32 field-grown plants of each homozygous genotype are shown in Table 1. For *cucubb*, *cucuBB*, and *CuCubb* leaves, differences in results obtained by the two extraction procedures were insignificant. As expected, leaves of the *cucu* genotypes contained very small amounts of either free or bound coumarin, and assays of *CuCubb* extracts indicated substantial amounts of coumarin, essentially all of which was in the bound form. In the case of *CuCuBB* leaves, on the other hand, extracts obtained by procedure "A" contained substantial amounts of free coumarin whereas "B" extracts contained little if any of the compound in this form. Total coumarin levels observed in the two types of extract were not appreciably different.

Application of the 2 extraction procedures to greenhouse-grown plants of the 4 doubly-homozygous genotypes yielded results similar to those obtained when field-grown material was used. In no case did procedure "B" produce an extract containing an appreciable amount of free coumarin.

Table 1—Mean levels of free and total coumarin in young leaves from 4 genotypes of sweetclover. Results are based on extracts made by two procedures; 32 plants of each genotype were assayed.

Genotype	Coumarin content (percentage of dry weight)			
	Procedure "A"		Procedure "B"	
	Free mean ± SE	Total mean ± SE	Free mean ± SE	Total mean ± SE
cucubb	0.02 ± 0.001	0.03 ± 0.001	0.01 ± 0.001	0.02 ± 0.001
cucuBB	0.01 ± 0.001	0.03 ± 0.001	0.01 ± 0.001	0.02 ± 0.001
CuCubb	0.01 ± 0.001	3.36 ± 0.164	0.02 ± 0.001	3.22 ± 0.133
CuCuBB	0.75 ± 0.050	3.35 ± 0.118	0.02 ± 0.002	3.21 ± 0.125

Table 2—Mean levels of free and total coumarin in young leaves from 9 varieties of sweetclover. Procedure "B" was used in preparing the extracts; 5 plants of each variety were assayed.

Variety	Coumarin content (percentage of dry weight)	
	Free mean ± SE	Total mean ± SE
Biennial yellow		
Common Yellow	0.02 ± 0.002	3.18 ± 0.226
Madrid	0.02 ± 0.003	3.02 ± 0.159
Goldtop	0.05 ± 0.020	3.02 ± 0.115
Erector	0.02 ± 0.001	2.84 ± 0.330
Biennial white		
Common White	0.04 ± 0.007	3.36 ± 0.206
Spanish	0.05 ± 0.014	4.35 ± 0.706
Evergreen	0.02 ± 0.002	3.72 ± 0.183
Arctic	0.02 ± 0.004	2.48 ± 0.230
Annual white		
Israel	0.02 ± 0.002	4.30 ± 0.353

Results of assaying extracts obtained by using procedure "B" on leaves of nine sweetclover varieties are shown in Table 2. The fact that very low free coumarin values were observed for each of these varieties, all of which have the *CuB* phenotype, indicates that the virtual absence of free coumarin is the rule rather than the exception in intact sweetclover tissues. Furthermore, in preliminary tests of procedure "B" on two coumarin-containing grasses, sweet vernal grass (*Anthoxanthum odoratum* L.), and sweet grass (*Hierochloe odorata* (L.) Beauv.) essentially all of the compound was found to be present in the bound form. The limited data available do not, of course, permit a general statement as to whether a similar situation exists in all species of plants which have been reported to contain coumarin.

Evidence presented by Rudorf and Schwarze (10) and Schaeffer[3] indicates that the bound coumarin present in sweetclover is the glucoside of *cis*-o-hydroxycinnamic acid.[4] Hydrolysis of this compound by β-glucosidase, an enzyme known to occur in tissues of the *CuCuBB* genotype (11), yields *cis*-o-hydroxycinnamic acid which undergoes spontaneous lactonization to produce free coumarin. Accordingly, the obvious conclusion to be drawn from results obtained with extraction procedure "A" is that β-glucosidase released a portion of the bound coumarin during the period of time between addition of *CuCuBB* leaflets to water and inactivation of β-glucosidase in the autoclave. In an attempt to determine the time of release of bound coumarin in pro-

[3] Schaeffer, G. W. Chemical genetics of coumarin metabolism in *Melilotus*. Unpub. Ph.D. thesis, University of Nebraska, 1960.

[4] The glucoside of *trans*-o-hydroxycinnamic acid also occurs in sweetclover tissues (7), and this isomer is included in the "total coumarin" figures obtained by the fluorometric procedure. However, Rudorf and Schwarze (10) report that only small amounts of the *trans* isomer are normally found in the plant.

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cedure "A," *CuCuBB* leaflets were added to water, treated with small amounts of alcohol to cause submersion, and allowed to stand at room temperature for various times up to one hour before removal from the water and subjection to extraction procedure "B." Little if any free coumarin was found, either in the leaflet extracts or in the water in which the leaflets were allowed to stand prior to extraction. Apparently, then, the release of free coumarin noted in procedure "A" occurred in the autoclave in advance of heat inactivation of β-glucosidase in the leaflet tissues. The possible involvement of alcohol in hydrolyzing bound coumarin during autoclaving in procedure "A" is precluded by the fact that omission of the alcohol did not prevent the release of free coumarin. It follows that in procedure "B" the boiling water inactivated β-glucosidase with sufficient rapidity to prevent appreciable hydrolysis of bound coumarin.

Hydrolysis of bound coumarin can be induced by several means other than extraction procedure "A". Thus, the following treatments of *CuCuBB* leaflets resulted in the formation of substantial amounts of free coumarin: mechanical maceration; freezing in liquid nitrogen followed by thawing in water, acetone, methyl ethyl ketone, methyl alcohol, 95% ethyl alcolhol, n-propyl alcohol, n-butyl alcohol, ethyl ether, ethyl acetate, benzene, or chloroform at room temperature; and brief submersion in the foregoing organic solvents followed by addition of water and autoclaving. In some instances the extent of hydrolysis exceeded 90%. Replacement of water in procedure "A" by 10^{-3} M solutions of the glucosidase inhibitors, $AgNO_3$, $HgCl_2$, and $CuCl_2$, failed to effect complete inhibition of hydrolysis. In these and other extraction trials, the treatments most effective in preventing hydrolysis of bound coumarin were those involving submersion of the tissue in a hot solvent; and, of the solvents tested, none was more effective than hot water as used in procedure "B." However, inhibition of hydrolysis can be effected by the use of low temperatures, as shown in an experiment in which leaves of the *CuCuBB* genotype were ground in liquid nitrogen in a mortar after which the frozen ground material was extracted first with 100% methyl alcohol and then with 50% methyl alcohol in a deep freeze. The extracts thus obtained accounted for approximately 99% of the total coumarin present in the leaves, and had free coumarin levels only slightly higher than those observed in extracts made by procedure "B."

The simultaneous existence of bound coumarin and β-glucosidase in intact tissues of the *CuCuBB* genotype apparently depends upon a pattern of cellular or tissue organization that limits hydrolysis of the bound coumarin by the enzyme. Anything that interferes with the postulated pattern of organization without immediate inactivation of the enzyme, then, may lead to rapid formation of free coumarin.

The virtual absence of free coumarin in intact sweetclover tissue of the *CuCuBB* genotype may result from either (a) failure of β-glucosidase to hydrolyze bound coumarin in such tissue, or (b) failure of free coumarin to accumulate because of rapid conversion to other compounds. Available evidence does not permit a conclusive choice between these alternatives. If the first is correct, the postulation that the *B/b* allelic pair controls the conversion of bound coumarin to the free form in the intact plant (5, 6) obviously is in error, as is the suggestion of Schaeffer et al. (11) that β-glucosidase is responsible for this conversion in the intact plant. If the second alternative is correct, on the other hand, no change in the suggested roles of the *B/b* alleles and β-glucosidase is required. The report of Kosuge and Conn (7) that exogenously-supplied coumarin is metabolized at a rapid rate, with melilotic acid being one of the principal products, gives some credence to this alternative. Regardless of which alternative is correct, or whether both are partially correct, there is no apparent reason to doubt the influence of the *B/b* alleles on level of β-glucosidase activity in preparations of sweetclover tissues, as demonstrated by Schaeffer et al. (11).

SUMMARY

In early work on coumarin in sweetclover, the assumption was made that the free form of the compound predominated in the intact plant. Subsequent investigations demonstrated the presence of bound coumarin in addition to free, and it was then thought that both forms were normally present. Recent work indicates that when suitable extraction procedures are used, virtually all the coumarin is obtained in the bound form. Such extraction procedures must provide for the rapid inactivation of β-glucosidase, thus preventing the hydrolysis of bound coumarin (apparently the glucoside of *cis*-o-hydroxycinnamic acid) during the extraction process.

In the present study, inactivation of β-glucosidase was achieved by submersion of the plant tissue in boiling water. When this step was incorporated into the extraction procedure, leaf samples of 4 homozygous genotypes (*cucubb, cucuBB, CuCubb,* and *CuCuBB*) and 9 coumarin-containing varieties of sweetclover, and of sweet vernal grass and sweet grass were found to be essentially devoid of free coumarin. In general, any treatment that caused disruption of cellular or tissue organization prior to inactivation of β-glucosidase resulted in conversion of bound coumarin to the free form. Therefore, care in handling the plant tissue is essential.

Implications of the apparent absence of free coumarin in the intact sweetclover plant are discussed with respect to the possible roles of the *B/b* alleles and β-glucosidase in coumarin biosynthesis.

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