take of 1.5 mg of NAAamide or NAA per person per day can be tolerated (Schlatter, 1973). Therefore, an intake of 1 kg of apples per day containing 1.5 ppm of NAAamide or NAA would represent an upper toxicological limit for adult persons. The detected amounts of NAAamide (0.049 ppm) and NAA (0.012 ppm) are far below this value. Since metabolic studies (Lethco and Brouwer, 1966) as well as carcinogenic investigations (Truhaut and Vermes, 1948; Innes *et al.*, 1969) have shown no indication that NAAamide or NAA may pose health hazards, there appears to be no objection to the agricultural use of these two growth regulators.

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A Method for Determination of Vicine in Plant Material and in Blood

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A colorimetric method for the determination of small quantities of vicine (2,6-diamino-4,5-dehydroxypyrimidine 5-(β-D-glucopyranoside)) in biological materials is described. Folin and Ciocalteu's phenol reagent is used as the chromatogen after removal of proteins, amino acids, and phenolic materials from extracts of plants and from blood plasma. The method makes possible the study of variations in vicine concentrations in fava beans, due to habitat and maturity. Its use will help elucidate the role of vicine as a causative factor in favism.

Favism is the term used for an acute hemolytic anemia which occurs in some people after the ingestion of the fava bean (*Vicia faba*). The condition exhibits a striking prevalence in the Mediterranean and Middle East area, but is rarely seen in other parts of the world although the bean is almost universally used as a cheap source of carbohydrate and protein. Susceptibility to fava bean is now accepted as being due to a glucose-6-phosphate dehydrogenase deficiency, but the causative agent is still not identified. One group of compounds thought likely to be implicated is the pyrimidine glycoside group found in considerable quantities in the fava bean (Mager *et al.*, 1965; Lin and Ling, 1962). The most common of these are vicine (2,6-diamino-4,5-dehydroxypyrimidine 5-(β-D-glucopyranoside)) and convicine (2,4,5-trihydroxy-6-aminopyrimidine 5-(β-D-glucopyranoside)), the former being present in greater amounts than the latter. The subject of favism has been recently reviewed (Mager *et al.*, 1969). To date, no method has been described to determine the concentration of these glycosides in small quantities of plant material or in plasma. This paper deals with such a method. Research on this subject was called for recently by the United Nations System (Protein Advisory Group Bulletin, 1973).

EXPERIMENTAL SECTION

Method. Vicine and convicine react with Folin and Ciocalteu's phenol reagent to give a blue color. Many other compounds react similarly with the reagent, and it is necessary to prepare a plant extract free from these. Extraction of the plant material with cold trichloroacetic acid solution gives a solution which is free from protein, nucleic acids, and other high molecular weight materials. Treatment of the extract with ether removes trichloroacetic acid together with fat soluble material. The resulting solution still contains tyrosine and dihydroxyphenylalanine, both of which give a strong reaction with phenol reagent, together with other water-soluble material. Treatment with copper carbonate forms copper complexes with amino acids and salts with acids. These are absorbed by alumina, together with other compounds. Under the conditions described, vicine and convicine are not absorbed by the alumina. After centrifugation the clear supernatant is used for the color reaction.

Reagents. *Vicine and Convicine.* These glycosides are not available commercially and were prepared from fresh fava beans by a published method (Bendich and Clements, 1953).

Procedure. *Standard Curve.* Vicine was dissolved in water to given concentrations of 5, 10, 15, 20, 25, 50, 75, and 100 μg/ml. One milliliter of each of the solutions and a water blank were mixed with 1 ml of phenol reagent (Folin and Ciocalteu, 1927) which had been diluted 30 times with water; 2 ml of aqueous 20% sodium carbonate was then added to each and the mixture was shaken for a few seconds. The mixture was left at room temperature for 30 min and its density was then read at a wavelength of 650 nm.

Plant Material. A mixture of 1 g of finely divided plant material, either fresh or dry, was shaken with 10 ml of

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Table I. Optical Densities Measured at 650 nm of Reaction Mixtures Containing Known Quantities of Vicine[a]

	Vicine, μg/ml										
	5	10	20	30	40	50	60	70	80	90	100
Optical density	0.010	0.019	0.038	0.057	0.076	0.095	0.113	0.134	0.153	0.169	0.188

[a] Mean values for ten sets of measurements.

Table II. Concentration of Vicine Equivalent (Vicine Plus Convicine) Found in Four Samples of the Same Fava Bean Flour)

Sample no.	OD at 650 mμ	Vicine, μg/ml	Vicine, mg/g of sample
1	0.152	76.8	9.6
2	0.150	75.2	9.4
3	0.149	75.1	9.4
4	0.154	78.0	9.7
Mean value			9.53 (sd ± 0.50)

Table III. Recovery from Samples of Fava Bean Flour of Added Vicine

Sample no.	Vicine content of sample, mg/g	Added vicine, mg	Total vicine, mg/g	Recovered vicine, mg
1	9.6	10.0	19.2	9.6
2	9.4	10.0	19.1	9.7
3	9.4	10.0	19.0	9.6
4	9.7	10.0	19.2	9.5

10% trichloroacetic acid for 30 min at room temperature. After centrifugation the supernatant was filtered and, after extracting twice with 10 ml of ether, 2 ml of the aqueous layer was diluted to 10 ml. From this protein and trichloroacetic acid free extract, 2 ml was removed and mixed with copper carbonate (50 mg) and aluminum oxide (1 g) and heated in a boiling water bath for 10 min. After cooling, the mixture was mechanically shaken at room temperature for 30 min and then centrifuged. One milliliter of the clear supernatant was then diluted with water to 2.5 ml. From this, 1 ml was treated with diluted phenol reagent and sodium carbonate solution, as described for making the standard curve, and, after 30 min, the color density was read at 650 mμ. The vicine content of the sample was then calculated by multiplying the reading from the standard curve by 125 (the dilution factor from the original sample).

Serum or Plasma. To 3 ml of serum or plasma was added 2 ml of 20% trichloroacetic acid. After mixing and centrifugation, the supernatant fluid was extracted twice with 5 ml of ether to remove the excess acid, and 2 ml of the aqueous layer was treated as for the protein and trichloroacetic acid free plant extract. The reading from the standard curve was equivalent to the vicine content of 0.24 ml of serum.

RESULTS AND DISCUSSION

Table I shows the optical densities found for vicine within the range likely to be present in the materials described. The method cannot differentiate between the two glycosides, but as the color produced by vicine is about double that of convicine, and the latter is present in much smaller amounts in the materials, most of the color produced by a sample will be due to vicine.

To test the reproducibility of the method, it was applied to four samples of the same batch of fava bean flour from Egypt. The results are shown in Table II. The mean value for these samples was 9.53 mg/g (sd ±0.50). Lin and Ling (1962) were able to separate 5 g of vicine from 1 kg of dried fava beans by chemical methods. Table III shows the extent of recovery of 10 μg of vicine added to four 1-g samples of bean flour before applying the method. The recovery of the added material was 96 ± 1%.

In view of the very variable water content of the plant material and the difficulty of completely drying plant material without application of heat, to which vicine is unstable, we suggest that vicine content be expressed in terms of nitrogen content rather than in terms of weight of material.

The simplicity of the method suggested that with slight modification it could be applied to serum or plasma. To date, the method has not been applied to the serum for subjects who have previously ingested fava products. Recovery from sera to which have been added amounts of vicine between 10 and 100 μg/ml has been approximately 95%. Serum or plasma not containing vicine gives a reading of between 0.009 and 0.014/3 ml treated in the described way. This is equivalent to approximately 5 μg of vicine/ml. The nature of the material causing the color formation is not known, and all attempts to remove it from the sample have failed. Its relatively constant level, and the smallness of the error it introduces are probably unimportant, and could be in part corrected for by subtracting 5 μg/ml from results obtained by the described method. This method makes it possible to measure the vicine content of the many varieties of fava beans grown throughout the world, and to determine the effect of climate, habitat, and maturity on vicine content. The effect of processing can be studied, and rate of absorption (if any) of vicine from the digestive system measured. The role of vicine as a causative factor in favism may therefore be more clearly determined.

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