The Enzymic Hydrolysis of Amygdalin

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Chromatographic examination has shown that the enzymic hydrolysis of amygdalin by an almond β -glucosidase preparation proceeds consecutively: amygdalin was hydrolysed to prunasin and glucose; prunasin to mandelonitrile and glucose; mandelonitrile to benzaldehyde and hydrocyanic acid. Gentiobiose was not formed during the enzymic hydrolysis. The kinetics of the production of mandelonitrile and hydrocyanic acid from amygdalin by the action of the β -glucosidase preparation favour the probability that three different enzymes are involved, each specific for one hydrolytic stage, namely, amygdalin lyase, prunasin lyase and hydroxynitrile lyase. Cellulose acetate electrophoresis of the enzyme preparation showed that it contained a number of enzymically active components.

Amygdalin, D(-)-mandelonitrile β -gentiobioside (Haworth & Wylam, 1923), is found in the tissues of species of *Prunus*, and is particularly abundant in the kernels. The kernels are also a rich source of the enzyme system, commonly known as emulsin, which attacks a wide variety of β -glycosidic bonds. The enzymic hydrolysis of amygdalin was first observed by Wohler & Liebig (1837). Other early studies with yeast extracts (Fischer, 1895) and almond emulsin (Armstrong, Armstrong & Horton, 1908; Auld, 1908; Kriebel, 1912) showed that the reaction took place in at least three steps: firstly the β -(1-6') bond of the gentiobiose portion of the amygdalin was split to yield D(-)-mandelonitrile β -glucoside, called prunasin, and glucose; next, prunasin was hydrolysed to (+)-mandelonitrile and glucose; finally the (+)-mandelonitrile was broken down to benzaldehyde and hydrocyanic acid. It was thought that three different enzymes, amygdalin lyase, prunasin lyase and hydroxynitrile lyase acted consecutively on the substrate molecule. Armstrong, Armstrong & Horton (1912) found a high concentration of prunasin lyase in cherry-laurel leaves, which displayed only slight enzymic activity towards amygdalin.

The evidence for the existence of hydroxynitrile lyase stemmed mainly from observations on the influence of emulsin on the synthesis of (+)-mandelonitrile from benzaldehyde and hydrocyanic acid (Feist, 1909; Kriebel & Wieland, 1921; Rosenthaler, 1922). Nordefeldt (1925) demonstrated that many of the earlier observations, made on unbuffered systems, were the result of changes in pH when the emulsin was added to the reaction mixture. However, he also concluded that hydroxy-

nitrile lyase was present in emulsin, and isolated and characterized the enzyme.

Weidenhagen (1932) reviewed the enzymic hydrolysis of amygdalin and, on the basis of his own kinetic measurements, concluded that the hydrolysis was brought about by the action of only one enzyme, β -glucosidase, acting consecutively on the 6-O- β -D-glucopyranosyl-D-glucose bond and the aglucone-O- β -D-glucose bond.

Later reviewers have cast doubt on Weidenhagen's hypothesis of a single enzyme of low specificity able to hydrolyse a wide range of β -glycosidic bonds. In view of the differences in specificity shown by β -glucosidase isolated from different sources (e.g. almond and snail emulsin), Pigman (1944) suggested that β -glucosidase comprised a class of closely related enzymes all showing a specific ability to hydrolyse β -glycosidic linkages. Veibel (1950) examined the evidence on the specificities of almond emulsin and showed that the existence of a pure homogeneous enzyme which would act on more than one glycosidic type had not been proved. Jermyn (1961), in a more general review, reached the same conclusion and, considering aglycone specificity, pointed out that the differences in the effect of *ortho*-substitution in phenyl- β -glucosides on enzymes from various sources precluded the existence of a single β -glucosidase of constant properties.

β -Glucosidase activity in plant tissues is frequently assayed by measuring the rate of hydrolysis of a standard solution of salicin (Veibel, 1950; Baruah & Swain, 1957), and we have used the salicin method for measuring the enzyme activity in the kernels of plums. In such experimental work

allowance must be made for the competitive effect of the natural substrate, amygdalin, which is present in appreciable amounts. As the enzymic hydrolysis of amygdalin is complex and appears to involve consecutive reactions, it was thought that the inhibitory effect of amygdalin on the hydrolysis of salicin might be significant. To explore this possibility, the kinetics of the hydrolysis of amygdalin have been re-examined. The results agree with the views of the earlier investigators, that different β -glucosidases of high specificity do exist.

MATERIALS

Enzyme. β -Glucosidase preparation from almonds, activity 1000 units/mg. (Baruah & Swain, 1957), was purchased from Koch-Light Laboratories Ltd. (Colnbrook, Bucks.).

Substrates. Mandelonitrile was obtained from B. Newton Maine Ltd. (North Walsham, Norfolk), estimated purity 89%. After distillation under reduced pressure, a colourless fraction, estimated purity 97%, was obtained.

(+)-Salicin, amygdalin and gentiobiose were commercial samples.

Prunasin was prepared by the acid hydrolysis of amygdalin, by using the method of Caldwell & Courtauld (1907). It was recrystallized from ethyl acetate until found pure by thin-layer chromatography.

METHODS

Enzymic hydrolyses. Hydrolyses were carried out in flasks in a shaking incubator bath maintained at 30°. All solutions were allowed to equilibrate in the bath before mixing.

Chromatography. Chromatographic separations were performed on thin layers of cellulose (Vomhof & Tucker, 1965) or silica gel (Adachi, 1965), and the components were identified either by treatment with silver nitrate and 0.5*N*-sodium hydroxide in ethanol, or by spraying with 0.5% thymol in 5% sulphuric acid in ethanol and heating for 15 min. at 120°.

Electrophoresis. Electrophoretic separations were carried out on cellulose acetate strips (Oxoid) with either acetate or barbitone-acetate buffers over the pH range 5.0–8.6. Buffer concentrations between 0.025 and 0.1 *M* and potential gradients of about 20 v/cm. were used. After drying, the strips were stained by soaking overnight in 0.001% nigrosine in 2% acetic acid.

Analyses. The expected products from the degradation of amygdalin were prunasin, gentiobiose, glucose, mandelonitrile, benzaldehyde and hydrogen cyanide, and the kinetics of the degradation could best be followed if the concentrations of all of these components were known throughout the course of the reaction. However, chromatographic investigation showed that gentiobiose was not produced in significant amounts during the enzymic degradation, and could be ignored in subsequent analysis.

Cyanide was determined by the colorimetric measurement of the complex formed by cyanogen bromide, pyridine and benzidine (Aldridge, 1945; Russell & Wilkinson, 1959). It was not found necessary to isolate the cyanide by steam-distillation, provided that appropriate blank values were

obtained with the buffers and substrates used. Reducing the bromination time to 30 sec. was found to minimize colour formation due to side reactions with the buffers, and this time was adequate for the complete conversion of cyanide into cyanogen bromide.

Glucose could not be determined by the usual copper methods for reducing sugars because of interference from the cyanide present, but an adaptation of the glucosazone method of Wahba, Hanna & El-Sadr (1956) was found to be convenient and accurate. A portion of the reaction mixture in 0.1 *M*-acetate buffer was pipetted into 3 ml. of acetic acid, which stopped the enzyme reaction. The volume was made up to 8 ml. with 0.1 *M*-acetate buffer, 2 ml. of 5% phenylhydrazine hydrochloride was added, and the solution was heated for 1 hr. in a boiling-water bath for full colour development. The glucosazone showed maximum absorption at 380 m μ . Under the same conditions any benzaldehyde present also formed a phenylhydrazone, absorption maximum 341 m μ . Extinctions were measured at both wavelengths against a reagent blank and calibration standards of glucose and benzaldehyde in 0.1 *M*-acetate buffer were used in each set of determinations. The concentrations of glucose and benzaldehyde were calculated by the usual two-component analysis procedure (Knudson, Meloche & Juday, 1940).

In the degradation of amygdalin, benzaldehyde and hydrogen cyanide are produced in equimolar amounts, and in our analyses the estimated benzaldehyde and cyanide contents were in good agreement.

To complete the analysis of the degradation products a method had to be devised for the estimation of mandelonitrile in the presence of hydrocyanic acid.

The formation of cyanohydrins, as catalysed by enzymes, acids and other compounds, has been extensively studied, but there has been no methodical examination of their decomposition. As the formation of cyanohydrins is an acid-catalysed reaction (Svirbely & Roth, 1953), the kinetics of the hydrolysis of mandelonitrile as a function of pH were studied. It was found that the nitrile decomposes instantaneously at high pH values, so that it could be estimated in a solution by measuring the cyanide concentration before and after alkaline hydrolysis.

A stock solution of mandelonitrile was prepared by dissolving a catch weight of about 0.1 g. in 250 ml. of distilled water. Portions (2 ml.) were immediately pipetted into 50 ml. of 0.2 *M*-acetate or -citrate buffer or 80 ml. of distilled water, and, after addition of hydrochloric acid or sodium hydroxide for the pH ranges 1–3 and 11–12, made up to a final volume of 100 ml. Blanks for each pH value were also prepared, and the pH value of each solution was checked both before and after the experiment.

The samples were placed in an agitating water bath at 30°, and the time was recorded. Portions (5 ml.) were withdrawn at intervals for estimation of their cyanide content by the method already outlined. The method is specific for free cyanide ions; mandelonitrile was found not to react. The alkaline and neutral solutions were acidified before addition of the bromine-water. At fixed pH, the hydrolysis of mandelonitrile conformed to first-order kinetics (eqn. 1).

$$t = \frac{2.303}{k} \log a - \frac{2.303}{k} \log (a-x) \quad (1)$$

With eqn. (1), where *a* is the initial concentration of

mandelonitrile, x is the degree of hydrolysis after time t and k is the specific rate constant, plots of $\log(a-x)$ against t were linear, and specific rate constants for each pH value could be calculated from the slopes.

Svirbely & Roth (1953) studied the effect of acid catalysis on the kinetics of formation of cyanohydrins, and proposed several mechanisms, all having the common rate-determining step

Hence the decomposition of the cyanohydrins is actually a bimolecular reaction, and a plot of the logarithms of the specific rate constants, calculated at fixed pH values, against pH should be linear. Values of the specific rate constant over the pH range 2-7 fitted the regression equation (2) with a correlation coefficient 0.995.

$$\log k = 0.844 \text{ pH} - 7.025 \quad (2)$$

Outside this pH range the reaction was either too slow or too fast to be measured accurately.

A series of twelve analyses on aqueous solutions of mandelonitrile carried out by making the solutions alkaline, neutralizing and estimating the cyanide content gave an average recovery of 99.8% (coefficient of variation 0.85%).

Hence, the concentration of mandelonitrile and hydrogen cyanide in the reaction mixtures could be determined from cyanide estimations before and after alkaline hydrolysis.

The influence of emulsin preparations on the hydrolysis of cyanohydrins has been studied most extensively in those cases where preferential attack on one optical isomer can be demonstrated (Feist, 1909; McKenzie, 1936). Bové & Conn (1961) observed the action of hydroxynitrile lyase on *p*-hydroxymandelonitrile in etiolated sorghum seedlings. Its presence in our sample of β -glucosidase was confirmed by comparing the degree of hydrolysis of mandelonitrile with and without added enzyme. With 0.067 mM-mandelonitrile in 0.1 M-acetate buffer, pH 5.0, the rate of hydrolysis at 30° was found to be 0.13 $\mu\text{mole/l./min.}$ In the presence of 2.5 mg. of β -glucosidase/l. the rate of hydrolysis increased to 1.66 $\mu\text{mole/l./min.}$

RESULTS

Production of cyanide during the hydrolysis of amygdalin. Early experiments with 0.022 M-amygdalin and various concentrations of β -glucosidase seemed to indicate a time-lag of up to 1 hr. before measurable amounts of cyanide appeared in the solution, after which the concentration increased at a steady rate. The rates of cyanide production at different enzyme concentrations did not conform to Michaelis-Menten kinetics.

Further investigation showed that in addition to 'free' cyanide, the hydrolysis solutions contained appreciable quantities of mandelonitrile. This could be readily converted into benzaldehyde and hydrocyanic acid by making the solution alkaline (pH 10), followed by neutralization. Re-examination of the hydrolysis products, estimating free cyanide and mandelonitrile, and the use of higher concentrations of enzyme, showed that the rate of production of both increased during the hydrolysis,

Fig. 1. Production of mandelonitrile and free cyanide during the hydrolysis of amygdalin (5.465 mM) by β -glucosidase (2.5 mg./l.) in 0.1 M-acetate buffer, pH 5.0. Total cyanide (i.e. free cyanide + mandelonitrile) (○) and free cyanide (▲) were estimated.

starting at zero and reaching a steady value after about 30-60 min. (Fig. 1).

Chromatographic examination of the products of hydrolysis. The possible products of the hydrolysis of amygdalin are prunasin, glucose, gentiobiose, mandelonitrile, benzaldehyde and hydrocyanic acid. The last-named three components are volatile, difficult to detect chromatographically and had already been identified and determined in the hydrolysis solutions. The examination was therefore confined to glucose and the various glucosides.

Satisfactory separation of these components was achieved by thin-layer chromatography on both cellulose and silica gel mixed with bisulphite. The definition of the spots was better on silica gel, and the glucosides were more easily detected. The R_f values on cellulose, with formic acid-butan-2-one-2-methylpropan-2-ol-water (15:30:40:15, by vol.) as solvent were gentiobiose 0.05, glucose 0.25 and amygdalin 0.60. On silica gel, with ethyl acetate-acetic acid-methanol-water (60:15:15:10, by vol.), the R_f values were gentiobiose 0.15, glucose 0.30, amygdalin 0.50 and prunasin 0.75. Faint spots were also produced by the acetate buffer, roughly coincident with the amygdalin spot in both cases.

After hydrolysing amygdalin with β -glucosidase in acetate buffer at pH 5.0 and in water, glucose and prunasin were detected. The course of the hydrolysis could be followed chromatographically, the intensity of the glucose and prunasin spots increasing steadily with time. Other sugars were not detected, even after prolonged times or when the enzyme concentration was increased 100-fold. When prunasin was hydrolysed by β -glucosidase, only glucose was found on the chromatograms.

On the other hand, when amygdalin was hydrolysed with 0.1 N-hydrochloric acid at 95°,

glucose, prunasin and gentiobiose were found in about equal amounts.

Gentiobiose was readily hydrolysed by 0.1N-hydrochloric acid, but was not attacked by our sample of β -glucosidase, even at high enzyme concentrations for times up to 30 min. in water and in acetate buffer, pH 5.0. The reference sample of gentiobiose contained a trace of glucose, so that the possibility of very slight enzymic hydrolysis could not be refuted.

Kinetic constants for the enzymic hydrolysis of amygdalin, prunasin and mandelonitrile. As gentiobiose is not produced during the reaction, the enzymic hydrolysis of amygdalin must proceed consecutively. Amygdalin is hydrolysed to prunasin and glucose; the prunasin is then hydrolysed to mandelonitrile and glucose; finally the mandelonitrile is hydrolysed to benzaldehyde and hydrocyanic acid. Thus, in the first stage, the amount of amygdalin hydrolysed is equivalent to the glucose produced. However, each mole of glucose is accompanied by a mole of prunasin, some of which is hydrolysed further to more glucose and its molecular equivalent of mandelonitrile. Hence the degree of hydrolysis of amygdalin can be calculated from the number of moles of glucose, less the number of moles of mandelonitrile, produced in the reaction.

The degree of hydrolysis of prunasin can be calculated directly from the amount of either mandelonitrile or glucose produced during the action of β -glucosidase on pure prunasin.

Similarly the enzymic hydrolysis of mandelonitrile can be estimated from the hydrocyanic acid produced, after allowing for the hydrolysis which occurs at pH 5.0 in the absence of the enzyme. However, as mandelonitrile is only sparingly soluble in water, its hydrolysis can only be examined at very low concentrations, making the exact determination of initial rates difficult.

Michaelis constants and maximum velocities for the three substrates, calculated by the method of least squares from plots of the reciprocals of the initial rates against the reciprocals of the substrate concentrations, are shown in Table 1. The values for mandelonitrile are only approximate, owing to

the difficulty of measuring initial hydrolysis rates, but the Michaelis constant is similar to that obtained by Bové & Conn (1961) for *p*-hydroxybenzaldehyde cyanohydrin (0.55 mm).

Estimation of the products of the enzymic hydrolysis of amygdalin. The concentrations of glucose and mandelonitrile in the hydrolysate were determined at intervals during the reaction, and hence the concentrations of prunasin and amygdalin could be calculated at each stage. The results obtained with an initial concentration of 10.93 mm-amygdalin are shown in Table 2.

Effect of buffer and pH on the enzymic hydrolysis. The behaviour of the enzyme towards different substrates varied with the pH and the type of buffer used in the experiment. Thus, at pH 5.0, amygdalin was hydrolysed more slowly in 0.1M-citrate than in 0.1M-acetate buffer, but with prunasin this effect was reversed. With 10.93 mm-amygdalin, the initial rates were 24.25 μ moles/min. in citrate and 30.75 μ moles/min. in acetate buffer, whereas with 3.39 mm-prunasin, the initial rates were 42.75 μ moles/min. in citrate and 25.25 μ moles/min. in acetate. This difference was reflected in the way in which cyanide was produced in the last step of the hydrolysis of amygdalin. In acetate buffer, the rate of production of cyanide was initially very slow, but

Table 2. *Products of the enzymic hydrolysis of amygdalin*

Results were determined in 0.1M-acetate buffer, pH 5.0, with 2.5 mg. of β -glucosidase/l. and 10.93 mm-amygdalin.

Time of hydrolysis (min.)	Concn. in solution (μ M)			
	Determined		Calculated	
	Glucose (g)	Mandelonitrile (m)	Amygdalin hydrolysed (g-m)	Prunasin (g-2m)
2	60	1	59	58
4	131	5	126	121
6	197	11	186	175
8	278	22	256	234
10	351	32	319	287

Table 1. *Kinetic constants for the hydrolysis of amygdalin, prunasin and mandelonitrile by β -glucosidase*

Constants were determined in 0.1M-acetate buffer, pH 5.0, with 2.5 mg. of β -glucosidase/l. (Coefficients of variation are given in parentheses.)

Substrate ...	... Amygdalin	Prunasin	Mandelonitrile
Michaelis constant, K_m (mm)	1.51 (23%)	1.69 (9%)	0.73
Maximum velocity, V (μ moles/l./min.)	34.67 (10%)	39.80 (7%)	20.28
No. of determinations	5	4	

increased rapidly as prunasin accumulated in the solution. In citrate buffer, the initial rate of production of cyanide was higher, and quickly reached a constant value.

The activity of the β -glucosidase preparation towards amygdalin, prunasin, mandelonitrile and salicin in 0.1 M-acetate buffer was examined over the pH range 3.6–5.8. With salicin, the activity was maximal over the pH range 5.0–5.5. The activity towards mandelonitrile was more sensitive to pH, and reached a well-defined peak at pH 5.4. With both amygdalin and prunasin, the activity increased steadily with pH, levelling off towards pH 5.8.

Electrophoresis of the β -glucosidase preparation. Solutions of the enzyme were subjected to low-voltage electrophoresis on cellulose acetate strips in barbitone buffer, pH 8.6, and acetate buffer, pH 5.0. After 2–5 hr., five distinct protein bands were observed, some of which had several components.

To test the bands for enzymic activity, strips were taken after electrophoresis and divided longitudinally. One half-strip was then stained to reveal the position of the protein bands, so that the bands in the untreated strip could be approximately located, and cut out. Each section was then tested for activity towards amygdalin, prunasin and salicin. Three of the bands, on and near the origin, were enzymically active, but there was too much overlap between bands to allow any definite assessment of their specificity, and too little material for an accurate determination of the ratios of the activities. However, the results indicated that a separation of the enzymes by electrophoresis might be feasible.

DISCUSSION

The chromatographic examination of the products of the enzymic hydrolysis of amygdalin showed that the reactions involved were the hydrolysis of amygdalin to prunasin and glucose, and prunasin to mandelonitrile and a further molecule of glucose. In contrast, when acid hydrolysis was used, the glycosidic bonds were attacked at random, so that in addition to prunasin, glucose and mandelonitrile, gentiobiose was also produced.

If a single enzyme was responsible for both hydrolytic steps, the kinetics could be represented as in eqn. (3) and eqn. (4) where E, A, P, M and G

represent enzyme, amygdalin, prunasin, mandelonitrile and glucose respectively. Considering the hydrolysis of the prunasin as in (4), the amygdalin present behaves as a competitive inhibitor, and

eqn. (5) (Dixon & Webb, 1958) can be derived for the rate of hydrolysis of prunasin (i.e. the rate of production of mandelonitrile) at any particular instant during the reaction. In eqn. (5) v_p is the

$$v_p = \frac{V_p}{1 + \frac{K_{mP}}{p} \left(1 + \frac{a}{K_{mA}}\right)} \quad (5)$$

velocity of hydrolysis of prunasin, p is the concentration of prunasin, a is the concentration of amygdalin, V_p and K_{mP} are the maximum velocity and Michaelis constant for the hydrolysis of prunasin, and K_{mA} is the Michaelis constant for the hydrolysis of amygdalin.

On the other hand, if each hydrolytic step was catalysed by a different enzyme, and competitive effects are for the moment ignored, the rate of hydrolysis of prunasin can be represented by the usual Michaelis equation (6).

$$v_p = \frac{V_p}{1 + \frac{K_{mP}}{p}} \quad (6)$$

In both cases the velocity of the hydrolysis of the prunasin will vary during the reaction as p , and to a much smaller extent a , change. From the analyses performed during the hydrolysis of amygdalin the concentrations of prunasin, mandelonitrile and amygdalin during the reaction could be calculated. Substituting these values in eqn. (5) and eqn. (6), the theoretical rates of hydrolysis of prunasin, v_p , for both one-enzyme and the two-enzyme mechanisms, were obtained at 2-min. intervals during the early stages of the reaction, and velocity-time curves constructed. Approximate values for the theoretical concentrations of mandelonitrile at various stages of the reaction were then obtained by the graphical integration of the velocity-time curves for each mechanism. In Fig. 2 the concentrations of mandelonitrile found experimentally during the hydrolysis of various concentrations of amygdalin are compared with the two theoretical curves.

The experimental data shows that increasing the initial concentration of amygdalin brought about an increase in the rate of production of mandelonitrile. As the kinetic constants for amygdalin and prunasin are only slightly different (Table 1), this would not be expected if both hydrolytic steps were performed by the same enzyme, because the competitive effect of the added amygdalin would be considerably greater than the augmentation of rates ensuing from the higher concentrations of the intermediate products. The calculated curves for the single-enzyme mechanism fall well below the experimental points, and the divergence is greatest at the highest concentrations of amygdalin.

Fig. 2. Production of mandelonitrile during the hydrolysis of amygdalin by β -glucosidase (2.5 mg./l.) in 0.1 M-acetate buffer, pH 5.0. Experimental values (O) are compared with the calculated values, assuming either a single-enzyme (---) or a two-enzyme (—) mechanism. Concentrations (mM) of amygdalin were (a) 10.931, (b) 5.465, (c) 2.733, (d) 1.640, (e) 1.093.

By contrast, the curves calculated assuming a different enzyme for each hydrolytic step are in reasonable agreement with the experimental points, particularly at the highest concentration of amygdalin used. One must conclude that the β -glucosidase preparation contains one enzyme which specifically catalyses the hydrolysis of amygdalin to prunasin, and another enzyme which catalyses the hydrolysis of prunasin to mandelonitrile.

The calculated curves for the two-enzyme mechanism tend to fall slightly below the experimental points. Although exact agreement between the curves is unlikely because of the approximations in the calculations and the cumulative experimental errors, it is surprising that the experimental values for the most part exceed the theoretical results, as this indicates that the presence of even a large excess of amygdalin has no effect on the hydrolysis of prunasin.

The results of a few experiments, with mixed

Table 3. Competitive effects of amygdalin and prunasin on the enzymic hydrolysis of each

Concn. (mM)		Initial velocity of hydrolysis (μ moles/l./min.)	
Amygdalin	Prunasin	Amygdalin	Prunasin
1.10	0	14.2	—
0	3.39	—	27.2
1.10	3.39	3.7	29.0
5.47	0	27.1	—
0	1.70	—	20.8
5.47	1.70	18.2	21.3
10.93	0	32.8	—
0	0.26	—	6.1
10.93	0.26	29.3	4.5

Table 4. Enzymic decomposition of amygdalin to hydrocyanic acid, benzaldehyde and glucose

Results were determined in 0.1 M-acetate buffer, pH 5.0, with 5.465 mM-amygdalin and 2.5 mg. of β -glucosidase/l.

Time of hydrolysis (min.)	Concn. of cyanide (μ M)	
	Calc. on the basis of three enzymes attacking consecutively	Experimental
2	0.04	0.03
6	0.68	1.68
10	2.73	5.95

substrates, to determine the competitive effect of amygdalin on the hydrolysis of prunasin and vice versa are shown in Table 3, and confirm that, in fact, the presence of amygdalin does not appreciably affect the hydrolysis of prunasin. On the other hand the presence of prunasin considerably depresses the hydrolysis of amygdalin, even at high amygdalin/prunasin ratios.

So far, only two stages of the hydrolysis have been considered. The last stage of the reaction, from mandelonitrile to benzaldehyde and hydrocyanic acid, proceeds at a measurable rate even in the absence of enzyme. However, it is also catalysed by a component of the β -glucosidase preparation. The calculated values for the production of hydrocyanic acid, from the three-stage hydrolysis of amygdalin assuming the consecutive action of three different enzymes, are compared with experimental results in Table 4. The calculated results are low, about half the experimental values, but this can probably be regarded as reasonable agreement in view of the possible errors involved. Calculated values for the hydrocyanic acid produced on the basis of a single enzyme catalysing all three hydrolytic stages are infinitesimal.

The inference that three different enzymes, amygdalin lyase, prunasin lyase and hydroxynitrile lyase catalyse successive stages of the total hydrolysis of amygdalin is supported by the other experimental data. Thus the β -glucosidase preparation can be separated by electrophoresis into several proteinaceous components, some of which are enzymically active and possibly represent distinct enzymic species. Similarly, the differences in the effect of citrate and acetate ions on the different stages of the hydrolysis might be expected when a different enzyme is operating at each stage.

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