material. If, however, reaction product is measured, as in the present investigation, a minute fraction of 1% of the original thiol groups can be determined with accuracy.

It is further of interest that although all the other amino-acids investigated (Dale *et al.* 1949*a*) show deamination by X-radiation in varying degree, cysteine was not deaminated. The radiation energy seems to be used, partly at least, for H_2S production and not for deamination.

The instability of glutathione, particularly its property of readily losing its sulphhydryl group as H_2S was commented on by Hopkins (1929). He showed, for example, that during prolonged aeration of an aqueous solution of the reduced form at pH 7.6 (in the presence of a trace of iron) 80% of the thiol form was converted to the disulphide form. The remaining 20% was proved to have lost some of its nitrogen and sulphur. It should be noted that under the conditions of our work no H_2S was detected in the control experiments.

The majority of experiments was deliberately done with cysteine because of its greater stability and the easy suppression of autoxidation in this case. However, the experiments with glutathione prove the essentially similar response to radiation with the one peculiar exception of a depression in the pH curve near pH 5.5. One has to bear in mind, however, that at pH values near neutrality the stability of glutathione is greatly diminished.

It seems that the liberation of H_2S may be of importance from the biological point of view since its occurrence within the cell is likely to have a toxic effect.

SUMMARY

1. Experiments are described on the production of hydrogen sulphide by X-radiation from cysteine and glutathione.

2. The influence of pH, oxygen, hydrogen peroxide, concentration of solute, etc., on the process, has been studied.

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An L-Glutamic Acid Decarboxylase from Barley

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Okunuki (1937) discovered an enzyme in powders prepared from various plant tissues which was specific for the decarboxylation of glutamic acid. He reported that pollen from *Lilium auratum* was a particularly good source of the enzyme, whereas barley seedlings were included in a list of tissues which did not yield active preparations by his method. Schales, Mims & Schales (1946) have since

shown that the enzyme can be extracted by water fairly readily from plant tissues and give a list of some thirty species from which it was obtained. Using aqueous extracts from carrots, Schales & Schales (1946*a*) have investigated the characteristics of the enzyme and the reaction kinetics. The present paper describes some experiments on a similar enzyme occurring in seedling barley roots.

EXPERIMENTAL

Preparation of enzyme. Spratt Archer barley seed, previously soaked for 24 hr., was germinated in thick layers in rectangular glass vessels with the minimum amount of water. In 3 days at room temperature the growing roots had bound the grains together in mats, which were then supported on bent glass stands 2.5 cm. high. The seedlings were maintained in an incubator at 21–24° and were well watered. In this way a heavy crop of roots was obtained which grew rapidly downwards away from the grain. The seedlings continued to grow well for about 15 days, but in most cases the roots were removed after 10 days. They were cut off with a razor, washed in several changes of distilled water and then either (a) treated in a Waring blender with water or a similar amount of 0.5M-phosphate buffer, or (b) ground with a minimum amount of water or buffer in a pestle and mortar. The fibrous residue was separated on muslin and discarded and the remaining solid material centrifuged down at 3000 rev./min. The cloudy supernatant was collected and stored at 0° until required. More concentrated extracts were occasionally prepared by method (a) by blending further roots in the liquid obtained in the first extraction.

Manometry. Conventional Warburg technique was employed to measure CO_2 outputs at 30° after addition of glutamate from the side arms at zero time. The solution containing the enzyme was placed in the main chamber of the vessel and was maintained at pH 5.2 by addition of 0.5M-phosphate buffer. The gas phase in the experiments reported here was air, since some preliminary comparisons had shown that there was little difference in activity under aerobic and anaerobic conditions.

RESULTS

Experiments with untreated aqueous extracts

Effect of amount of enzyme on decarboxylation. The extracts alone showed a very slow evolution of CO_2 and the addition of glutamate immediately led to substantial increases in the rate, whereas additions to boiled extracts were without effect. Fig. 1 shows the result of an experiment in which sufficient glutamate to give a final concentration of 0.01M was added to different amounts of extract. In all cases the decarboxylation slowed down well before the theoretical yield of CO_2 (560 μl .) had been evolved; there was only 56% decarboxylation in *a* which received 2 ml. of extract, four times the amount supplied in *c*. Okunuki (1937) and Schales *et al.* (1946) observed that curves of this type were typically produced in their experiments with aqueous extracts and that the CO_2 yields were frequently less than one-third of those equivalent to the added glutamate. In considering their suggestions for the cause of this, it is significant that, with increasing amounts of enzyme, the decarboxylation proceeded further. More concentrated extracts have since been obtained in which the CO_2 output in 60 min. was 80% of that calculated (Fig. 2).

Stability of the enzyme during the reaction. Samples from different batches of roots varied in their ability to withstand a prolonged period at 30°, even in the

absence of substrate. It was sometimes found that, if the addition of the glutamate was delayed for 2–3 hr., considerable thermal inactivation had

Fig. 1. The effect of decreasing amounts of untreated extract on the decarboxylation of 0.01M-glutamate: *a*, 2 ml. extract; *b*, 1 ml. extract; *c*, 0.5 ml. extract. Volumes adjusted with 0.5M-phosphate, pH 5.2.

Fig. 2. The effect of delayed addition of glutamate to 2 ml. extract: *a* and *b* received 0.5 ml. 0.02M-glutamate at the outset, *c* had no addition; 0.5 ml. 0.02M-glutamate was added to *a* and *c* after 60 min., but not to *b*.

occurred, whilst in other cases only small losses were encountered during the same period. The results of an experiment designed to test the possibility that the enzyme was inactivated during the reaction or inhibited by accumulation of end product are shown

in Fig. 2. To two samples, *a* and *b*, of a concentrated extract was added 0.5 ml. of 0.02M-glutamate, while to a third, *c*, there was no addition at the outset. There was a vigorous CO_2 output from *a* and *b* and at the end of 60 min., when this was slowing down, a further 0.5 ml. of glutamate was added to *a* and also to *c*. The CO_2 output from *c* showed that some thermal inactivation had occurred during the first hour (30 min. output 115 μl ., initial value for *a* and *b* 143 μl .). The similar output from *a* after the second addition (99 μl . in the period 60–90 min.) shows that the enzyme had not been appreciably inactivated during the decarboxylation and was not markedly inhibited by the accumulation of end product which had occurred in the first 60 min.

The effect on activity of age of roots. Extracts made by method *a* from roots of different ages did not vary greatly. The activity of samples taken daily from a crop between the fifth and thirteenth days of growth, expressed as $\mu\text{l. CO}_2$ from 0.002M-glutamate/mg. dry weight of root/10 min., ranged between 2.0 and 3.2. Second extracts made from the residues showed about one-third of the activity of the initial extracts.

Attempts to precipitate the enzyme. Gale (1946) has employed the technique of drying bacterial suspensions with acetone as the principal method of preserving the decarboxylases in a solid state. When water extracts containing enzyme from barley were treated with acetone, a precipitate formed which was quite inactive towards glutamate when dried and redissolved in buffer. Similarly, dry powders prepared from roots by acetone treatment did not contain active enzyme. Ethanol treatment also yielded inactive preparations.

The only precipitant found to be effective was ammonium sulphate. Precipitates obtained by adding an excess of ammonium sulphate to extracts showed considerable activity when redissolved, even without prior removal of salt included with the unwashed precipitate. In one such experiment 62% of the total activity of the extract was found in the ammonium sulphate precipitate. Since no increase in activity occurred when the solutions of the precipitates were dialysed against water for 24 hr., it seems that small amounts of ammonium sulphate do not affect the activity of the enzyme. It has, in fact, been shown that ammonium sulphate in concentrations as high as 0.2M has little inhibitory effect; in one experiment the CO_2 output from 0.004M-glutamate in 30 min. was reduced from 117 to 103 μl . Prolonged contact with the saturated salt solution is not injurious, and the best means of preserving the enzyme found so far has been to leave it as a precipitate in the ammonium sulphate solution at 0° until required. It is stable for at least 14 days in this condition. Little separation from other proteins was achieved by using different ammonium sulphate

concentrations, as almost all of the proteins, including the decarboxylase, precipitated at 25–50% saturation. Attempts to separate the enzyme by precipitation at different pH values were made with little success. Some precipitates formed at pH 3.2 and 8.5, but they did not contain the enzyme, and experiments on the supernatant re-adjusted to pH 5.0 showed that losses in activity of over 70% had occurred at these pH levels. Most of the further work was carried out using the crude precipitate, prepared by saturating the water extract with ammonium sulphate, dissolved in 0.5M-phosphate buffer pH 5.2. Such preparations had the following advantages over aqueous extracts: (1) Samples of closely similar activity could be taken from a bulk preparation on successive days. (2) Considerable concentration of the enzyme could be achieved. (3) Water-soluble amino-acids, known to be present in the original extracts, did not occur in the precipitate.

Experiments using redissolved precipitated enzyme

Course of reaction. When glutamate was added, the initial part of the CO_2 output curve was linear and, in contrast to the behaviour of untreated extracts, did not show early flattening. In Fig. 3 are shown CO_2

Fig. 3. The course of decarboxylation by 2 ml. redissolved $(\text{NH}_4)_2\text{SO}_4$ precipitate of three different preparations of glutamate. Concentration of substrates 0.01 M.

outputs with three different (laboratory reagent) preparations of glutamate, and it is seen that the decarboxylation was maintained at a falling rate until 88–94% of the calculated yield of CO_2 had been produced in 240 min.

Effect of pH. Samples (1 ml.) of a precipitate which had been redissolved in water were added to 1 ml. of 0.5M-buffer at various pH values in manometers. CO₂ outputs during the first 10 min. after tipping 0.5 ml. of 0.1M-glutamate were recorded and the amounts by which these exceeded outputs from control systems at the same pH have been used to establish the pH activity curve shown in Fig. 4. No correction has been made for CO₂ retained at the higher pH levels. The optimum pH range is 5.0-5.5, which is near the values previously reported, and there is little activity outside the range pH 3.5-7.5.

Fig. 4. The effect of pH on the activity of the enzyme. The points are 10 min. CO₂ outputs from 0.02M-glutamate.

Effect of substrate concentration. This section has been written in conjunction with W. L. Howard, Rhodes Scholar from the University of Oklahoma, U.S.A., at present in the Department of Botany, Oxford. Schaales and his colleagues have generally used concentrations of glutamate of about 0.06M in their experiments and have reported (Schaales & Schaales, 1946a) that the enzyme from carrots has a Michaelis constant of $3.6 \pm 0.4 \times 0.001$ M. In making their calculations these authors have introduced methods to correct for the fact that their preparations gave rates of CO₂ output which were already falling during the first few minutes of the reaction.

With the previously precipitated enzyme the CO₂ outputs were virtually linear at the outset and 10 min. values provide a valid measure of the initial rate. A series of experiments with graded glutamate concentrations gave the results shown in Fig. 5. The points are the observed initial rates at the corresponding glutamate levels and it is clear that they increase to a maximum and then decrease as the substrate concentration increases, indicating inhibition by excess substrate. The activity-substrate

concentration curve is clearly not a rectangular hyperbola, and calculations based on the application of the simple Michaelis formula are not possible in the case of the barley enzyme.

Fig. 5. The effect of glutamate concentration on the rates of decarboxylation. The points are the observed 10 min. CO₂ outputs. The line is calculated from the equation

$$v = V[S]/([S] + K_s + [S]^n/K_2),$$

using the values given in the text.

Using the analysis proposed by Lineweaver & Burk (1934), the data can be shown to agree with the mechanism of inhibition by formation of a complex of one enzyme molecule with two of substrate,

the kinetics of which is described by the equation

$$v = V[S]/([S] + K_s + [S]^n/K_2),$$

where v = initial velocity, V = maximum initial velocity, K_s = dissociation constant of the enzyme-substrate complex (ES), n = number of substrate molecules in the inactive complex and K_2 = dissociation constant of this inactive complex. The curve in Fig. 5 is calculated from this equation using the values $V = 245 \mu\text{l./10 min.}$, $K_s = 9.6 \times 0.001$ M, $K_2 = 1.93$ M and $n = 2$ which have been determined from the primary data. The close agreement of the observed points and the calculated curve strongly indicates the correctness of the proposed mechanism and the reliability of the calculated constants.

Effect of temperature on stability. When redissolved, the enzyme lost activity over a period of days, even when stored at 0°, and was very rapidly inactivated at temperatures above 50°. Fig. 6 shows the effect of pretreatment for (a) 5 min. and (b) 10 min. at various temperatures on the activity of the enzyme when it was subsequently measured at 30°. It is clear that some destruction results at temperatures well below 50° during relatively short exposures. Attention has already been drawn to the losses which occur in experiments at 30°. At 37°, therefore, the temperature used by the American authors, even greater losses might be expected.

Specificity. The enzyme did not decarboxylate the closely related compounds glutaric acid and glutamine, and also had no measurable effect on the following amino-acids: L-arginine, L-aspartic acid, L-histidine, L-tryptophan and L-tryosine.

Inhibitors. In its reactions to inhibitors the behaviour of the enzyme was closely similar to the examples already described. Thus, at pH 5.2 in the presence of 0.002M-semicarbazide the rate of decarboxylation was reduced by 86% and, in 0.001M-semicarbazide, to 63% of the control. 0.00012M-Hydroxylamine produced 94% inhibition and 0.0004M-cyanide complete inhibition. Carbon monoxide was without effect.

Fig. 6. The effect of pretreatment at the temperatures indicated for (a) 5 min. and (b) 10 min. on the subsequent activity of the enzyme measured at 30°. The points represent 10 min. CO₂ outputs from 0.02M-glutamate.

Identity of the product of decarboxylation. Gale (1940) has shown that γ -aminobutyric acid is the product when glutamic acid is attacked by the bacterial carboxylase, and this acid has now been satisfactorily identified from barley enzyme digests.

On paper chromatograms with phenol-water as the mobile phase, it was found that crude water extracts from barley contained several amino-acids, or ninhydrin-reacting substances, which tended to mask on the paper the effects of added glutamate. The previously precipitated enzyme, however, did not contain any free amino-acids and gave clear blanks on the chromatograms. Samples were removed at intervals from a flask in which decarboxylation by this enzyme preparation was proceeding at a known rate, and run on chromatograms. It was found that as the reaction progressed a ninhydrin-reacting substance appeared at R_F 0.72 and increased in intensity while the glutamate spot (R_F 0.27) was disappearing. When decarboxylation was complete, the sample showed that the glutamate had entirely disappeared and that the spot at R_F 0.72 was then strongly developed and was the only one present. Parallel runs with γ -aminobutyric acid showed identical R_F values and only one spot was observed on the chromatogram when this acid was added to a digest sample which had completely decarboxylated the added glutamate.

DISCUSSION

The demonstration of an active glutamic acid decarboxylase in barley, after Okunuki's negative finding, can probably be explained by the fact that different methods of extraction were employed. Schales *et al.* (1946) have already shown that Okunuki failed to detect the enzyme in some tissues because he used preparations from which soluble matter had been removed, and the barley enzyme is quite soluble.

The enzyme is fairly stable for short periods at 30°, but thermal inactivation may be one of the contributory factors when incomplete decarboxylation is observed with untreated extracts. Schales & Schales (1946a) suggested that the accumulation of end product might be the cause of the falling rates, but the inhibitory action of the end product (now shown to be γ -aminobutyric acid) has been demonstrated to be small, since glutamate added after a period of decarboxylation was attacked at a rate almost equal to that in a control system. A further possibility is that, in extracts possessing low decarboxylase activity, other reactions which also consume glutamic acid may significantly reduce the substrate concentration and therefore the yield of CO₂.

It seems, however, that if sufficiently active preparations (e.g. the redissolved precipitates mentioned) are used, quantitative decarboxylation can be nearly approached without difficulty. Schales & Schales (1946b) have now shown that complete and specific decarboxylation of glutamate can be achieved using preparations obtained by freeze drying squash tissue.

In calculating a value for the Michaelis constant, using experimental data for the redissolved enzyme, it was found necessary to make allowance for inhibition by excess substrate. A value somewhat higher than that recorded for the carrot enzyme was obtained. A consequence of the relatively high Michaelis constant is that the rate of CO₂ output begins to fall some time before decarboxylation nears completion. The evidence of the different Michaelis constants alone does not show that the carrot and barley enzymes are basically different, although this remains a possibility. In many of their other properties the enzymes are quite similar.

The wide occurrence of glutamic acid decarboxylase (Schales *et al.* 1946) and its substrate in plants in all probability explains the origin of γ -aminobutyric acid. This acid has now been isolated in quantity from beetroot (Westall, 1950) and also from apples (Hulme & Arthington, 1950). Dent (1948) gives evidence for supposing that an unidentified component of the amino-acids from potato (described earlier by Dent, Stepka & Steward, 1947) is γ -aminobutyric acid. It might be expected that the more

precise methods of analysis now available will reveal the widespread occurrence of γ -aminobutyric acid in small amounts.

SUMMARY

1. Aqueous extracts from barley roots contain an active L-glutamic acid decarboxylase, but do not decarboxylate L-aspartic acid, L-arginine, L-histidine, L-tryptophan or L-tyrosine at measurable rates.

2. Active precipitates can be obtained from aqueous extracts by treatment with ammonium sulphate, and the enzyme is stable in this state.

3. Almost quantitative decarboxylation can be achieved using redissolved precipitates and the

product has been identified by chromatography as γ -aminobutyric acid.

4. A calculation of the Michaelis constant has given a value of 9.6×0.001 M, allowance being made for inhibition by high substrate concentrations.

5. The pH relationships and reactions to inhibitors of the barley enzyme are similar to those previously described.

6. It is suggested that the natural occurrence of γ -aminobutyric acid may be ascribed to the functioning of the enzyme *in vivo*.

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The Component Acids and Glycerides of a Horse Mesenteric Fat

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Until recently no comprehensive account of the component acids of fats from the horse has been recorded. Nevertheless, the general studies of Heiduschka & Steinruck (1921) and of Schuette, Garvin & Schwoegler (1934) showed that horse body fats differed strikingly from those of oxen or sheep in containing relatively small proportions of stearic acid and definite amounts of the linoleic (octadeca-9-12-dienoic) and linolenic (octadeca-9:12:15-trienoic) acids which are present in grass fats (and indeed in many other vegetable fats):

acids of lower molecular weight than C_{16} acids, and 11 % of unsaturated acids of lower molecular weight than C_{18} acids): the low proportion of stearic acid, and the presence of linoleic and linolenic acids, are again noteworthy and contrast with the higher proportions of the former acid, and the substantial absence of linoleic and linolenic acids, in cow or sheep milk fats.

It was thus felt desirable to undertake a further detailed examination of a horse body fat, not only in relation to its component fatty acids, but especially

	% (w/w)				
	Palmitic	Stearic	Oleic	Linoleic	Linolenic
Horse body fat (Heiduschka & Steinruck, 1921)	29	7	55	7	2
Horse abdominal fat (Schuette <i>et al.</i> 1934)	28	5	50	13	4

A detailed analysis of horse milk fat (Hilditch & Jasperson, 1944) showed its acids to contain 16 % palmitic, 3 % stearic, 19 % oleic, 8 % linoleic and 16 % linolenic acids (in addition to 22 % of saturated

in regard to its component glycerides. Whilst this work was in progress two further important studies of horse fat component acids have appeared, in both of which the more recent techniques of separa-