CARBOXYLASE AND COCARBOXYLASE IN BARLEY

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The presence of carboxylase in barley powders prepared by rapid drying and grinding has been shown previously by James & Norval (1938). Further information about the enzyme, especially its separation into an apo- and co-enzyme, is reported in this paper.

Methods

Sap and other preparations described below, together with additional substances to serve as substrates and a few crystals of thymol as antiseptic, were placed in incubation tubes. These wide tubes were fitted with a rubber bung carrying finer inlet and outlet tubes with screw clips, as used by James & Norval in their original experiments. The atmosphere of the tube was then replaced with nitrogen by exhausting, refilling with nitrogen bubbled through the sap or other preparation, and repeating this process a second and a third time. The commercial nitrogen used was freed from oxygen and carbon dioxide by passing it through three Pettenkofer tubes filled with alkaline pyrogallol. The clips were then closed and the tube shaken in a bath at 30° C. for the experimental period of 3 hr. This period was taken from the moment of closing the exhaustion flask after the last exhaustion, to the moment of opening the tube for the CO_2 determination. It was not possible to ensure that this period was always precisely 180 min., but the period was accurately timed, and if it exceeded 185 min. a proportional correction was made in the results. Below this time, the correction involved (5 %) was less than the overall accuracy of the method.

At the end of the experimental period, CO_2 was measured by the micromethod of James & James (1936). A CO_2-free air stream (passed through a soda-lime tower and a baryta tell-tale) is drawn through the liquid by means of an evacuated suction flask containing 25 ml. of $N/150$ baryta, and the CO_2 absorbed by the baryta is determined by titration with $N/150$ HCl.

For the present series of experiments the method was varied at two points. First, the liquid in the tube was strongly acidified before the estimation of CO_2, by allowing 2–3 ml. of 2 % phosphoric acid to be drawn into it from a 10 ml. graduated pipette. This was inserted into the inlet tube, before connection was made for the flushing air stream. The possibility of retention of CO_2 by the liquid was thus reduced. The addition of acid in this way also enables the first slight opening of the tube to be detected immediately by the movement of the liquid in the pipette, and so aids materially in preventing a sudden rush of air which otherwise

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may ruin a determination by carrying baryta from the tell-tale into the tube, or material from the tube into the suction flask.

Secondly, capryl alcohol is added to stop frothing. This is done by adding 3 ml. capryl alcohol to 100 ml. stock 2 % phosphoric acid, which is then vigorously shaken before the acid is taken up into the pipette.

As a precaution against gas leaks, it was found advisable to seal all permanent rubber-to-glass joints with collodion and to smear all stoppers lightly with vaseline before fitting. The rubber pressure tubing used to join the incubation tubes into the titration apparatus was similarly treated. All the glass parts, such as the burette, incubation tubes, gas inlet and outlet tubes and especially the suction flask in which the titration is finally carried out, must be cleaned regularly with hot chromic acid. Failure to do this soon results in loss of accuracy. Using the precautions named, determinations on duplicate tubes containing aliquots of a given preparation usually agreed within 0·1 ml. *N*/150 HCl. Purely chemical duplicates, as in the determination of titration blanks, checked to within one part in a thousand. Under these circumstances, the method could be used over a range of 0·03 —→ 1·0 mg. CO_2 with 5 % accuracy at 0·3 mg. The pulpy consistency of some of the preparations used made manometric methods unsuitable, but did not affect the accuracy of the method employed.

Glass-distilled water was used both for final washings of the apparatus and for all experiments.

The use of thymol. In spite of the relatively short incubation period of 3 hr., satisfactory duplicates could not always be obtained without the use of thymol as antiseptic. The addition of thymol only slightly reduced the amount of CO_2 obtained from the tubes, showing, in agreement with the experience of James & Norval (1938), that action by micro-organisms over such a period was small. The use of thymol in the present series of experiments did somewhat reduce the experimental error. No reason was found to suppose that it interfered in any way with with enzymatic reactions being examined.

Material

Barley sap was prepared from green seedling plants, 12–14 days old and 6–7 in. high. These were cut in bulk and in the majority of experiments were wrapped in small lots in muslin and frozen solid in the refrigerator at temperatures down to − 12° C. They were then thawed out at room temperature and pressed out by hand through muslin. The sap so obtained was light brown to yellow in colour and slightly turbid. It was left overnight in a tall cylinder at about 2° C. when slight sedimentation occurred and the sap became quite transparent although still retaining its colour. This sap was used without further treatment.

In certain later experiments sap was prepared by grinding the young plants to a pulp in a mechanical pestle and mortar and pressing it out through muslin. This yielded a dark green liquid which slowly deposited a bulky green precipitate after some hours' standing, or after a period of centrifuging, leaving a clear brown or yellow liquid. This sap was either shaken up and used raw, or else was centrifuged

free from the green solids. No qualitative difference was noticed in the experimental behaviour of the sap whichever of these procedures was adopted, but the pulped sap appeared, when fresh, to be more active than that obtained by freezing. This difference was reduced by keeping.

Thymol, at the rate of 0·3 g./100 ml., was added to all liquid preparations from barley, to minimize deterioration on keeping.

RESULTS

Carboxylase activity of frozen sap

The CO_2 emission of sap pressed from thawed barley is low, ranging from 0·03 to 0·15 mg./5ml./3 hr.

Table 1, column 3 gives figures of several estimations with different samples of sap.

Table 1. CO_2 *emissions in mg./5 ml./3 hr. of frozen barley sap, with and without pyruvic acid*

Exp. no.	Mg. pyruvic acid added	Emission without pyruvic acid	Emission with pyruvic acid
1 (fresh sap)	—	0·14	—
2 ,,	—	0·08	—
3 ,,	—	0·15	—
4 ,,	50·0	0·03	0·13
5 ,,	10·0	0·12	0·20
6 (sap kept 5 days)	15·0	0·08	0·09
7 (sap kept 6 days)	15·0	0·08	0·08

(All CO_2 figures in the above table represent the mean of closely checking duplicates.)

The increase of activity on addition of pyruvic acid (columns 3 and 4) is variable in amount, but is always observed in freshly prepared sap. It falls off considerably on keeping, and sap stored for 5 or 6 days in the refrigerator (experiments 6 and 7) showed no increase over the spontaneous CO_2 emission rate when pyruvic acid was added. It is probable that the carboxylase system itself had broken down on standing. It could not have been already saturated with pyruvic acid in the sap itself, since these saps have been shown to contain no appreciable amount of the acid (James *et al.* 1941). An unduly prolonged period of freezing of the barley during the preparation of the sap also appears to have a slight adverse effect on its enzymes, though it is impossible to particularize which enzyme is affected, since CO_2 emission is reduced both with and without addition of pyruvic acid.

Table 2. *Milligrams* CO_2 *emitted by 5 ml. sap + 0·2 g. barley powder in 3 hr. at* 30° C.

Duration of freezing in hr.	Without pyruvic acid		With 15 mg. pyruvic acid	
	Digest A	Digest B	Digest C	Digest D
10	0·27	0·27	0·45	0·44
24	0·20	0·21	0·42	0·41

The carboxylase activity of barley powder

The low CO_2 emissions observed in the experiments with sap + pyruvic acid suggested that the extraction of the carboxylase system was very incomplete. There was even a possibility that the pressing out of the sap might have brought out a soluble prosthetic group (cocarboxylase), leaving the remainder of the system (apo-enzyme) wholly or partly in the solid residue.

In powder prepared from leaves without the removal of anything but water, the system should be complete and its full activity able to be developed. Such a powder was prepared by drying young green leaves rapidly in a fast air stream warmed to 30° C. As soon as the leaves were brittle they were ground in a mechanical mortar to a fine powder which was suspended in glass-distilled water during incubation. The amount of fresh barley which yielded 5·0 ml. of sap gave approximately 0·5 g. of this powder, and these quantities of sap and powder are therefore treated as equivalent. The carbon dioxide emissions of sap and powder simultaneously prepared from a single picking of barley leaves, are shown in Table 3.

Table 3. *Milligrams* CO_2 *emitted in* 3 *hr. at* 30° C.

	Without pyruvic acid			With 15 mg. pyruvic acid		
	A	B	Mean	C	D	Mean
0·3 mg. powder + 5 ml. H_2O	0·17	0·17	0·17	0·29	0·27	0·28
5 ml. sap	0·09	0·06	0·08	0·09	0·10	0·10
0·5 g. powder (calculated, ≡ 5 ml. sap)	—	—	0·28	—	—	0·47

It is clear, therefore, that the powder is very much more active in releasing CO_2 than the sap, both from its own contained substrates and from the added pyruvic acid (= carboxylase activity).

Cocarboxylase

The separation of a soluble co-enzyme (cocarboxylase) and an insoluble apo-enzyme ('protein') was achieved as follows: Seedling barley was frozen, thawed and its sap pressed out in the usual way. The solid residue was washed out with five times its own weight of water and the washing repeated four times in all. The four extracts thus obtained were combined and reduced to a small volume by evaporation under reduced pressure and then added to the sap. The washed residue

Table 4. *Milligrams* CO_2 *emitted in* 3 *hr. at* 30° C. *from* 15 *mg. pyruvic acid*

	A	B	Mean
5 ml. sap	0·06	0·06	0·06
0·2 g. washed powder suspended in 5 ml. glass-distilled water	0·08	0·09	0·09
Sum	0·14	0·15	0·15
0·2 g. powder suspended in 5 ml. sap	0·18	0·21	0·20

was dried in a rapid air stream at 30° C., ground and sieved through muslin. Three digests were set up in duplicate. The first pair contained 5 ml. sap; the second 0·2 g. of the washed powder suspended in 5 ml. glass-distilled water; and the third the same amount of powder suspended in 5 ml. sap. 15 mg. pyruvic acid were added to each digest as a substrate for carboxylase activity.

The sap and powder when incubated together gave off substantially more CO_2 than the sum of their emissions when incubated apart. In other words, there was at least a partial separation of a soluble cocarboxylase in the sap and washings, from a necessary apo-enzyme left in the powder.

The action of cocarboxylase from yeast

Yeast cocarboxylase in solution[1] was first tested in a washed yeast suspension and found to be active in increasing its CO_2 output. Further aliquots of the same preparation were then applied to barley as follows: Sap was pressed out from frozen barley in the usual way and the residue rapidly dried and ground. Part of the powder was then given two rapid washings with $N/10$ Na_2HPO_4 followed by one with distilled water, to complete the removal of the barley cocarboxylase. This procedure greatly reduced the powder's carboxylase activity, which was only very partially restored by an addition of the yeast cocarboxylase.

Table 5. *Milligrams* CO_2 *emitted in* 3 *hr. at* 30° C. *from* 15 *mg.* Na *pyruvate*

	A	B	Mean
0·5 g. unwashed powder + 12 ml. water	0·26	0·26	0·26
0·5 g. *washed* powder + 12 ml. water	0·12	0·12	0·12
0·5 g. *washed* powder + 12 ml. water + 10γ cocarboxylase	0·15	0·15	0·15

It was thought that the failure to restore full activity by the addition of yeast cocarboxylase might result from damage to the barley apo-enzyme by the alkaline washings. The conditions were, therefore, varied by using only a single brief alkaline washing on dried seedling leaves, thus omitting the freezing and pressing and one alkaline washing. The alkaline washing was followed by a distilled water washing as in the previous experiment and a similar series of digests was set up. While a similar decline of activity was found to follow the washing no restoration of activity could be brought about in this case by the addition of yeast cocarboxylase, possibly owing to a deficiency of Mg or other divalent metals. We are therefore unable without further work to compare the identities of the yeast and barley co-enzymes.

The thiochrome test for cocarboxylase

The presence of a cocarboxylase in barley sap was next successfully investigated by means of the thiochrome reaction. 70 g. barley seedlings, grown for 14 days in the dark to prevent the formation of the pigments of the chlorophyll com-

[1] Given to us by Prof. R. A. Peters to whom we are greatly indebted.

plex, were frozen and thawed out and the sap expressed. The sap was neutralized to litmus with barium hydroxide solution (0·35 *N*) and brought rapidly to the boil for a few seconds. It was then cooled and filtered and a drop of saturated ammonium sulphate solution was added, to remove excess barium. The precipitate of barium sulphate was removed on the centrifuge, and the clear yellow liquid thus obtained was examined in the light of a screened mercury-vapour lamp after oxidation by alkaline ferricyanide. The existence of the oxidation product with blue fluorescence was observed. The writers are indebted to Mr Kinnersley, of the Department of Biochemistry, for the estimation of the amount of cocarboxylase present; he found the equivalent of 30γ cocarboxylase per 100 g. original tissue.

Summary

1. The carboxylase activity of barley powders was confirmed, and a smaller activity demonstrated in clear saps.

2. The activity of a mixture of sap + residual powder was greater than the sum of their separate activities. From this it was concluded that the carboxylase system of barley includes an insoluble apo-enzyme and a soluble co-enzyme, like that of yeast.

3. Cocarboxylase from yeast did not fully restore the activity of washed barley powders.

4. Clear barley saps gave a positive fluorescence test for cocarboxylase. This method yielded an estimate of 30γ (as diphosphothiamine) per 100 gm. of original barley tissue.

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