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Methionine sulphoximine and the growth of the wheat embryo

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Besides having a neurotoxic action methionine sulphoximine, the toxic principle produced by the action of nitrogen trichloride (agene) on the protein of wheat (Bentley, McDermott, Moran, Pace & Whitehead, 1950), has been reported to depress or inhibit the germination of seeds, the larval development of insects, the growth of certain bacteria (Pace & McDermott, 1952), the synthesis of protein by cells in culture (Rabinovitz, Olson & Greenberg, 1956), the growth in culture of bone rudiments and the development of chick embryo (Mellanby, 1956). It has also been shown that the depressing effect on the growth of bacteria and the synthesis of protein by cells in culture can be prevented by the provision of glutamine or methionine to the growing cells (Heathcote, 1949; Heathcote & Pace, 1950; Rabinovitz *et al.* 1956). The experiments described in this paper show that methionine sulphoximine is also markedly toxic to the wheat embryo and that this toxicity can be nullified by the presence of glutamine.

EXPERIMENTAL

The effects were demonstrated by growing the embryos on sterile media containing methionine sulphoximine. Grain, variety Squarehead's Master, of good germinating vigour was sterilized for 1 sec in 70% alcohol, allowed to dry and then placed in a moist chamber for 2 or 3 days until the embryos, inclusive of scutellum, were just moist enough to be removed cleanly without damage. They were allowed to dry and immediately before being placed on the medium were sterilized in 70% alcohol, the excess being removed on sterile filter-paper. Two types of medium were used, one a simple synthetic similar to that described by Purvis (1944), containing dextrose 2%,

agar 0·7%, and mineral salts to which methionine sulphoximine or some other substance was added. The other type contained dextrose 2%, agar 0·5% together with 10% of wheat flour or a hydrolysate prepared from wheat flour. The hydrolysate was prepared by making a 10% suspension of flour in water, adding 0·5% of Armour's pancreatin, adjusting to pH 8 with ammonia, adding a few drops of toluene and digesting for a week at 25°, maintaining the pH at 8. The starchy residue was removed by centrifuging and the supernatant liquid was used as the liquid part of the medium. The pH was adjusted to 5 with citric acid, since bacteria were difficult to exclude while the embryos were being placed on the media.

Tubes were set up containing slopes of the synthetic medium, the embryos being placed on the surface. With the hydrolysate medium, however, such a high concentration of agar (1·5%) was needed to prepare slopes that the roots could not penetrate it and the embryos were lifted from the surface. The embryos were therefore supported 4 or 5 mm above the surface on strips of filter-paper sterilized in the tubes and pushed into the medium (Pl. 2). With this method of support solid medium is unnecessary; root growth, however, though satisfactory on liquid synthetic medium was only successful on the hydrolysate medium when agar was added.

Further, a total acid hydrolysis of the protein was carried out by boiling the supernatant liquid after enzyme digestion for 24 h with the addition of HCl at a concentration of 6N. Removal of HCl by distillation, neutralizing with ammonia, decolorizing and desalting by electrodialysis were carried out by the usual methods, but satisfactory growth was never obtained with this hydrolysate.

The embryos, shaded from direct sunlight, were grown at normal temperatures in the laboratory; further shading of the roots appeared to have no effect.

D- and L-methionine sulphoximine were prepared by Dr D. G. H. Daniels in this laboratory from D- and L-methionine obtained by methods described (Bentley, McDermott & Whitehead, 1951; Brenner & Kocher, 1949; Wretlind, 1950). Judged by its optical rotation, the D-methionine contained a small amount, of the order of 5%, of the L-form, which would be converted to sulphoximine and contaminate the D-sample. L-glutamine was obtained from a commercial sample; its rotation agreed with published figures (Fruton, 1946).

RESULTS

The effect of methionine sulphoximine could be demonstrated at a concentration in the medium of 2 p.p.m., although the concentration required to stop growth completely appeared to depend upon the vigour of the seedlings. Thus, in one series 2 p.p.m. caused growth to cease more abruptly than 10 p.p.m. with a different sample of grain (Pl. 1, 1). Likewise, two of the seedlings shown in Pl. 1, 1 had developed to the stage of emergence of the first leaf and one or two secondary roots, whereas growth had stopped at an earlier stage in the remainder. The only detectable morphological change in the affected seedlings was a swollen and roughened appearance in the normally smooth surface of the roots at the meristematic region just behind the tip, apparently the result of uneven proliferation of tissue. It was not confined to the cortex but was visible also in the stelar cylinder, which is

readily separated by dissection. Secondary roots were initiated within 1 or 2 mm of the tip at about the same time and the same distance from the base as the first secondaries in the controls. The tip in the controls, however, was 2 or 3 cm ahead of the secondaries at this time. The extent of growth of the secondaries varied with the vigour of the seedling; it frequently did not proceed beyond the stage of a swelling on the main root, many more initials being formed than in the controls. Higher concentrations, e.g. 100 p.p.m., prevented any effective growth in all seedlings.

This inhibition of growth was obtained with L-methionine sulphoximine, the D form, which as previously noted contained a small amount of the L form, having only a slight retarding effect (Pl. 1, 2; control, Pl. 1, 3).

The effect of L-methionine and L-glutamine on this growth inhibition by L-methionine sulphoximine was tested by adding them in solution, sterilized by filtration, to the medium containing 4 p.p.m. of sulphoximine after autoclaving and partially cooling.

At a concentration of 40 p.p.m. each had a slight protective action, the seedlings growing about half as long again before dying.

At 400 p.p.m. methionine intensified the effect of methionine sulphoximine. At this concentration methionine alone had a depressing effect that was not seen at lower concentrations. At 400 p.p.m. glutamine had a marked protective action, the seedlings becoming twice as long, with the lateral roots five times longer before growth ceased.

At 4000 p.p.m. glutamine almost completely protected the embryos. The first leaf withered before the controls and secondary roots grew more slowly, which probably means that root growth in general, difficult to assess visually, proceeded more slowly; there was, however, no distortion of the tips, and for 6 weeks growth as a whole continued along with that of the controls. After this period the treated plants failed.

Glutamine added to the simple medium had a stimulating effect on growth. With the addition of 4000 p.p.m. the plants produced three leaves during 4 weeks, whereas the controls had produced two, leaf and root growth being also more luxuriant. This effect was almost eliminated by the presence of 4 p.p.m. of sulphoximine, though development had proceeded to the partial emergence of the third leaf.

When 10% of flour treated with varying amounts of nitrogen trichloride was incorporated in the medium, normal growth was always obtained. That it was due to the absence of free methionine sulphoximine and not to a protective action of the endosperm was shown by the fact that the further addition of 2 p.p.m. of sulphoximine inhibited growth. On the other hand, 10% of the pancreatic hydrolysate did affect growth, though only when the level of treatment of the flour with nitrogen trichloride corresponded to 20 p.p.m. of sulphoximine in the medium (Pl. 2, 1); at a level of 2 p.p.m. there was no effect. An attempt made to find out whether this was due to incomplete liberation of the methionine sulphoximine, by using the complete acid hydrolysate, unfortunately failed, in spite of various refinements, because satisfactory growth was never obtained in the control series (with untreated flour). The hydrolysate itself, however, had a protective action, since the addition of 4 p.p.m. of sulphoximine to the medium made with untreated flour hydrolysate had a much weaker effect than on the simple medium. The protective action was equivalent to that of about 200 p.p.m.

of glutamine in the simple medium. Likewise, the addition of 400 p.p.m. of glutamine to the heavily treated flour hydrolysate had no more protective action than 40 p.p.m. added to the simple medium.

<center>DISCUSSION</center>

The characteristic action of methionine sulphoximine, to inhibit growth, has been demonstrated at concentrations in the medium ranging from 100 to 2 p.p.m.; at the lower concentrations an initial period of normal growth intervened. There was probably a delay while an effective concentration was being reached in the embryo; once it was established, growth ceased. On the other hand, at similar concentrations *Leuconostoc mesenteroides* (Heathcote & Pace, 1950) and chick-bone rudiments in culture (Mellanby, 1956) continued to grow, though at a reduced rate. This may point to a greater susceptibility of the embryos, but it is also possible that the concentration in the synthesizing cells may reach higher levels than that in the medium. The behaviour of the roots suggests that secondary initials were formed and made slight growth at a time when the apical meristem had stopped growing, which might suggest that concentration to an effective level had occurred in the regions of growth and was only attained in other parts when growth began. It is of interest to note that the potential amount of sulphoximine in flour treated with the normal commercial dose of nitrogen trichloride is 2 p.p.m. (Moran, 1952).

There were apparent differences in susceptibility between embryos, but there is no evidence to show whether they followed from differences in the ease with which the concentration in the growing cell was attained or were due to true differences in susceptibility resulting from differences in vigour or, again, were a reflection of differences in the rate of growth, the susceptibilities being equal.

It has been noted that L-methionine sulphoximine is about twice as toxic to rabbits as the DL form (Bentley *et al.* 1951), and twice as effective in acetylcholine synthesis by brain slices (McLennan & Elliott, 1951). In the experiment reported here D-methionine sulphoximine had only a very slight depressing effect on the growth of the embryos and was known to contain a small amount of the L form. It is probable that this slight effect was due to the contamination and that D-methionine sulphoximine is biologically inactive.

The toxic action of methionine sulphoximine could be neutralized in these experiments only by a concentration of glutamine 1000 times that of sulphoximine. *Leuconostoc mesenteroides*, on the other hand, had an acid production equal to that of the control with a concentration of ten times that of the sulphoximine (Heathcote & Pace, 1950), whereas less than equimolecular proportions were sufficient to repair the process of protein synthesis (Rabinovitz *et al.* 1956). Glutamine, supplied externally, readily enters the nitrogen metabolism of plant cells and when supplied to the embryos stimulated growth and was clearly utilized as a source of nitrogen. This stimulus almost disappeared in the presence of sulphoximine, whose toxic action, however, was nearly eliminated by the glutamine. This might indicate two competing demands for the glutamine, each at the centre of protein synthesis.

The existence of more than one pathway in the metabolism of glutamine has

recently been suggested, whereby it enters either the structural protein, or protein broken down in katabolic processes, becoming metabolically available again (Steward, Bidwell & Yemm, 1956). The effect of the increasing doses of glutamine was to extend the time for which the embryos were able to grow normally, corresponding, perhaps, to a period in which the available glutamine was built into the structural protein, after which further protein synthesis failed. This interpretation would agree with the suggestion that methionine sulphoximine, which presumably persists in the medium, inhibits protein formation because it interferes with the stage of glutamine synthesis (Pace & McDermott, 1952; Rabinovitz *et al.* 1956). The failure of normal glutamine synthesis could also account for the absence of the glutamine stimulus in the sulphoximine-treated embryos.

The higher concentration of glutamine required may also follow from the earlier suggestion that there may be some concentration of sulphoximine in the meristematic cells, which might require increased amounts of glutamine; further, the bacterial cell must stand in a different relationship to its glutamine supply from the meristematic cell organized in a tissue through which a restriction of movement is to be expected.

The normal growth occurring on the medium containing undigested flour heavily treated with nitrogen trichloride was not unexpected since it was improbable that free methionine sulphoximine would be present in the medium. Nevertheless, it was possible that some sulphoximine might be liberated and absorbed by the roots as a result of their interaction with the medium. From the experimental results, it seems clear that the roots had little or no proteolytic action. The pancreatic hydrolysate, on the other hand, possessed the toxic properties typical of methionine sulphoximine. The toxic effects were only obtained at a high level of treatment, which is at least in part due to a protective action of the protein hydrolysates. It is probably due to glutamine, known to be present in enzymic digests of wheat protein (Sullivan & Payne, 1951), since the further addition of 400 p.p.m. of glutamine to the hydrolysate had a much weaker effect than when it was added to the simple medium.

At the same time it is probable that pancreatin does not liberate the whole of the sulphoximine or produce a high concentration of free amino-acids, although the attempt to investigate this failed, as the complete acid digest was an unsatisfactory medium. The addition of hydrolysed casein or a mixture of amino-acids to the medium has been reported to stimulate growth of the oat embryo (Harris, 1953). The concentrations employed were much lower than that involved in the present acid-digest experiments, which was about 1% of hydrolysed protein. The poor growth of the embryos may be due to this higher concentration or to the production of some harmful substance during acid hydrolysis.

Agene as a flour improver has now been replaced by chlorine dioxide (commonly known as Dyox). Slight changes in the amino-acids and lipids are known to follow from the use of this gas, but feeding experiments with animals have so far failed to detect any toxic properties of treated flour (Moran, Pace & McDermott, 1953; Meredith, Sammons & Frazer, 1956; Frazer, Hickman, Sammons & Sharratt, 1956). During the work reported here wheat embryos have been grown normally on the protein hydrolysate of flour treated with ClO_2 at 100 times the usual level (Pl. 2, 2).

This is a further indication that toxic substances, if any, are not produced in any significant amount.

SUMMARY

1. Wheat embryos growing in sterile culture were killed, after a short initial period of growth, by the addition of 2 p.p.m. of L-methionine sulphoximine to the medium. The slight effect noted with D-methionine sulphoximine was probably due to contamination with the L form.

2. A pancreatin protein hydrolysate of wheat flour treated with nitrogen trichloride had the same toxic effect when used as the culture medium only if the level of flour treatment corresponded to a concentration of 20 p.p.m. of methionine sulphoximine in the medium.

3. The addition of glutamine at 1000 times the concentration of methionine sulphoximine neutralized the latter's toxic effect. This concentration is 100 times greater than is recorded as being effective with *Leuconostoc mesenteroides*.

4. The higher concentration of sulphoximine necessary to demonstrate the toxic effect with the protein hydrolysate was due in part to a protective action of the hydrolysate, which probably follows from the presence of glutamine.

5. Embryos have grown normally on medium containing 10% hydrolysed protein from flour treated with chlorine dioxide at 100 times the level at which it is used as an improver.

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EXPLANATION OF PLATES

PLATE 1

1. Growth of the wheat embryo in the presence of 10 p.p.m. of L-methionine sulphoximine.
2. Growth in the presence of 10 p.p.m. of D-methionine sulphoximine.
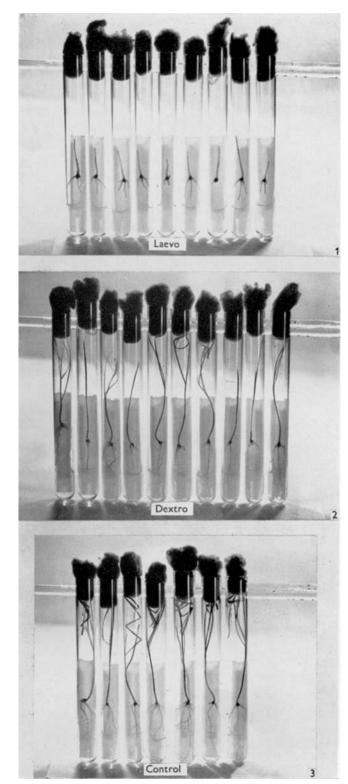3. Growth in control medium.

PLATE 2

1. Effects on growth of the wheat embryo of a protein hydrolysate from flour treated with nitrogen trichloride (ten times the commercial level).
2. Effects on growth of a protein hydrolysate from flour treated with chlorine dioxide (100 times the commercial level).
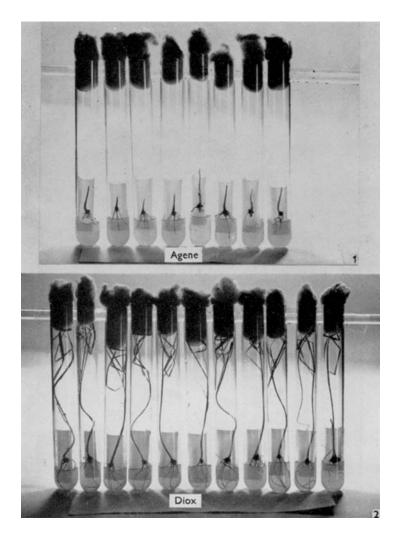
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Plate 2