

## Metabolism of excised embryos of *Lupinus luteus* L. I. Effect of metabolic inhibitors and growth substances on the water uptake

J. CZOSNOWSKI

Very little research on metabolism of excised embryos was carried out up to the present (Oota 1958). More often seedlings with cotyledons abscised after a given period of germination (McRary 1940, Maróti 1957) and embryos excised from soaked seeds but cultured for some time in a mineral-organic nutrient solution (Rogozinśka 1960) were investigated.

The vast majority of research on excised embryos is concerned with conditions of their further development on artificial media. The present paper starts a series of investigations on metabolism of excised embryos. Short experiments, under possibly simplest conditions, without the use of media, shall constitute the main line of research. Metabolism, therefore, of excised embryos, taking place during the few days after the immersion of seeds, or after the excision of the dry embryo in water — shall be the subject of our interest.

The present communication describes some results of experiments on water uptake and its relation to respiration in isolated embryos of yellow lupine.

A very comprehensive review of the literature on cell growth, water uptake during growth, the relation of these processes to metabolism, on the influence of growth substances and related problems is given by Thimann (1960). While analysing recent papers on relations between respiration and water uptake in growing cells he declares: "It will be seen that the influence of the inhibitor on respiration is always much smaller than on growth".

Scores of papers of many authors report on the influence of metabolic inhibitors and growth substances on water uptake by tissues of potato or Jerusalem artichoke tubers, sections of young stems of pea and sections of oat coleoptile etc. Not many papers, however, are concerned with water uptake by embryos (Stiles 1949, Oota 1958).

The present work is aimed at ascertaining to what degree water uptake by excised embryos is dependent on respiration, and to what degree Thimann's statement given above is right in this case.

#### MATERIAL AND METHODS

Seeds of *Lupinus luteus* var. "Express" originated from the Research Centre of the Institute of Soil Science, Plant Cultivation and Manuring at Przebedowo. Embryos were excised from the cotyledons after removing the testa. Two kinds of embryos were employed:

a. "dry embryos" — excised from normal, dry seeds. Average weight 4.8 mg.

b. "soaked embryos" — excised from seeds soaked in distilled water for 24 hours. The seeds were spread in one layer in shallow vessels and hardly covered with water. The average weight of these embryos ranged from 9.4 to 10.4 mg.

In each test 10—25 dry embryos were treated. In the case of soaked embryos samples with an initial weight of 250 mg were used. The course of water uptake and the influence of inhibitors and growth substances was investigated under conditions of aeration. Given samples of embryos were, therefore, placed in penicillin bottles of ca 10 ml capacity; 5 ml of distilled water or a 5 ml solution was given and the samples were aerated with a moderate stream of air moistened in a washer with distilled water.

Solutions of inhibitors and growth substances were prepared on distilled water. The solutions were adjusted with KOH or HCl to a pH value of 5.5. The experiments lasted for 24 hours and took place at a room temperature (17—21°C). No precautions were taken to provide asepsis; no bacterial contaminations were noted. After a 24 hour exposure to the tested solutions the embryos were taken out of the vessels, dried with blotting paper and weighed on an torsion balance to the nearest mg.

After that they were transferred to a Warburg equipment where the intensity of respiration was measured. The measured  $QO_2$  was referred each time to the initial weight of 250 mg — not to the weight at the end of the experiment, as it was different at varying concentrations of the substance in question.

From 2 to 5 samples were taken for each treatment. Embryos left for 24 hours in distilled water were used for control. These control samples (250 mg) of soaked embryos weighed after 24 hours  $500 \pm 40$  mg (average from 20 experiments). Each experiment had separate control samples.

Respiration measurements were carried out with the use of standard Warburg respirometers, at 25°C. Embryos soaked for 24 hours in test solutions were employed. Samples were weighed and placed in Warburg's flasks, in 2 ml of the same solutions or in distilled water — as the case might be. 0.2 ml of 10 per cent KOH was added to the center well.

Excess of weight of control samples over the initial weight of 250 mg (determined as water uptake) and the oxygen absorption by controls were regarded as 100 per cent.

## RESULTS

### 1. Water uptake of embryos in the presence and absence of oxygen

Thimann and Bonner (1948) showed that growth of excised sections of oat coleoptile is dependent upon favourable conditions of aeration. Even an immersion of these sections 1 mm under the surface of the solution inhibited considerably the growth.

Hackett, Schneiderman and Thimann (1953) have determined the dependence of cell enlargement in potato slices on oxygen pressure.

The degree of dependence, therefore, of growth of a completely different type of plant material, viz. of excised embryos, on aeration of the environment seemed to constitute an interesting problem. Soaked embryos in samples of 250 mg were used. One of the samples was aerated with a flow of air, in 5 ml of distilled water, in penicillin bottles. Another sample was spread on a very moist paper in a Petri dish. The third was exposed to identical conditions as the first, with the exception of aeration. The surface of the water-layer was 14—16 mm over the embryos spread on the bottom. The experiment was conducted in two replications. Average results are represented in Table 1.

Table 1  
Influence of oxygen relations on water uptake of embryos

25 October 60 Embryos	Growth percentage after	
	27 hrs	48 hrs
Aerated	129	196
On moist filter paper	92	145
Submerged	7	9

Another time, in a test lasting 24 hours the growth percentage of aerated and submerged embryos was respectively 137 and 16 (see Fig. 1).

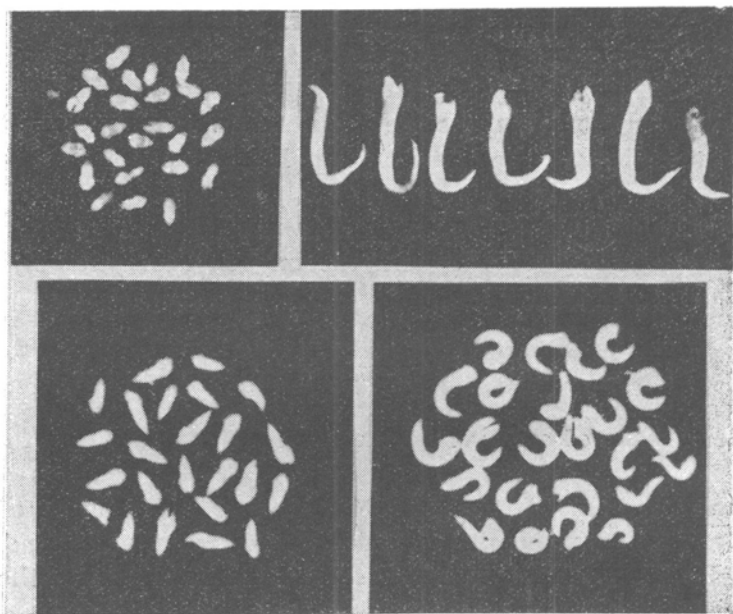


Fig. 1. Top row: Isolated dry embryos (left) and after 8 days growth on filter paper moistened with distilled water. Bottom row: Isolated soaked embryos after 24 hours under submerged (left) and aerated conditions (1:1)

These data show that the growth of excised lupine embryos is extremely dependent on the aeration of the environment. A normal diffusion of oxygen through the water layer covering embryos on the depth of 15 mm enables them to grow but very slightly and only for a short time.

## 2. The course of water uptake and oxygen absorption

Stiles (l.c.) examined water uptake by individual parts of seeds of a few species of *Phaseolus* for 4 days. Analyses were carried out every 24 hours. It soon became clear that the embryo absorb water in a most effective manner when compared with other parts of the seed. Cotyledons and the seed testa absorb water much less vigorously and only for a short time.

The present experiments being concerned with excised embryos which are organs with a very limited food supply — it seemed necessary to analyse carefully the course of water uptake by excised dry embryos. Obtained data were to render possible a choice of a limit of time during which water uptake is a continuous process. Within this particular time-limit it was planned to investigate the influence of inhibitors and growth substances on water uptake.

The dry embryos were divided into samples of 10 embryos each (48 mg). They were placed in penicillin bottles in 5 ml of distilled water and aerated. At given intervals the embryos were taken out of water, dried with blotting paper, and weighed on an torsion balance; after this they were placed back in water. The weighing was performed after 5, 10, 15, 20, 30, 45 and 60 minutes; later at intervals from 1 to 2 hours; towards the end at longer even periods. Average results of three experiments are represented in Fig. 2.

Embryos excised from dry seeds absorb water in a very peculiar manner, especially during the first 12 hours. Immediately after the immersion in water a rapid absorption takes place; it lasts for 45 minutes. During the second phase lasting 7—8 hours the weight of the embryos remains unchanged. The third phase begins after 8 or 9 hours by

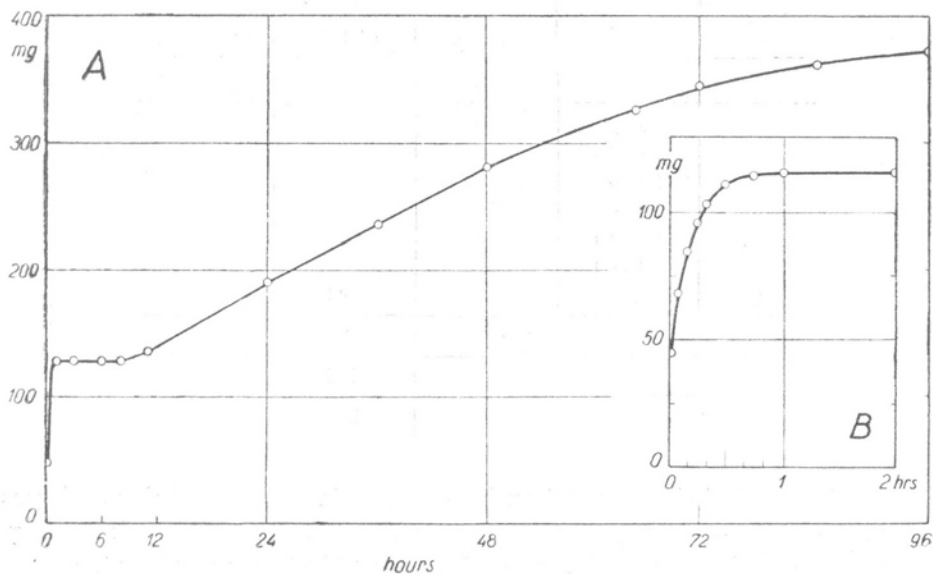


Fig. 2. The time course of increase in fresh weight of isolated dry embryos. Graph B represents the detailed course during the first two hours

a further absorption of water, at a much smaller rate, however, than during the first phase. The third and last phase persists for 5—7 days, the absorption rate slowly approaching zero. In one case 10 dry embryos (48 mg) weighed after 8 days 490 mg (Fig. 1). The dry weight amounted to 28 mg, i.e. 58 per cent of the former. The embryos required for metabolic processes, or perhaps released, 20 mg of dry matter, i.e. 42 per cent of the initial dry matter.

During the first phase water is absorbed by imbibition. Embryos killed by a 1-hour treatment with a 110°C temperature also absorb water for 45 minutes; after this, of course, they discontinue to imbibe water. Embryos placed in cytological aqueous fixatives behave in a similar manner.

The third phase consists of water absorption related to metabolism. It is dependent on the influence of metabolic inhibitors (see below). The experiments concerning the action of inhibitors and of growth substances, based on the character of the curve in Fig. 2, were carried out in the time interval of from 24 to 48 hours after the beginning of imbibition.

Table 2

Respiration and water uptake by dry embryos

Hours	Min.	25 embryos		
		$\mu\text{l O}_2/10 \text{ min}$	$\mu\text{l O}_2/\text{hr}$	weight mg
0		0	0	105 (dry)
	0—10	0		
	10—20	8		
	20—30	15		
	30—40	20		
	40—50	20		
	50—60	22		
1			85	270
2			124	270
3			145	270
20			205	460

In order to find the correlation between water uptake of dry embryos and respiration — the following experiment was conducted: 25 dry embryos were placed in sidearm of Warburg flask. 2 ml of water were added to the main chamber; 0.2 ml of 10 per cent KOH and a strip of filter paper were placed in the center well. After 20 minutes of thermal equilibration (25°C) the three-way stopcock was closed. During the next 30 minutes the embryos did not reveal any signs of respiration. After this the embryos were moved into the main chamber and placed in water where the first measurements took place. During the first hour readings were carried out every 10 min., later every 30 min. At the same time a second set of Wargurg's equipment was established. Embryos of this series were used for measuring water absorption. Every hour the embryos were taken out, weighed, put back and shaken together with the previous series. Measurement of oxygen and water uptake were

performed in 5 parallel replications and the average results are represented in Table 2.

It becomes evident that respiration processes begin as early as 10—15 minutes after the commencement of water absorption. From this moment the rate of respiration becomes continuously larger for the whole tested period of 20 hours. The increase, however, in oxygen absorption is not accompanied by a proportionate increase in the rate of water uptake. After three hours the weight of the embryos is the same as after one hour — this situation being in conformity with the above reported data concerning water uptake during the first 7—8 hours. After 20 hours the water uptake is at its greatest (the third phase) and is accompanied by a great increase in the rate of oxygen absorption.

On the basis of the above described experiments it became interesting to ascertain how much water is absorbed by dry embryos during 24 hours from the beginning of imbibition, under conditions of aeration and submerged. The initial weight of 10 dry embryos was 48 mg. After 24 hours the aerated embryos weighed 247 mg while the submerged — 118 mg (average from two replications). All these data show that submerged embryos absorbed only imbibition water (1-st phase) and remained in this state till the end of the trial. Aerated embryos, on the contrary, entered normally the 3-rd phase of water absorption. And so, in this case too, the indispensability of oxygen for normal water absorption of physiologically active embryo protoplasts became evident.

Anatomical and cytological analyses of embryos from the commencement of water absorption until 48 hours later were carried out. It was found that during this period growth is limited to a swelling in size, mostly in length, of hypocotyl and root cells which are the farthest from the growing point. No mitoses were observed.

### 3. Water uptake and oxygen absorption under the influence of metabolic inhibitors

Iodoacetate was applied in concentrations of from  $10^{-4}$  to  $10^{-2}$  M (Fig. 3). In concentrations lower than  $4 \times 10^{-4}$  M it increases water uptake; in concentrations lower than  $8 \times 10^{-4}$  M it stimulates respiration as compared with the control. In concentrations higher than the mentioned ones — both processes are inhibited, the water uptake however to a greater degree. Excised lupine embryos are much more resistant to the action of iodoacetate than e.g. sections of *Avena* coleoptile, where at a concentration of  $3 \times 10^{-5}$  M there occurs a 50 per cent inhibition (Thimann and Bonner 1948).

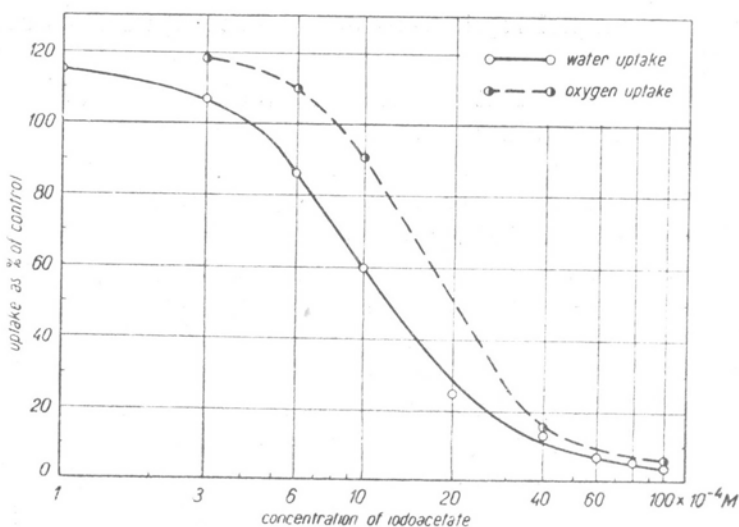


Fig. 3. The effect of iodoacetate on water uptake and oxygen absorption

Arsenite was applied in concentrations of  $10^{-5}$  —  $10^{-3}$  M (Fig. 4). Within the whole range of these concentrations it inhibits absorption of water and oxygen. A 50 per cent inhibition of growth occurred at

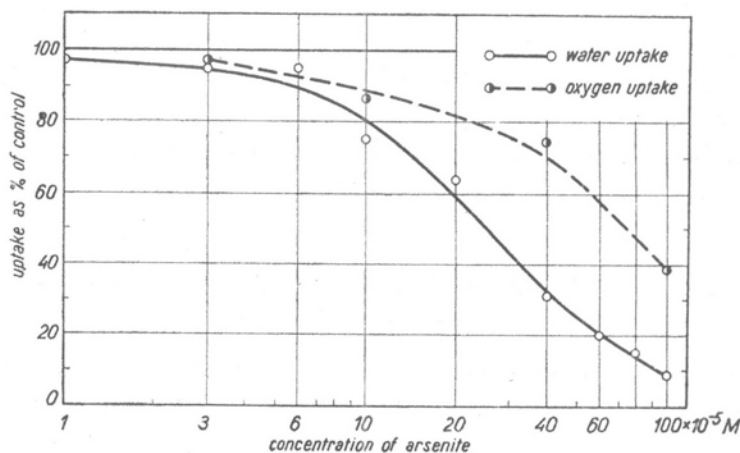


Fig. 4. The effect of arsenite on water uptake and oxygen absorption

a  $2.5 \times 10^{-3}$  M conc., i.e. at a much higher concentration than in the case of *Pisum* stems and *Avena* coleoptiles ( $10^{-4}$  and  $10^{-3}$  M — respectively) (Thimann and Bonner 1949).



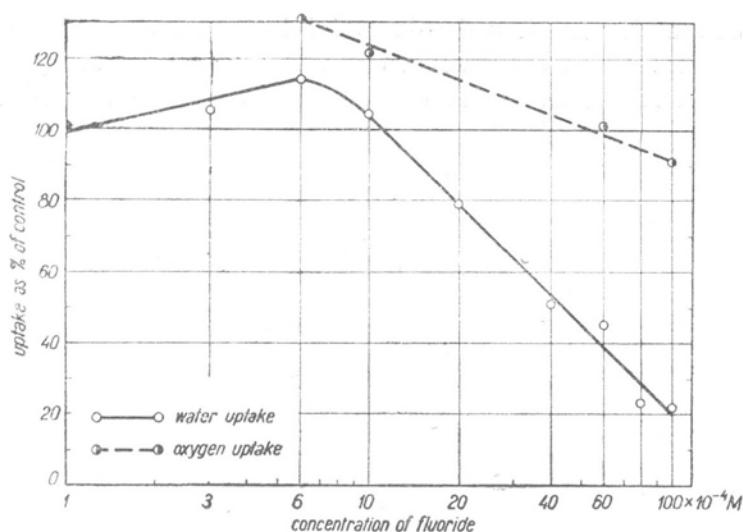


Fig. 5. The effect of fluoride on water uptake and oxygen absorption

Fluoride were applied in concentrations of from  $10^{-4}$  to  $10^{-2}$  M (Fig. 5). Below the concentrations of  $10^{-3}$  M a faint stimulation of water uptake occurs — the hardly notable maximum taking place at  $6 \times 10^{-4}$ . Above  $10^{-3}$  M there occurs a greatly increasing inhibition of water absorption. Fluoride, on the other hand, greatly stimulate respiration of embryos — over, however,  $6 \times 10^{-3}$  M. At  $6 \times 10^{-4}$  M the respiration rate is about 30 per cent higher than in control samples. Fluoride start to inhibit respiration only in high concentrations. At  $10^{-2}$  M respiration of embryos is inhibited in ca 10 per cent, water uptake — in 80 per cent.

Azide (Fig. 6) were applied in concentrations of from  $10^{-5}$  to  $10^{-3}$  M. At concentrations higher than ca  $3 \times 10^{-5}$  M they inhibit water uptake which at  $10^{-3}$  M nearly approaches zero. The inhibition of respiration is much weaker and at the highest concentration amounts to ca 40 per cent.

$\alpha\alpha'$ -dipyridyl was applied at from  $10^{-5}$  to  $10^{-3}$  M concentrations (Fig. 7). At  $3 \times 10^{-5}$  M it stimulates water uptake to about 15 per cent as compared with the control. Above concentrations of about  $10^{-4}$  M it inhibits the process.  $\alpha\alpha'$ -dipyridyl does not stimulate respiration of embryos. Concentrations higher than  $3 \times 10^{-5}$  M bring about an inhibition which however in higher concentrations is weaker than inhibition of water uptake.

Ethylenediaminetetraacetic acid (disodium) was applied at  $10^{-6}$  to  $10^{-3}$  M concentrations (Fig. 8). This factor chelating

a series of metals reveals a decidedly inhibiting action on both processes in question. As in the case of other inhibitors, however, inhibition of respiration occurs in higher concentrations than inhibition of water uptake.

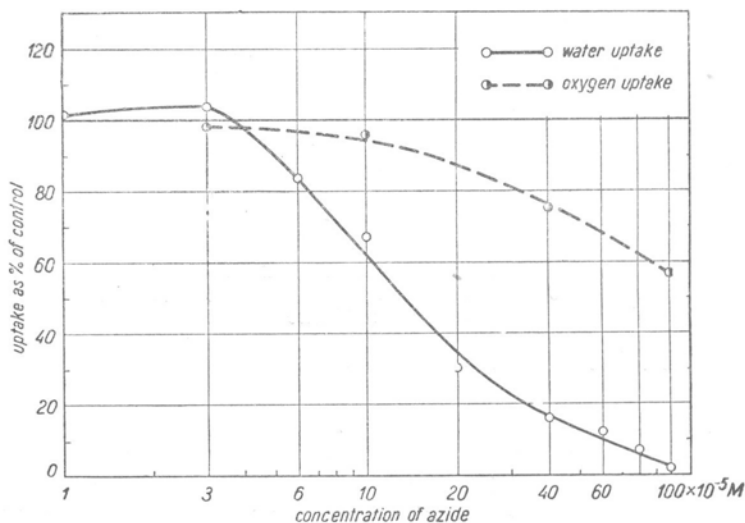


Fig. 6. The effect of azide on water uptake and oxygen absorption

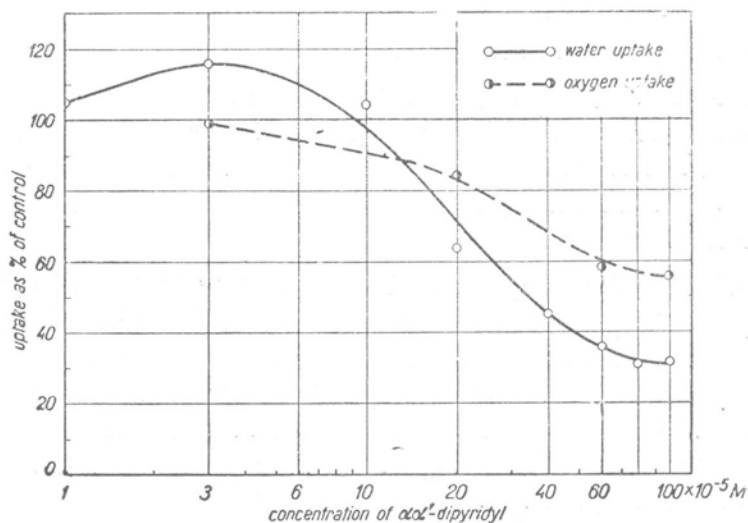


Fig. 7. The effect of  $\alpha\alpha'$ -dipyridyl on water uptake and oxygen absorption

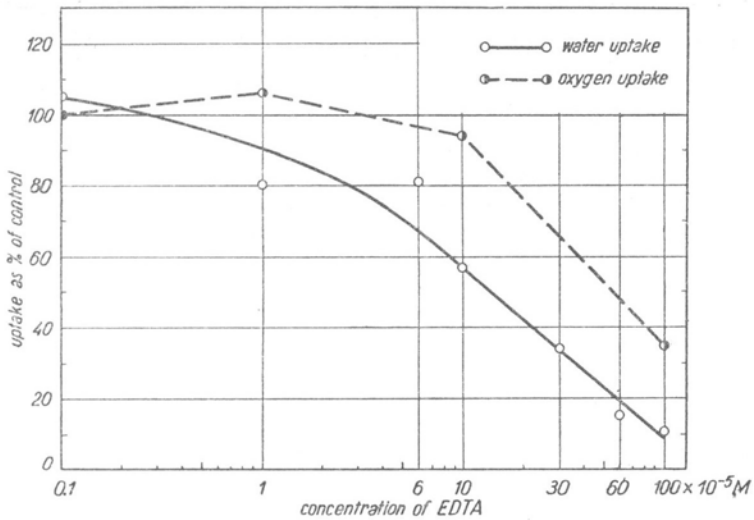


Fig. 8. The effect of ethylenediaminetetraacetic acid on water uptake and oxygen absorption

Diethyldithiocarbamate was applied in concentrations of from  $10^{-5}$  to  $10^{-3}$  M (Fig. 9). In comparison with other inhibitors the mentioned concentrations result in a relatively very slight inhibition of respiration. Water uptake is inhibited in a greater degree, concentrations, however, above  $4 \times 10^{-4}$  M bring about in both cases nearly similar results.

Fluoroacetate (Fig. 10). Initial research revealed that lupine embryos are not very susceptible to the action of this particular inhibitor. Rather high concentrations of from  $10^{-4}$  to  $10^{-2}$  M, therefore, were applied in further work. Within these limits of concentration inhibition of water uptake and oxygen absorption was also very faint in comparison with other inhibitors. At concentrations up to  $10^{-3}$  M water uptake proceeds normally and at a concentration of  $10^{-2}$  M it is inhibited in about 25 per cent. The latter concentration lowers respiration 13 per cent.

The above given data show that excised lupine embryos are much more resistant to the action of fluoroacetate than potato tubers or sections of pea stems or oat coleoptiles (Hackett and Thimann 1952).

2,4-dinitrophenol was applied in concentrations of from  $5 \times 10^{-6}$  to  $3 \times 10^{-4}$  M (Fig. 11). The whole range of concentrations

decidedly inhibited water uptake. In respect to oxygen absorption the situation is different. The lowest conc. of DNP brings about a 30 per cent increase in oxygen absorption as compared with the control. It equals the control at  $10^{-4}$  — water uptake is inhibited in over 90 per cent.

A 50 per cent inhibition of water uptake occurs at a concentration of  $3 \times 10^{-5}$  M — this being in conformity with results obtained by Hackett and Thimann (l.c.) with tissues of potato tubers and by Bonner (1949) with *Avena* coleoptile.

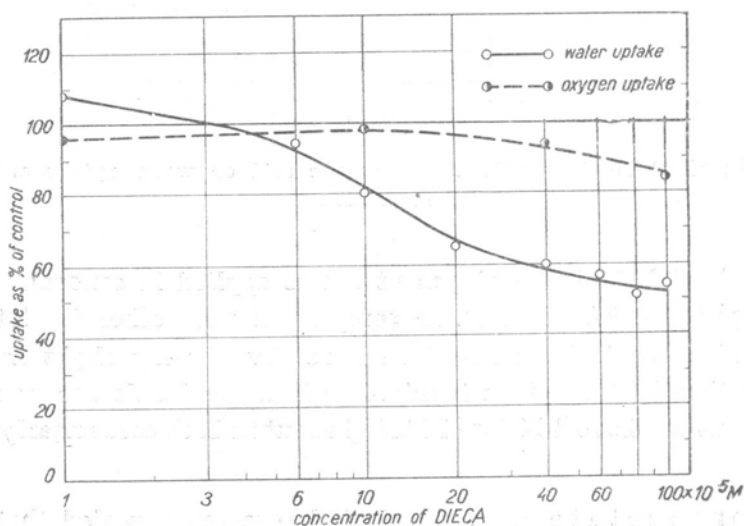


Fig. 9. The effect of diethyldithiocarbamate on water uptake and oxygen absorption

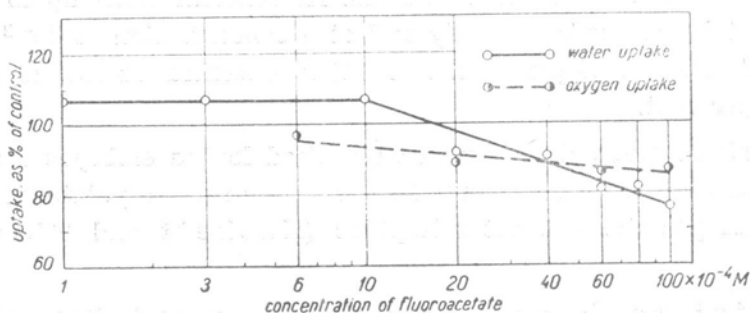


Fig. 10. The effect of fluoroacetate on water uptake and oxygen absorption

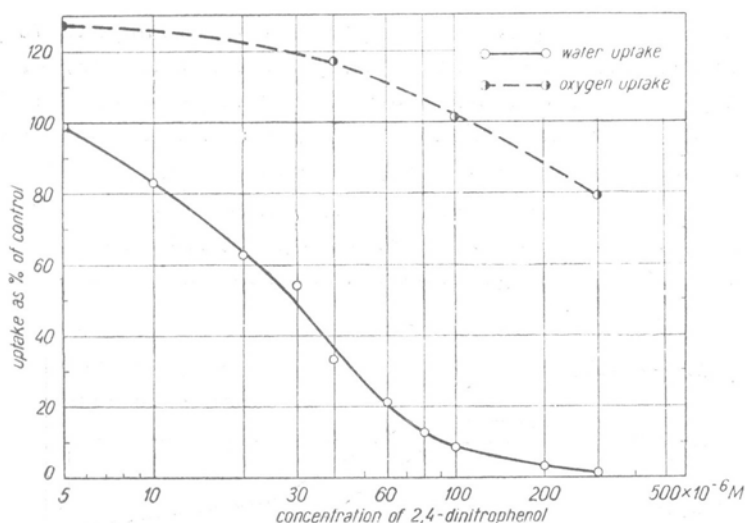


Fig. 11. The effect of 2,4-dinitrophenol on water uptake and oxygen absorption

#### 4. Water uptake and oxygen absorption under the influence of growth substances

$\beta$ -indolylacetic acid was applied at concentrations of  $1 \mu\text{g}$  to  $10 \text{ mg/l}$  ( $5.7 \times 10^{-9}$  to  $5.7 \times 10^{-5}$  M) (Fig. 12). IAA within the limits of these concentrations inhibits water uptake of embryos. The concentra-

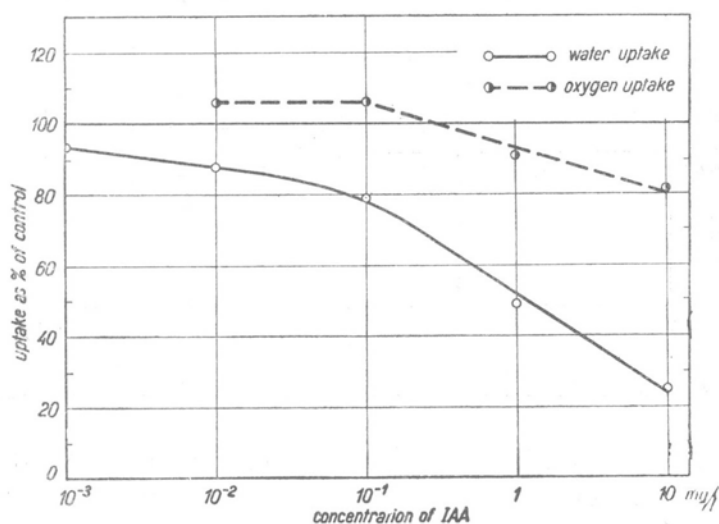


Fig. 12. The effect of indolylacetic acid on water uptake and oxygen absorption

tion of 1 mg/l inhibits growth in 50 per cent and 10 mg/l in 75 per cent in comparison with the control samples. At concentrations 0.01 — 0.1 mg/l there occurs a slight stimulation of respiration, and at higher concentrations — an inhibition which is, however, much weaker than the inhibition of water uptake (20 per cent at 10 mg/l).

$\alpha$ -naphthylacetic acid was used in concentrations of from 1  $\mu$ g to 1 mg/l ( $5.4 \times 10^{-9}$  to  $5.4 \times 10^{-6}$  M) (Fig. 13). NAA in higher concentrations than 10  $\mu$ g/l brings about a violent inhibition of water uptake.

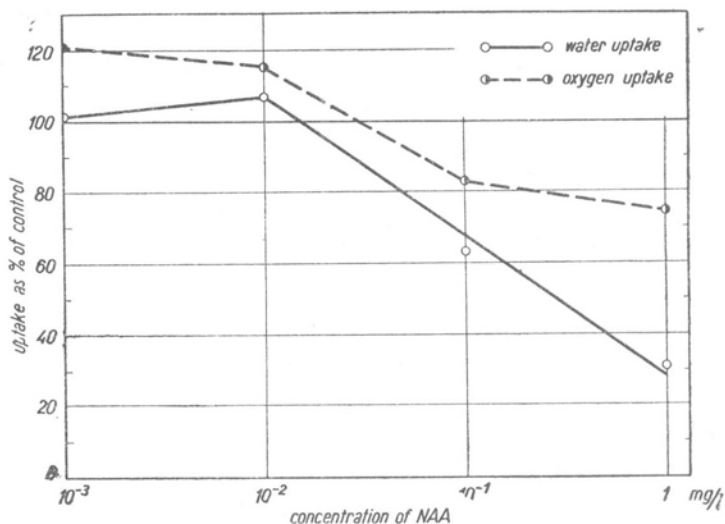


Fig. 13. The effect of naphthylacetic acid on water uptake and oxygen absorption

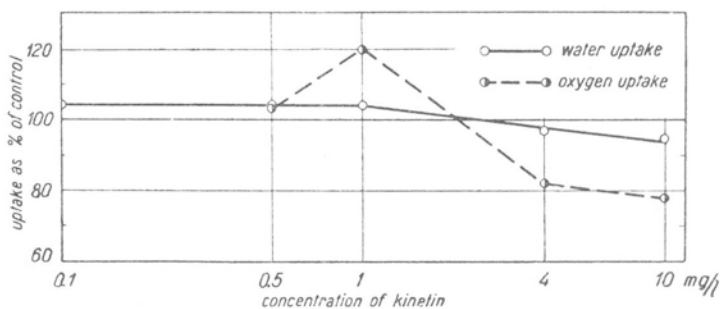


Fig. 14. The effect of kinetin on water uptake and oxygen absorption

Respiration, on the other hand, at conc. from 1  $\mu$ g/l to 10  $\mu$ g/l becomes increased (about 20 per cent). Only at concentrations higher than ca 30  $\mu$ g/l respiration becomes inhibited — much less markedly, however, than water uptake.

Kinetin (Fig. 14) in conc. of 0.1 to 10 mg/l inhibits slightly the water uptake of embryos — but only at the higher concentrations. The concentration of 1 mg/l increases the respiration rate up to 20 per cent over the control value, but concentrations higher than about 2 mg/l inhibit respiration to about 20 per cent when the highest concentration is applied.

Gibberellin (Fig. 15) in the same concentrations as kinetin in respect to water uptake by embryos brings about similar effects. Yet,

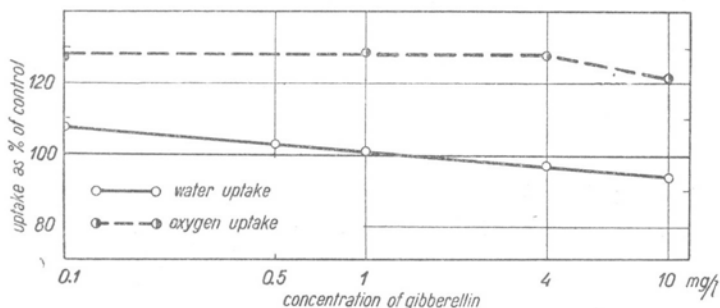


Fig. 15. The effect of gibberellin on water uptake and oxygen absorption

at conc. of from 0.1 to 4 mg/l it increases greatly respiration (about 28 per cent in comparison with the control samples); at 10 mg/l it falls slightly.

#### DISCUSSION

Excised embryos, as an object of studies on water uptake differ greatly by their physiological and anatomical characters from other objects like e.g. tuber tissues, sections of young shoots or grass coleoptiles examined usually up to the present. Mature tissues, at the moment of their being employed in trials, contain relatively much water; whereas tissues of a dry embryo contains hardly any water. Absorption of the right amount of water by a dry embryo is a basic condition of its entering an active physiological state, it seemed, therefore, that analyses of the processes of water absorption by embryos should provide in certain respects interesting results. During these analyses, of course, only the phase of embryo development when growth consists of an increase in cell-size was taken into consideration. Only at that moment it is possible (to a certain degree) to compare embryo relations with such relationships like in e.g. tuber tissues or coleoptile sections.

Water uptake by dry embryos is a process during which the first

phase, lasting barely one hour and consisting of swelling, is separated from the third phase by a few-hour cessation in water absorption. The third phase takes place only in the presence of oxygen. From the beginning of this phase there occurs an increase in cell-size. Oota (l. c.) also observed the existence of three similar phases of water uptake in *Vigna*, the pause in water uptake however in this case is very short and lasts less than an hour.

Respiration processes do not have such stages in their development. An embryo placed in water begins to respire in about a quarter-of-an-hour, already during its swelling. When the increase in size ends — respiration is by that time quite efficient. There takes place then a pause in water uptake, but metabolic processes continue. It is, therefore, a period when respiration is not accompanied by water uptake. 8 to 9 hours later, under aerated conditions, further growth takes place.

These facts might be interpreted on the same basis as data concerning tissues of potato tubers (Hackett and Thimann 1953): "a small fraction of the respiration, over and above that required for maintenance, is in control of water uptake". Most probably this is the case in excised lupine embryos, the effects of metabolic inhibitors on water uptake and oxygen absorption seem to provide evidence in favour of this supposition.

From data reported in the experimental part and illustrated by Figs. 3—11 a general conclusion can be drawn: water uptake is restrained by inhibitors in a greater, not rarely much greater degree, than respiration (see Thimann 1960, p. 787—88). Only  $\alpha\alpha'$ -dipyridyl and fluoroacetate in lower concentrations bring about a significant increase in water uptake. In higher concentrations, however, this rule does not hold true.

Under the influence of DNP embryos behave in a peculiar manner. As in the case of other objects DNP, in concentrations which bring about a nearly complete suppress of growth, stimulates strongly oxygen absorption. It is therefore concluded that in lupine embryos, like in other tested objects, water uptake is dependent on phosphorylations and phosphate transfer.

Out of 4 growth substances, the influence of which on water uptake and respiration was examined, viz. IAA, NAA, kinetin and gibberellin, only the two former substances bear a decisive influence on the processes in question. IAA and NAA in the applied concentrations did not reveal any significant beneficial influence on water uptake — on the contrary, they had a marked suppressing effect. Respiration was also weakened, but in a much lesser degree. In general, therefore, the



effects of the action of IAA and NAA in the given set of conditions were similar to those brought about by metabolic inhibitors. Summarizing the above one must conclude that water uptake of excised lupine embryos is a process dependent on the inflow of energy originating from respiration and thus closely related to metabolism. This relation, however, is not direct and one must assume that only certain fractions of respiration energy are active in this case.

### SUMMARY

Dry embryos, excised from dry seeds, and soaked embryos excised from seeds soaked for 24 hours constituted the object of examinations.

1. Water uptake by excised embryos is only possible under conditions of good aeration.

2. In dry embryos placed in an aerated water-environment water uptake is not a continuous process; three phases are discernible: a. a short-time (less than an hour) swelling, b. a few-hour cessation of water uptake, c. the third phase of water uptake, taking place 8—9 hours from the beginning, and dependent on aeration. Under conditions of insufficient aeration dry embryos absorb water only by swelling and further uptake does not occur.

3. Respiration processes in dry embryos start as soon as a quarter-of-an-hour from the moment of the commencement of swelling, and increase continuously.

4. The influence of 9 metabolic inhibitors and 4 growth substances on water uptake and respiration of embryos was examined. It is found, in general, that the tested substances bear a much stronger inhibiting influence on water uptake than on respiration (Thimann 1960).

5. The obtained results show that processes of water uptake in lupine embryos are closely related to respiration; not, however, in a direct manner. One fraction of respiration processes, sufficient for the basic metabolism, may be insufficient for the processes of water uptake (cf. p. 2—3); These conclusions are in conformity with the ones put forth by Hackett and Thimann (1953) in respect to a completely different material, viz. to tissues of potato tubers.

6. For 48 hours from the time of placing seeds or dry embryos in water, mitoses do not occur, and growth is limited to cell enlargement.

*Laboratory of Plant Physiology  
University of Poznań*

(Entered: 22.9.1961)

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