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STUDIES ON THE INCREASED RESPIRATION OF POTATO-TUBER TISSUE AFTER INFECTION WITH GIBBERELLA SAUBINETII (MONT.) SACC.

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CHAPTER I

GENERAL INTRODUCTION

It has been known for many years that structural and physiological changes take place in plants as a result of an infection with a parasitic micro-organism. These changes are markedly different dependent on the host-parasite combination. The course of an infectional disease is determined by the influence of the parasite on the plant and the reaction of the host to the attack. GÄUMANN (1946) extensively discussed this subject. More recent reviews have been published by ALLEN (1954) and CHEVAUGEON (1957).

From the literature it appears that the physiological changes in the host cells may be of various kinds and are not limited to the tissue invaded by the pathogen. In several objects an increased temperature was observed in tissue at some distance from the centre of infection (EGLITS 1933, FISCHER 1950 and YARWOOD 1953). THATCHER (1939, 1942) investigated changes in the osmotic value and in the permeability of the host cells. An increase in the amount of growth-promoting substances in infected tissue was demonstrated by DALY and INMAN (1958) and by SHAW and HAWKINS (1958). Like KIRALY and FARKAS (1957) the latter authors reported a higher activity of certain enzymes after the infection. It appeared that the chemical composition of the host cells may show a great change. ALLEN (1954) mentions changes in nitrogen-containing compounds, lipids and carotenoids, carbohydrates and phosphate. Transpiration, photosynthesis and respiration may be affected too. In general it may be said that, when a parasitic micro-organism penetrates into a plant, the metabolism of the host cells is disturbed.

The increase of the respiration rate after infection is the subject of this investigation. To explain this phenomenon an attempt may be made to detect an agent excreted by the pathogen. However, DIMMOND and WAGGONER (1953) rightly point out the necessity that the toxins demonstrated *in vitro* should be proved to be active *in vivo* too. These authors distinguish between toxins and vivotoxins. With

Gibberella saubinetii (*Mont.*) *Sacc.* as a pathogen HELLINGA (1942) reported an increased O_2-uptake by disks of potato-tuber tissue after addition of extracts from infected tissue, from mycelium or from the culture-solution in which the fungus had been grown, but it was not demonstrated that the "active principle" which was found was active *in vivo*.

It may be asked whether a respiratory increase in the host cells is only due to compounds excreted by the parasite, or whether it may be partly or wholly attributed to a change in the metabolism of the host which is independent of these compounds (wound reaction, reaction to dying cells). Therefore it seemed desirable to investigate, what happens in the host cells, and in this way to try to find an explanation for the respiratory increase. After this it may be possible to explain the rôle played by the parasite.

With the potato tuber (*Solanum tuberosum L.*) as host and *Gibberella saubinetii* (*Mont.*) *Sacc.* as a pathogen it will be studied, whether a respiratory increase can be demonstrated in the host cells after infection of the tissue, and whether this increase is accompanied by a qualitative change in the respiratory pathway. In the second place an attempt will be made to obtain information concerning the mechanism by which the respiration rate is regulated.

CHAPTER II

RESPIRATORY INCREASE IN THE HOST CELLS

1. INTRODUCTION

For many host-parasite combinations the CO_2-production and the O_2-uptake of the host-parasite complex or one of them were found to be greater than the corresponding values found in healthy tissue (ALLEN 1954, CHEVAUGEON 1957). However, owing to the often high metabolic activity of micro-organisms, this might be attributed to the respiration of the pathogen. Indeed, MARESQUELLE (1928, 1930) reported in rust-infected plant parts a strict localization of the increased O_2-uptake to the places where the parasite was present. From this observation the author concluded that a higher respiration rate in the host cells is out of the question.

After infection with *Gibberella saubinetii* (*Mont.*) *Sacc.* cylinders of potato-tuber tissue developed a greater respiration rate (HELLINGA 1942). This was attributed to an increased O_2-uptake by the potato tissue, but experiments showing the extent to which the metabolism of the fungus was responsible for the accelerated gas-exchange were not described.

First of all it is necessary to separate the metabolism of host and parasite. How far this is possible, is determined by the nature of the micro-organism concerned, by the way in which it grows on the host tissue, and by the properties of the tissue attacked. The comparison of the respiration rate of infected tissue with that of the parasite cultured on synthetic media (FISCHER 1950), has the drawback that

the growing-conditions for the parasite on the host are quite different from those which it finds in nutritional solutions. From the ecto-parasite *Erysiphe graminis var. hordeï* growing on barley leaves only the haustoria remain in the leaves when the mycelium is brushed off. Still the O_2-uptake and the CO_2-production of these leaves are distinctly greater than they are in healthy leaves (MILLERD and SCOTT 1956). ALLEN and GODDARD (1938), comparing the respiration rate of *Erysiphe*-infected wheat leaves with that of the carefully isolated epidermides of leaves with or without parasite, ascertained that the greater part of the extra O_2-uptake is to be attributed to the metabolism of the mesophyll cells of the leaves.

If it is impossible to remove the parasite from the host, an attempt may be made to find out whether in infected plant parts a reaction occurs in the tissue not invaded by the pathogen. A higher CO_2-production from cells next to the centre of infection is reported in potato tubers infected with *Bacillus phytophthorus* (EGLITS 1933). In tubers of sweet potato (AKAZAWA and URITANI 1956) and of white potato (AKAZAWA 1956a) infected with *Ceratostomella fimbriata* it is the same with the O_2-uptake of the tissue adjacent to the areas invaded by the pathogen.

As *Gibberella saubinetii* (*Mont.*) *Sacc.* growing on potato-tuber tissue penetrates only the outer cell layers and slowly spreads inward with simultaneous collapse of the superficial cells, the respiration of the tissue next to the parts invaded by the fungus can be investigated.

2. MATERIAL AND METHODS

a. *Material*

Potato tubers var. Bintje were bought on the market and stored in a cool room. *Gibberella saubinetii* (*Mont.*) *Sacc.* was obtained from the Centraal Bureau voor Schimmelcultures at Baarn and grown in culture tubes on oat meal agar.

b. *Inoculation and incubation*

Sound tubers were brushed in tapwater, externally sterilized in 4 % formalin during 2 min., and washed in running tapwater for 15 min., after which the tubers were cut by means of a sterilized knife into two halves, the cut running perpendicular to the longitudinal axis. These halves, one of which was inoculated over the whole surface of the cut with a suspension of spores in distilled water, were each transferred to a sterile glass jar (volume 0.5 L.) and placed at 25° C. Unless otherwise stated, the experiments were carried out after 5–10 days incubation.

c. *The preparation of tissue disks*

With the help of the apparatus drawn in Fig. 1 disks 1 mm. thick and 6 mm. in diameter were cut from the rather homogeneous tissue of the tubers found inside

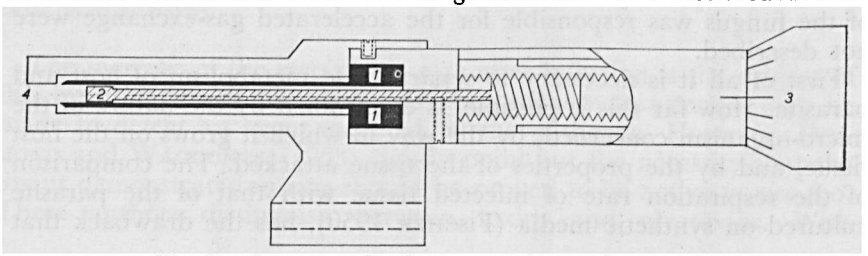

Fig. 1. Apparatus for the preparation of tissue disks.

the ring of vascular bundles. All parts of the apparatus which came into contact with the tissue, were of stainless steel. With the cork-borer (1) a tissue cylinder was bored out, which after the borer had been arrested in the apparatus, was pressed out by means of a rod (2), which was moved by turning a knob (3) provided with a scale graduated up to 0.1 mm. To prevent torsion tension in the tissue, the rod itself was not turned round. The disks were cut off along the opening of the apparatus (4) with a sterile razor blade.

From the healthy and the infected tuber halves some tissue cylinders were successively bored out perpendicular to the surface[1]) and divided into disks. From each tuber half the disks cut at the same distance from the surface were put together as tissue samples. The zonation in the infected halves was found to be parallel to the surface which was hollowed out by the fungus. The parts of the tissue cylinders which proved to contain mycelium and which showed discolorations, varied but little in extent within a tuber half, and these parts were removed. The disks from the first zone that proved to be macroscopically indistinguishable from healthy tissue, were used as the sample nearest to the area invaded by the fungus. Within each sample the variation in distance to the surface was less than 1 mm.

d. *Measurement of respiration rate*

Respiration rates, as indicated by oxygen uptake, were determined by the Warburg manometric technique, using flasks of about 15 ml. with a centre well and a side arm. They are expressed in $\mu l.O_2$ absorbed per hour per gram of tissue (fresh weight; determined before the Warburg estimation). Unless stated otherwise in the text, each flask contained 10 disks submersed in 1.5 ml. distilled water, and in the centre well 0.2 ml. KOH 10 % and a piece of filter paper (2 × 2 cm.) folded fanwise. When the CO_2-production was also to be determined, the KOH was omitted. The temperature of the waterbath was 25° C. The flasks were shaken at 130 oscillations per minute through a stroke of 4 cm. After 30 min. equilibration readings were taken at 1 hour intervals.

Preliminary experiments showed that a considerable O_2-uptake can be measured using the method described above without the experimental conditions becoming rate-limiting. Although in preparing the disks a full sterility was not pursued, interference by infections was never observed.

As there are indications that alterations in the metabolism have developed after a 24 hours' washing-period (STEWARD and STREET 1946, SCHADE and LEVY 1949, THIMANN, YOCHUM and HACKETT 1954, HACKETT and HAAS 1958, GRIFFITHS and HACKETT 1957, LOUGHMAN 1957, CALO and VARNER 1957), the disks were washed in distilled water a few times only and used practically immediately after cutting. The O_2-uptake of such disks appeared to increase gradually during the experimental period.

e. *Test for the presence of mycelium*

In order to be sure that only tissue not penetrated by the fungus was used, series of disks from infected tuber halves were laid out on moistened filter paper in petri dishes at 25° C. Mycelial development was observed only on macroscopically discolorated tissue that had been cut at the most 2–3 mm. from the surface. This is in accordance with observations made on microtome sections of infected potato-tuber tissue (Richter, unpublished) according to which, dependent on the infection-period (3–11 days), the mycelium occurred up to a distance of 1.3–3.2 mm. from the surface. So it may be concluded that the fungus does not penetrate into the intact tissue to a greater depth than about 3 mm.

Disks that are macroscopically indistinguishable from healthy tissue, do not contain mycelium. In a number of experiments all disks were laid out on moistened filter paper at 25° C. after measurement of the respiration rates. Mycelial development was never observed on these disks.

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[1]) The surface of the cut by which the tuber was divided into two halves, is always called "the surface".

3. Results and discussion

Preliminary experiments showed that, when a great number of disks were cut from the central part of a potato tuber and divided at random into samples of 10 disks, the differences between the amounts of oxygen absorbed per hour per gram (fresh weight) were small and did not exceed 10 %.

To calculate the extent of a change in respiration rate which might occur after fungal infection, one may compare the O_2-consumption of samples from the infected tuber half with that of tissue from the non-infected half of the same tuber. However, the respiration intensity of the tissue from non-infected tuber halves which had been stored at 25° C. for some days, appeared to be dependent on the place from which the disks were cut. As shown in table 1, the tissue close below

TABLE 1. μl. O_2-uptake/hour/gram (fresh weight) of samples cut at various distances from the surface of the non-infected half of two tubers. Tuber halves stored at 25° C. for 18 days. O_2-uptake average over 2 hours.

mm. from the surface	μl. O_2-uptake I	II
½–1½	100.1	97.7
1½–2½	59.1	59.2
2½–3½	55.7	57.0
3½–4½	51.4	55.9
4½–5½	48.8	55.2
5½–6½	52.7	54.9

the cutting-surface which was blocked by a cork layer, absorbed 80–90 % more oxygen than the adjacent tissue. The zone with increased respiration did not extend beyond a line 2–3 mm. from the surface. The differences between the O_2-consumption of the other samples did not exceed the variation in respiration intensity of parallel samples from an untreated tuber. The average O_2-uptake of these samples may be considered to represent the normal respiration rate of healthy tissue.

An attempt may also be made to determine the normal respiration rate with tissue from the infected tuber half itself, and to calculate the extent of the changes in respiration intensity by comparison with this rate of O_2-uptake. Assuming that the influence of the parasitically growing fungus on the metabolism of the host cells decreases when the distance to the zone with mycelium increases, it may be expected that at a certain distance from this area no change in the respiration rate is caused by the disease.

In table 2 the results are given of a representative experiment during which the O_2-consumption was determined in a number of samples taken from successive zones out of the infected half and of the non-infected half of a tuber. The respiration was distinctly accelerated near the area invaded by the fungus, and decreased with increasing distance to this area, to reach a nearly constant level in the zones at greater distance. In this experiment this level of O_2-uptake appeared to be higher than in the non-infected half of the

same tuber, but in other experiments this level was often lower than or differed but slightly from the respiration rate of healthy tissue.

In preliminary experiments with non-infected tuber halves after storage at 25° C. in a number of tubers similar differences were observed between the normal respiration rate of the tissue from the two halves. Averaged for 20 successive experiments the O_2-uptake

TABLE 2. μl. O_2-uptake/hour/gram (fresh weight) of samples from the non-infected and the infected halves of a tuber, incubated after infection at 25° C. during 10 days. O_2-uptake average over 3 hours. Dry weight estimated after heating to 100° C. overnight.

mm. from the surface	non-infected tuber half				infected tuber half				
	μl. O_2	fresh weight mg.	dry weight mg.	water content %	μl. O_2	%	fresh weight mg.	dry weight mg.	water content %
6–8	50.3	328	33	90	122.7	192	286	27	91
8–10	48.0	325	31	90	86.7	135	294	28	90
10–12	50.3	324	32	90	70.1	110	281	27	90
12–14	49.5	327	33	90	65.2		299	29	90
14–16	51.8	326	34	89	63.1		290	30	89
16–18					61.6	100	294	33	89
18–20					66.0		294	33	89

of tissue from the non-infected half and that of tissue from the area of the infected half which was supposed to be unaffected by the disease, was the same, *viz.* 58.8 μl. and 58.7 μl. O_2/hr./gram respectively. The conclusion may be drawn that the nearly constant level of O_2-uptake of samples from the infected tuber halves taken at greater distance from the surface represents the respiration rate which had not been altered by the disease.

In the experiments the extent of the respiratory increase was calculated by comparing the O_2-absorption with the average respiration rate of some samples cut from the same tuber half, but from the area which had not been affected by the fungal infection, as appeared from the nearly constant level of respiration (table 2). In addition the O_2-uptake of some samples from the non-infected half of the tuber was determined.

Table 2 shows an accelerated O_2-uptake per gram (fresh weight) after infection and incubation at 25° C. As the water content of all samples was the same, this respiration-gradient can not, either partly or wholly, be due to a decrease of the water content of the tissue near the area invaded by the fungus, which at calculating the O_2-uptake per gram of tissue would result in a respiration-gradient independent of the occurrence of a real increase of the respiration rate.

In the infected tuber halves an ever increasing respiration rate was usually found with decreasing distance to the surface. However, in some tubers the respiratory increase of the tissue adjacent to the zone with mycelium appeared to deminish again and sometimes the O_2-uptake dropped to a level lower than the normal respiration rate. This phenomenon was accompanied by a loss of turgescence in the

cells of the area adjacent to the infected parts. The development of such a zone with soft tissue in some tubers may be accompanied by a higher intensity of the fungal attack and a greater susceptibility to infection.

It was difficult to ascertain the exact relation between the extent of the respiratory increase and the intensity of infection, as a reliable standard of comparison has not yet been found. In fact the density of the aerial mycelium on the infection surface in relation to the depth over which the potato tissue had collapsed, could be observed at the end of the incubation, but these two phenomena could only be roughly estimated. When this was done, the results of many experiments suggested that in tuber halves without a zone with soft tissue the differences in respiratory increase as well as the distance from the surface where a respiratory increase could still be demonstrated, corresponded with the differences in intensity of infection and with the length of the period of incubation at 25° C. This is illustrated in Fig. 2 and 3.

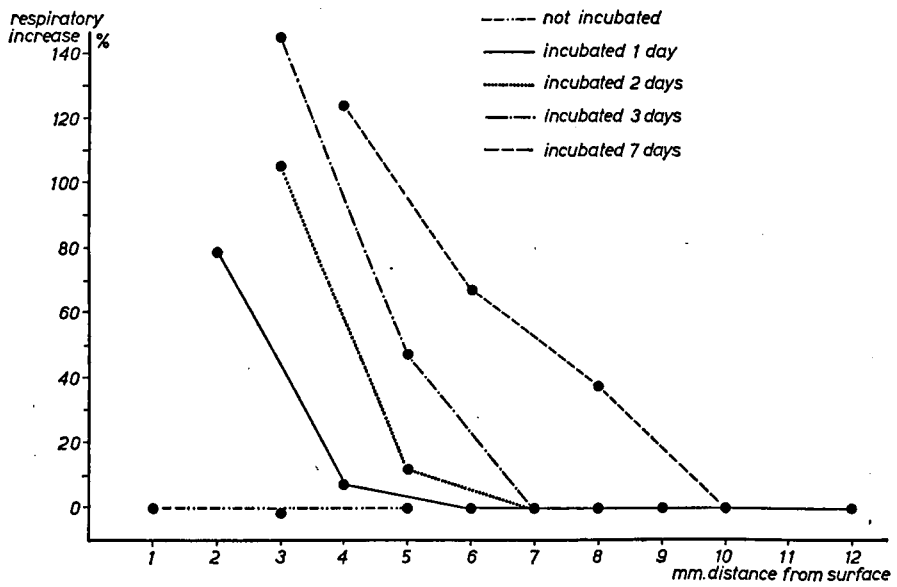

Fig. 2. Relation between respiratory increase after infection and the length of the period of incubation at 25° C.

It may be concluded that after infection of a tuber half with *Gibberella saubinetii* (*Mont.*) *Sacc.* the respiration of host tissue which was not invaded by mycelium, is accelerated. The extent of the acceleration is dependent on the distance to the area invaded by the fungus and on whether or not a zone with soft tissue had developed.

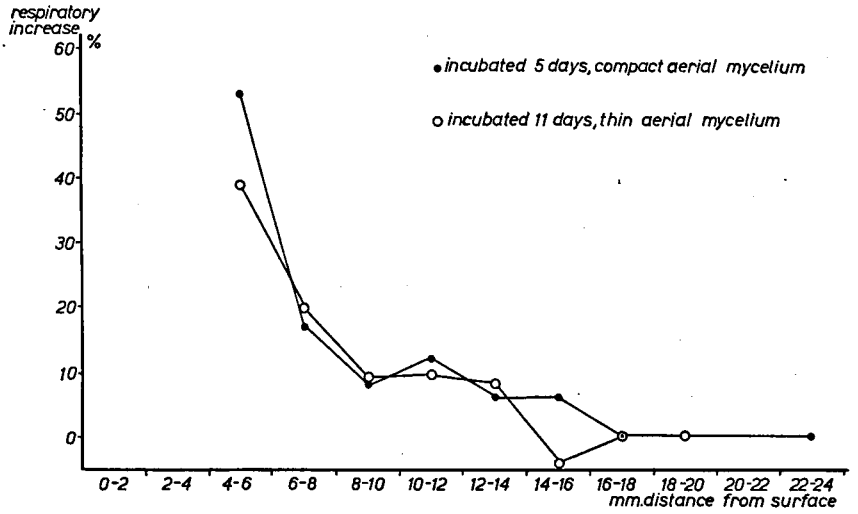

Fig. 3. Relation between respiratory increase after infection and the density of the aerial mycelium as well as the period of incubation at 25° C.

CHAPTER III

COMPARISON OF THE PATHWAYS FOLLOWED BY AEROBIC RESPIRATION IN HEALTHY AND IN DISEASED TISSUE

1. Introduction

A respiratory increase may be caused by an acceleration of the normal processes of which the respiration chain consists, by the development of pathways that under normal conditions are not present or are but slightly used, or by a combination of these possibilities. This aspect of the host-parasite relation has been scarcely investigated. Many investigations on the respiration after infection are not concerned with the respiratory pathway. Allen (1953) discusses a number of factors which might be rate-limiting in respiration and which might be changed under the influence of toxins excreted by the parasite, but this are factors which would allow the respiration to increase without altering the pathway.

Farkas and Kiraly (1955) and Kiraly and Farkas (1955) think that the respiratory mechanism of wheat leaf disks infected with *Puccinia graminis* is changed between the glycolysis and the terminal oxidation, as malonate much stronger inhibited the O_2-uptake of healthy tissue (60–70 %) than of infected disks (30–40 %). By iodoacetate and fluoride both tissues were equally affected. The latter observation was confirmed for other varieties of wheat infected with *Puccinia graminis* by Shaw and Samborski (1957), who studied in addition the rôle of the pentosephosphate cycle (hexosemonophosphate shunt, direct oxidative pathway; Horecker 1953) in respiration.

In the Embden-Meyerhof-Parnas pathway C_6 and C_1 from glucose are equally converted to CO_2 (C_6/C_1 ratio 1), but in the pentosephosphate cycle the C_6/C_1 ratio is <1, as in the beginning C_1 is more rapidly liberated in the form of CO_2 than C_6 is (BEEVERS and GIBBS 1954). In experiments with added glucose–1–C^{14} or glucose–6–C^{14} the C_6/C_1 ratio in the respiratory CO_2 of infected leaf parts appeared to be lower than that of healthy parts, indicating an increasing importance of the cycle in the respiration after infection. However, the cycle is reputedly resistant to the action of fluoride and iodoacetate. The % inhibition of the respiration of infected tissue caused by these compounds should have been lower than it is in healthy tissue and not equal, as was observed. Authors do not give a satisfactory explanation of these data.

A lower C_6/C_1 quotient in hypocotyls of safflower (*Carthamus tinctorius L.*) infected with *Puccinia carthami Cda.* especially in tissue containing sporulating pathogen (pycnospores, uredospores) might suggest an increase of the oxidation via the hexosemonophosphate shunt. This is in agreement with the decrease of the NaF-sensitiveness of the respiration (DALY and SAYRE 1957; DALY, SAYRE and PAZUR 1957).

All three investigations mentioned above were carried out with the host-parasite complex. It is not known to what extent the metabolism of the parasite is responsible for the change in the respiratory pathway of the complex. However, according to SHU and LEDINGHAM (1956) the hexosemonophosphate shunt plays an important part in the respiration of uredospores of wheat-stem rust. The supposed changes in the respiratory mechanism of safflower, which were especially noticeable with sporulating pathogen, probably should be attributed to an increase of the metabolism of the parasite.

In this chapter an attempt will be made to obtain evidence which may throw light on the question whether or not the respiratory mechanism is qualitatively changed in potato slices obtained from areas adjacent to the cells invaded by *Gibberella.*

2. RESPIRATORY QUOTIENT

The respiratory quotient is not only dependent on the substrate used up, but is also determined by the products formed from it (TAMIYA 1932). Although R.Q.-values are of secondary importance only, they may indicate changes in the metabolism appearing after infection.

The respiratory quotient was estimated by means of the direct method of Warburg. The gas exchange of each sample was measured first in the absence of alkali during two intervals of one hour; then 0.2 ml. KOH 10 % was added to the centre well and after 15 min. equilibration the O_2-uptake was measured for some hours. The R.Q.-values were calculated from the readings of the last hour before and the first hour after addition of KOH to the flask; in this manner the influence of the gradual increase in respiration rate during the experimental period remained small.

In table 3 the values are given for three experiments with both non-infected halves of some tubers which have been stored at 25° C. for some days after cutting. In general the R.Q. was <1.0 and showed a considerable variation. This agrees with other investigations (BOSWELL and WHITING 1938, HELLINGA 1942). The variation is chiefly to be attributed to individual differences between the tubers. Usually the differences within a tuber half were small. The halves of a tuber gave nearly equal average values of the R.Q.

As appears from table 4, it was found for samples from the infected halves of potato tubers that the R.Q. was not affected in spite of the accelerated respiration of the tissue adjacent to the area invaded by the fungus.

It may be concluded that the R.Q. of the host cells which were not penetrated by the mycelium, was not changed.

TABLE 3. Respiratory quotients of samples from the two non-infected halves of three tubers. Tuber halves stored at 25° C. for resp. 10, 13 and 18 days.

storage at 25° C.	10 days		13 days		18 days	
tuber halves / mm. from the surface	I	II	I	II	I	II
5–7	0.79	0.85	0.98	0.98	0.80	0.87
7–9	0.84	0.84	0.93	1.01	0.82	0.84
9–11	0.92	0.81	0.97	1.05	0.80	0.84
11–13	0.91	0.81	—	1.00	—	0.85
13–15	0.85	0.84	—	1.00	—	0.80
15–17	—	—	—	0.95	—	0.82
average	0.86	0.83	0.96	1.00	0.81	0.84

TABLE 4. R.Q. and μl. O_2-uptake/hour/gram (fresh weight) of samples from the non-infected and the infected halves of two tubers, incubated after infection at 25° C. Infected halves without soft tissue.

mm. from the surface		incubated 15 days		incubated 22 days	
		R.Q.	μl. O_2	R.Q.	μl. O_2
non-infected half	5–7	0.96	85.8	0.95	98.8
	7–9	0.95	85.6	0.96	98.5
	9–11	0.95	84.7	0.99	99.0
	11–13	0.92	88.5	0.98	101.5
infected half	5–7	0.91	126.4	0.88	131.8
	7–9	0.91	107.3	0.90	102.8
	9–11	0.94	108.2	0.89	94.7
	11–13	0.93	94.8	0.92	90.8
	13–15	0.96	90.6	0.90	91.6
	15–17	0.96	90.2	0.90	92.4
	17–19	0.85	86.7	0.90	85.4

3. Experiments with respiratory inhibitors

The mechanism of respiration and its changes may be studied by the determination of the effect on the O_2-uptake of an addition of compounds which are inhibitory to one or more reactions of the respiratory chain. The value of conclusions from such experiments is highly dependent on the specificity of the inhibitory action. From the extensive review by James (1953) on the use of respiratory inhibitors, it is clear that no inhibitors are known that specifically interfere with one enzyme reaction, though the specificity might be enhanced by a careful choice of the dosage and the experimental conditions.

In experiments with intact cells it is necessary that the compounds should be taken up well. The great influence exercised by the pH of the medium on the inhibitory action of certain compounds, which by some authors is attributed to the effect of the pH on the entrance of the molecules into the cells (James 1953), made it necessary to replace distilled water as a medium for the potato slices by a buffer solution. For this a 1/15 M. phosphate buffer was selected (mixture of 1/15 M. KH_2PO_4 and 1/15 M. Na_2HPO_4 in water). In this buffer the O_2-uptake of the disks is only slightly enhanced. Within certain limits the pH of the medium appeared to have little effect on the respiration rate. In accordance with Boswell and Whiting (1938) and with Hellinga (1942) it was found that the O_2-consumption was the same at pH 5.5–6.8. Table 5 shows that this O_2-uptake (pH 6.2) was somewhat greater than in distilled water, and scarcely differed from that at pH 7.7 and pH 4.2.

Unless otherwise stated, a buffer pH 6.2 was used in experiments with added iodoacetate, sodium fluoride or sodium azide. When malonate was added, the disks were submersed in a buffer pH 4.3.

TABLE 5. μl. O_2-uptake/hour in distilled water or 1/15 M. phosphate buffer of samples of 10 disks from an untreated potato tuber.

medium	μl. O_2-uptake			
aqua dest.	16.5	16.0		
buffer pH 4.2	16.6	16.2	15.7	16.5
buffer pH 6.2	18.9	19.1	18.1	18.5
buffer pH 7.7	19.4	18.2	15.2	

In the experiments the additions of the inhibitor solved in water and adjusted to the proper pH, were made from the side-arms of the Warburg flasks after measurement of the respiration rate during the two preceding hours. After that the course of the O_2-uptake was watched for some hours. To the control samples corresponding amounts of distilled water were added.

In calculating the effect of the inhibitors the gradual increase of the respiration rate during the experimental period had to be taken into account. Therefore, in all experiments from each tissue zone a control sample was prepared besides the disks to which the compound was added. The same procedure was followed for the determination of the stimulatory effect of 2,4–dinitrophenol (Chapter v). For each

period of one hour after addition of the compound, the effect was calculated in % of the average respiration rate during the two hours before addition, and corrected for the increase of the respiration rate in the course of the experiment.

$$\% = 100 - \frac{\mu l.\ O_2 \text{ after addition}}{\mu l.\ O_2 \text{ before addition}} \times \frac{\mu l.\ O_2 \text{ control before addition}}{\mu l.\ O_2 \text{ control}} \times 100.$$

An inhibition is indicated by a positive %, a stimulation by a negative %.

a. *Experiments with mono-iodoacetate*

Iodoacetate is generally considered to be an inhibitor of glycolysis. This action is thought to be due to an irreversible reaction with –SH groups of enzyme protein. Although several enzymes contain these groups, and an inhibitory action on the activity of a number of these enzymes is known, *in vivo* the 3–phosphoglyceraldehyde (triose-phosphate) dehydrogenase would be affected preferentially, and the reaction in low concentrations of the inhibitor would be reasonably specific (JAMES 1953).

The experiments were carried out with potato-tissue slices in 1/15 M. phosphate buffer pH 6.2 to which iodoacetate was added, so that a concentration of 0.002 M. was obtained. The O_2-uptake was considerably reduced to a nearly constant, low level, which was reached 4–5 hours after addition of the inhibitor. In table 6 the data of 4 experiments are reviewed. It is clear that the % inhibition of the O_2-uptake of the samples from the infected tuber halves of experiments 2 and 4 were nearly the same, whether the respiration rate was increased by the disease or not, and were in close agreement with the inhibition in disks from non-infected tuber halves. The O_2-absorption was suppressed with 90–95 %. However, in the infected halves of the experiments 1 and 3 a zone with soft tissue had developed. The respiration rate of samples from this area (expt. 1:I, II and expt. 3:I) decreased when the samples were approaching the cells invaded by the fungus, and this decrease was accompanied by a

TABLE 6. . $\mu l.\ O_2$-uptake/hour/gram (fresh weight) before addition of iodoacetate to 0.002 M. pH 6.2 and % inhibition 4–5 hrs. after addition. Samples cut from zones of non-infected and infected halves of 4 tubers. Zones I nearest the surface at > 3 mm. distance.

samples		expt. 1		expt. 2		expt. 3		expt. 4	
		$\mu l.$ O_2	% inhibition	$\mu l.$ O_2	% inhibition	$\mu l.$ O_2	% inhibition	$\mu l.$ O_2	% inhibition
non-infected	I	54.6	94	50.7	96	54.4	95	53.6	99
	II	53.8	94	49.1	95	54.9	95	46.1	98
infected	I	*40.1*	*66*	111.1	91	*55.2*	*78*	88.5	92
	II	*53.5*	*85*	87.4	92	69.4	92	79.1	91
	III	56.7	89	75.0	93	55.0	93	70.1	93
	IV	53.2	92	68.1	94	42.7	93	66.6	86
	V	44.4	95	67.8	94	50.4	92	62.2	94
	VI	35.9	96	53.5	95	51.8	92	—	—

decreasing inhibition by iodoacetate. The % inhibition in the other samples had not changed.

b. *Experiments with sodium fluoride*

Sodium fluoride has an inhibitory effect on several enzymes, but enolase, which catalyses a reaction of the glycolysis chain, would be the most sensitive of them and this enzyme would be nearly specifically inhibited in low concentrations of the inhibitor. The inactivation of enolase is dependent on the presence of inorganic phosphate and magnesium (SUMNER and MYRBÄCK 1951, MILLER 1958).

In the experiments sodium fluoride was added to potato slices submersed in 1/15 M. phosphate buffer pH 6.2, so that a concentration of 0.02 M. was obtained. The development of full inhibition required 4–5 hours, after which the O_2-uptake was suppressed with about 80 %. Just as in the experiments with iodoacetate the % inhibition after addition of NaF were the same in samples from the non-infected and the infected tuber halves, except in those samples that has been cut from the area with soft tissue that occurred in some infected tuber halves (table 7). The O_2-uptake of these disks was inhibited to a lower degree.

TABLE 7. μl. O_2-uptake/hour/gram (fresh weight) before addition of sodium fluoride to 0.02 M. pH 6.2 and % inhibition 4–5 hrs. after addition. Samples cut from zones of the non-infected and the infected halves of a tuber. Zones I nearest the surface at 5 mm. distance.

tuber halves	non-infected		infected					
samples	I	II	I	II	III	IV	V	VI
μl. O_2-uptake	53.9	53.5	*47.9*	56.3	51.4	45.0	43.7	39.9
% inhibition	80	82	*68*	80	78	80	81	79

c. *Experiments with sodium azide*

According to JAMES (1953) sodium azide influences the metabolism by the formation of metal complexes, by which cytochrome oxidase may be markedly inhibited. Polyphenol oxidase is reported to be partially inhibited *in vitro*. Moreover, sodium azide interferes with the phosphate transfer and the formation of high-energy phosphate. Both actions are reversible after removal of the azide. The degree of inhibition of the metal-containing enzymes is at pH 5–8 highly dependent on the pH of the experimental medium. Now the latter phenomenon was studied with potato tissue too.

As shown in table 8, the inhibition of the O_2-uptake of potato disks was much greater at a lower pH. As in all experiments with azide the inhibition rapidly developed and the respiration rate reached a nearly constant level already 2–3 hours after addition of the inhibitor, it seems reasonable to suppose that the differences in inhibition are a result of the effect exercised by the pH on the metal-complex formation rather than of its effect on the entrance of the azide into the cells. In conclusion, the inhibition of the terminal oxidase(s) was probably dominant.

The experiments with disks from non-infected and infected tuber halves were carried out in phosphate buffer pH 6.2 to which sodium azide was added, so that a concentration of 0.002 M. was obtained.

TABLE 8. Inhibition of the O_2-uptake of healthy potato-tuber tissue after addition of sodium azide to 0.002 M. to media (1/15 M. phosphate buffer) with pH 7.7, 6.2 or 4.2.

pH medium	% inhibition					
7.7	50	59	53	54	56	
6.2	83	81	84	84	88	89
4.2	94	95				

From the experiment of table 9 it is clear that a stimulation of the respiration occurred in the samples of the infected half near the area invaded by the fungus, but that the % inhibition were nearly the same in all samples and in both halves.

Data on the effect exercised by sodium azide on the respiration of samples from tuber halves with a zone with soft tissue are not available.

TABLE 9. μl. O_2-uptake/hour/gram (fresh weight) before addition of sodium azide to 0.002 M. pH 6.2 and % inhibition 3 hrs. after addition. Samples cut from zones of the non-infected and the infected halves of a tuber. Zone I nearest the surface at 6 mm. distance.

tuber halves	non-infected		infected					
samples	I	II	I	II	III	IV	V	VI
μl. O_2-uptake	53.7	53.4	75.9	70.5	59.5	50.0	48.3	54.8
% inhibition	86	89	87	87	91	91	89	86

d. *Experiments with malonate*

In the argumentation for the participation of the tricarboxylic acid cycle in the respiration of a tissue the inhibition of the O_2-uptake by malonate plays an important part. However, malonate is not a specific inhibitor, although it is generally assumed that it primarily gives a competitive inhibition of succinic dehydrogenase with *in vitro* an affinity ratio malonate/succinate of about 50/1 in favour of the inhibitor (JAMES 1953). HANLY, ROWAN and TURNER (1952), discussing the literature, conclude that it seems quite likely, at least in experiments with higher concentrations of malonate and especially where succinate accumulation can not be demonstrated, that its effect on respiration can not safely be ascribed to an effect on succinic dehydrogenase.

Whether the tricarboxylic acid cycle is active in the respiration of potato-tuber tissue is not certainly known. Succinic dehydrogenase is reported to occur in the potato (MILLERD 1951, HACKETT 1956). The inhibition by malonate of the O_2-absorption of potato slices, as discussed below, would be in agreement with this view. However, evidence has been obtained for a not entirely specific inhibitory action of malonate, at least when the inhibition is greater than about 25 %.

In the experiments a solution of malonic acid, the pH of which was

adjusted with NaOH, was added to potato-tuber slices submersed in 1/15 M. phosphate buffer with the same pH. In accordance with the results obtained with carrot disks by HANLY, ROWAN and TURNER (1952), the degree of inhibition depended on the concentration of the inhibitor and on the pH of the medium, a lower pH promoting the inhibition. At pH 6.5 the O_2-uptake of healthy tissue was inhibited by 0.1 M. and 0.17 M. malonate with about 25 % and 40 % respectively. At pH 4.3 0.01 M. malonate caused an inhibition of about 30 % already, whereas the O_2-consumption was suppressed with about 80 % by 0.06 M. malonate.

If the tricarboxylic acid cycle is active in potato-tuber tissue and if malonate inhibits the O_2-uptake exclusively by a competitive action on the succinic dehydrogenase, one may expect to observe a reversal of the inhibition as a result of the accumulation of succinic acid in the cells owing to this inhibition or after addition of this acid to the medium. However, table 10 shows that a self-recovery of the O_2-uptake was only observed when the respiration was but slightly inhibited.

From table 11 it appears that in agreement with these results a greater inhibition by malonate of the O_2-uptake was not reversed after addition of succinate. Nevertheless the development of the

TABLE 10. % Inhibition of the O_2-uptake of healthy potato-tuber tissue by malonate in buffer pH 6.5 for each hour after addition of the inhibitor. Self-recovery in 0.1 M. malonate pH 6.5.

hrs. after addition	1st	2nd	3rd	4th	5th	6th
0.1 M. malonate	—11 %	27 %	18 %	10 %	0 %	—
	— 4 %	20 %	14 %	10 %	6 %	—
0.17 M. malonate	— 8 %	27 %	33 %	33 %	40 %	40 %
	—16 %	22 %	23 %	37 %	35 %	41 %

TABLE 11. Effect of succinate or fumarate on % inhibition of the O_2-uptake of healthy potato-tuber tissue by malonate added simultaneously or 4 hrs. before. Medium: 1/15 M. phosphate buffer pH 4.3.

hrs. after addition	1st	2nd	3rd	4th	5th	6th	7th
0.04 M. malonate	11 %	53 %	64 %	64 %	82 %	72 %	76 %
	16 %	51 %	61 %	66 %	79 %	69 %	73 %
0.04 M. malonate 0.05 M. succinate	—19 %	3 %	42 %	47 %	61 %	68 %	73 %
					+ 0.05 M. succinate		
0.04 M. malonate	18 %	54 %	65 %	71 %	77 %	75 %	73 %
	11 %	51 %	64 %	72 %	73 %	73 %	70 %
0.03 M. malonate 0.012 M. fumarate	2 %	17 %	21 %	37 %	55 %	64 %	75 %
					+ 0.012 M. fumarate		
0.03 M. malonate	8 %	42 %	62 %	71 %	78 %	74 %	70 %

inhibition was distinctly retarded by simultaneous addition of succinate and malonate, yet after a few hours the degree of inhibition was nearly equal to that obtained with the same concentration of malonate without succinate. Similar results were obtained with fumarate.

That in these experiments succinate had no effect on the ultimate inhibitory effect exercised by malonate on the O_2-absorption might be explained from the great affinity of the inhibitor to the succinic dehydrogenase. However, the experiments with fumarate as well as the absence of a self-recovery when the inhibition was greater than 25 %, suggest that the results are to be understood as an indication that malonate did not attack succinic dehydrogenase exclusively.

Experiments with disks from the non-infected and the infected halves of potato tubers were performed with 0.06 M. malonate pH 4.3. The respiration rates and the inhibition by malonate are represented for two experiments in table 12. Clearly the inhibition of the O_2-uptake of the samples from the infected halves was not the same. A stimulation of the respiration after infection was not immediately accompanied by a change in the degree of inhibition by malonate. Only when the influence of the infection continued, and the respiration rate was accelerated to a greater extent, the % inhibition distinctly decreased.

TABLE 12. μl. O_2-uptake/hour/gram (fresh weight) before addition of malonate to 0.06 M. pH 4.3, and % inhibition after addition. Samples cut from the zones of non-infected and infected halves of 2 tubers. Medium: 1/15 M. phosphate buffer pH 4.3.

tuber halves	mm. distance from surface	expt. 1		expt. 2	
		μl. O_2	% inhibition	μl. O_2	% inhibition
non-infected	5–7	43.0	77	39.6	72
	7–9	45.4	79	38.8	76
infected	5–7	81.8	21	118.8	38
	7–9	66.7	52	83.1	52
	9–11	57.6	67	66.9	74
	11–13	34.8	66	54.6	73
	13–15	37.3	67	52.1	75

The O_2-consumption of the two samples from the non-infected halves was equally retarded. The differences in the inhibition of the normal respiration of tissue from both halves of these tubers did not exceed the differences in % inhibition observed in preliminary experiments with disks from two non-infected halves of a tuber.

Data concerning the inhibition by malonate in samples from infected tuber halves containing a zone with soft tissue are not available.

4. Discussion

From the experiments with inhibitors described above no definite conclusions can be drawn concerning the pathway of aerobic respiration, as it is not known, whether the compounds have affected enzymes other than those that would have been attacked preferentially. The data obtained after addition of malonate suggest that the inhibitory action of this compound was not limited to the succinic dehydrogenase.

Concerning the question whether a qualitative change occurs in the respiratory pathway in consequence of the disease, it is clear that in tissue from the infected tuber halves the % inhibition after addition

of iodoacetate, sodium fluoride or sodium azide were the same for all samples, whether or not the respiration rate was increased after infection, provided that the samples were cut from apparently normal tissue. In conclusion, these experiments provided no evidence of a qualitative change of the respiratory pathway.

On the other hand, the inhibition by malonate did not prove to be the same for all samples. Although stimulation of the respiration after infection was not immediately accompanied by a change in the % inhibition, yet when the influence of the infection continued and the O_2-uptake was increased to a greater extent, the % inhibition considerably decreased. This indicates that with continued influence of the infection the respiratory pathway was qualitatively changed too.

In the zone with soft tissue which developed in some tubers after infection the tissue-structure was affected and the cells had lost their turgescence. The desintegration of tissue not penetrated by mycelium was accompanied by a decrease of the inhibition by iodoacetate and sodium fluoride, whereas the respiration rate was increased to a smaller extent than in the adjacent normal cells. So, within this area changes in the respiratory pathway had developed which were not observed outside this area. Maybe these changes are connected with the dying of the cells.

Of the apparently normal tissue it may be said that at least during the initial stages of respiratory stimulation after infection no evidence of a qualitative change of the pathway was obtained, whereas after addition of iodoacetate, sodium fluoride or sodium azide no evidence at all was found in support of such a change. The unaltered R.Q. after respiratory increase was in agreement with these facts.

CHAPTER IV

CHANGES IN CARBOHYDRATE CONTENT AND THEIR RELATION TO THE PARASITICALLY INCREASED RESPIRATION

1. Introduction

In the preceding experiments with infected potato tubers evidence for a qualitative change of the respiratory pathway was only obtained after addition of malonate, and then only in advanced stages of respiratory stimulation. The onset of the stimulation is likely to be merely quantitative, and might be attributed to an increase of the intensity or/and the capacity of the processes which regulate the respiration rate.

It is generally assumed that in potatoes carbohydrates are the ultimate respiratory substrate. Hopkins (1927) found that after wounding potato tubers the content of total sugars or reducing sugars was altered parallel with the respiration rate. Appleman and Miller (1926) studied tubers in various stages of development, and Barker (1935/36) treated mature tubers with low temperatures. These authors observed a similar behaviour of the respiration intensity and the sucrose

content. In infected tuber halves starch disappeared from the tissue adjacent to the area invaded by *Gibberella* (Richter, unpublished) and it is clear from the experiments described in Chapter II that in this tissue the O_2-uptake is stimulated.

In this chapter experiments will be described bearing on the question whether there exists a close relation between the O_2-consumption and the sugar content, and whether the respiration rate is regulated by the amount of substrate. As the respiration intensity may be related with one special sugar, it is necessary to determine not only the amount of total sugars or reducing sugars in relation to the O_2-uptake, but, if possible, also to determine the content of the various sugars separately.

Therefore, after measurement of the O_2-absorption, extracts were prepared from disks out of the non-infected and the infected tuber halves, and the amounts of the respective sugars were determined after quantitative separation using paper-chromatography.

2. Methods

a. *Colorimetric estimation of sugars*

Dissolved carbohydrates were estimated in 1 ml. samples using the colorimetric method with anthrone reagent according to Mokrasch (1954). In view of the rapidity of colour formation with the respective sugars, the mixture of the reagent with fructose was heated to 80° C. $\pm$ 0.5° C. for exactly 5 min. and the mixture with glucose or sucrose for exactly 25 min.

The colour densities were measured with a Gallenkamp colorimeter (King 1951) provided with a heat-filter. A colour-filter (Ilford nr. 607) transmitted from the entering light mainly the light with a wave length of 620 mμ. The lightpath through the solution in the absorption cells was 1 cm.

From estimations with watery solutions of sucrose, glucose or fructose with a concentration of up to resp. 80 μg./ml., 40 μg./ml. and 40 μg./ml. it appeared that after reaction with anthrone the extinctions were proportional to the amount of sugar. It proved to be possible to carry out the estimations correct to 1–2 μg.

b. *The preparation of extracts from potato-tuber tissue*

The tissue disks were killed rapidly in boiling ethanol 80 % to which was added a little $CaCO_3$ and a few drops of a satured solution of $CuSO_4$ in water, and cooled after about 10 min. boiling. The disks were extracted further in a Soxhlet apparatus containing 80 % ethanol.

Preliminary experiments showed that the extraction of 100 and 500 disks was complete after 3–4 hours. Therefore after 3–4 hours extraction the solution was removed from the Soxhlet into an Erlenmeyer flask, the ethanol was distilled off, and the remaining watery extract was concentrated to about 2 ml. by slightly heating in vacuo. After transference of the extract to a little flask, and rinsing the flask in which it was concentrated, the end-volume was about 3 ml. These extracts could be used in paper-chromatography without further treatment.

TABLE 13. Recovery of sucrose, glucose and fructose from 20 ml. of a solution of each sugar in water concentrated to about 3 ml. and estimated in 0.1 ml. (fructose 0.2 ml.) diluted with water to 1 ml. Values in μg./0.1 ml. (fructose 0.2 ml.) of the concentrated solution.

μg. sucrose		μg. glucose		μg. fructose	
calculated	recovered	calculated	recovered	calculated	recovered
46	46	44	42	44	43
46	46	44	44	44	43

As appears from table 13, it could be ascertained in experiments with known sugar solutions that no sugar was lost in concentrating 20 ml. to about 3 ml. provided that the flask in which it was concentrated, was rinsed with warm, distilled water after transference of the concentrated solution to a small flask. The differences observed correspond with the normal variation of estimations using anthrone.

c. *Paper-chromatography*

Chromatograms were prepared on water-washed Whatman nr. 1 paper and run with propanol-ethylacetate-water (7:1:2; v/v/v) as a solvent in closed glass jars placed in a room with a constant temperature of 21° C. The descendent technique was used. On the starting-line 0.25 ml. extract was applied in a narrow strip 2 cm. in length (Fig. 4) using a micro-pipette (AGLA Micrometer Syringe, Burroughs Wellcome & Co., London). Before applying extract again on the same place, the paper was dried with warm air (40°–50° C.). On both sides at some distance from the extract a mixture of sucrose, glucose and fructose was applied. A complete separation of the sugars required about 40 hours. The solvent front ran from the teeth cut in the end of the paper.

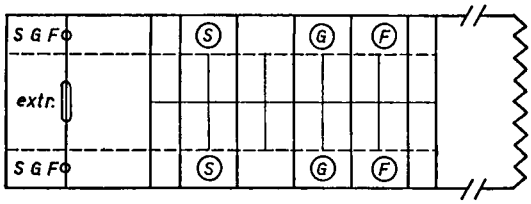

Fig. 4. Scheme of a chromatogram.
S = sucrose G = glucose F = fructose

To determine the running-speed of some sugars and to identify the sugars that occur in potato extract, chromatograms with extract and known sugars were run with propanol-ethylacetate-water (7:1:2; v/v/v) or n-butanol-ethanol-water (9:1:10; v/v/v) for 40 and 60 hours respectively. After drying the paper was sprayed with an aniline phthalate-, a naphthoresorcinol- or a benzidine-reagent (CRAMER 1953, LINSKENS 1955). The coloured spots obtained with extracts appeared indistinguishable from those formed by known sucrose, glucose and fructose. In the concentrations of extract used, never more than these three spots were detected, which is in agreement with the observations of LE TOURNEAU (1956). It is assumed, that the sugars detected in the extracts were sucrose, glucose and fructose.

d. *Quantitative estimation of sugars from paper-chromatograms*

With water the sugars could be easily eluted from the chromatogram papers. However, as Whatman nr. 1 paper appeared to contain water-soluble compounds which produced a colour with the anthrone reagent, the paper was thoroughly washed with distilled water and dried before use. After this the interference was slight and was corrected by comparison with blank estimations.

The sugars of the extracts were localized by means of the mixture of sucrose, glucose and fructose chromatographed on the same paper. Blank estimations were carried out with pieces of paper cut from the parts of the chromatogram just before the area with sucrose and just after that with fructose (Fig. 4). The strips containing sugars and the strip between those with sucrose and with glucose were cut out and divided into 4 equal parts. All pieces were eluted separately with distilled water to obtain 1 ml. eluate. This treatment had to be carried out with extreme care, as paper fibrils after hydrolysis in the anthrone reagent would react as sugars.

Some estimations were carried out with chromatograms on which mixtures of known amounts of sucrose, glucose and fructose had been separated. The amounts which were applied and recovered, are given in table 14. It appears that the determination after chromatographic separation was mostly accompanied by some loss of sugar, but the differences did not exceed 10–15 %.

In table 15 are represented the results of estimations in two extracts from 250 potato slices, all cut out of one tuber. To one extract known amounts of sucrose, glucose and fructose were added, after which both extracts were concentrated to 3.4 ml. From chromatograms with 0.01 ml. extract the amounts of sugars were determined and calculated in μg. per disk.

Assuming that the content of the various sugars originally was the same in both extracts, it may be concluded that the % sugar which was recovered agreed with the % found with a solution of pure sugars.

From the values of table 15 it can be calculated that 10 disks contain sufficient extractable sugars for an estimation using paper-chromatography, if 0.25 ml. of the extracts concentrated to about 3 ml. was used.

TABLE 14. Recovery of sucrose, glucose and fructose from chromatograms of mixtures of these sugars.

sugars	μg. applied	μg. recovered			% recovery
sucrose	70	70	63	64	90–100
	35	32	38	33	90–110
glucose	40	38	37	41	90–105
	20	17	20	17	85–100
fructose	40	41	36	37	90–105
	20	18	19	20	90–100

TABLE 15. Sucrose, glucose and fructose in μg./disk of potato-tuber tissue estimated in two parallel extracts of 250 disks, concentrated to 3.4 ml. To one extract known amounts of the sugars were added before concentration. Estimations from chromatograms of 0.01 ml.

sugars	extract I	extract II + sugars	μg. recovered	μg. added	% recovery
sucrose	45 μg.	69 μg.	24	27	89
glucose	51 μg.	66 μg.	15	16	94
fructose	38 μg.	52 μg.	14	16	88

3. Results and discussion

Experiments were carried out in spring (April–June) and in autumn (September). In all tubers a clear respiratory increase was observed after infection. The amounts of sucrose, glucose and fructose were determined in duplo, and the average values calculated in μg. per 0.1 gram of tissue were plotted against the O_2-consumption in μl. per 0.1 gram.

The results of two experiments in spring are drawn in Fig. 5. Although in each experiment, as a result of the relatively narrow area with stimulated respiration after infection, few observations were obtained of samples with different respiration rates, it is clear that the fructose content is not altered parallel with the respiration rate. Experiment 1 shows a more or less parallel behaviour of the O_2-consumption and the amounts of sucrose and glucose, but from experiment 2 it appears that this relation is absent.

If it is assumed that the observations obtained with samples from different tubers may be taken together, and for all experiments carried out in spring (5 expts. including those of Fig. 5) the average O_2-absorption is calculated of samples with a sucrose content of 120–140 μg.,

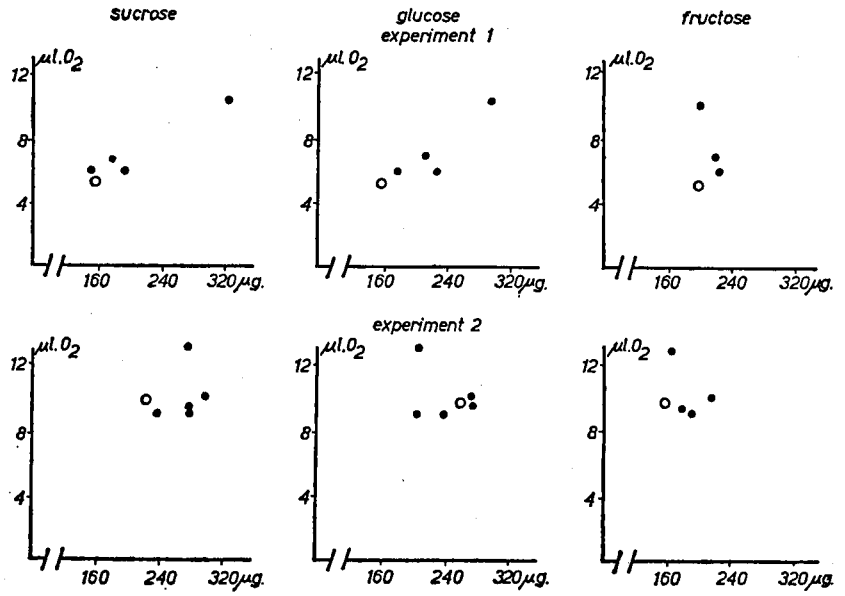

Fig. 5. µl. O_2-uptake and µg. sucrose, glucose and fructose per 0.1 gram (fresh weight). The sugar values are averages of duplicate determinations.
Experiments carried out in spring.
• values of samples from the infected halves.
o average values of samples from the non-infected halves.

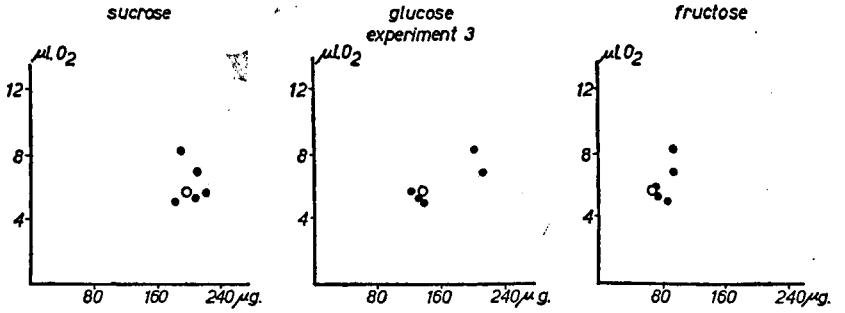

Fig. 6. Legends as in Fig. 5. Experiment carried out in September.

140–160 µg., 160–180 µg. etc. per gram of tissue, it appears from Fig. 7A that the relation between O_2-uptake and sucrose content might be understood as a relation between the rapidity of an enzyme reaction and the concentration of the substrate. This is not true for the relation between the O_2-uptake and the glucose content, when calculated and plotted in the same way (Fig. 7B).

However, from the experiment of Fig. 6 it is clear that in September an acceleration of the respiration after infection is not accompanied by an increasing sucrose content.

These conflicting results indicate that the extra O_2-consumption after infection is not due to an increase of the content of sucrose and glucose. Moreover, in the literature there is evidence that the respiration rate in the potato tuber is limited by other factors than the concentration of the substrate. HUELIN and BARKER (1939) found that the increase of the O_2-uptake of ethylene-treated potato tubers was accompanied by an increase of the sugar content, except when the experiments were carried out with tubers shortly after lifting. CALO and VARNER (1957) concluded from the influence of chloramphenicol on the acceleration of the O_2-absorption of potato-tuber disks during aeration that the protein synthesis is a necessary part of the mechanism by which the increased respiration develops. Moreover, the experiments with 2,4-dinitrophenol as described in Chapter v do not support the view that the respiration rate is regulated by the amount of available substrate.

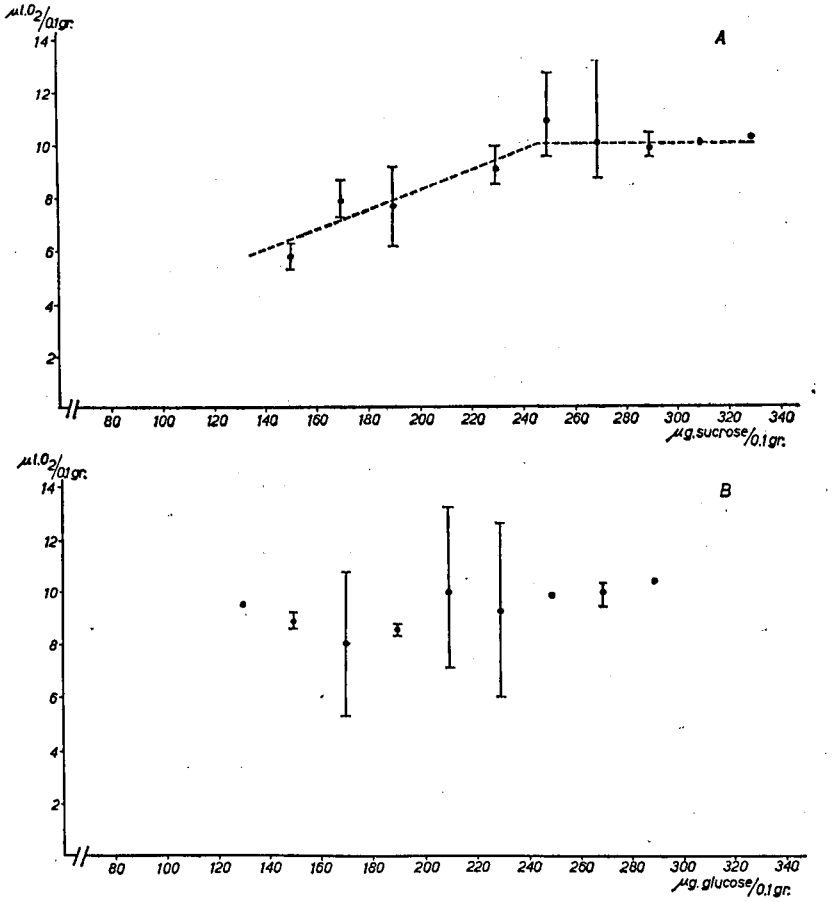

Fig. 7. Average μl. O_2-uptake/0.1 gram of the samples of all 5 experiments carried out in spring, calculated for the samples containing 120–140 μg., 140–160 μg. etc. sucrose (A) or glucose (B) per 0.1 gram (fresh weight).

On account of these data it is thought that the changes in the sugar content after infection are a parallel phenomenon resulting from the general activation of metabolism which includes the increase of the O_2-uptake, rather than the regulating mechanism of the respiration rate.

The importance of an increase in sugar content might be that a considerable stimulation of the respiration is made possible because in this case there is no danger that the substrate supply will become rate-limiting.

CHAPTER V

THE EFFECT OF 2,4-DINITROPHENOL ON THE OXYGEN CONSUMPTION

1. Introduction

The preceding experiments yield no evidence for a qualitative change of the respiratory pathway during the initial stages of the reaction to infection. Therefore, the most simple explanation of the increase of the O_2-uptake is an influence of the disease on the regulating mechanism of the respiration rate.

In preparations of cellular particles (mitochondria ?) from avocado fruits (Millerd, Bonner and Biale 1953), cauliflower (Laties 1953) and sweet potato (Akazawa and Uritani 1954) it appeared that there exists a coupling between the oxidative and the phosphorylating processes. After addition of adenylate to cauliflower preparations oxidizing succinate or α-ketoglutarate, the O_2-absorption increased (Laties 1953). This may indicate a regulation of the respiration rate by the oxidative phosphorylation, also in intact plant tissue. Then the respiration would proceed dependent on the presence of sufficient inorganic phosphate, which is bound to high-energy phosphate ($\sim$P), and on the regeneration of phosphate acceptors, the latter being determined in turn by the activity of transphosphorylating systems and the rate of utilization of phosphorylated acceptors in cellular reactions. If in potato-tuber tissue the respiration rate is limited by the phosphate-acceptor regeneration, it may be expected that influences which accelerate the regeneration and the $\sim$P-consumption, will increase the respiration rate.

Much of the evidence on the regulation of the respiration in plant tissue by a coupled phosphorylating-mechanism has been obtained from experiments with "uncoupling agents", especially with 2,4-dinitrophenol (DNP). According to Laties (1957) substrate level phosphorylation is shown to be resistent to DNP, and its action applies only to phosphorylation during electron transport. There is no unanimity of opinion whether DNP really uncouples the respiration from the phosphorylation (Loomis and Lipmann 1948, 1949; Bonner 1949), or whether this compound interferes in the metabolism after the first phosphorylation-reaction has taken place and $\sim$P-compounds may have been formed already (Teply 1949; Lardy and Wellman

1952, 1953; LATIES 1953; ELIASSON and MATTHIESEN 1956). BRONK and KIELLEY (1958) and Löw et al. (1958) studied the stimulation by DNP of the ATP [1])-ase action and the inhibition by DNP of the ATP-inorganic P^{32} and the ATP-ADP^{32} exchange reactions in preparations of rat-liver mitochondria. It is suggested that the stimulated ATP-ase action is the reversal of the phosphorylating reactions, and that DNP uncouples the phosphorylation by stimulating the breakdown of a high-energy, non-phosphorus-containing intermediate in the enzyme complex. COHN (1953), COHN and DRYSDALE (1955) and DRYSDALE and COHN (1958) conclude from their experiments with the same object, using O^{18}-labelled inorganic phosphate or water, that all effects of DNP can not yet be explained by the assumption that there exists one place of action in the electron-transport system. There is evidence in favour of a DNP-action after the formation of a $\sim$P-bond, but an action before the formation of this bond could not be excluded.

Whatever the details of the mechanism of action of DNP may be, all authors agree that DNP abolishes or reduces the rate-limiting action of the phosphorylation. If the O_2-uptake of a tissue is stimulated after addition of DNP, this indicates that the respiration rate is limited either by the amount of endogenous phosphate acceptor and its regeneration rate or by the amount of inorganic phosphate. LATIES (1957) mentions a number of investigations with plant tissues in which after addition of DNP the respiration was distinctly increased a.o. with potato-tuber tissue (Sharpensteen 1953).

In this chapter experiments will be described concerning the effect of DNP-treatment on the O_2-uptake of potato-tuber disks after stimulation of the respiration due to infection.

2. METHODS

The effect of DNP on the O_2-uptake depends on the concentration of DNP in the medium and on the pH. Whereas DNP in certain concentrations stimulates the O_2-uptake, the latter is inhibited by higher concentrations. A lower pH of the medium intensifies the DNP-effect. In preliminary experiments with potato disks at pH 5.0 the greatest stimulation of the O_2-uptake was observed in 10^{-3} M. DNP, whereas 2.5×10^{-3} M. was inhibitory.

From each of a number of zones of the infected tuber halves 20 disks were cut and divided at random into two samples of 10 disks, one of which was used as a control by addition of distilled water instead of DNP. The respiration rate of the disks submersed in water was measured during two hours, and then DNP was added from the sidearms of the Warburg flasks to obtain a concentration of 10^{-3} M. After addition the course of the O_2-uptake was watched for some hours. The pH of the DNP-medium was 5.0.

As the O_2-uptake had reached its highest value in the second hour after addition of DNP, the DNP-effect was calculated in μl. O_2-

[1]) ATP = adenosinetriphosphate.
ADP = adenosinediphosphate.

uptake/hour/gram (fresh weight) and in % by comparison of the respiration rate during this hour with the average rate during the two hours before addition. Both values were corrected for the respiratory increase of the control samples during the same period as follows:

DNP-effect μl. O_2=increase μl. O_2 in DNP—increase μl. O_2 control

$$\text{DNP-effect } \% = 100 \times \frac{\mu\text{l. O}_2 \text{ in DNP}}{\text{initial O}_2\text{-uptake}} \times \frac{\text{initial O}_2\text{-uptake control}}{\mu\text{l. O}_2 \text{ control}} - 100$$

3. Results and discussion

In experiments with healthy potato-tuber tissue the O_2-uptake was markedly stimulated by 10^{-3} M. DNP at pH 5.0, the R.Q. being 1.0–1.1, a fraction higher than in water. This reaction to the addition of DNP indicates that the respiration rate of healthy tissue is regulated by the coupled oxidative phosphorylation, which in its turn depends on the amount of phosphate acceptor and its rate of regeneration or on the inorganic phosphate content.

It is not likely that the amount of inorganic phosphate is the rate-limiting factor. As has already been stated in Chapter III (table 5) the O_2-uptake in 1/15 M. phosphate buffer was slightly larger than in water. This extra O_2-uptake is considered to be a salt respiration rather than a consequence of the increased concentration of inorganic phosphate in the cells. For the respiration is also accelerated in solutions of other salts (Steward, Stout and Preston 1940). In conclusion, the phosphorylation rate is most likely dependent on the trans-phosphorylating system and the regeneration of phosphate-acceptor sites.

In table 16 the results are represented of 4 experiments with tubers the two halves of which were placed at 25° C. during resp. 0, 3, 8 and 11 days after infecting one half of each tuber. For all zones the O_2-uptake of the control samples without addition of DNP is also given.

The samples of experiment I were cut immediately after infecting one of the two halves. As appears from column A, their O_2-uptake was equal. After incubation at 25° C. a distinct respiratory increase was found near the surface of the infected tuber halves of the expts. II–IV. In expt. IV this increase was lower in sample inf. 1 than in sample inf. 2 as a result of the occurrence of a zone with soft tissue. In column B the respiration rates during the second hour after addition of DNP or distilled water are presented. The O_2-absorption of all samples, except that of sample inf. 1 of expt. IV, appears to have been stimulated by DNP. In DNP-medium the O_2-uptake of the samples inf. 3–5 was practically equal within each tuber half, but the uptake of samples with a much increased respiration after infection reached higher values (expts. II and III inf. 1 and 2; expt. IV inf. 2).

The stimulation by 10^{-3} M. DNP was calculated in μl. O_2 (column C) and in % (column D). The O_2-uptake of the samples from the infected half of expt. I, which did not show a respiratory increase, was stimulated to the same extent. In the other experiments the stimulation by DNP decreased with the acceleration of the respiration after infection.

TABLE 16. The effect of 10^{-3} M. 2,4-dinitrophenol (DNP) at pH 5.0 on the O_2-uptake in μl./gram (fresh weight) in 4 experiments. Samples cut from the non-infected and the infected tuber halves, incubated at 25° C. for resp. 0–3–8–11 days. Samples inf. 1 nearest the surface. For all zones the O_2-uptake of the control samples without addition of DNP is given too.

			initial μl. O_2-uptake (A)				μl. O_2-uptake after addition of DNP or water (B)				Stimulation by DNP corrected for increase of the controls: μl. O_2 (C)				Stimulation by DNP corrected for increase of the controls: % (D)			
experiments			I	II	III	IV	I	II	III	IV	I	II	III	IV	I	II	III	IV
non-infected tuber halves		+ DNP	46	66	52	54	127	138	135	125	60	52	69	55	89	59	101	74
		+ aq. dest.	*46*	*65*	*48*	*49*	*67*	*85*	*62*	*65*								
infected tuber halves	zones																	
	1	+ DNP	47	155	135	**96**	128	181	160	**99**	47	4	7	**0**	61	2	5	**0**
		+ aq. dest.	*49*	*147*	*136*	**85**	*83*	*169*	*154*	**88**								
	2	+ DNP	42	115	98	107	106	162	139	141	42	24	20	19	69	17	15	15
		+ aq. dest.	*45*	*113*	*89*	*107*	*67*	*136*	*110*	*122*								
	3	+ DNP	45	65	67	73	105	119	125	109	44	34	35	23	70	41	36	26
		+ aq. dest.	*43*	*68*	*62*	*70*	*59*	*88*	*85*	*83*								
	4	+ DNP	45	51	56	41	101	99	129	100	41	42	55	44	68	74	75	80
		+ aq. dest.	*44*	*54*	*57*	*42*	*59*	*60*	*75*	*57*								
	5	+ DNP	—	—	50	42	—	—	125	101	—	—	52	43	—	—	69	71
		+ aq. dest.	—	—	*48*	*40*	—	—	*71*	*56*								

It is conspicuous that the respiratory stimulation by DNP in the samples not affected by the infection (inf. 4 and 5) differs from that in tissue from the non-infected tuber halves. From preliminary experiments with some tubers the two non-infected halves of which had been stored at 25° C. for some days, it appeared that the respiration rate and the stimulation by DNP in the tissue from the two halves of a tuber showed similar differences. These differences are caused by the variation existing between the two halves of a tuber, and are not a result of the infection. Therefore, the respiration rates and their stimulation should be compared within each tuber half.

In all samples from the infected tuber halves, except in sample inf. 1 of expt. IV, a stimulation by DNP was observed. This suggests that the respiration rate in this tissue was limited by the phosphorylation, even after a large increase of the respiration due to infection. The DNP-effect calculated in % as well as the effect in μl. O_2 decreased after the acceleration of the respiration appearing after infection. A decrease of the % stimulation does not necessarily point to a decrease of the DNP-effect and the degree of limitation of the respiration by the phosphorylation, as a higher initial respiration rate must show a greater increase of the O_2-uptake to obtain an equal % stimulation. Consequently it is difficult to draw conclusions from the percentages. Therefore, in the following discussion the stimulation calculated in μl. O_2 will be compared.

The decrease of the DNP-effect in μl. O_2 indicates that the rate-limiting action of the phosphorylation was smaller after an increase of the respiration due to infection. In sample inf. 1 of expt. IV, cut on the border between the soft and the apparently normal tissue, this limitation would be absent.

Within the various tuber halves the O_2-uptake of the samples inf. 3–5 before addition of DNP was not equal. Yet it was stimulated by DNP to about the same level, especially in the expts. III and IV. This suggests that DNP accelerated the respiration, till a factor which had not changed under the influence of the infection became rate-limiting for the respiration. As it is generally assumed that DNP accelerates or uncouples the phosphorylation, and as the DNP-effect decreased with the increase of the respiration after infection, these observations might be interpreted as an indication that, at least initially, the phosphate-acceptor regeneration, which regulates the respiration rate, is accelerated under the influence of the infection without a change of other factors which may become rate-limiting in consequence of this acceleration.

With the exception of sample inf. 1 of expt. IV, the O_2-uptake of the samples inf. 1 and 2, which was already markedly increased after infection, reached higher values after addition of DNP than the O_2-uptake of samples not influenced by the infection did. The latter values of O_2-uptake remained lower than (expt. II) or hardly increased to (expts. III and IV) the values of the samples with the greatest respiratory increase before addition of DNP. So with continued influence of the infection more changes take place. Possibly this might

be connected with an increase of the intensity and/or capacity of the processes which may become rate-limiting after acceleration of the phosphorylation, or/and with the development of a pathway causing an extra O_2-absorption which is not regulated by the rate of phosphorylation.

In DNP-milieu the O_2-uptake of the samples without and with a slight respiratory increase after infection (inf. 3–5) was equal, especially in the expts. III and IV. This makes it unlikely that already in the initial stages of respiratory stimulation after infection some oxygen was taken up as a result of the development of such a pathway.

The above reasoning is based on the assumption that DNP accelerates or uncouples the phosphorylation to such a degree that it is no longer rate-limiting for respiration. Now HONDA (1956) already remarked that possibly DNP alone is not suitable for determining the exact degree with which the coupled phosphorylation is rate-limiting. On the grounds of experiments with preparations of cauliflower-mitochondria LATIES (1953) also mentioned this possibility. When α-ketoglutarate was oxidized by these preparations, a much greater O_2-uptake was observed after addition of a sufficient quantity of hexokinase and glucose than when the regeneration of phosphate-acceptor sites was accelerated by DNP. These experiments *in vitro* suggest that the metabolic mechanisms are more effective than DNP in liberating these sites. Consequently, in intact tissue too the O_2-uptake after stimulation by DNP may be considerably lower than in tissue in which the phosphorylation is no longer rate-limiting for the respiration due to a complete uncoupling or an accelerated phosphate-acceptor regeneration. The differences in O_2-uptake remaining after the action of DNP and amounting to resp. 82, 35 and 40 μl. O_2 in the expts. II–IV (column B: inf. 1–4; expt. IV inf. 2–5), possibly have to be attributed to a stimulation of the respiration by DNP, without the phosphorylation ceasing to be rate-limiting, and to a more efficient phosphate-acceptor regeneration by the endogenous processes as compared with the regeneration after addition of DNP.

As appears from table 16 (column C), a considerably increased O_2-uptake was still stimulated by DNP, although sometimes the effect was very slight. So DNP was capable of increasing a high respiration rate. Consequently, if DNP *in vivo* in potato-tuber tissue only accelerates or uncouples the phosphorylation to such a degree that the phosphate-acceptor regeneration remains rate-limiting, it is not to be expected that the effect of DNP on a respiration which has slightly increased after infection, will be considerably smaller than the effect on a normal respiration.

Now a considerable decrease of the DNP-effect was observed indeed. Moreover, within each tuber half the O_2-uptake of the samples with a slight respiratory increase after infection or with a normal respiration rate was increased by DNP to the same level. Therefore, it is more likely that DNP completely abolishes the limitation of the respiration rate by the phosphate-acceptor regeneration and stimulates the O_2-uptake to a rate at which other factors become limiting.

From the preceding discussion the following picture is obtained. The respiration rate of healthy tissue is limited by the coupled phosphorylation, probably by the activity of the transphosphorylating system and the regeneration of phosphate-acceptor sites. When the respiration increases after infection, the phosphate-acceptor regeneration becomes less limiting, even when the respiratory increase is slight. One observation made in tissue cut from the border between the soft and the apparently normal tissue (expt. IV inf. 1), the O_2-absorption of which was not enlarged by DNP, indicates that in such tissue the phosphorylation is no longer rate-limiting.

It is likely that no pathway for an O_2-uptake uncontrolled by the phosphorylation, developed during the initial stages of respiratory increase after infection. In later stages this may indeed be the case, or the intensity and/or capacity of the processes which may become rate-limiting after acceleration of the phosphate-acceptor regeneration, may have increased.

Now the hypothesis is put forward that after infection of potato-tuber tissue the regeneration of phosphate-acceptor sites is accelerated, in consequence of which the respiration rate can increase, while probably also the intensity and/or capacity of other processes which may become rate-limiting after acceleration of the phosphorylation, increases with continued influence of the infection. It is possible that in the later stages of respiratory increase after infection the respiratory pathway does change qualitatively. This would be in agreement with the experiments with malonate (Chapter III), in which a decrease of the % inhibition was observed in apparently normal tissue which had been strongly influenced by the infection.

CHAPTER VI

CHANGES IN PHOSPHATE COMPOSITION AFTER INFECTION

1. Introduction

If the hypothesis is correct that respiration is stimulated after infection because of an accelerated regeneration of phosphate-acceptor sites, in tissue with increased O_2-absorption the consumption of high-energy phosphate ($\sim$P) will be enhanced. This may be achieved either by direct breakdown of these $\sim$P-compounds or by stimulation of energy-requiring synthetic processes. The phosphate groups are liberated as inorganic phosphate or incorporated in organic compounds.

In experiments with sweet potato (Akazawa and Uritani 1956) and potato tubers (Akazawa 1956a) Akazawa and Uritani compared sound tissue adjacent to parts infected with *Ceratostomella fimbriata* with non-infected tissue. Some evidence was provided in support of a stimulation of synthetic processes after infection. In ethanol-$HClO_4$ extracts of tissue homogenates from both objects an increase of the acid-soluble organic phosphate fraction and the acid-insoluble nitrogen

fraction with concomitant decrease of respectively the inorganic phosphate and the acid-soluble nitrogen was reported after infection. The changes in the phosphate composition and the acceleration of the respiration by the disease were less considerable in the potato than in the sweet potato. AKAZAWA (1956b) observed a greater O_2-uptake per unity of mitochondrial nitrogen in mitrochondria preparations from infected sweet potato oxidizing α-ketoglutarate. A greater activity of ATP-ase and of acid and alkaline phosphatase was also reported. Authors think it possible that the accelerated respiration after infection is accompanied by an increase of the activity of various enzymes and of the synthesis of functional proteins, possibly also of enzyme proteins. The observation of AKAZAWA, UMEMURA and URITANI (1957) that the electrophoretic picture of protein extracts obtained from sound tissue adjacent to the infected parts differs from that of healthy sweet potato tissue, seems to support this view.

In this chapter experiments will be described that were undertaken in order to see whether after infection with *Gibberella saubinetii* (*Mont.*) *Sacc.* changes are to be found in the composition of the phosphate fraction of potato-tuber tissue in relation to the respiratory stimulation. In addition to the estimation of total phosphate and free inorganic phosphate attention will be paid to the easily hydrolysable organic phosphate and the norit-adsorbed phosphate. According to CRANE and LIPMANN (1953) and BORST PAUWELS (1956) in acid solution chiefly the nucleotides are adsorbed to norit, whereas inorganic phosphate and the hexosephosphates are not adsorbed.

2. METHODS

In the experiments described in this chapter only pyrex glass equipment and pro analyse chemicals (Merck) were used.

a. *Tissue extracts*

The tissue was killed in 5 ml. ethanol 96 % by heating to 80° C. for 5–10 min. After homogenization in 3 ml. ethanol 96 % the homogenate was quantitatively transferred to a centrifuging tube and after 15 min. extraction at room temperature centrifuged at 0° C. The supernatant was added to the ethanol in which the disks had been killed. The residue was resuspended in 2 ml. distilled water and centrifuged after 15 min. This was repeated four times. The supernatants were added to the ethanol extract. As ethanol interfered with the phosphate estimations, all ethanol was removed by concentrating the extract to less than 5 ml., after which the pH was adjusted to 4.0–4.2 with acetate buffer pH 4.2 (equal volumina 1 N. acetic acid and 0.025 N. sodium acetate). Now the extract was ready for use.

b. *Estimation of inorganic phosphate*

Inorganic phosphate was estimated in extracts using the colorimetric method according to LOWRY and LOPEZ (1946). In a Unicam Spectrophotometer (glass absorption cell 3.5 ml., light path 10 mm.) the intensity of the developing colour was measured at 5 min. intervals at 800 mμ., till it was constant. In each series a blank was included. The amount of phosphate was calculated in μg. phosphorus.

c. *Estimation of total phosphate in tissue disks*

Destruction of the tissue in sulfuric acid at high temperature liberates all phosphate as inorganic phosphate. In a pyrex glass tube with 2 ml. 10 N. H_2SO_4 five potato

disks were heated to about 160° C. on a sand bath. After some hours 2 drops of a 30 % H_2O_2-solution were added to accelerate the discoloration of the liquid and the destruction was continued. This was repeated every hour, until a complete discoloration was obtained. After cooling, 1 N. sodium acetate was added till the pH was 4.0, and then the mixture was diluted with acetate buffer pH 4.2. Now the inorganic phosphate could be estimated.

As an excess of H_2O_2 inhibits the colour formation with the molybdate-ascorbic acid reagent, only so much H_2O_2 was added during the destruction as was strictly necessary for complete discoloration.

d. *Estimation of easily hydrolysable phosphate*

The inorganic phosphate, liberated in the extracts by a short hydrolysis in 1 N. HCl at 100° C., was estimated after heating tubes containing 2 ml. extract mixed with 2 ml. 2 N. HCl in a boiling water bath during 8 min. After cooling and adjusting the pH to 4.0 with 1 N. sodium acetate, the inorganic phosphate was determined, if necessary, after dilution with acetate buffer pH 4.2.

e. *Estimation of norit-adsorbed phosphate and not-adsorbed phosphate*

Before use norit SX II was freed from interfering substances. A suspension in 10 % trichloroacetic acid (TCA) was boiled for 20 min., filtered and the norit thoroughly washed with distilled water.

From an extract a 5 ml. sample was acidified with 2 ml. 10 % TCA and mixed well with 300 mg. norit. This was shaken a few times. After 30 min. the norit was centrifuged. In order to remove the not-adsorbed phosphate, the norit was twice resuspended in 3 ml. acetate buffer pH 4.2 and centrifuged after 15 min. The supernatants were mixed and the pH was adjusted to 4.0 with 1 N. sodium acetate and the inorganic and easily hydrolysable phosphate was estimated. The amount of easily hydrolysable phosphate adsorbed to the norit was determined as inorganic phosphate, liberated by suspending the norit in 4 ml. 1 N. HCl and heating to 100° C. during 8 min. The pH of the liquid was adjusted to 4.0 with 1 N. sodium acetate, the norit centrifuged and the supernatant diluted, if necessary, with acetate buffer pH 4.2.

The methods described were tested with a solution of adenosinediphosphate (ADP) containg 54 μg. phosphorus per ml. and with a potato-tissue extract. In table 17 the results are presented of estimations in the untreated ADP-solution (1–3) and after adsorption to norit in the supernatant (4–5) and the adsorbed fraction (6–7). The total phosphate found agreed with the calculated amount. In the solutions a small part of the ADP appeared to be hydrolysed (2). The ADP was completely adsorbed to norit (5). Only the terminal phosphate group of ADP is easily hydrolysable (UMBREIT c.s. 1951). As might be expected, about half of the adsorbed phosphorus was liberated by weak hydrolysis (6–7). From the agreement of the values of columns 2 and 4 and of columns 3 and 6 we may conclude that the norit-method

TABEL 17. Phosphate fractions in μg. phosphorus/ml. of a solution of adenosinediphosphate (ADP) containing 54 μg. P/ml. (pH 4.0).

untreated ADP-solution			after adsorption with norit			
			supernatant		norit	
total P (1)	inorganic P (2)	easily hydrol. P (3)	inorg. P (4)	easily hydrol. P (5)	easily hydrol. P (6)	not-easily hydrol. P (7)
55 μg.	5 μg.	26 μg.	5 μg.	0 μg.	27 μg.	24 μg.

is suitable. Identical results were obtained with 200 mg., 300 mg. and 500 mg. norit per 5 ml. solution. In the experiments 300 mg. norit was used.

From the potato extract only a slight fraction was adsorbed to norit. From table 18 it appears that similar values were obtained with parallel extracts from tissue of the same tuber. The agreement of the values obtained with and without norit treatment for both inorganic phosphate (1 and 3) and easily hydrolysable phosphate (2 and 4+5) shows that also with extracts the norit-method may be employed. What compounds are adsorbed was not investigated.

TABLE 18. Phosphate fractions in μg. phosphorus/20 disks in two parallel extracts from each 20 disks of one tuber.

extracts	untreated extracts		after adsorption with 300 mg. norit		
			supernatant		norit
	μg. inorganic P (1)	μg. easily hydrolysable P (2)	μg. inorganic P (3)	μg. easily hydrolysable P (4)	μg. easily hydrolysable P (5)
I	67	17	65	7	9
II	64	17	63	7	9

3. EXPERIMENTAL RESULTS

In discussing the changes in the phosphate composition of tissues with a respiration stimulated after infection, it is of importance to study whether the parasitic fungus, which for its nutrition depends on the tissue constituents, withdraws phosphorus-containing compounds from non-invaded cells.

The samples were analysed after measuring the O_2-uptake during two one hour intervals. The amounts of phosphate were calculated in μg. phosphorus per gram of tissue (fresh weight).

From the experiments it appears that a distinction should be made between tuber halves in which a zone with soft tissue has developed after infection, and halves in which this is not so. The values of table 19 show that when no soft tissue occurs, the content of total phosphate (2) in the samples of a tuber half is the same. Consequently, there is no question of a withdrawal of phosphorus-containing compounds from non-invaded cells by the pathogen. The amount of inorganic phosphate (3) distinctly decreases with increased stimulation of the respiration. After infection the organic phosphate (4), calculated as the difference between total phosphate and inorganic phosphate, increases with concomitant acceleration of the respiration.

In samples from infected tuber halves provided with a zone of soft tissue changes in total phosphate and inorganic phosphate are negligible, except in the tissue (inf. 1) situated on the border between the soft and the apparently normal tissue, the inorganic phosphate content of which is distinctly lower. However, the total phosphate also decreases.

TABLE 19. Total phosphate content and the amount of inorganic phosphate in relation to the respiration rate estimated in samples from non-infected tuber halves and infected halves with and without a zone with soft tissue after incubation at 25° C. Organic phosphate calculated from total and inorganic phosphate. Values averaged for 3 experiments. Phosphate in μg. P/gram (fresh weight).

tissue zones		% respiratory stimulation (1)	μg. total P (2)	μg. inorganic P (3)	μg. organic P (calculated) (4)
non-infected		0	485	102	383
infected halves without soft tissue	infected 1	170	522	57	465
	infected 2	71	517	73	444
	infected 3	21	529	92	437
	infected 4	0	522	95	427
infected halves with soft tissue	infected 1	*18*	*479*	*71*	*408*
	infected 2	50	558	110	448
	infected 3	21	567	115	452
	infected 4	0	590	120	470

By the estimation of the easily hydrolysable phosphate and the norit-adsorbed fraction of this an attempt was made to obtain further information on the nature of the organic phosphate fraction that increases after infection, when no soft tissue has developed. From table 20 it is clear that no increase of these fractions was observed in infected tuber halves. On the contrary, the norit-adsorbed, easily hydrolysable phosphate tends to decrease at higher respiration rates. The reality of these differences, however, is questionable in view of the slight amount of phosphate of this fraction and the accuracy of the method of estimation. In tuber halves with a zone of soft tissue the

TABLE 20. Fractions of easily hydrolysable phosphate in relation to the respiration rate estimated in samples from non-infected tuber halves and infected halves with and without a zone with soft tissue after incubation at 25° C. Zones inf. 1 nearest to the surface. Values averaged for 3 experiments, in infected halves with soft tissue averaged for 2 experiments. Phosphate in μg. P/gram (fresh weight).

tissue zones		% respiratory stimulation	μg. easily hydrolysable P	μg. norit-adsorbed easily hydrolysable P
non-infected		0	36	16
infected halves without soft tissue	infected 1	87	31	17
	infected 2	30	34	18
	infected 3	11	33	21
	infected 4	0	27	22
infected halves with soft tissue	infected 1	*12*	*11*	*9*
	infected 2	33	26	18
	infected 3	16	23	20
	infected 4	0	22	22

same is true, except that the decrease of both fractions in the tissue situated on the border between the soft and the apparently normal tissue (inf. 1) seems to be real indeed.

4. Discussion

For its nutrition the parasitically growing fungus is dependent on the constituents of the host tissue. Indeed, in cells of the soft tissue from infected potato-tuber halves a decrease of all estimated phosphate fractions was observed, indicating a general decrease of the phosphate content. On the other hand it appeared clearly from the experiments that no phosphorus-containing compounds had been withdrawn from non-invaded cells of the potato tuber, as far as these cells are apparently normal and not situated in a zone with soft tissue. Consequently, changes in the phosphate composition of apparently normal, non-invaded cells should be understood as internal shiftings that occur simultaneously with the respiratory reaction to the infection.

From the stimulation of the O_2-uptake of healthy potato tissue by added DNP it was concluded that the coupled oxidative phosphorylation is likely to be rate-limiting for the normal respiration. As the somewhat increased O_2-absorption in 1/15 M. phosphate buffer was considered to be a salt respiration, the conclusion was drawn that the rate of phosphorylation in turn is probably not limited by the amount of inorganic phosphate in the cells. The observation that in infected tuber halves without a zone with soft tissue the amount of inorganic phosphate in the samples distinctly decreased with concomitant acceleration of the respiration, supports this view.

Therefore it is most likely that the normal respiration of potato-tuber tissue is limited by the amount and/or the regeneration rate of phosphate-acceptor sites, the latter depending on the dephosphorylation of the phosphorylated acceptors, either by direct breakdown leading to the formation of free inorganic phosphate or by the activity of the transphosphorylating system. The increase of the amount of organically bound phosphate at the cost of the inorganic phosphate which was demonstrated parallel to the acceleration of the respiration, points to the latter possibility, and may be interpreted as evidence in support of the hypothesis that the synthetic activity in the cells is enhanced. As the fractions of easily hydrolysable phosphate show hardly any change, the conclusion may be drawn that the increase of the organic phosphate must be ascribed to the formation of phosphate compounds from which the phosphate can not easily be hydrolysed.

However, the increase of organic phosphate does not always occur when the respiration is stimulated. In tuber halves in which after infection a zone with soft tissue has developed, the phosphate composition of cells outside the area with soft tissue does not seem to be affected or is only very slightly affected, and this is also found when the respiration has been relatively slightly stimulated at greater distance from the surface of infected tuber halves without soft tissue. This is not necessarily in conflict with the proposed hypothesis on the regulating mechanism of the respiration rate. For it is possible 1° that

energy-requiring processes show an increased activity and cause an accelerated phosphate-acceptor regeneration by which just as much phosphate is liberated as is bound by the phosphorylation of the acceptor sites, or 2° that an increased synthesis of organic phosphate is accompanied by an accelerated breakdown of phosphate compounds by which the over-all organic phosphate fraction remains equal. However, the possibility that an accelerated regeneration of acceptor sites is caused by a direct breakdown of the phosphorylated acceptor(s) can not be precluded.

In conclusion, the results of the phosphate analyses of disks from infected tuber halves without soft tissue are in support of the hypothesis that the respiration rate in non-invaded cells can increase after infection by an acceleration of the regeneration of phosphate-acceptor sites. If after infection a zone with soft tissue has developed, or if the respiration rate is relatively slightly stimulated, the results for non-invaded, not-soft cells are not in conflict with this hypothesis, but they do not support it either.

CHAPTER VII

SOME EXPERIMENTS WITH P^{32}-LABELLED PHOSPHATE

1. Introduction

From literature it is known that inorganic orthophosphate can be taken up by potato-tuber tissue and incorporated into organic compounds (Loughman 1957, Calo and Varner 1957, Lundegårdh 1958a, b). In the following experiments with the radioactive phosphorus-isotope P^{32} an attempt will be made to obtain direct data concerning the phosphate metabolism of tissue affected by the parasitically growing fungus in comparison with that of normal tissue.

It is assumed that the isotopes P^{31} and P^{32} are chemically identical, and are taken up and metabolised by the cells at the same rate, without the radioactive radiation affecting the metabolism.

2. Material and methods

Carrier-free radioactive phosphate was obtained from the N.V. Philips Duphar as a 10^{-3} M. solution of $Na_2HP^{32}O_4$. Before use this solution was brought to an activity of 1 mC. per ml. by addition of 10^{-3} M. inactive Na_2HPO_4. It was confirmed by paper-chromatography that all P^{32} was in the form of inorganic orthophosphate.

Samples of 20 disks were prepared from successive zones of the infected potato-tuber halves. These samples were submersed separately in 1.5 ml. of a solution of pH 6.0, containing 1.5 mC. P^{32} and placed at 25° C. during 1.5 hours. The phosphate uptake was terminated by removing the disks from the medium, after which they were washed thoroughly 6 times with water and homogenised in 1 ml. ethanol 96 %. The homogenate was quantitatively transferred to a centrifuging tube and centrifuged after 30 min. The tissue residue was

resuspended in 1 ml. water and centrifuged again after 30 min. This was repeated once more. The extract of each sample was made up to 4 ml.

The radioactivity of the extracts was measured, after which chromatograms were prepared with a total activity of 2–5.10^5 counts/min. Strips (6 × 56 cm.) of acid-washed paper (Schleicher and Schüll 2043b Mgl. ausgew.) with at the start over the full width of the paper 0.1–0.4 ml. extract, dependent on the radioactivity per 0.1 ml., were equilibrated in closed glass jars at 21° C. and irrigated for about 70 hours with propanol-ammonia-water (60:30:10; v/v/v) in which 1 ‰ EDTA (disodium-ethylene-diamine-tetraacetate) was solved. The descendent technique was used. After drying in warm air, a strip of 2.8 cm. width was cut out in the length from the middle of the chromatogram and the radiation of each zone of 0.5 cm. was counted. The same strips were used for autoradiography on Kodak X-ray (Kodirex) film. The exposure-time was 3–4 days.

The radiation intensity was counted at 600 Volt with a mica-window Geiger-Müller tube-counter, 3 cm. in diameter (plateau region 500–800 Volt). The counter tube was fixed at 6 mm. distance from the paperstrip under a 3 mm. thick lead plate with a diaphragm 0.5 × 3 cm. As P^{32} is a β-particle emitter, it was not necessary to cut the 0.5 cm. zones from the strips, provided these were pressed against the diaphragm while counting. The "shadow-radiation" from adjacent zones was absorbed in the lead. When the activity/zone was high, an aluminium filter, which was ascertained to absorb 50 % of the radiation, was placed between the strip and the counter tube.

The radiation in counts/min. was corrected for the resolving time of the tube (dead time) and the background radiation, and was plotted in a diagram. The radioactivity of the various radiation peaks was calculated and corrected for the decay since the killing of the cells by homogenisation in ethanol.

3. Experimental results and discussion

A detailed discussion of the results is only possible when the various radiation peaks have been identified. However, a chromatographic identification of P-compounds is difficult and uncertain (Borst Pauwels 1956). In order to obtain at least some information concerning the position of the various compounds, a mixture of known phosphate esters was separated, using the method described above. Potato-tissue extract appeared to have no influence on the separation of the esters and on their position in the chromatogram. The compounds were localized by means of the molybdate spray reagent according to Bandurski and Axelrod (1951). In addition the nucleotides ATP and ADP were detected by making an ultraviolet print of the chromatogram with a Philips TUV fluorescent tube as a light source. As the solvent front had run from the paper, the position of the compounds was calculated with respect to the position of inorganic phosphate (Rp-values). The hexosephosphates glucose-1-phosphate, glucose-6-phosphate and fructose-6-phosphate and the 3-phosphoglyceric acid ran faster than inorganic phosphate (Rp 1.12–1.39), whereas fructose-1,6-diphosphate was slower (Rp 0.54). ATP had a Rp 0.74. ADP ran about as fast as inorganic phosphate (Rp $\pm$ 1.0).

The concentration of the phosphate esters in the potato-tissue extracts was so low that they could not be detected either with the spray reagent or by UV-photography. Only inorganic phosphate was detected.

When a chromatogram of an extract from a P^{32}-treated sample was prepared, a number of radiation peaks were found in addition to

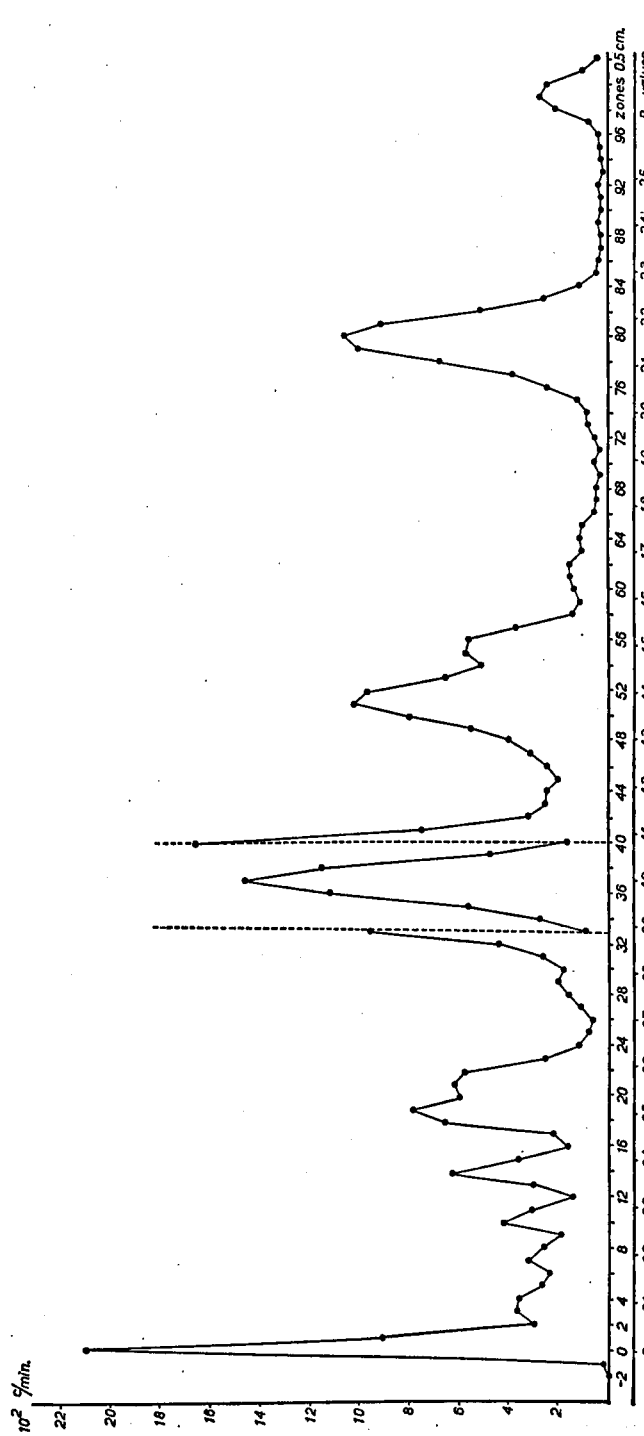

Fig. 8. Distribution of P^{32} on a chromatogram of extract from tissue disks cut near the region invaded by the fungus. Values corrected for dead time and background radiation. Of the inorganic phosphate peak the values of the zones 33–40 are drawn at scale 1/10. Total radioactivity of the paperstrip about 85,000 c./min.

inorganic phosphate. The fastest running peak observed had a Rp-value of about 2.7. This is illustrated by Fig. 8. After chromatographic separation of a norit-treated part of such an extract, the peaks with Rp < 1.0, except that with Rp 0.54 (fructose-1,6-diphosphate?), appeared to be absent, whereas in comparison with the untreated part the decrease of the compound with Rp 0.54, inorganic phosphate and the faster running compounds was negligible.

On account of the preceding data and the often incomplete separation of the peaks, the P^{32}-distribution in the extracts was calculated for some groups of compounds instead of for each peak separately. It was supposed that the compounds with Rp < 1.0, except peak Rp 0.54, are mainly nucleotides and that in the region Rp 1.10–1.50 chiefly hexosephosphates are localized. This is in agreement with the experience of BORST PAUWELS (1956). The compound with Rp 0.54 is probably fructose-1,6-diphosphate, and was included in the group "hexosephosphates".

Using the inorganic phosphate content in potato-tuber tissue as given in Chapter VI (table 19) and the radioactivity of the extracts obtained in the experiments with P^{32}, it could be calculated that the total amount of inactive and labelled phosphate taken up during the experimental period had hardly any effect on the concentration of inorganic phosphate in the cells. This concentration will have increased by at most a few %. Consequently, it may be assumed that at a certain concentration of inorganic phosphate in the cells the amount of organically bound P^{32}-labelled phosphate, which is dependent on the concentration-ratio P/P^{32}, is more or less proportional to the amount of P^{32}-phosphate taken up.

From the radioactivity of the extracts it appeared that the uptake of P^{32}-phosphate by tissue adjacent to the area invaded by the fungus, was greater than the uptake by tissue at a greater distance. This may be related to the increased respiration rate after infection, and then this is in accordance with the observations of LUNDEGÅRDH (1958a, b) according to which the entrance of organic phosphate into cells of potato-tuber tissue after a rapid initial absorption depends on the metabolic activity of the cells.

As a consequence of the differences in uptake, the absolute amounts of organically bound P^{32}-phosphate in counts/min./unity of extract can not be used as a measure for the activity of the phosphate metabolism in the cells. Therefore, the amounts of P^{32} in the compounds were calculated from the distribution of the radiation on the chromatograms as % of the total activity of the paperstrip, that is as % of the ethanol-water-soluble labelled phosphate.

In table 21 the P^{32}-distribution over the groups of "nucleotides", inorganic phosphate, "hexosephosphates" and compounds with Rp-values > 1.50 is given for extracts of tissue from five successive zones of infected tuber halves. The % are average values of 4 experiments. For comparison the values for non-infected tissue are also given.

Although a detailed quantitative comparison of the P^{32}-distribution

in the various extracts is impossible, yet some general features find expression in table 21. The P^{32}-distribution over the groups of compounds was nearly the same in extracts from non-infected tissue and from tissue of the infected halves at a great distance from the zone with mycelium. However, within the infected tuber halves marked differences occurred in the % "nucleotides" and "hexosephosphates". These percentages increased, resp. decreased with increasing influence

TABLE 21. Distribution of P^{32} in extracts from tissue of infected and non-infected tuber halves incubated at 25° C. Zones inf. 1 nearest to the surface. % mean values for 4 experiments.

fractions	non-infected	inf. 1	inf. 2	inf. 3	inf. 4	inf. 5
"nucleotides"	3.0 %	7.7 %	5.1 %	3.9 %	3.3 %	2.9 %
inorganic phosphate	73.9 %	72.8 %	72.5 %	71.9 %	72.9 %	71.4 %
"hexosephosphates"	13.3 %	9.7 %	12.2 %	13.8 %	14.2 %	15.2 %
Rp > 1.50	8.1 %	8.9 %	8.7 %	8.9 %	8.4 %	9.3 %
Total	98.3 %	99.1 %	98.5 %	98.5 %	98.8 %	98.8 %

of the disease. As it was not ascertained whether the P/P^{32} ratio in the various compounds had become constant at the end of a 1.5 hours experimental period, differences in % may indicate either differences in the amount of the compounds or differences in the rate of conversion.

The influence of the differences in phosphate uptake on the P/P^{32} ratio in the cells induced us to calculate the P^{32}-distribution in % of the total radioactivity of the soluble P^{32}-fraction. Now the P/P^{32} ratio is not only determined by the uptake of phosphate but also by the inorganic phosphate content in the cells at the start of the P^{32}-treatment. Table 19 in Chapter VI showed that this content may decrease under the influence of the infection. However, a decrease by more than about 40 % was never found.

Starting from this greatest decrease of the inorganic phosphate content, it might be supposed that in the experiments with P^{32} this content in the samples inf. 1 was 40 % lower than in the samples inf. 5. If this is true, and if the rate of incorporation of inorganic phosphate was the same in all samples of a tuber half, it might be expected that the % P^{32} in the various groups of organic phosphate compounds should be 10/6 times higher in inf. 1 than in inf. 5. According to table 21 the average % P^{32} in the "nucleotides" of the samples inf. 1 was 2.5 times that of the samples inf. 5. When the % of inf. 1 is corrected for the increase with respect to the % of inf. 5 which may be expected after this decrease of the inorganic phosphate content after infection, the % P^{32} in the "nucleotides" in inf. 1 still remains 1.5 times as high as in inf. 5.

The amount of P^{32} incorporated in the in ethanol-water-insoluble compounds was not determined in the experiments and was not included in the calculation of the % of table 21. If this fraction is taken into account, the % of table 21 should be corrected by multi-

plying the % by the factor $\frac{\text{soluble } P^{32}}{\text{soluble + insoluble } P^{32}}$. If the % P^{32} in the insoluble fraction of the samples of each infected tuber half (inf. 1–5) was the same, this factor will be equal within a tuber half, and the ratio of the % P^{32} in each group of compounds will remain equal to the ratio without applying this correction. If, however, the % P^{32} in the insoluble fraction of the samples near the area with mycelium was greater than it was in the tissue at greater distance from the infection-surface, the factor will decrease when approaching the surface. In that case the differences in the % P^{32} in the "nucleotides" will be relatively reduced as a result of the correction.

So the increase of the % P^{32} in the "nucleotides", which might be interpreted as an indication that the rate of conversion and/or the amount of these compounds had increased under the influence of the infection, might also be explained in another way. Only a part of the increase of the % in inf. 1 as compared with the % in inf. 5 might be attributed to the possible decrease of the inorganic phosphate content in the cells by at most 40 % (Chapter VI) under the influence of the infection. That the ethanol-water-insoluble P^{32}-fraction was not included in the calculation of the % of table 21, may only be put forward to explain the difference in the % P^{32} in the "nucleotides" or a part of it, if it is assumed that the % P^{32} in the insoluble fraction increased with increasing influence of the infection. The latter assumption suggests an increase in the amount of the compounds of this fraction and/or of their rate of conversion. Assuming that the inorganic phosphate content in the samples inf. 1 had decreased to 60 % of the content in the samples inf. 5 (the largest decrease observed in Chapter VI) and that the higher % P^{32} in the "nucleotides" does not indicate an increase of the amount and/or the rate of conversion of these compounds, the correction-factor $\frac{\text{soluble } P^{32}}{\text{soluble + insoluble } P^{32}}$ should be 1.5 times as high in the samples inf. 5 as in the samples inf. 1. This implies that the insoluble P^{32}-fraction in inf. 1 should have been at least more than half of the soluble P^{32}-fraction or a third of the total amount of the P^{32} taken up.

Anyhow, it may be concluded that the increase of the % P^{32} in the "nucleotides", as found in table 21, is an indication for the increase of the amount and/or the rate of conversion of the "nucleotides" and/or of the compounds of the ethanol-water-insoluble P^{32}-fraction.

When the above corrections are also applied to the % of the compounds with Rp > 1.50 and of the "hexosephosphates", it appears that under the influence of the infection the % P^{32} in these groups of compounds decreased, resp. decreased even more than is indicated by table 21.

Especially the decrease after infection of the % P^{32} in the "hexosephosphates" is considerable. This suggests that the amount of these compounds and/or their rate of conversion in the samples inf. 1 was less than in the samples inf. 5. A lower rate of conversion of the hexosephosphates after infection seems unlikely, as in potato-tuber

tissue these compounds are said to be closely connected with the respiration and consequently it is more probable that an accelerated conversion of hexosephosphates will take place when the respiration is increased after infection. Therefore, it is more likely that the amount of these compounds decreased. This does not necessarily mean that the synthesis of hexosephosphates was retarded too. For, if with a higher respiration rate the consumption of hexosephosphates increases to a greater extent than the rate of synthesis, the amount of the compounds will decrease, even if the rate of synthesis is enhanced.

It is not known what compounds correspond with the radiation peaks with Rp $>$ 1.50. So, about this group of compounds no further information can be given.

The general picture obtained from the experiments with P^{32} is that the amount and/or the rate of conversion of the "nucleotides" and/or possibly of the ethanol-water-insoluble P-containing compounds increased after infection. Of the organic phosphate-compounds it is especially the nucleotides that are closely connected with the metabolism of high-energy phosphate and the regeneration of phosphate-acceptor sites. So the P^{32}-distribution in the extracts of the samples inf. 1–5 indicates an accelerated formation of $\sim$P-bonds and phosphate-acceptor regeneration. If the amount of the "nucleotides" increased, a greater capacity of the $\sim$P-metabolism after infection is indicated. A possible increase of the insoluble P^{32}-fraction after infection would mean an enlarged formation of insoluble organic phosphate-compounds, and this enlarged formation might be accompanied by a greater consumption of high-energy phosphate and by an accelerated phosphate-acceptor regeneration.

The results of the experiments with P^{32}-labelled phosphate are considered to support the hypothesis that the regeneration of phosphate-acceptor sites is accelerated after infection, in consequence of which the respiration rate can increase.

CHAPTER VIII

GENERAL DISCUSSION

For the investigation of the reaction of the host cells to the infection it was necessary to separate the gas-exchange of the host from that of the parasite. Therefore, in the experiments with infected tuber halves tissue samples were cut from the area not penetrated by the fungus. What happened in the host cells invaded by the mycelium, was not investigated.

The question may be asked whether the respiratory increase observed in the mycelium-free tissue of the infected tuber halves, was a result of the infection with the fungus, or whether a wound-reaction which occurs after cutting the tubers into two halves before inoculation, played a more or less important part. For after incubation at 25° C. a respiratory increase was also observed in the non-infected tuber halves (table 1). This increase was restricted to a zone of 2–3 mm. immediately below the surface of the cut, which was blocked by

a cork layer, and was already at its maximum after a 24 hours' incubation (increase 80–90 %). In the infected tuber halves no cambial activity was ever observed under the places where the fungus was growing (Richter, unpublished). A blocking of the surface by the formation of a cork layer did not occur. Moreover, the respiratory increase in the mycelium-free tissue often proved to be much larger than 80–90 % (table 16), and often an increase of the O_2-uptake could still be demonstrated at a distance from the surface which was much greater than 2–3 mm. When the penetration of the mycelium into the tissue is taken into account, the distance to the zone with mycelium was still greater than 2–3 mm. The graphs reproduced in Fig. 2 and 3 further suggested that differences in intensity of infection and in the duration of the incubation at 25° C. corresponded to differences in the extent of the respiratory increase. Therefore it seems reasonable to conclude that the increase of the respiration in infected tuber halves was caused by the infection with the fungus. Yet it remains possible that a wound-reaction played a part, as the growing mycelium continually penetrated into new cells, which resulted in a continual wounding of cells. On the contrary, in the non-infected tuber halves cells were wounded only once. Whether compounds excreted by the fungus have a function in causing the respiratory reaction, has not yet been ascertained, although HELLINGA (1942) thought he had found evidence for this assumption.

Concerning the respiratory increase in the tissue adjacent to the zone with mycelium, according to the hypothesis submitted in Chapter v this increase is caused by an acceleration of the phosphate-acceptor regeneration. This would be in agreement with the decrease after infection of the stimulating DNP-effect. However, this decrease might also indicate a partial uncoupling of the respiration and the phosphorylation. Now, in the experiments with P^{32}-phosphate always 10–15 radiation peaks were found on the chromatograms also after a strong influence of the infection. From the P^{32}-distribution on the paperstrips it was concluded that the amount and/or the rate of conversion of the "nucleotides" and/or the insoluble P^{32}-containing organic phosphate had increased under the influence of the infection. By means of the phosphate analyses (Chapter vi) an increase of the organic phosphate was indeed found in infected tuber halves without a zone with soft tissue. As, in addition, the O_2-uptake of samples with greatly enhanced respiration was still stimulated by DNP, it seems unlikely that in the apparently normal tissue of infected tuber halves a complete or partial uncoupling occurred under the influence of the infection.

The O_2-uptake may be considerably enlarged after infection. It is not likely that a qualitative change of the respiratory pathway developed in the initial stages of respiratory increase. For in these stages no differences in % inhibition were found after addition of respiratory inhibitors, and the O_2-uptake of samples with a slightly increased respiration was stimulated by DNP to the same level as the O_2-uptake of samples with a normal respiration rate was. After addi-

tion of malonate only in the later stages of respiratory increase evidence was obtained for a qualitative change of the respiratory pathway.

When in an infected tuber half a zone with soft tissue had developed, not only the tissue-structure appeared to be affected in this area, but the cells had also lost their turgescence and the respiration rate in this tissue proved to be lower than that in the adjacent apparently normal tissue with increased respiration. This was accompanied by changes in the reaction shown by the O_2-uptake to addition of some inhibitors, whereas the O_2-uptake was not stimulated by DNP (one observation). So, when the tissue becomes soft, more changes take place than were observed in the apparently normal tissue. This might be connected with the dying of the cells.

SUMMARY

I

The respiratory increase observed in many infected plant parts might be partly or wholly attributed to the gas-exchange of the pathogen. With potato-tuber tissue infected with *Gibberella saubinetii (Mont.) Sacc.* investigations were made in order to find out whether a respiratory increase could be demonstrated in the host cells, and whether this increase was accompanied by a qualitative change of the respiratory pathway. In the second place the mechanism by which the respiration rate is regulated was investigated.

II

As the fungus only penetrates the outer cell layers (2–3 mm.) of potato-tuber tissue, the respiration of host cells which did not contain mycelium, could be investigated in the tissue next to the invaded parts. From the two halves of each tuber, one of which was inoculated on the surface of the cut while both were incubated at 25° C., tissue samples were cut at various distances from the surface of the original cut. In the infected halves the respiration was distinctly accelerated in the proximity of the area invaded by the fungus, and decreased with increasing distance to this area, to reach a nearly constant level in the zones at greater distance. Occasionally a zone with soft tissue was found close below the area with mycelium. The O_2-uptake of this soft tissue was lower than that of the adjacent apparently normal tissue, the respiration of which was accelerated. The results of many experiments suggested that in tuber halves without a zone with soft tissue differences in respiratory increase and in the distance from the surface where this increase could still be demonstrated, corresponded with differences in intensity of infection and in duration of the incubation at 25° C. (Fig. 2 and 3). In the non-infected halves an increased respiration was observed in a narrow area (2–3 mm.) immediately below the surface of the cut, which was blocked by a cork layer.

III

In order to find out whether the respiratory mechanism had changed qualitatively after infection, R.Q.-values were determined and the effect of respiratory inhibitors was examined in tissue from non-infected and infected tuber halves. The R.Q.-values of non-infected tissue and those of host cells which were not penetrated by the mycelium, were equal. The O_2-uptake of all samples from apparently normal tissue of the infected and the non-infected halves of the tubers was retarded to the same extent after addition of various inhibitors, namely with 90–95 % by 0.002 M. iodoacetate pH 6.2, with about 80 % by 0.02 M. sodium fluoride pH 6.2 and with 85–90 % by 0.002 M. sodium azide pH 6.2. In samples with a large increase of respiration after infection the inhibition by 0.06 M. malonate pH 4.3 proved to be lower (20–40 %) than in the other samples (65–75 %). Probably malonate did not attack succinic dehydrogenase exclusively.

When a zone with soft tissue developed after infection, the softening of the tissue was accompanied by a decrease of the respiration rate and its inhibition by iodo-

acetate and sodium fluoride. Data on the effect of the other inhibitors on the respiration of this tissue are not available.

For the apparently normal tissue without mycelium it was concluded that the experiments with malonate were the only ones which indicated a qualitative change of the respiratory pathway; this effect however, was noticed only after continued influence of the infection. In the zone with soft tissue changes in the pathway had developed which were not observed outside this area.

IV

In the infected tuber halves starch disappeared from the tissue adjacent to the area with mycelium. In this tissue the fructose content did not change. Occasionally a more or less parallel behaviour of the respiration and of the amounts of sucrose and/or glucose was observed. The changes in the sugar content after infection were considered to be a parallel phenomenon resulting from the general activation of the metabolism, which also caused the increase of the O_2-uptake, rather than the regulating mechanism of the respiration rate.

V

As the O_2-uptake of healthy potato-tuber tissue was markedly stimulated by 10^{-3} M. 2,4-dinitrophenol (DNP) pH 5.0, the respiration rate was supposed to be regulated by the coupled phosphorylation, which in its turn depends on the activity of the transphosphorylating systems and the regeneration of phosphate-acceptor sites. The stimulation by DNP of the O_2-uptake of apparently normal tissue from infected tuber halves decreased with the increase of the respiration after infection, which suggested a decrease of the rate-limiting action of the phosphorylation. Only in the tissue cut from the border between soft and apparently normal tissue the O_2-uptake was not increased by DNP, indicating that the respiration was no longer limited by the phosphorylation.

Although, especially in later stages of respiratory increase after infection, a qualitative change of the respiratory pathway could not be excluded, the hypothesis was put forward that in the apparently normal tissue of the infected tuber halves the regeneration of phosphate-acceptor sites was accelerated under the influence of the infection, in consequence of which the respiration rate could increase. Probably also the intensity and/or capacity of other processes which may become rate-limiting after acceleration of the phosphorylation increased with continued influence of the infection.

VI

Determinations of the total phosphate content showed that the fungus withdrew phosphorus-containing compounds from the non-invaded cells of soft tissue, not, however, from apparently normal tissue. In infected tuber halves without a zone with soft tissue a decrease of the inorganic phosphate and an increase of the organic phosphate was observed when respiration was markedly accelerated. In the apparently normal tissue of infected halves with soft tissue these two fractions were unchanged. In all infected halves the easily hydrolysable phosphate and its norit-adsorbed fraction tended to decrease with increasing respiration. Both fractions distinctly decreased in tissue cut from the border between soft and apparently normal tissue, which was probably a result of the withdrawal of phosphate-compounds by the fungus.

The increase of organic phosphate after infection suggested an enhanced synthetic activity in the host cells, which may cause an accelerated phosphate-acceptor regeneration. The absence of this increase of organic phosphate in tuber halves with a zone with soft tissue is not necessarily in conflict with this view.

VII

The influence of the infection on the esterification of inorganic phosphate was investigated using P^{32}-labelled phosphate ($Na_2HP^{32}O_4$). After the P^{32} taken up during 1.5 hours had been extracted with ethanol and water, the compounds n the extracts were separated by paper-chromatography. The distribution of the

radioactivity on the paperstrip was determined. The radiation of the "nucleotides" (Rp < 1, except Rp 0.54), inorganic phosphate (Rp 1.0), "hexosephosphates" (Rp 1.10–1.50) and the compounds with Rp > 1.50 was calculated in % of the total radioactivity of the paperstrips.

The % P^{32} in the "nucleotides" and the "hexosephosphates" appeared to increase, resp. decrease after infection. The influence exercised on the interpretation of these differences by a decrease after infection of the inorganic phosphate content in the host cells (Chapter VI), and by the fact that the P^{32}-fraction which is insoluble in ethanol-water, was not included in the calculation of the %, was discussed. It was concluded that the increase of the % P^{32} in the "nucleotides" is an indication for the increase of the amount and/or the rate of conversion of the "nucleotides" and/or of the compounds of the insoluble P^{32}-fraction. The decrease of the % P^{32} in the "hexosephosphates" does not necessarily mean that the synthesis and the rate of conversion of these compounds were retarded after infection. The results of the experiments with P^{32}-labelled phosphate were considered to support the hypothesis that after infection the regeneration of phosphate-acceptor sites was accelerated, in consequence of which the respiration was increased.

VIII

The rôle which the wound-reaction that occurred after the tubers were cut into two halves, may have played in the respiratory increase in infected tuber halves, was discussed. Although the respiratory increase in the infected halves was most likely a result of the infection, a wound-reaction caused by the continual wounding of cells by the penetrating mycelium, might have played a part.

From the results of the Chapters V, VI and VII the conclusion was drawn that it was not likely that in the apparently normal tissue of infected tuber halves an uncoupling of respiration and phosphorylation occurred. At least in the earlier stages of respiratory increase no evidence was obtained for a qualitative change of the respiratory pathway. In the soft tissue which developed in some infected tuber halves more changes were observed than in the apparently normal tissue. This might be connected with the dying of the cells.

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