Observations on the Oxygen Uptake of Isolated Plant Tissue

II. The Effect of Inhibitors

BY

JOHN CALDWELL

AND

JANE MEIKLEJOHN

(*Departments of Plant Pathology and General Microbiology, Rothamsted Experimental Station, Harpenden*)

With three Figures in the Text.

Introduction

DURING an attempt to study the possible effects of aucuba mosaic virus upon the respiratory activity of infected plants a number of observations were made on the oxygen uptake of excised tissue from the stem of the tomato (*Lycopersicum esculentum*). Some of these observations have been reported in a previous paper (Caldwell and Meiklejohn, 1937), which deals with the effect of phosphate and of added carbohydrate. In the present paper are described a further series of experiments, in which substances known or thought to inhibit enzyme action were added to the tomato tissue.

Methods

The methods and material used have been described in detail in the earlier paper. The stem tissue was cut into slices 0·5 mm. thick, which were suspended in 3 ml. of M/30 acid potassium phosphate (KH_2PO_4), and their oxygen uptake at 26° C. was measured by means of Barcroft differential manometers over a six-hour period. The method, previously described, of providing a separate control for each experiment, was followed throughout; the inhibitor was added to three of each set of six Barcroft bottles after weighing, and just before they were attached to the manometers. These bottles contained either 2·9 or 2·7 ml. of phosphate solution, and either 0·1 or 0·3 ml. of a solution of the poison of appropriate strength was added to bring the volume up to 3 ml. (e.g. to obtain a concentration of M/300 KCN in the bottles, 0·1 ml. of an M/10 solution of potassium cyanide was added to 2·9 ml. of phosphate solution). The amount of poison added is expressed throughout in terms of the concentration in the bottles at the beginning of the experiment.

[Annals of Botany, N.S., Vol. I, No. 3, July 1937.]

The amount of oxygen taken up is expressed as μl. of gas (dry at N.T.P.) per hour per gramme fresh weight of tissue, and each figure given in the tables is the mean of three parallel samples. The degree of inhibition of oxygen uptake, where this occurred, is expressed as a percentage of the uptake of the control, i.e. by the expression $\frac{(a-b)}{a}$ 100, where a is the mean oxygen uptake of the control, and b of the treated material. The reality of the observed differences between the control and the treated material was tested by calculating the standard errors of these mean values (Fisher, 1934). As was stated in the previous paper, the variation of the individual readings with this material is such that differences of the order of 10 per cent. of the control cannot be detected. In a few cases small differences between the mean values for control and treated material have been found not to be significant, and are marked accordingly in the tables. The differences not so marked have been tested and found to be highly significant.

Results

(a) *Cyanide.*

The addition of potassium cyanide to the phosphate solution in which the stem slices were placed produced a very marked inhibition of oxygen uptake. As will be seen by reference to Table I, an inhibition amounting to 85 per

Table I

Effect of Cyanide on the Oxygen Uptake of Tomato Tissue

Concentration of cyanide.	Solution in.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
M/30	M/30 KH_2PO_4	Healthy young	127	19	85
M/300	,,	,,	97	21	79
,,	,,	,,	124	20	84
,,	,,	Virus young	77	15	80
,,	Water	Healthy young	98	14	85
,,	M/30 K_2HPO_4	,,	85	12	85
M/3,000	M/30 KH_2PO_4	,,	114	46	61
M/30,000	,,	,,	104	97	5 (not sig.)
,,	M/30 K_2HPO_4	,,	76	85	0
,,	,,	,,	74	72	0

cent. of the uptake of untreated material was produced by M/300 cyanide; on raising the concentration of cyanide to M/30 no further inhibition was produced, so that the cyanide-stable part of the respiratory system accounts for about 15 per cent. of the total oxygen uptake. These figures are very similar to those obtained by van Heyningen (1935) with animal tissue, both in the amount of cyanide required for maximal inhibition, and in the degree of inhibition (80–90 per cent. for most animal organs) produced.

It will be seen that the inhibition produced by M/300 cyanide is of the same order with virus material as with healthy, and is the same in water or alkaline phosphate as in the acid phosphate solution used as a standard. The disadvantage of using an acid solution for these experiments, however, is that there is a loss of cyanide from the solution during the experiment, which

affects the results with low concentrations of cyanide. (A method for avoiding this loss has been described by Krebs (1935), but it was not employed in our experiments.) This point is brought out in Fig. 1, which represents measurements of total oxygen uptake during the course of an experiment, from single samples of tissue of approximately equal weights. It will be noticed that in presence of M/3,000 cyanide, the oxygen uptake increased during the experiment; the effect of the cyanide wore off, as more and more of it was lost from the solution. In the case of M/30,000 cyanide there was an initial inhibition only; but with M/300 or M/30 there was so much cyanide present that the loss by evaporation was not noticeable.

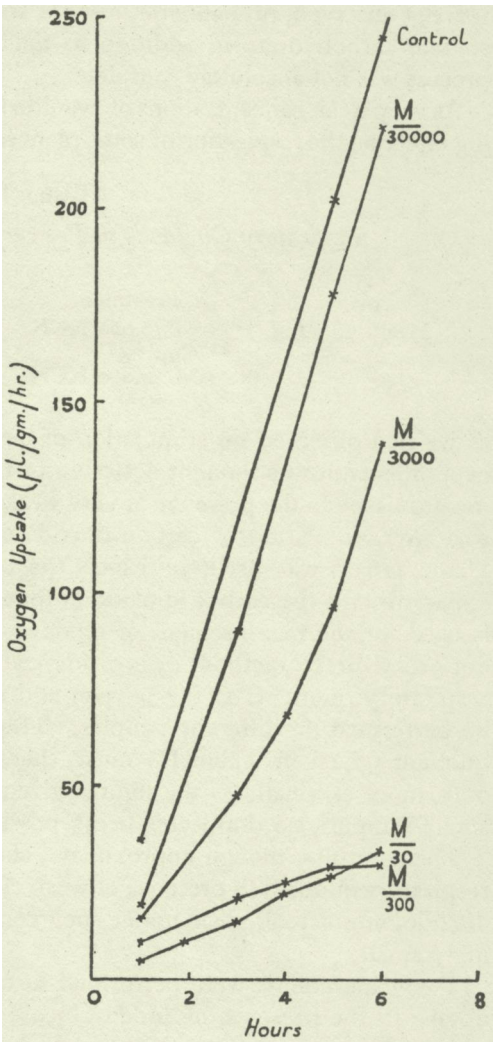

FIG. 1. Oxygen uptake of single samples of tissue in the presence of KCN.

This diagram also illustrates the fact, to which reference has previously been made, that the oxygen uptake of the control tissue was constant throughout the period of the experiment (i.e. successive readings give a straight line when plotted as a graph); this has been found to hold for untreated material throughout these experiments.

The action of cyanide in lowering the oxygen uptake is at least partly reversible, i.e. the effect disappears on the removal of the cyanide, as is shown by an experiment carried out with tobacco stem tissue (no tomato tissue being available). In this experiment the average oxygen uptake of three control samples (in M/30 KH_2PO_4) was 76 μl./gm./hr. The three treated samples were allowed to respire for one hour in an M/300 solution of cyanide in M/30 phosphate, when their average oxygen uptake was 8 μl./gm./hr., giving an inhibition of 89 per cent. The treated samples were then removed, washed in two changes of sterile phosphate solution, and placed in fresh bottles with 3 ml. of phosphate solution in each. Their average oxygen uptake, measured

over four and a half hours, was then 33 μl./gm./hr., or four times the uptake in presence of cyanide, a recovery of nearly half the original rate. Total recovery was not observed, probably because the washing process removed some of the soluble carbohydrate in addition to the cyanide, and because the washing process was not absolutely complete.

As very weak concentrations of cyanide have been shown to have a stimulating effect on the respiration of some plant tissues (Hanes and Barker, 1930), and

TABLE II

Respiratory Quotients in Presence and Absence of Cyanide

Type of plant.	Treatment.	Oxygen uptake (μl./gm./hr.).	R.Q. $(CO_2)/O_2$.
Healthy young	+M/30,000 KCN	114	1·02
,,	Control	158	1·02
,,	+M/30,000 KCN	139	1·02
	Control	144	1·01

as we had observed no stimulation of oxygen uptake due to cyanide in our experiments, it was thought desirable to measure the respiratory quotient of tomato tissue in the presence of very weak cyanide, to ascertain whether there was any stimulation of carbon-dioxide output. The method described by Dixon (1934) was used, in which the oxygen uptake is measured in one apparatus and the carbon-dioxide output measured in another, while a third is used for the measurement of retained carbon dioxide. As Dixon pointed out, this direct method is not adapted to accurate measurement of the respiratory quotient, as the oxygen and carbon-dioxide estimations have to be performed on different samples. The values obtained for the respiratory quotient (given in Table II) must, therefore, be regarded as approximate only, more especially as the figures given for oxygen uptake are taken from single samples, no duplicates being possible.

These results, though approximate, show no perceptible alteration of the respiratory quotient in presence of weak cyanide; if any stimulation in carbon-dioxide output took place under such conditions, it must have been exceedingly small.

Two experiments were performed to test the effect of cyanide on oxygen uptake in the presence of added glucose; for this purpose tissue from very young plants was employed, as it was known to respond to the addition of glucose (Caldwell and Meiklejohn, 1937).

In both cases, although the respiration in absence of cyanide was raised on the addition of glucose, the residual respiration in presence of cyanide was the same with or without glucose. These results agree with those obtained by Genevois (1929).

(b) *Sodium fluoride.*

Sodium fluoride produced a marked inhibition of the oxygen uptake, but only in relatively high concentrations, the maximal effect being observed in

the presence of M/30 fluoride; the effect in water was the same as in phosphate solution. This inhibition is not reversible; tissue subjected to treatment with M/300 fluoride for an hour showed no recovery of oxygen uptake after washing. The results are given in Table IV.

TABLE III

Effect of Cyanide in Presence of Glucose (Very Young Plants)

Type of plant.	Treatment (in M/30 KH_2PO_4).	Oxygen uptake (μl./gm./hr.). No KCN.	Oxygen uptake (μl./gm./hr.). KCN present.	Change in uptake (per cent. of control).
Healthy	Control	41		
	+M/75 KCN		13	−68
,,	0·5 per cent. Glucose	59		+44
	+M/75 KCN		11	−80
Virus	Control	34		
	+M/150 KCN		15	−56
,,	0·5 per cent. Glucose	83		+144
	+M/75KCN		14	−82

TABLE IV

Effect of Sodium Fluoride on the Oxygen Uptake (Healthy Young Plants)

Concentration of NaF.	Solution in.	Oxygen uptake (μl./gm./hr.). Control.	Oxygen uptake (μl./gm./hr.). Treated.	Inhibition per cent.
M/30	M/30 KH_2PO_4	99	8	92
,,	Water	122	11	91
M/300	M/30 KH_2PO_4	130	32	75
M/3,000	,,	101	92	9
M/300	,,	106	44 (before washing)	
	(no recovery)		37 (after washing)	

The course of respiration of individual samples of tissue in presence of different concentrations of fluoride is shown in Fig. 2. There was no diminution in effect due to loss of inhibitor during the experiment in this case; on the contrary, an increase in inhibition as the experiment proceeds may be seen in the curve for M/300 fluoride.

(c) *Iodoacetic acid.*

Iodoacetic acid strongly inhibited the oxygen uptake of tomato tissue at very weak concentrations.

The inhibition caused by iodoacetic acid is not constant throughout the period of observation; in Fig. 3 are given the actual readings from control and treated tissue samples of approximately equal weight, which show that the inhibition increases with time. This is particularly noticeable in the curve for M/10,000 iodoacetic acid. Apparently this particular inhibitor penetrates into the tissue far more slowly than, for instance, cyanide. This inhibition

is not reversible; the results obtained by allowing the tissue to respire for one hour in presence of iodoacetate, washing it with sterile phosphate, and measuring the oxygen uptake in fresh phosphate, are given in Table VI.

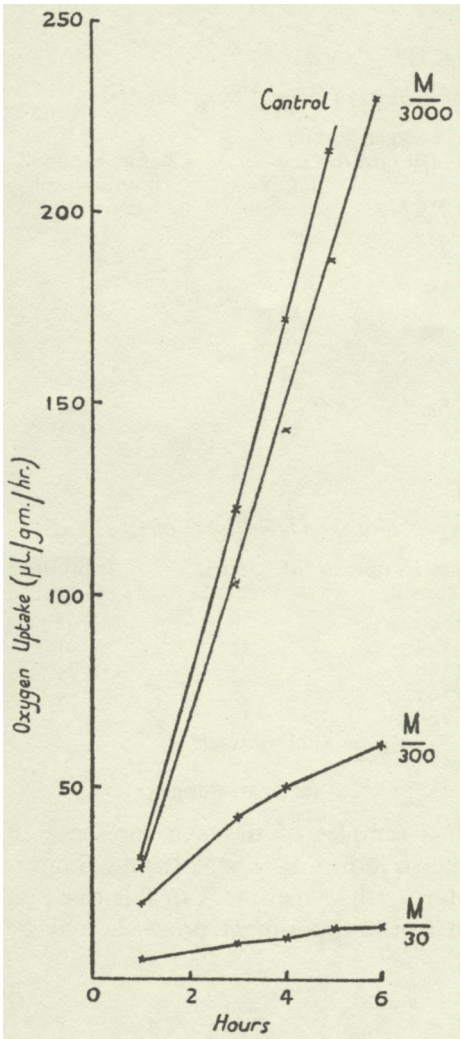

FIG. 2. Oxygen uptake of single samples of tissue in the presence of sodium fluoride.

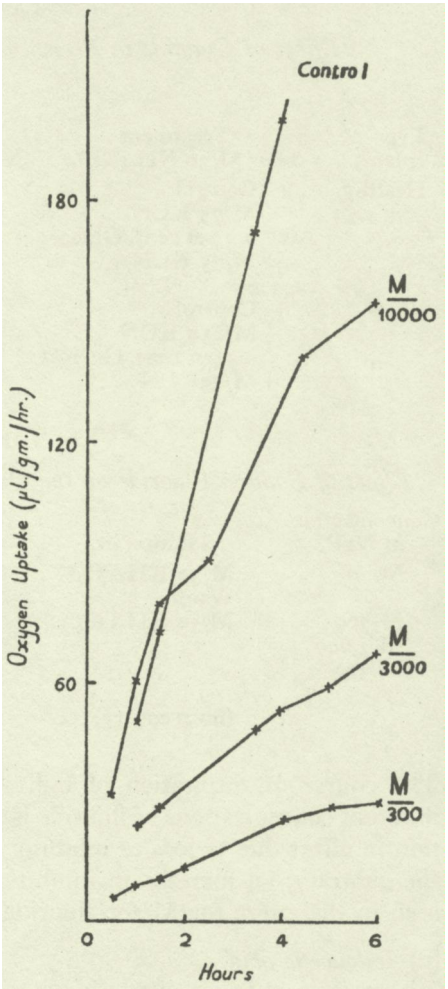

FIG. 3. Oxygen uptake of single samples of tissue in the presence of iodoacetic acid.

(The first experiment was done with tobacco stem tissue, owing to a shortage of tomato plants.) No recovery was observed after washing, even at M/10,000 iodoacetate; in fact, washing appears to reduce the oxygen uptake still further. This may be because the inhibition increases with time.

As, in some animal tissues, the oxygen uptake in presence of iodoacetate can

be restored to its original value by the addition of lactate (Needham, 1932), it was thought of interest to examine the effect of iodoacetic acid on tomato stem tissue in presence of lactic acid. Two preliminary experiments with

TABLE V

The Effect of Iodoacetic Acid on the Oxygen Uptake of Tomato Tissue

Concentration of iodoacetate.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
M/300	Old healthy	83	8	91
M/3,000	,,	54	12	77
,,	Young healthy	132	30	77
,,	,,	109	33	70
M/10,000	,,	141	51	64
M/30,000	Old healthy	46	31	32
M/300	Young virus	103	2	98
,,	,,	140	8	94
M/3,000	,,	109	19	82
M/30,000	Old virus	60	53	(12) (not sig.)

TABLE VI

Effect of Washing Tissue treated with Iodoacetate (Healthy Plants)

Concentration of iodoacetic acid.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated. (before washing).	Treated. (after washing).
M/300	Old tobacco	92	30	11
M/3,000	Young tomato	122	57	24
,,	,,	141	26	18
M/10,000	,,	141	98	72

TABLE VII

Lactic Acid and Iodoacetic Acid (Young Healthy Plants)

Concentration of iodoacetic acid.	pH.	Concentration of lactic acid	Oxygen uptake (μl./gm./hr.). Without lactic acid.	With lactic acid.
0	4·6	0·1 per cent.	67	77
0	4·6	0·5 ,,	40	10
M/3,000	3·0	0·5 ,,	38	0
,,	4·8	0·5 ,,	28	17
,,	7·2	0·5 ,,	*56	*60
0	7·2	0·5 ,,	*73	

* 2 samples.

lactate alone in acid phosphate solution showed that it was only utilized to a limited extent as a respiratory substrate (first two lines of Table VII). When lactic acid was added to the phosphate solution (M/30 KH_2PO_4) without neutralization, the resulting solution had a pH value of 3·0, and this, in conjunction with iodoacetic acid, completely killed the tissue, making it flaccid, and stopping all uptake of oxygen. Another experiment was therefore performed in which the lactic acid in the acid phosphate solution was neutralized

to pH 4·8; and a third experiment was performed in a neutral buffer solution (equal parts of M/30 KH_2PO_4 and M/30 K_2HPO_4, pH 7·2).

In acid solution the lactic acid depressed still further the lowered oxygen uptake produced by iodoacetic acid; even in neutral solution, iodoacetate and lactate together do not give so great an uptake as that recorded in the absence of both.

(d) *Sodium azide.*

Sodium azide (NaN_3) is known to have a pronounced inhibiting action on catalase (Keilin and Hartree, 1934). In these experiments it was found to inhibit oxygen uptake to a marked degree in the standard acid phosphate

TABLE VIII

Effect of Sodium Azide on the Oxygen Uptake of Healthy Tissue

Concentration of azide.	Solution in.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition. per cent.
M/300	Acid phosphate	Old tomato	70	4	94
M/3,000	,,	Old tobacco	85	6	92
,,	,,	Young tomato	170	24	86
M/30,000	,,	Old tobacco	85	49	42
M/300,000	,,	,,	84	81	0
M/300	Alkaline phosphate	Young tomato	144	55	61
M/30,000	,,	,,	125	114	(9) (not sig.)

solution; under these conditions the maximal inhibition was attained already at a concentration of M/3,000, and M/30,000 azide gave a considerable inhibition. In alkaline solution (M/30 K_2HPO_4), on the other hand, the effect was much reduced, M/300 azide giving 60 per cent. inhibition only instead of over 90 per cent. as in acid solution. The results are given in Table VIII; as before, some of the experiments were performed on tobacco plants.

The action of sodium azide was reversible; tobacco stem tissue treated with M/3,000 sodium azide for an hour, then washed and allowed to respire in fresh phosphate, showed a recovery of 35 per cent. When M/300 azide was used, no recovery on washing was observed; but as very dilute azide gives a considerable inhibition, M/300 is probably too strong a solution to be adequately removed by washing.

(e) *Malachite green.*

Malachite green is typical of the basic dyestuffs, which are known to be powerful inhibitors of the dehydrogenases (Quastel and Wheatley, 1931). When applied to tobacco stem tissue it was found to have a powerful inhibiting action on the oxygen uptake at extraordinarily low concentrations. It was the only substance used which gave a considerable inhibition at a concentration as low as 1/300,000 (about M/100,000).

(f) *Narcotics.*

The narcotics urethane and phenyl urethane are known to inhibit dehydrogenase systems, but only in relatively strong concentrations (Warburg, 1930; Sen, 1931). With tobacco tissue quite strong solutions gave only partial

TABLE IX

Effect of Washing Tissue treated with Sodium Azide (Healthy Plants)

Concentration of azide (in M/30 KH_2PO_4).	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated (unwashed).	Treated (after washing).
M/300	Old tomato	33	0	4
,,	Young tobacco	130	3	4
M/3,000	Old tobacco	93	10	33

TABLE X

Effect of Malachite Green on the Oxygen Uptake of Tobacco Tissue

Concentration of malachite green.	Type of plant.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
1/3,000	Young healthy	109	23	79
1/30,000	,,	104	39	61
1/300,000	,,	114	54	44
1/3,000,000	,,	111	105	(5) (not sig.)

TABLE XI

Effect of Urethanes on the Oxygen Uptake of Tobacco Tissue (Healthy)

Substance.	Concentration.	Oxygen uptake (μl./gm./hr.). Control.	Treated.	Inhibition per cent.
Urethane	M/3	85	30	64
,,	M/30	79	71	(10) (not sig.)
Phenyl Urethane	saturated	93	22	76
	M/300	110	75	31

inhibition of oxygen uptake; in fact with phenyl urethane the insolubility of the substance made it difficult to get a solution strong enough to show any pronounced action, and a saturated solution with some undissolved solid still present was added to the stem slices.

(g) *Amyl alcohol.*

The vapour of amyl alcohol has been found to have a stimulating effect on the carbon-dioxide output of apples (Caldwell, 1931). When added to the solution in the Barcroft manometer vessel, a strong solution of iso-amyl alcohol (1/30) had a very strongly inhibiting effect on oxygen uptake; the control samples of tomato stem tissue had an average oxygen uptake of 126 μl./gm./hr.; in presence of 1/30 iso-amyl alcohol this was reduced to 5μl./gm./hr., an inhibition of 96 per cent. A much weaker solution (1/3,000) had no effect, neither stimulating nor inhibiting; the average oxygen uptake of both control and treated material was 97 μl./gm./hr.

Discussion

The substances applied to the tomato tissue in these experiments were all known to be poisons with an inhibiting effect on one or more enzyme systems. They were all found to affect the oxygen uptake of the tissue in substantially the same way; they gave an inhibition increasing with their concentration (in some cases to a limiting value, e.g. cyanide), and if the concentration was reduced below the point where inhibition occurred, no effect was produced. Now it is well known (see e.g. Irving, 1911, Hanes and Barker, 1930) that poisons applied to green plant material as vapour in the air respired have an inhibiting effect upon respiration at high concentrations, but a stimulating effect in very small doses. With the very different technique employed in our experiments, where the poisons were applied in solution to cut material, no stimulation of oxygen uptake was observed to result from the application of smaller doses than those which produced inhibition. This point is further illustrated by the case of amyl alcohol, which stimulates the carbon-dioxide output of apples when applied as vapour (Caldwell, 1931), but in solution was found to have no effect on oxygen uptake at a low concentration and to inhibit it almost completely at a high concentration.

The effects observed on the addition of individual poisons to tomato stem slices show a very close resemblance, both in kind and in degree, to results obtained by other workers with animal tissues and bacteria.

The results which we have obtained with cyanide are very strikingly similar to those recorded by Dixon and Elliot (1929) and van Heyningen (1935) on the action of cyanide on the oxygen uptake of slices of various animal tissues. As in animal tissue slices, about 85 per cent. of the total oxygen uptake is accounted for in plant tissue slices by a system (the 'atmungsferment' of Warburg) which is reversibly poisoned by cyanide. The results obtained on the addition of cyanide in presence of added glucose agree with those of Genevois (1929), and might be regarded as confirming the conclusion he draws from them—that the oxidation of carbohydrate is effected only by that part of the respiratory system which is sensitive to cyanide. It is possible, however, that the small fraction of the respiratory enzyme system which is resistant to cyanide is also concerned with the oxidation of glucose, but is saturated by a very minute quantity which would presumably be present even in conditions of extreme starvation, and the addition of more glucose would have no effect on the oxygen uptake.

The analogy between the respiratory systems of animal and plant tissue must not be pushed too far; the results obtained on the addition of fluoride and iodoacetic acid to tomato tissue illustrate this point. Both these reagents inhibit to a marked degree the oxygen uptake of plant as well as of animal tissue. But in animal tissue they produce their effect by interrupting a process —the conversion of glucose to lactic acid—which probably does not take place in plant tissue (see Genevois, 1929). This conclusion is supported by the observation that the inhibition of oxygen uptake produced in tomato

tissue by iodoacetate was not removed by the addition of lactate, which removes the inhibition observed with chicken embryos in presence of iodoacetic acid (Needham, 1932). It is of interest to note that the slowness of penetration into tissues exhibited by iodoacetic acid in Needham's experiments was observed by us at certain concentrations, see the curve for M/10,000 iodoacetic acid in Fig. 3.

The marked inhibition of oxygen uptake in tomato tissue produced by sodium azide, which has been shown by Keilin and Hartree (1934 and 1936) to act as a specific inhibitor of catalase, may indicate that catalase is an essential part of the respiratory system in plants (see Stiles and Leach, 1932). The fact that azide has a more powerful inhibiting action in acid solutions (Keilin and Hartree, 1936) has also been observed in our experiments.

The results obtained with malachite green and with urethanes are very similar to those formerly recorded (Quastel and Wheatley, 1931; Sen, 1931) with animal tissues and with bacteria.

Taken together, the effects of poisons recorded in this paper show the very great similarity that must exist between the cellular respiration of green plants and that of animals, and also of colourless plants such as yeast.

Summary

Substances known to inhibit enzyme action were added to slices of tomato stem tissue, and their effect on the oxygen uptake of the tissue was measured. All the substances showed an inhibiting action which increased with their concentration. Concentrations lower than those which inhibited oxygen uptake were found to have no stimulating effect.

Cyanide (M/300) produced a reversible inhibition of about 85 per cent. of the total oxygen uptake; no greater inhibition was produced by M/30 cyanide than by M/300.

Sodium fluoride and iodoacetic acid had an irreversible inhibiting action, and sodium azide a reversible one stronger in acid than in alkaline solution.

Malachite green was effective in very small doses, but the urethanes only in high ones.

Amyl alcohol was ineffective at 1/3,000, but produced almost complete inhibition at 1/30.

Literature Cited

Caldwell, J., 1931: Studies in Plant Respiration. (Ph.D. Thesis Cambridge Univ.)

—— and Meiklejohn, J., 1937: Observations on the Oxygen Uptake of Isolated Plant Tissue. I. The Effect of Phosphate and of Added Carbohydrate. Ann. Bot., N.S. iii. 477.

Dixon, M., 1934: Manometric Methods. Camb. Univ. Press.

—— and Elliott, K. A. C., 1929: The Effect of Cyanide on the Respiration of Animal Tissues. Bio-chem. Journ., xxiii. 812–30.

Fisher, R. A., 1934: Statistical Methods for Research Workers. 5th ed. Oliver & Boyd.

GENEVOIS, L., 1929: Sur la Fermentation et sur la Respiration chez les Végétaux Chlorophylliens. Rev. gén. Bot., xli. 252–71.

HANES, C. S., and BARKER, J., 1930: The Physiological Action of Cyanide. I. The Effects of Cyanide on the Respiration and Sugar Content of the Potato at 15° C. Proc. Roy. Soc. B, cviii. 95–118.

HEYNINGEN, W. E. VAN, 1935: The Inhibition of Respiration by Cyanide. Bio-chem. Journ., xxix. 2036–9.

IRVING, A. A., 1911: The Effect of Chloroform upon Respiration and Assimilation. Ann. Bot., xxv. 1077–99.

KEILIN, D., and HARTREE, E. F., 1934: Inhibitors of Catalase Reaction. Nature, cxxxiv. 933

—— —— 1936: Coupled Oxidation of Alcohol. Proc. Roy. Soc. B, cxix. 141–59.

KREBS, H. A., 1935: Metabolism of Amino-Acids. III. Deamination of Amino-Acids. Bio-chem. Journ., xxix. 1620–44.

NEEDHAM, J., 1932: A Manometric Analysis of the Metabolism in Avian Ontogenesis. II. The Effects of Fluoride, Iodoacetate, and other Reagents on the Respiration of Blastoderm, Embryo, and Yolk-sac. Proc. Roy. Soc. B, cxii. 114–38.

QUASTEL, J. H., and WHEATLEY, A. H. M., 1921: The Action of Dyestuffs on Enzymes. I. Dyestuffs and Oxidations. Bio-chem. Journ., xxv. 629–38.

SEN, K. C., 1931: The Effect of Narcotics on some Dehydrogenases. Bio-chem. Journ., xxv. 849–57.

STILES, W., and LEACH, W., 1932: Respiration in Plants. Methuen.

WARBURG, O., 1930: The Metabolism of Tumours, trans. Dickens. Constable.