Specificity of Cycloheximide in Higher Plant Systems

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ABSTRACT

Although cycloheximide is extremely inhibitory to protein synthesis *in vivo* in higher plants, the reported insensitivity of some plant ribosomes suggests that it may not invariably act at the ribosomal level. This suggestion is reinforced by results obtained with red beet storage tissue disks, the respiration of which is stimulated by cycloheximide at 1 microgram per milliliter. Inorganic ion uptake by these disks is inhibited by cycloheximide at 1 microgram per milliliter while the uptake of organic compounds, by comparison, is unaffected. Ion uptake by all nongreen tissues tested is inhibited by cycloheximide, but leaf tissue is unaffected, indicating that the ion absorption mechanism in the leaf may differ fundamentally from that in the root. It is concluded that cycloheximide can affect cellular metabolism other than by inhibiting protein synthesis and that the inhibition of ion uptake may be due to disruption of the energy supply.

Ribosomal inhibitors such as chloramphenicol and cycloheximide are frequently used to determine which metabolic changes in higher plants are linked directly to protein synthesis. The value of any inhibitor depends on the specificity of its action, but there is a tendency to assume specificity in higher plants from studies with microorganisms. The danger of this approach is well illustrated by chloramphenicol. Although this antibiotic inhibits protein synthesis by chloroplast and mitochondrial ribosomes (but not by cytoplasmic ribosomes), it cannot be concluded that effects of chloramphenicol *in vivo* are due solely to interference with the activity of such ribosomes unless effects on energy production can be eliminated. Chloramphenicol does in fact inhibit oxidative phosphorylation by maize mitochondria (7). Fortunately the inhibition of chloroplast ribosomal activity is stereospecific for the D-threo isomer of chloramphenicol (4), while the inhibition of oxidative phosphorylation is not (8); this stereospecificity provides a simple means of establishing for a given system whether chloramphenicol is acting directly at the ribosomal level or on the energy supply.

In much recent work (see 13) cycloheximide rather than chloramphenicol has been used since this antibiotic inhibits protein synthesis by cytoplasmic ribosomes from yeast and mammalian cells. However, remarkably few reports have appeared of the effect of cycloheximide on cytoplasmic ribosomes from higher plants, and the evidence is conflicting. Ribosomes from beet

disks (6), peanut cotyledons (9), and pea epicotyls (3) are inhibited by cycloheximide, whereas ribosomes from castor bean embryos (16), tobacco leaf cytoplasm (4), and wheat embryos (14) are not. This variation in the effect of cycloheximide on isolated ribosomes stands in marked contrast to its uniformly inhibitory effect on protein synthesis in intact plant cells (13). This paper reports in some detail the effect of cycloheximide on respiration and ion uptake by a variety of higher plant tissues, from which it is concluded that cycloheximide can sometimes disrupt cellular metabolism other than by inhibiting protein synthesis.

MATERIALS AND METHODS

Disks (10 mm × 1 mm) were cut from storage tissue of beetroot (*Beta vulgaris* L.), carrot (*Daucus carota* L.), potato (*Solanum tuberosum* L.) and artichoke (*Helianthus tuberosus* L.) and aged aseptically (12) in water at 27 C on a New Brunswick gyrotory shaker. Pea radicles were taken from *Pisum sativum* var. Alaska seeds germinated in moist Perlite for 3 or 4 days. Wheat (*Triticum vulgare*) roots were obtained from seed germinated for 5 or 6 days in stainless steel trays suspended over distilled water. Leaf disks 10 mm in diameter were punched from leaves of cabbage (*Brassica oleracea* var. capitata) and tobacco (*Nicotiana tabacum* L.) with a cork borer. Plants of *Lemna gibba* were harvested after growing aseptically in nutrient solution (5).

Oxygen uptake of these tissues was determined, unless otherwise specified, in aqueous solution by Warburg manometry at 25 C. Each vessel contained 10 disks (approximately 1 g fresh wt of material.) Inhibitors were added to the side arm and tipped in after the basic respiration rate had been established over 60 min. The Na salt of 2,4-dinitrophenol was used, and the pH of solutions containing dinitrophenol or cycloheximide was 5.2 approximately.

Inorganic ion uptake was measured at 25 C from 10 mM $K^{36}Cl$, pH 5.5, as previously described (12). Uptake of organic compounds was determined by following the loss of activity from labeled solutions of 1 mM leucine, 1 mM glycine, and 0.5 mM glucose. Fourteen disks were added to 10 ml of solution containing approximately 1 μc of activity. The flasks were shaken as for inorganic ion uptake on a reciprocating shaker at 25 C and 0.1-ml samples were plated out on lens paper at intervals of 30 and 60 min. Radioactivity was determined with a gas flow Micromil window detector, with the use of an automatic sample changer (Nuclear-Chicago). At the end of the uptake period the disks were rinsed for 5 min to remove label in the free space and extracted with 2.5% (w/v) trichloroacetic acid at 100 C for 20 min. The activity in the residue and soluble extract was then determined separately.

All values reported are the means of duplicate or triplicate determinations within an experiment, and all experiments have been repeated at least once.

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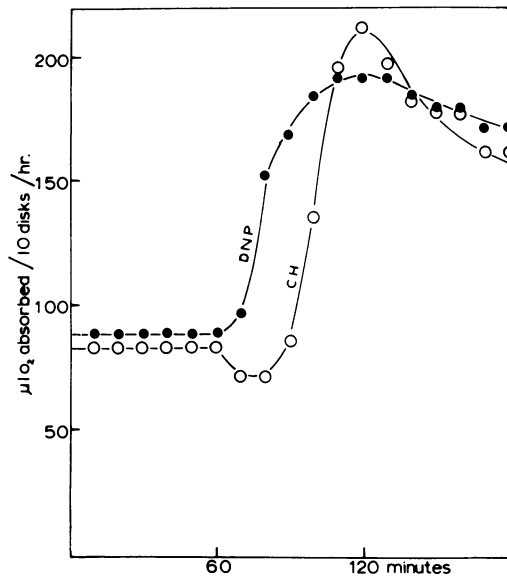
FIG. 1. The respiration of 4-day aged sterile beet disks was established over 1 hr whereupon 2,4-dinitrophenol or cycloheximide was added to give a final concentration of 50 μM DNP or 3.5 μM CH (1 μg/ml). Readings were taken every 10 min. and the changing rate was plotted as O_2 uptake per hr.
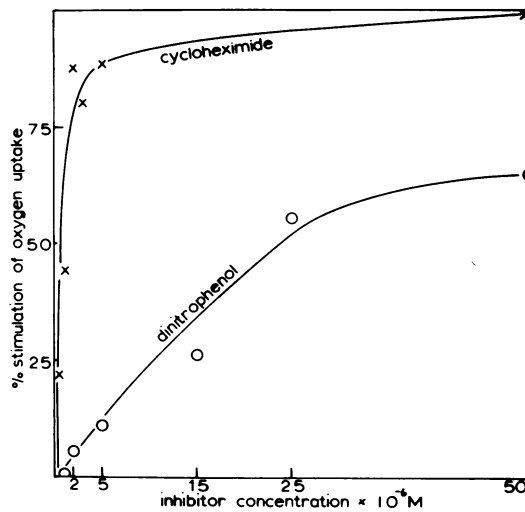

FIG. 2. The respiration of 3-day aged sterile beet disks was established over 1 hr whereupon 2,4-dinitrophenol or cycloheximide was added to give final concentration as shown. Readings were then taken every 15 min over a further 2 hr. The highest values obtained in any 15-min period are plotted, expressed as a percentage stimulation of the basic respiration over a similar period. Maximal values are generally obtained 75 min after addition of CH and 45 min after addition of DNP.

RESULTS

Comparison of the Effects of CH[2] and DNP on Oxygen Uptake by Aged Beet Disks. The addition of 1 μg/ml CH to aged red beet disks markedly stimulates oxygen uptake. The stimulation produced is quantitatively similar to that induced by DNP, but the effect is not so immediate and indeed is preceded by a brief inhibition (Fig. 1). CH, however, is maximally effective at a molar concentration less than $\frac{1}{10}$ that at which DNP exerts its maximum effect (Fig. 2). A characteristic of CH is the narrow-

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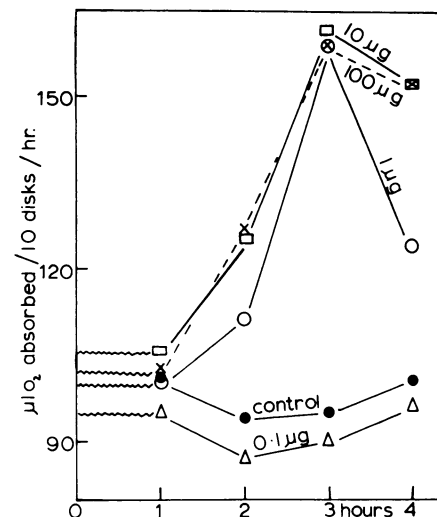[2] Abbreviations: CH: cycloheximide; DNP: 2,4-dinitrophenol.

FIG. 3. The respiration of 3-day aged sterile beet disks was determined over 1 hr whereupon cycloheximide was added to give a final concentration ranging from 0.1 μg/ml (0.35 μM) to 100 μg/ml. Respiration was measured over a further 3 hr and the oxygen uptake was plotted hourly.
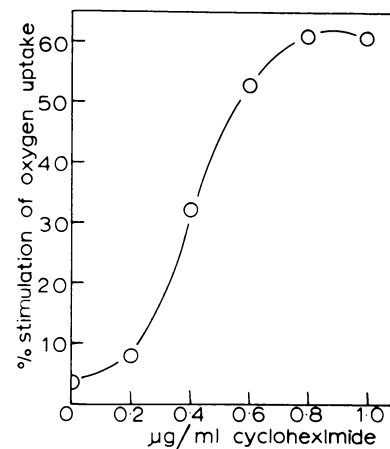

FIG. 4. The respiration of 3-day aged sterile beet disks was measured over 1 hr. Cycloheximide was then added to give a final concentration varying between 0.2 and 1.0 μg/ml and oxygen uptake was followed over a further 2 hr. The average respiration rate during this period is expressed as a percentage of the basic respiration.

ness of the concentration range from no effect to maximal effect. Figure 3 shows that 0.1 μg/ml produces no stimulation whereas 1 μg/ml exerts maximal effect. Within this narrow concentration band a steep response curve is obtained (Fig. 4).

Effect of CH on the Oxygen Uptake of Various Tissues. The effect of CH is not uniform on all tissues or even on beet disks under all circumstances. Of six tissues tested in addition to beet disks, only carrot and artichoke disks gave a marked response to CH (Table I). A failure to affect the respiration of leaf disks has already been reported (20). Its effect on storage tissue generally tends to become more pronounced with aging. This is well illustrated by beet disks of different ages (Table II).

Effect of CH on Cl Uptake. The active uptake of inorganic ions by beet disks is progressively inhibited, maximal inhibition being achieved in 2 hr with 1 μg/ml (Fig. 5). This pattern is typical of the effect of CH on Cl$^-$ uptake by nongreen tissues. Table III gives the percentage inhibition of Cl$^-$ uptake induced by cycloheximide on a variety of tissues. All showed marked

Table I. *Effect of Cycloheximide on the Respiration Rate of a Variety of Tissues*

Oxygen uptake was determined manometrically at 25 C in H_2O over 60 min, and CH was then added to give a final concentration of 1 μg/ml. Readings were taken every 15 min for a further 2 hr, and the highest reading subsequent to adding the inhibitor was expressed as a percentage of the basic respiration.

Tissue	Oxygen Uptake
	% of control
Pea radicles	93
Wheat roots	105
Cabbage leaf disks	104
Carrot root disks aged 1 day	153
Potato tuber disks aged 1 day	113
Artichoke tuber disks aged 1 day	164

Table II. *Effect of Cycloheximide on the Respiration Rate of Sterile Beet Disks of Varying Age*

Oxygen uptake was determined manometrically at 25 C in H_2O over 60 min, and CH was then added to give a final concentration of 5 × 10^{-6} M (1.4 μg/ml). Readings were taken every 15 min for a further 2 hr, and the highest reading subsequent to adding the inhibitor was expressed as a percentage stimulation.

Age of Tissue	O$_2$ Absorbed		Stimulation
	Before addition	After addition	
days	*μl/10 disks·hr*		*%*
Freshly cut	40	37	−7
1	64	91	42
2	95	190	100
3	83	187	125
4	61	150	146

sensitivity with the exception of *Lemna* and leaf disks. Increasing the CH concentration to 100 μg/ml did not significantly decrease Cl^- uptake by leaf disks, and the result was unaffected by light or darkness, or by vacuum infiltration of the tissue. Cl^- uptake was, however, markedly decreased by low temperature, cyanide, or 2,4-dinitrophenol. CH was also found to have no effect on Cl^- uptake by leaf disks from lettuce (*Lactuca sativa*) and leek (*Allium ampeloprasum*) plants.

Effect of CH on the Uptake of Organic Molecules. The time course of CH inhibition of leucine uptake by beet disks (Fig. 6) is quite different from Cl^- uptake. At 1 μg/ml, CH causes an initial disruption of leucine uptake, but after an hour or so uptake proceeds linearly with the control. In contrast to Cl^- uptake, inhibition becomes progressively less with time. An identical pattern is obtained with glycine uptake and with glucose uptake (Fig. 7). Relatively speaking, the major effect of CH at 1 μg/ml is exerted not on the amount of leucine absorbed but on the amount incorporated into trichloroacetic acid-insoluble material in the tissue (Table IV). This is also true for glucose incorporation (Table V).

Effect of Salt Concentration on CH-stimulated Oxygen Uptake of Beet Disks. Beet disks were aged aseptically in water for 3 days, and their respiration rate was then determined in the presence of different salt concentrations. As expected, the respiration rate rises with increasing ionic concentration (Table VI), especially with the phosphate salt. A further stimulation is obtained on adding CH, but at high salt concentrations the effect is less marked, and with phosphate it can drop to 10% or
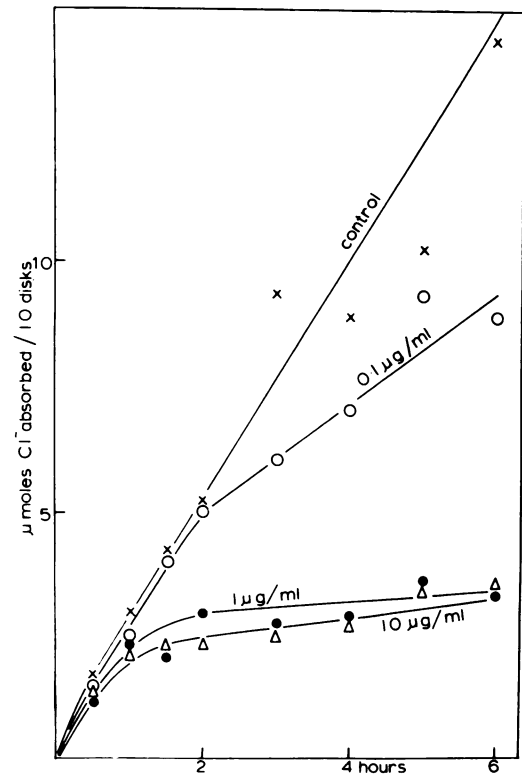

FIG. 5. The effect of cycloheximide on Cl^- uptake at 25 C from 10 mM $K^{36}Cl$, pH 5.5, by 3-day aged beet disks.

Table III. *Effect of 1 μg/ml Cycloheximide on the Absorption of $^{36}Cl^-$ from 10 mM $K^{36}Cl$ by a Variety of Tissues*

Total influx was determined after 6 hr of absorption at 25 C with and without CH.

Tissue	CH-treated Uptake
	% of control
Beet root disks, 3 days aged	24
Carrot root disks, 3 days aged	55
Potato tuber disks, 1 day aged	36
Artichoke tuber disks, 1 day aged	43
Excised wheat roots	59
Excised pea radicles	27
Cabbage leaf disks	97
Lemna	102
Tobacco leaf disks	98

less. Similar results are obtained with Ca^{2+} or Na^+ as the counter ion. Aging the tissue in the presence of salt also has the effect of decreasing the CH-induced stimulation of oxygen uptake (Table VII). Experiments with carrot tissue showed similar salt effects. It is interesting that Rhodes *et al.* (18), who aged apple peel disks in 50 mM KH_2PO_4, subsequently found no effect of 5 μg/ml CH on oxygen uptake.

DISCUSSION

The curious feature of these results is that CH stimulates oxygen uptake of some tissues under suitable conditions, and the question which this prompts is how significant is this finding in relation to the claim generally made for CH that it is a specific inhibitor of protein synthesis in the metabolism of higher plants
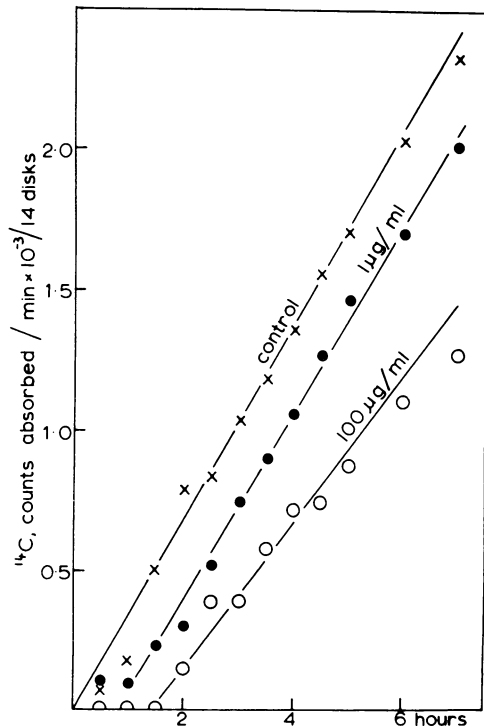
FIG. 6. The effect of cycloheximide on the absorption of ¹⁴C-leucine by 1-day aged sterile beet disks from 1 mM leucine solution, pH 5.5 Aliquots of the external solution were taken at intervals for counting, and the decrease in activity was plotted as cpm absorbed. The control rate shown is equivalent to 1.06 μmoles of leucine absorbed per 14 disks per hr.
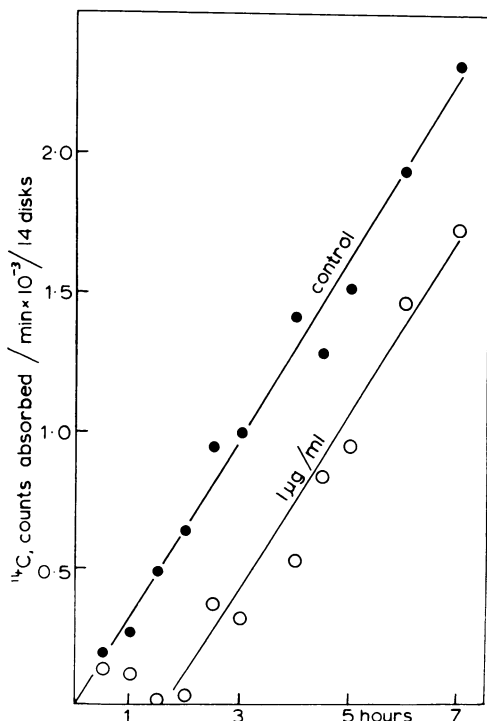

FIG. 7. The effects of cycloheximide on the absorption of ¹⁴C-glucose by 1-day aged sterile beet disks from 0.5 mM glucose. Aliquots of the external solution were taken at intervals for counting, and the decrease in activity is plotted as cpm absorbed. The control rate shown is equivalent to 0.42 μmole of glucose absorbed per 14 disks per hr.

Table IV. *Effect of Cycloheximide on the Absorption and Incorporation of ¹⁴C-Leucine by Beet Disks*

Fourteen 1-day aged sterile disks were added to 10 ml of 1 mM leucine solution, pH 5.5, containing 1 μc of activity. Absorption continued for 7 hr at 25 C.

	Control	+1 μg/ml CH	+100 μg/ml CH
	cpm/14 disks		
Activity lost from solution	232,900	201,000	127,100
Activity in trichloroacetic acid-soluble fraction	127,030	129,030	88,130
Activity in trichloroacetic acid-insoluble fraction	5,670	2,360	1,070

Table V. *Effect of Cycloheximide on the Absorption and Incorporation of ¹⁴C-Glucose by Beet Disks*

Fourteen 1-day aged sterile disks were added to 10 ml of 0.5 mM glucose solution containing 1 μc of activity. Absorption continued for 7 hr at 25 C.

	Control	+0.1 μg/ml CH	+1.0 μg/ml CH	+10 μg/ml CH
	cpm/14 disks			
Activity lost from trichloroacetic acid solution	231,600	225,700	172,700	110,600
Activity in trichloroacetic acid-soluble fraction	71,430	84,480	69,180	62,180
Activity in trichloroacetic acid-insoluble fraction	29,580	28,440	15,570	7,620

Table VI. *Effect of Salt Concentration during Assay on CH-stimulated Oxygen Uptake of Beet Disks Aged in H₂O*

Beet disks were aged aseptically in H₂O for 3 days. Oxygen uptake was determined at 25 C in H₂O or salt solution, pH 5.2 of varying concentration. The basic rate was determined over 60 min, and then CH was added in appropriate salt solution to give a final concentration of 1 μg/ml. Respiratory assay was continued for a further 2 hr, and the percentage stimulation was calculated on the highest 15-min reading recorded after side arm addition.

Salt Concn	O₂ Absorbed								
	KH₂PO₄			KCl			KNO₃		
	Basic rate	CH rate	Stimu-lation	Basic rate	CH rate	Stimu-lation	Basic rate	CH rate	Stimu-lation
mM	*μl/10 disks·hr*		%	*μl/10 disks·hr*		%	*μl/10 disks·hr*		%
0	123	231	87	132	239	74	115	204	77
5	156	212	36	139	248	79	119	208	74
10	161	211	31	132	247	88	120	205	71
20	174	218	26	144	208	44	135	193	43
40	179	207	15	152	221	46	131	187	43
50	191	205	7	147	200	36	131	192	47

It does not seem reasonable to argue that the CH-stimulated respiration results from a primary inhibition of protein synthesis. Puromycin inhibits protein synthesis in beet disks without stimulating respiration (12). The stimulation is more likely to be due to interference with oxidative phosphorylation in a manner analogous to that of DNP. Romani (19) reported a stimulatory effect of CH on isolated pear and avocado mitochondria. Chloramphenicol has also been shown to interfere with oxidative

Table VII. *Effect of Varying Salt Concentration during Aging on the CH-stimulated Oxygen Uptake of Beet Disks*

Beet disks were aged aseptically for 3 days in salts as shown at pH 5.2. Oxygen uptake was assayed at 25 C. The basic rate was determined over 60 min, and then CH was added to give a final concentration of 1 μg/ml. Respiratory assay was continued for a further 2 hr, and the percentage stimulation was calculated on the highest 15-min reading recorded after side arm addition.

Salt Concn	O_2 Absorbed								
	KH$_2$PO$_4$			KCl			KNO$_3$		
	Basic rate	CH rate	Stimu-lation	Basic rate	CH rate	Stimu-lation	Basic rate	CH rate	Stimu-lation
mM	$\mu l/10$ disks·hr		%	$\mu l/10$ disks·hr		%	$\mu l/10$ disks·hr		%
0	97	251	159	109	257	135	95	252	167
5	98	212	116	125	213	71	97	238	146
10	105	187	78	129	217	68	102	204	100
20	124	178	44	144	168	17	111	170	53
40	144	163	13	134	150	11	141	177	26
50	160	171	7	145	165	14	150	155	4

phosphorylation quite apart from its effect on protein synthesis (7, 12). CH, however, surpasses DNP in two respects: it is maximally effective as a respiratory stimulant at a molar concentration only $\frac{1}{10}$ that of DNP; and a 100-fold increase in CH concentration is without further effect, whereas with DNP superoptimal concentrations are inhibitory. Of course, the outstanding difference between DNP and CH is that DNP is ubiquitous in its activity. The selectivity shown by CH in inducing respiratory stimulation is a characteristic property of the drug, although the basis for this selectivity is by no means clear. It is not due to differences in penetrability since ion uptake is inhibited in tissues in which oxygen uptake is apparently unaffected. The indifference of some tissues and the susceptibility of others could be accounted for in terms of different respiratory pathways in leaves, roots, and storage tissues. Similarly, this could account for the increasing sensitivity of aged storage tissue since it has been shown that in such tissue the respiratory pathways alter with aging (1, 10, 11). Or it may be that CH itself brings about a switching from one pathway to another, as suggested by the initial inhibition of oxygen uptake on addition of CH (Fig. 1).

The apparently decreased sensitivity of tissue in high salt concentrations raises another query. In this instance it may be significant that the absolute level of respiration attained in the presence of CH is approximately the same in every salt concentration (Table VI). The percentage stimulation decreases with increasing salt concentration because the basic respiratory level prior to the addition of CH is already stimulated by the presence of salt. In other words, the level of oxygen uptake attained after the addition of CH represents the algebraic sum of a CH-stimulated basal respiration and a CH-inhibited salt respiration. This is not improbable since CH inhibits ion uptake. The conclusion to be drawn from these results is that CH may be interfering with energy transfer even when it has little or no observable effect on oxygen uptake.

The effect of CH on ion uptake is particularly interesting. In many instances it interferes with ion uptake without apparent effect on oxygen uptake. But it cannot thereby be assumed that CH is inhibiting ion uptake by inhibiting protein synthesis and not by affecting energy transfer. Ion uptake by barley roots is markedly inhibited by concentrations of DNP which have no effect on oxygen uptake (15), and beet disks similarly respond to chloramphenicol (12). With both these compounds, one of which may be classed as a protein synthesis inhibitor, the inhibitory

effect on ion uptake is mediated via interference with energy transfer and oxidative phosphorylation. But that apart, it still seems unlikely, because of the time scale involved, that a protein synthesis inhibitor will have a rapid effect on ion absorption. To explain, in these terms, an effect of CH on ion uptake in 1 hr demands a protein turning over with a half-life less than 1 hr. Such a high rate would be quite exceptional even in mammalian membranes where turnover rates are measured in days rather than hours (21). Thus it would appear that the effect of CH on ion uptake in short term experiments is not due to a direct effect on protein synthesis.

Although ion uptake by root tissue is rapidly inhibited by CH, ion uptake by leaf tissue is unaffected. This finding encouraged the thought that the difference could be due to light-generated ATP production in leaf tissue but so far it has not proved possible to demonstrate any effect of light on ion uptake by green tissue when measured over 6 hr. It was also found that the time course of the effect of CH on the uptake of organic compounds was quite the reverse of that on inorganic ion uptake. With the latter, inhibition becomes progressively more severe with time whereas an inhibitory effect of CH on sugar or amino acid uptake is very short-lived and thereafter uptake proceeds at the same rate as in the controls. On the other hand, while having little effect on the absorption of organic compounds, CH does reduce drastically the amount incorporated into trichloroacetic acid-insoluble compounds. This differential effect of CH on organic and inorganic ion uptake can also be seen in results published by Polya (17) although he did not comment on the distinction. It may be that CH will prove to be a useful tool in comparative studies on ion uptake by leaf and root tissue and between organic and inorganic uptake. This last comparison is a particularly neglected aspect of absorption studies with higher plant tissue.

Despite the fact that CH is toxic to a broad spectrum of organisms, it nevertheless displays a marked specificity in toxicity for even closely related organisms. For example, within the genus *Saccharomyces* CH may inhibit the growth of one species (*pastorianus*) but not another (*fragilis*) (23); and even within the one normally sensitive species (*cerevisiae*) insensitive mutants may be found (2). In contrast to chloramphenicol, which effectively inhibits all 70 S ribosomes, CH by no means inhibits all 80 S ribosomes. It is thought that the difference in susceptibility between species is related to differences in the affinity of the ribosomes to bind cycloheximide (22). The results presented here further illustrate the variety of response of different tissues to CH and force the conclusion that to establish that CH is acting via an effect on protein synthesis, it is necessary to demonstrate an effect of CH *in vitro* on amino acid incorporation by ribosomes from the tissue in question. Furthermore, in view of its effect on respiratory regulation, it is necessary to exclude disruption of energy transfer as the primary cause of inhibition of growth and protein synthesis. From a study of the effect of CH on the nucleotide metabolism of cocklebur leaf disks, Ross (20) also stressed that conclusions regarding the role of protein synthesis based on the use of CH are valid only where it has been shown that no other process has been affected or that, if affected, the consequences are unimportant.

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