Phytochemistry, 1964, Vol. 3, pp. 221 to 228. Pergamon Press Ltd. Printed in England

EFFECTS OF D-SERINE AND CHLORAMPHENICOL ON AMINO ACID METABOLISM

R. J. ELLIS*

Department of Biochemistry, Oxford

(*Received* 2 *September* 1963)

Abstract—The uptake of L-serine, L-alanine, L-leucine and glycine by washed beet slices is inhibited by D-serine, but unlike the inhibition caused by chloramphenicol this effect is demonstrable only when the ratios of the concentrations of D-serine to L-amino acid are about ten to one or greater. The inhibitory effect of D-serine on potassium and sulphate uptake by beet slices is reduced from 40–50 per cent to 0–25 per cent by the addition of an equimolar concentration of L-serine, L-alanine, L-threonine, or glycine. The evidence favours the view that both these effects are due to competition between D-serine and the L-amino acids for the amino acid transporting system of the slices. When the complications introduced by these effects of D-serine and chloramphenicol on amino acid uptake are avoided, it can be shown that both these inhibitors of salt uptake also inhibit the incorporation of L-^{14}C-serine into the trichloro-acetic acid-insoluble fraction of beet slices. However the incorporation of L-^{14}C-leucine and L-^{14}C-threonine into the same fraction is not affected by either D-serine or chloramphenicol, suggesting that the synthesis of new protein molecules is not being inhibited. The relevance of these results to the inhibition of salt uptake is discussed.

INTRODUCTION

D-SERINE, like chloramphenicol, inhibits salt uptake by plant cells without affecting the concomitant oxygen uptake.[1] Chloramphenicol is stereochemically related to D-threo-β-phenylserine.[2] It has been suggested[3] that the inhibition of salt uptake by chloramphenicol indicates some connexion between protein synthesis and salt uptake in plant cells because chloramphenicol is known to inhibit protein synthesis specifically in bacteria. Some experiments have been reported which are interpreted to show that chloramphenicol inhibits the incorporation of labelled amino acids into the soluble protein of carrot slices,[4] but the interpretation of most of these experiments is complicated by the inhibitory effect of chloramphenicol on the uptake of labelled amino acids by the tissue. In this paper experiments designed to separate the effects of D-serine and chloramphenicol on the uptake of amino acids from their effects on the incorporation of these amino acids are described. Both compounds inhibit amino acid uptake but neither compound appears to be a general inhibitor of amino acid incorporation and hence no support can be found for the assumption[3] that the action of chloramphenicol on plant tissues is mediated in the manner anticipated from studies on bacterial systems.

RESULTS

Inhibition of Amino Acid Uptake

Chloramphenicol inhibits the uptake of amino acids by carrot slices.[4] Figure 1 illustrates the inhibition by D-serine of the uptake of L-^{14}C-alanine by beet slices. Similar inhibitions

* Agricultural Research Council Fellow.

[1] R. J. ELLIS, K. W. JOY and J. F. SUTCLIFFE, *Phytochemistry* 3, 213 (1964).
[2] G. CARRARA and G. WEITNAUER, *Gazz. chim. ital.* 79, 856 (1949).
[3] J. F. SUTCLIFFE, *Nature, Lond.* 188, 294 (1960).
[4] B. JACOBY and J. F. SUTCLIFFE, *J. exp. Bot.* 13, 335 (1962).

of the uptake of L-serine, L-leucine and glycine were found. However this inhibitory effect of D-serine on amino acid uptake is obtained only when the ratio of D-serine to L-amino acid is high (about ten to one or greater); chloramphenicol is effective at inhibiting the uptake when present in an equimolar concentration to the amino acid (Fig. 2).

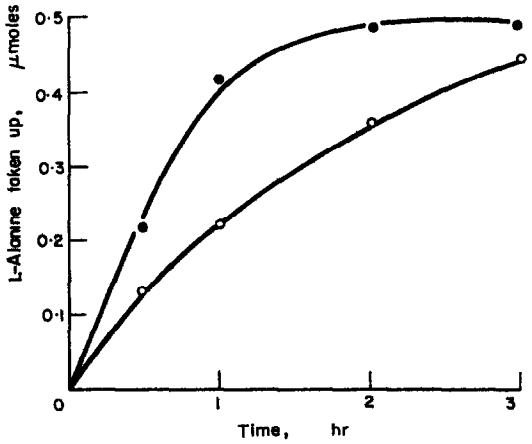

FIG. 1. INHIBITION OF L-ALANINE UPTAKE BY D-SERINE.

Samples of 15 three-day-washed beet slices were shaken in 5 ml 0·1 mM L-^{14}C-alanine with (○) and without (●) 5 mM D-serine at 25°. Aliquots (50 μl) of the solution were removed at intervals to estimate the uptake of ^{14}C-alanine. The figures are averages derived from duplicate flasks.

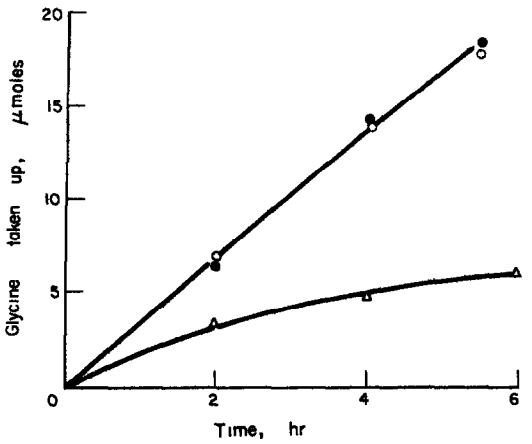

FIG. 2. EFFECT OF D-SERINE AND CHLORAMPHENICOL ON GLYCINE UPTAKE.

Samples of 30 two-day-washed beet slices were shaken at 25° in 5 ml 5 mM ^{14}C-glycine alone (●), with 1 mM D-serine (○), or with 5 mM chloramphenicol (△). Aliquots of the external solution were removed at intervals for counting. The figures are averages derived from duplicate flasks.

Antagonism of the Effect of D-*Serine on Salt Uptake*

The use of DL-serine in place of D-serine revealed that the inhibition of salt uptake by D-serine is reduced if L-serine is present at an equimolar concentration. This antagonism was found to be unspecific as regards the L-amino acid. Table 1 shows the restoration of sulphate

uptake when L-serine, L-alanine or L-threonine were added. Similar results were obtained when the uptake of potassium was followed. The L-amino acids when supplied alone do not affect ion uptake. A possible explanation of these effects is that the L-amino acids compete

TABLE 1. EFFECT OF AMINO ACIDS ON SULPHATE UPTAKE.

Samples of 30 three-day-washed beet slices were shaken in 5 ml 1 mM $Na_2{}^{35}SO_4$ with amino acids for 3 hr at 25°. The uptake of sulphate was estimated as described in the text. The figures are averages derived from duplicate flasks

Amino acid added	Concentration (mM)	$^{35}SO_4$ Taken up (μm moles/30 slices)
None	—	265
D-Serine	5	143
L-Serine	5	280
L-Alanine	5	274
L-Threonine	5	270
D-Serine + L-serine	5+5	203
D-Serine + L-serine	5+0·1	153
D-Serine + L-alanine	5+5	256
D-Serine + L-threonine	5+5	218

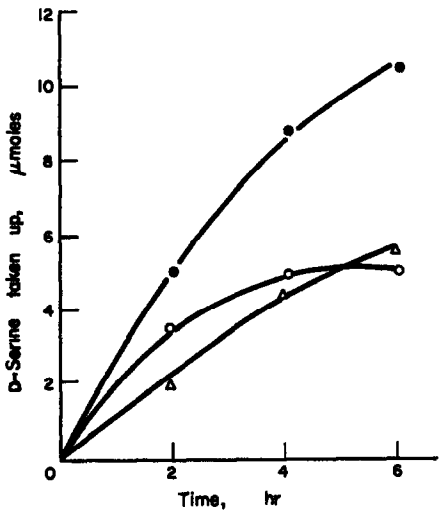

FIG. 3. INHIBITION OF D-SERINE UPTAKE BY L-AMINO ACIDS.

Samples of 25 three-day-washed beet slices were shaken in 5 ml 5 mM D-serine alone (●), with 5 mM L-^{14}C-serine (○), or with 5 mM L-^{14}C-threonine (△) at 25°. Aliquots of the solutions were removed at intervals for the determination of total amino acid uptake and ^{14}C-amino acid uptake as described in the text. The uptake of D-serine was determined by subtraction of the ^{14}C-amino acid uptake from the total amino acid uptake. The figures are averages derived from duplicate flasks

with the D-serine and reduce the amount of inhibitor taken up by the tissue. Figure 3 illustrates the reduction in the uptake of D-serine by equimolar concentrations of L-serine and L-threonine.

This competition between D-serine and the L-amino acids for uptake necessitates that experiments carried out to determine whether D-serine inhibits protein synthesis (i.e. amino

acid incorporation) must be designed so as to avoid this competition. If this is not done the inhibition of the uptake of the labelled L-amino acid will mask any additional effect on the incorporation of this amino acid into protein.

Effect of D-*serine and Chloramphenicol on Amino Acid Incorporation*

In looking for an effect of either D-serine or chloramphenicol on the incorporation of labelled amino acids into the protein of beet slices, it is essential to supply the L-amino acid to the tissue at a low concentration before the tissue is exposed to the inhibitor. The low concentration of the amino acid is necessary because high concentrations counteract the D-serine effect (Table 1). The pretreatment is necessary because both D-serine and chloramphenicol inhibit the penetration of L-amino acids into the cells. Failure to observe these

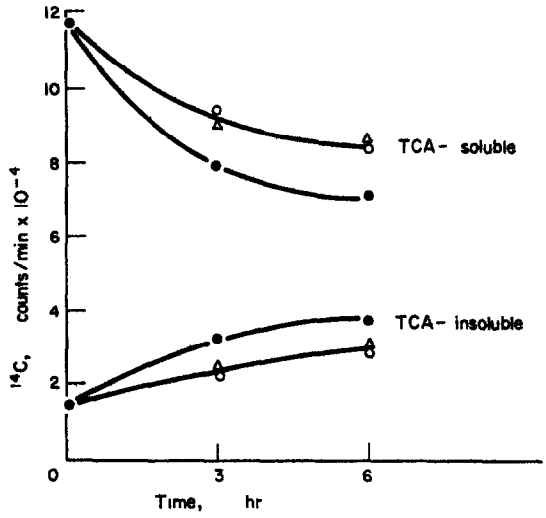

FIG. 4. INHIBITION OF L-SERINE INCORPORATION.

Samples of 20 three-day-washed beet slices were shaken in 5 ml L-^{14}C-serine (0·25 mM, 1 μC, 20·54 x 10^4 c.p.m.) for 2 hr at 25°. The samples were washed on a Buchner funnel for 1 min and some samples (zero time on graph) homogenized at once in cold 5% TCA. Other samples were placed in 5 ml water (●), 5 mM D-serine (○), or 6·2 mM chloramphenicol (△) and shaken for a further 3 or 6 hr before being homogenized. The homogenates were fractionated as described in the text.

conditions in attempts to show that chloramphenicol inhibits protein synthesis in plants has produced results which are difficult to interpret.[5] The results obtained in this type of experiment when L-^{14}C-serine is used are shown in Table 2 and Fig. 4. There is a transfer of radioactivity from the TCA-soluble to the TCA-insoluble fraction such that the protein radioactivity doubles in the first 3 hr. This transfer is reduced in the second 3 hr even though there is still a high proportion of ^{14}C-serine in the soluble fraction (Fig. 4); this decrease in the rate of serine incorporation may reflect the transfer of serine from the cytoplasm into the vacuoles of the cells. Both D-serine and chloramphenicol inhibit the transfer of label from the soluble to the insoluble fraction by about 50 per cent. An inhibition of similar magnitude is seen in the transfer of label from the soluble fraction to the fraction soluble in ethanol–ether (Table 2). It is clear from a consideration of the radioactivity recovered in all the fractions that

[5] C. PEAUD-LENOEL and C. DE GOURNAY-MARGERIE, *Phytochemistry* **1**, 267 (1962).

although some radioactivity is lost these results cannot be explained by supposing that D-serine or chloramphenicol cause an increase in the loss of radioactivity as $^{14}CO_2$ (Table 2). Autoradiography of chromatograms of hydrolysates of the TCA-insoluble fraction reveals that about 70 per cent of the radioactivity is in the serine–glycine area with traces in alanine,

TABLE 2. EFFECT OF D-SERINE AND CHLORAMPHENICOL ON L-SERINE METABOLISM.

Samples of 20 three-day-washed beet slices were shaken in 5 ml L-^{14}C-serine (0·25 mM, 1 μC, 21·63 × 10^4 c.p.m.) for 2 hr at 25°, during which time each sample took up about 19·9 × 10^4 c.p.m. The slices were washed on a Buchner funnel with distilled water for 1 min and some samples (zero times) homogenized at once in cold 5% trichloro-acetic acid (TCA). Other samples were placed in 5 ml water, D-serine (5 mM) or chloramphenicol (6·2 mM) and shaken at 25° for a further 3 hr. The samples were then homogenized in 5% TCA and fractionated as described in the text. The figures are averages derived from triplicate flasks

Treatment	c.p.m. × 10^{-4}			
	TCA-soluble ^{14}C	Ethanol/ether-soluble ^{14}C	TCA-insoluble ^{14}C	Sum of all fractions
Zero time	13·46	0·12	1·2	14·78
Water	11·27	0·24	2·7	14·21
D-Serine	12·42	0·18	2·1	14·70
Chloramphenicol	12·62	0·17	2·0	14·79

valine and threonine. Since it has been established that slices of red beetroot carry out a net synthesis of protein during washing,[6] these results could be taken to indicate that both the inhibitors of salt uptake are also inhibitors of protein synthesis. However, the results obtained when L-^{14}C-threonine or L-^{14}C-leucine are used do not support this conclusion (Table 3).

TABLE 3. L-LEUCINE AND L-THREONINE INCORPORATION.

Samples of 20 three-day-washed beet slices were shaken in 5 ml L-^{14}C-leucine or L-^{14}C-threonine (both 0·25 mM, 1 μC, 18·54 × 10^4 c.p.m.) for 2 hr at 25°. The experiment was then carried out in the manner described in Table 2. The figures are averages derived from triplicate flasks

Treatment	TCA-insoluble ^{14}C (c.p.m. × 10^{-4})	
	L-^{14}C-leucine	L-^{14}C-threonine
Zero time	0·34	0·27
Water	0·69	0·51
D-Serine	0·66	0·50
Chloramphenicol	0·69	0·56

The incorporation of these amino acids into the protein fraction is less than that of L-serine but there is again an approximate doubling of the protein radioactivity in the first 3 hr after the pretreatment. There is no inhibition by either D-serine or chloramphenicol of this rise in protein radioactivity. To make certain that this was a significant result, samples of slices from the same batch as were used in these experiments were tested for the effect of the two inhibitors

[6] I. R. MACDONALD, A. H. KNIGHT, and P. C. DEKOCK, *Physiol. Plantarum.* **14**, 7 (1961).

on sulphate uptake and the usual inhibitions demonstrated.[1] It was also shown that pre-treatment of slices with 0·25 mM L-leucine or L-threonine did not affect this inhibition of sulphate uptake. Autoradiography of hydrolysates of the TCA-insoluble fraction showed that in the case of L-^{14}C-leucine experiments only the leucine area was radioactive and in the case of the L-^{14}C-threonine experiments only the threonine and isoleucine areas were radioactive. D-Serine and chloramphenicol do not therefore appear to inhibit protein synthesis in beet slices.

Bacterial Contamination of Beet Slices

It has been found recently that slices of storage tissue can develop rather large endogenous populations of bacteria during washing which are not apparent on visual examination of the slices (Dr. J. S. D. Bacon, personal communication). In view of the inhibitory action of both chloramphenicol[7] and D-serine[8] on the growth of some bacteria, this point was examined in the case of beet slices to determine whether the effects of these compounds were mediated by an effect on bacteria. Freshly cut slices were found to contain very few bacteria, but in slices washed for three days the number had risen to 10^7–10^8/g fresh weight. When it was found that the growth of these bacteria in broth was inhibited by both D-serine and chloramphenicol the suspicion arose that the effects of these compounds on the slices were effects on the bacteria and not on the plant cells. This possibility was ruled out by utilizing the large difference in the concentration of chloramphenicol required to inhibit the growth of the bacteria on the one hand and the uptake of salt by the slices on the other. Table 4 shows that washing the slices in chloramphenicol at the low concentration of 0·12 mM reduces the bacterial count by about 150-fold compared with the number obtained from slices washed in distilled water. However, the oxygen uptake of this tissue and the inhibition of sulphate uptake caused by D-serine and chloramphenicol (6·2 mM) was not significantly different from the values obtained with slices washed in water; the somewhat different values for the amount of sulphate taken up by the two sets of slices may reflect differences in the degree of aeration of these two sets during washing. It therefore appears that the endogenous bacteria are not responsible for the effects under study, a conclusion supported by the failure of chloramphenicol to inhibit the incorporation of L-leucine or L-threonine (Table 3). The amino acid incorporation experiments were repeated with slices washed in 0·12 mM chloramphenicol but no significant differences from the previous results were noted.

DISCUSSION

D-Serine is much less effective than chloramphenicol at inhibiting the uptake of amino acids by beet slices; this fact, coupled with the lack of effect of D-serine on respiration,[1] suggests that this compound may be the most specific inhibitor of salt uptake by plant tissues that has yet been reported. D-Serine and chloramphenicol show some structural similarities[2] but the results presented here do not support the view that the action of either compound on salt uptake is mediated by an effect on protein synthesis.[3] There is little convincing evidence that chloramphenicol specifically inhibits protein synthesis in plant tissues in the same time period as it inhibits salt uptake; in animal tissues also, chloramphenicol either does not affect protein synthesis or does not do so specifically.[9] A metabolic effect of both D-serine and chloramphenicol in beet slices is an inhibition of the incorporation of L-serine into the TCA-insoluble fraction (Table 2). It has not been ruled that this effect in the case of D-serine is due

[7] T. D. BROCK, *Bacteriol. Rev.* **25**, 32 (1961).
[8] E. A. GRULA and M. M. GRULA, *J. Bacteriol.* **83**, 981 (1962).
[9] R. RENDI and S. OCHOA, *J. biol. Chem.* **237**, 3710 (1962).

to its racemization by the tissue to L-serine, thus lowering the specific activity of the absorbed L-^{14}C-serine. This does not seem likely in view of the fact that all the soluble serine of beet slices has the L- configuration.[1] It is therefore suggested that the action of D-serine and chloramphenicol on salt uptake may be mediated by an effect on L-serine metabolism.

Although the effects described with beet slices were shown not to be due to the endogenous bacterial population (Table 4), it is suggested that workers who use this type of tissue should be careful to check that the bacterial population that develops during washing is not responsible for some of their results. Experiments on the inhibitory effect of chloramphenicol

TABLE 4. BACTERIAL CONTAMINATION OF BEET SLICES.

A batch of beet slices was divided into two lots; one lot was washed in water as usual and the other lot in chloramphenicol (0·12 mM). After two days washing, samples of 20 slices from each lot were shaken in 5 ml 1 mM $Na_2{}^{35}SO_4$ with and without 5 mM D-serine or 6·2 mM chloramphenicol for 3 hr at 25°. The sulphate uptake was measured as described in the text. The rate of oxygen uptake of samples of both lots of slices was measured by shaking 20 slices in 3 ml water in Warburg flasks for 90 min at 25°. The bacterial count was made by plating out serial dilutions of homogenates of slices made in sterile water as described in the text

Washing medium	$^{35}SO_4$ taken up μm moles			O_2 taken up (μl/hr/g fresh wt.)	Bacterial count (per g fresh wt.)
	Control	+D-Serine	+Chloramphenicol		
Water	107	66	56	125	1×10^7
Chloramphenicol	130	86	72	134	7×10^4

on glucose uptake by wheat roots were carried out under sterile conditions,[5] confirming that effects of chloramphenicol on plant tissues cannot necessarily be explained in terms of associated micro-organisms.

EXPERIMENTAL

Chemicals

L-[U-^{14}C]serine, L-[U-^{14}C] leucine, L-[U-^{14}C] threonine and [U-^{14}C] glycine were obtained from the Radio-Chemical Centre, Amersham. All other chemicals were obtained as previously described.[1]

Plant Tissues

Slices of red beetroots were prepared as previously described.[1] A sample of 25 slices has a fresh weight of about 740 mg.

Uptake of Ions and Amino Acids

The uptake of sulphate and potassium was measured as previously described.[1] The uptake of ^{14}C-amino acids was measured by counting aliquots (50–200 μl) of the external solution. The uptake of unlabelled amino acids was determined by a ninhydrin procedure.[10]

Incorporation of ^{14}C-Amino Acids

The incorporation of ^{14}C-amino acids into protein was estimated by measuring the radioactivity of the fraction of the tissue insoluble in ice-cold 5% trichloro-acetic acid (TCA). Attempts to use the fraction of the tissue insoluble in hot 80% ethanol gave widely scattered replicates, perhaps due to the presence of ethanol-soluble protein.[11] After washing for 1 min

[10] E. W. YEMM and E. C. COCKING, *Analyst* **80**, 209 (1955).
[11] D. RACUSEN and M. FOOTE, *Nature, Lond.* **197**, 697 (1963).

with distilled water on a Buchner funnel, each sample of 20 slices was homogenized to a fine paste in 7 ml of ice-cold 5% TCA in a Potter-Elvehjem type glass homogenizer. The homogenizer was rinsed out with 3 ml 5% TCA and the rinsing solution added to the homogenate. The insoluble material was centrifuged down and the supernatant solution decanted. The precipitate was resuspended in 10 ml ice-cold 5% TCA and centrifuged; this washing was repeated twice more with 10 ml 5% TCA. The white precipitate was resuspended in 10 ml ethanol/diethyl ether (1 : 1, by vol.) and incubated at 37° for 20 min to remove TCA and lipids. The suspension was centrifuged, the supernatant solution decanted, and the precipitate incubated once more with 10 ml ethanol/ether. The final precipitate was resuspended evenly in 5 ml 80% formic acid and aliquots (100–200 μl) counted at infinite thinness on 3/4-in. duralumin planchettes under an end window counter of about 5 per cent efficiency. Duplicate samples of each suspension were counted and at least 2000 counts above background recorded. The figures shown in the tables are averages derived from triplicate flasks.

Control experiments were carried out with each amino acid to check the efficacy of the washing procedure by adding the ^{14}C-amino acid to the slices immediately before homogenization in TCA. Experiments were also performed to determine the stability of the incorporated label in the presence of a large excess of unlabelled amino acid. The results of these experiments indicated that the washing procedure removed the soluble radioactivity to below the limits of detectability in every case, and that the incorporated label could not be removed from the precipitate by adding a 200-fold excess of unlabelled amino acid to the TCA homogenizing solution.

Some of the labelled precipitates were not resuspended in formic acid but hydrolysed in 6 N HCl (about 200 ml/8 mg α-amino nitrogen) for 17 hr. The hydrolysate was reduced to dryness several times on a rotary evaporator to remove HCl and then taken up in water. The solution (pH 1–2) was passed through a column of cation exchange resin (Zeokarb 225) in the H^+ form and the column washed with water. The amino acids were eluted with 6 N HCl, evaporated to dryness and stored *in vacuo* over NaOH; 90–95 per cent of the radioactivity of the hydrolysate was recovered in this fraction. Aliquots (50–100 μg α-amino nitrogen) of the eluted amino acid fraction were chromatographed in two dimensions on Whatman 3MM paper as described by Wolfe.[12] Amino acids were revealed by spraying the dried chromatograms with 0·1% ninhydrin in butan-1-ol and heating at 100°. Radioactivity was revealed by placing the chromatogram against Kodirex X-ray film for 1–3 weeks.

Bacterial Contamination of Beet Slices

Slices were washed in distilled water in the usual way and then rinsed twice in sterile distilled water to remove surface contaminants. The slices were homogenized in sterile distilled water and the whole homogenate plated out at serial dilutions on to Oxoid nutrient agar (CM4) and incubated at 37° for 2 days.

Acknowledgements—Thanks are due to Dr. K. W. Joy for help with some of the experiments and to the Agricultural Research Council for financial assistance.

[12] M. Wolfe, *Biochem. biophys. Acta* 23, 186 (1957).