Biochemical Pharmacology, 1963, Vol. 12, pp. 303–316. Pergamon Press Ltd., Printed in Great Britain.

STUDIES OF THE THE MECHANISM OF ACTION OF 1-AMINO*CYCLO*PENTANE-1-CARBOXYLIC ACID*

WILLIAM R. STERLING† and J. FRANK HENDERSON

Department of Pharmacology, The George Washington University School of Medicine, Washington, D.C., U.S.A.

(*Received* 10 *October* 1962. *accepted* 7 *December* 1962)

Abstract—The mechanism of action of the nonmetabolizable α-amino acid, 1-amino-*cyclo*pentane-1-carboxylic acid (ACC), was investigated in ascites tumor cells *in vitro*. ACC replaced no natural amino acid in protein synthesis nor did it interfere with the endogenous synthesis of natural amino acids from glucose. ACC appeared to be concentrated in the cells by an active transport system utilized by some natural amino acids. Participation in a heteroexchange diffusion process with DL-leucine and DL-valine was demonstrated. Although high concentrations of ACC partially inhibited the incorporation of amino acids into proteins in ascites tumor cells *in vitro*, evidence was presented that this was due to inhibition of the transport of these amino acids into the cells. The relation of the inhibition of transport of amino acids into neoplastic cells to the mechanism of carcinostatic action *in vivo* is discussed.

1-AMINO*CYCLO*PENTANE-1-CARBOXYLIC acid has been reported to inhibit the growth of adenocarcinoma 755, 6C3HED ascites lymphosarcoma, and ascites leukemias L4946[1] and L1210[2] in mice, and Walker carcinoma 256 in rats,[3] but had no effect on sarcoma 180 and Ehrlich ascites carcinoma.[1] Clinical trials of ACC have given variable, but generally negative, results.[4–6] Its small degree of efficacy in the clinic may be ascribed at least in part to suboptimal dose schedules.[7] Studies on the metabolism of ACC have shown that it is excreted very slowly, and constant blood and tissue levels may be maintained for several days by a single injection of drug.[7] These studies have also demonstrated that ACC is itself the active inhibitor, since it is not metabolized by normal or neoplastic tissues.[7] The lack of metabolism of ACC has also been observed by Christensen and Jones[8] and by Berlinguet *et al.*[9]

Previous studies on the mechanism of action of ACC have shown that it does not inhibit the transamination of natural amino acids by liver homogenates or their oxidation by purified enzymes.[10] Inhibition of amino acid incorporation into proteins by ACC has been observed in rat tissues,[11] but not in Ehrlich ascites cells *in vitro*.[12]

This paper presents the results of studies on the mechanism of action of ACC in ascites tumor cells *in vitro*. The results indicate that an observed inhibition of the incorporation of certain radioactive amino acids into proteins is due to the inhibition of their transport into the ascites tumor cells by ACC.

* This research was aided by Research Grant CY-2978 from the National Cancer Institute, Bethesda, Md.

† Part of this work was carried out during the tenure of a predoctoral fellowship (8771) from the Division of General Medical Sciences, U.S. Public Health Service. Subsequently it was supported by Training Grant 2G-26. Present address: Dept. of Chemical Pharmacology, Lederle Laboratories, Pearl River, N.Y.

MATERIALS AND METHODS

ACC was obtained from the Cancer Chemotherapy National Service Center, National Cancer Institute, and ^{14}C-(carboxy)-ACC was synthesized in this laboratory, as previously described.[7]*

Male C3H mice, 6 to 10 weeks old, were given intraperitoneal inoculations of 2×10^5 6C3HED ascites lymphosarcoma cells and male BDF_1 mice, 6 to 10 weeks old, received approximately 7×10^5 adenocarcinoma 755 ascites cells. Mice were allowed water and laboratory chow *ad libitum*.

Ascites tumor cells were removed from mice by capillary pipet after laparotomy, were centrifuged to remove ascitic fluid and resuspended, without washing, in 3 volumes of medium. This concentrated cell suspension contained 0·25 ml packed cell volume (30 mg dry weight) per ml. Calcium-free Krebs-Ringer phosphate medium was used as the incubation medium and usually contained 5·6 mM glucose. Incubations were routinely conducted in a total volume of 10 ml in 50-ml Erlenmeyer flasks with constant shaking. Air was the gas phase and the temperature of the water bath was adjusted as desired. All compounds to be added to the incubation mixture were dissolved in medium and kept frozen until used.

The incorporation of radioactive precursors into the perchloric acid-insoluble fraction of cells was measured by the membrane filtration technique.[13, 14] Flasks containing 0·25 ml packed cell volume were incubated at 37·5° and duplicate 0·1- or 0·2-ml aliquots removed at intervals for analysis.

The study of amino acid uptake into ascites tumor cells was based on the measurement of radioactivity in an extract of the packed cell mass after incubation of the cells with radioactive amino acids and removal of the medium. Flasks containing 0·5 ml packed cell volume were incubated at 26° with various amino acids. At intervals after the addition of the cells, the contents of the flask were poured into 15-ml graduated centrifuge tubes and centrifuged. The supernatant was removed and aliquots taken for radioactivity measurements. Because intracellular amino acids are lost from ascites tumor cells upon washing, [15, 16] the centrifuge tubes containing the packed cells were inverted, drained thoroughly, and swabbed successively with balls of wet and dry absorbent cotton, without disturbing the cell pellet. The packed cells were extracted by stirring with 3 ml distilled water in a boiling water bath for 5 min. This extract was removed after centrifugation, and the cell debris was washed with 2 ml distilled water. The combined extract and wash contained the total hot water-extractable radioactive amino acids in the cell pellet. The studies of Heinz[17] and of Kit[18, 19] have established that the radioactivity extracted under these conditions is the free

* Uniformly labeled ^{14}C-glucose (1·79 mc/mmole) was obtained from Isotopes Specialties Co., Inc.; ^{14}C-inulin, carboxy-labeled (2·15 μc/mg), 1-^{14}C-L-alanine (7·5 mc/mmole), 3-^{14}C-DL-serine (0·83 mc/mmole), 4-^{14}C-DL-valine (3·0 mc/mmole), 1-^{14}C-L-isoleucine (12·5 mc/mmole), 2(ring)-^{14}C-L-histidine (9·95 mc/mmole), ^{14}C-(guanido)-L-arginine hydrochloride (5·1 mc/mmole), and 1-^{14}C-DL-α-aminoisobutyric acid (7·0 mc/mmole) from California Corp. for Biochemical Research; 1-^{14}C-DL-alanine (1·16 mc/mmole), 3-^{14}C-DL-serine (1·4 mc/mmole), and 3-^{14}C-DL-phenylalanine (1·1 mc/mmole) from Isotopes Specialties Co., Inc.; 2-^{14}C-DL-glutamic acid (5·4 mc/mmole) and 4-^{14}C-DL-aspartic acid (0·77 mc/mmole) from Tracerlab, Inc.; 2-^{14}C-glycine (2·3 mc/mole), and 1-^{14}C-DL-leucine (1·8 mc/mole) from Volk Radiochemical Co.; 1-^{14}C-DL-lysine hydrochloride (0·88 mc/mmole) from New England Nuclear Corp.; and ^{14}C-L-proline (1·45 mc/mmole) from Schwartz Bioresearch, Inc.

The nonradioactive amino acids used were of the same stereoisomeric configuration as their radiolabeled counterparts and were obtained from Nutritional Biochemicals Corp., except L-histadine, which was obtained from California Corp. for Biochemical Research, and DL-serine, DL-valine, and L-arginine hydrochloride, supplied by Sigma Chemical Co.

amino acid added. Aliquots of this solution were plated for radioactivity measurements. When uptake was measured at short time periods, radioactivity in the hot water-insoluble fraction was negligible.

The volume of entrapped medium in the cell pellet was determined by the use of radioactive inulin, which does not enter ascites cells.[17] Cells were incubated in medium containing ^{14}C-inulin, and the hot water extract of the cell pellet was prepared. The results indicated that the trapped fluid volume was 16% of the packed cell volume, which is in agreement with the results of Heinz[17] and Crane *et al.*[20] using Ehrlich ascites cells.

The volume of intracellular water was determined by placing 2-ml portions of a concentrated cell suspension containing 0·5 ml packed cells in tared centrifuge tubes; the supernatant was removed after centrifugation, the wall of the tubes dried, and the wet weight determined. The cells were then dried to constant weight at 110° and the dry weight measured. It was established that 80% of the cell weight was water, which is in agreement with previous findings with Ehrlich ascites cells.[15]

The intracellular concentration of radiolabeled amino acids transported into the cells was calculated from the following formula.

$$\frac{\mu\text{moles amino acid transported}}{\text{ml intracellular water}} = \frac{\left[\frac{\text{cpm in packed cell mass}}{\text{0·5 ml packed cell mass}} - \frac{\text{0·16} \times \text{cpm}}{\text{0·5 ml medium}}\right] \times 2{\cdot}5}{\text{specific activity (cpm/}\mu\text{mole)}}$$

RESULTS

The effect of ACC on the incorporation of a series of natural amino acids into the acid-insoluble fraction of ascites tumor cells was measured. The results are shown in Fig. 1. ACC and amino acids were added to the cell suspension simultaneously and were present in a ratio of 3·86:1. In the presence of ACC there was a slight inhibition of incorporation of all the amino acids studied except lysine. Glycine incorporation was inhibited to the greatest extent, and the degree of inhibition of incorporation of the other amino acids ranged between the extremes of those of lysine and glycine.

Similar experiments were conducted in which the concentration ratio of ACC to the radiolabeled amino acids was increased to 50:1. The results illustrated in Fig. 2 indicate that, although the extent of inhibition increased, the same relationship was found as at the lower ratio in the degree of inhibition of incorporation of the various amino acids into the cells. Glycine incorporation was inhibited to the greatest extent, lysine the least; the inhibition of leucine and valine were intermediate.

The inhibition of amino acid incorporation into protein caused by ACC might be due to direct inhibition of protein synthesis, to replacement of natural amino acids in protein by ACC, to inhibition of some steps in the intermediary metabolism of amino acids, or to inhibition of the entry of amino acids into the cells.

The variability in the percentage of inhibition by ACC of the incorporation of different amino acids (Fig. 1, 2) suggests that there was no direct inhibition of protein synthesis.

The ability of ^{14}C-ACC to replace natural amino acids in the proteins of 6C3HED ascites lymphosarcoma cells *in vitro* was examined. Fig 3 shows that there was no incorporation of ^{14}C-ACC into the macromolecules of the cells during the 45-min

incubation. The incorporation of radioactive glycine was used as a positive control in this experiment.

The next possibility examined was that ACC might block amino acid incorporation into proteins by interfering with the endogenous synthesis and intermediary metabolism of amino acids. This was tested by studying the effect of ACC on the incorporation of ^{14}C-glucose, which is converted to several amino acids and to ribose.[18] As may

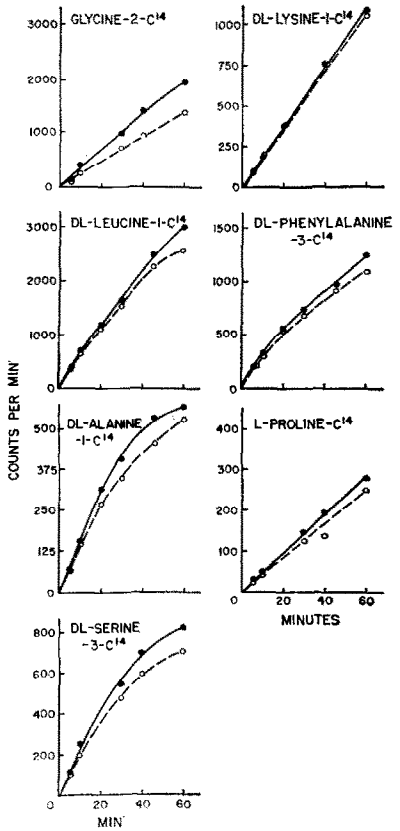

FIG. 1. Effect of ACC on the incorporation of amino acids into the acid-insoluble fraction of 6C3HED ascites cells *in vitro*. Tumor cells, 30 mg dry weight, were incubated in 10 ml of Krebs-Ringer phosphate medium at 37·5° and 0·2 mM radioactive amino acids with (○) or without (●) 0·78 mM ACC.

be noted in Fig. 4, ACC apparently did not interfere with these processes. The fact that there was no depression of the radioactivity incorporated into the acid-insoluble fraction of the cell suggested that ACC did not interfere with at least some of the metabolic interconversions of glucose or with the incorporation of the endogenously formed precursors into proteins and nucleic acids. It was therefore necessary to establish whether ACC was transported into cells in a manner similar to that of other amino acids, whether it competed with the transport of other amino acids, and whether such competition could account for the observed effects on amino acid incorporation into protein.

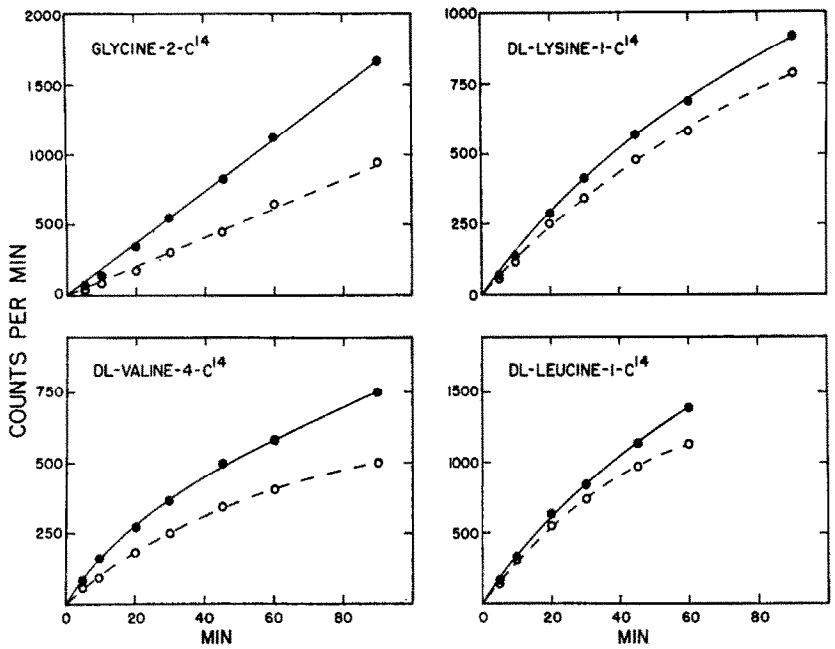

FIG. 2. Effect of ACC on the incorporation of amino acids into the acid-insoluble fraction of 6C3HED ascites cells *in vitro*. Tumor cells were incubated as described in Fig. 1 with 0·2 mM radioactive amino acids with (○) and without (●) 10·0 mM ACC.

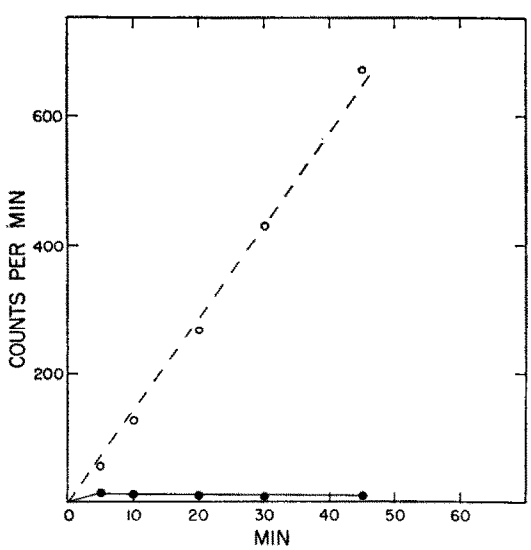

FIG. 3. Incorporation of ^{14}C-ACC and 2-^{14}C-glycine into the acid-insoluble fraction of 6C3HED ascites cells *in vitro*. Tumor cells were incubated as described in Fig. 1 with 0·2 mM 2-^{14}C-glycine (○) or 0·78 mM ^{14}C-ACC (●).

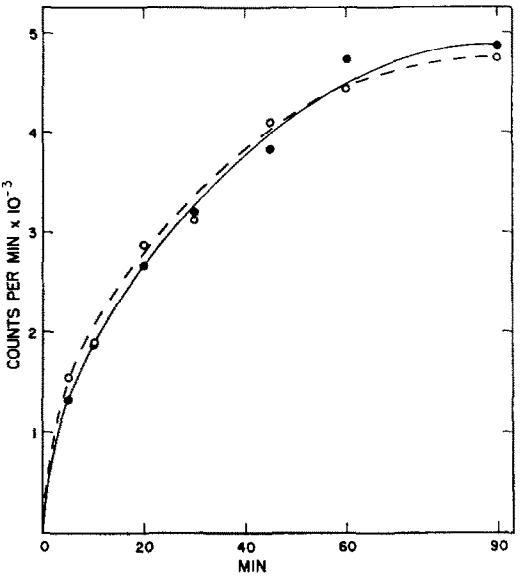

FIG. 4. Effect of ACC on the incorporation of ^{14}C-glucose into the acid-insoluble fraction of 6C3HED ascites cells *in vitro*. Tumor cells were incubated as described in Fig. 1 with 0·5 mM ^{14}C-glucose with (○) and without (●) 20·0 mM ACC.

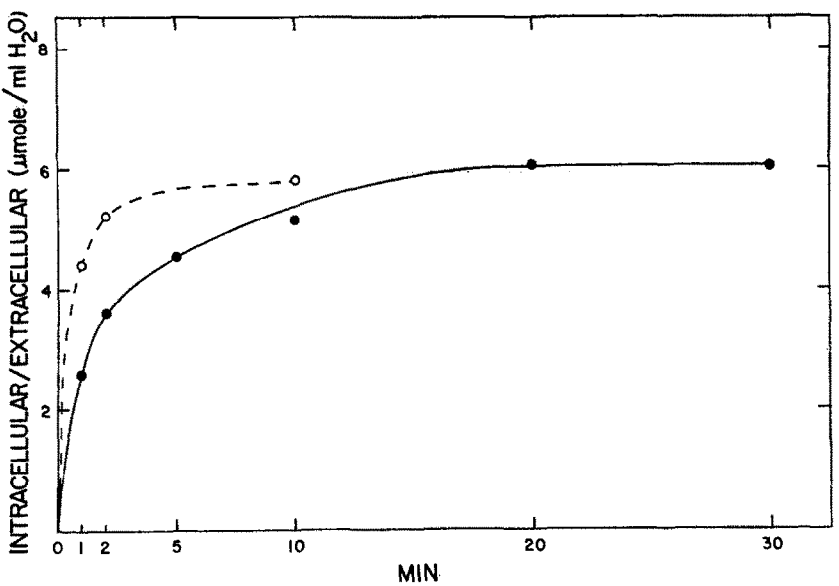

FIG. 5. Uptake of ACC into 6C3HED ascites cells *in vitro*. Tumor cells, 60 mg dry weight, were incubated in 10 ml of Krebs-Ringer phosphate medium in air with 5·6 mM glucose and 0·5 mM ^{14}C-ACC at 37·5° (○) or 26° (●).

The time course of ACC entry into ascites tumor cells is shown in Fig. 5. It is seen that ACC was initially rapidly transported into the cells before an equilibrium was attained. The same intracellular concentration was achieved at both 26° and 37·5°—namely a sixfold concentration over the extracellular level. This degree of

concentration was part of the evidence that an active transport process was involved in the translocation of ACC.

Further experiments conducted on the transport of ACC involved the determination of the "apparent initial influx" rate of amino acids, following Heinz.[16] This represents the influx at time intervals short enough to reflect near-linear uptake, and minimal efflux, and at the same time long enough that the time involved in sampling is reproducible. The apparent initial influx rate was therefore defined for these studies as the influx during a 2-min incubation period. Previous results (Fig. 1) showed that very little amino acid was incorporated into protein during this time. There is probably very little metabolism or interconversion of the amino acids during this short interval.[19]

Fig. 6 shows the rates at which different external concentrations of ACC were transported into ascites cells. When ACC was present in the external medium in low

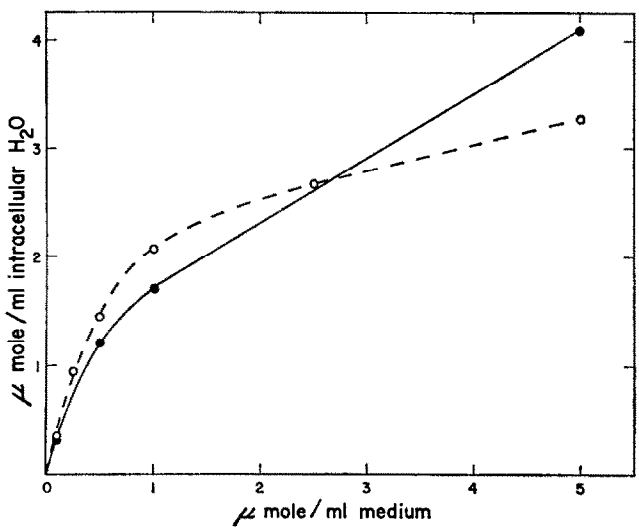

FIG. 6. Uptake of ACC into ascites cells *in vitro*. 6C3HED ascites lymphosarcoma cells (●) and adenocarcinoma 755 ascites cells (○), 60 mg dry weight, were incubated for 2 min in 10 ml of Krebs-Ringer phosphate medium at 26° in air, with 5·6 mM glucose and varying concentrations of ^{14}C-ACC.

concentrations, the intracellular concentration was in excess of the concentration in the medium. However, at higher external concentrations, the rate of transport was reduced, suggesting that the active transport process became saturated, and that ACC probably then entered the cells in part by a diffusion process.

The rate of efflux of ^{14}C-ACC from ascites tumor cells is presented in Fig. 7. In this experiment the cells were first incubated with ^{14}C-ACC for 25 min to achieve equilibrium. The cells were then centrifuged, the supernatant removed, the cells resuspended, and incubation continued as usual in the absence of ACC. At intervals, the radioactivity in a cell-free supernatant of a 0·5-ml aliquot of suspension was measured. It can be seen that ACC leaves the cell when exposed to a large concentration gradient so that by 10 min almost one-half of the radioactivity originally present intracellularly has left the cells, although ACC is still concentrated in the cells. The rapid rate of efflux from the cells indicates that ACC is not tightly bound to any intracellular component.

The results presented above have indicated that an active transport process was involved in the uptake of ACC. It has been established in other systems that such a transport process requires energy[15, 21] and that the availability of energy may be reduced by certain metabolic inhibitors.[22] The data in Table 1 show that energy is required for the uptake of ACC. 2,4-Dinitrophenol (2,4-DNP) reduced the transport of ACC and of glycine to a similar extent. The combination of iodoacetic acid and 2,4-DNP inhibited the transport of ACC to the same extent as did iodoacetic acid

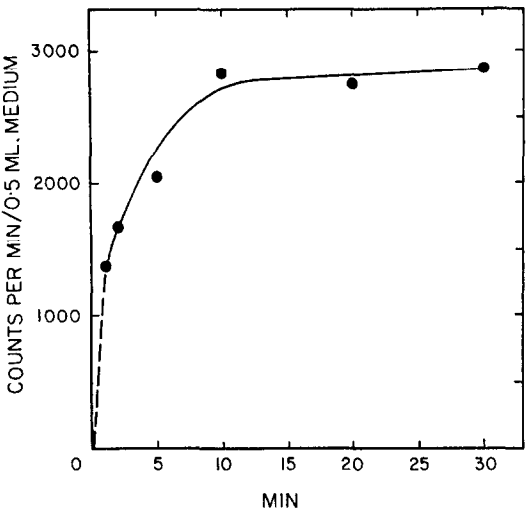

FIG. 7. Rate of efflux of ^{14}C-ACC from 6C3HED ascites lymphosarcoma cells. Tumor cells were incubated as described in Fig. 6 with 0·5 mM ^{14}C-ACC for 25 min. The medium was removed after centrifugation, and fresh medium without ACC was added, the cells resuspended, the incubation continued, and the radioactivity in the medium measured.

TABLE 1. EFFECT OF METABOLIC INHIBITORS ON ACC AND GLYCINE UPTAKE INTO ASCITES TUMOR CELLS*

Inhibitor	Conc. (mM)	Glucose	ACC uptake (μmole/ml H_2O/2 min)	Glycine uptake (μmole/ml H_2O/2 min)	Control (%)
None		+	0·99		(100)
None		−	0·98		99
2,4 DNP	1	+	0·71		72
2,4 DNP	1	−	0·72		73
Iodoacetate	5	−	0·69		70
Iodoacetate +2,4 DNP	5 1	+	0·68		69
Azide	25	+	0·56		57
Azide	25	−	0·57		58
Versene	1	+	0·62		63
None		+		0·45	(100)
2,4 DNP	1	+		0·35	78

* 6C3HED ascites cells were incubated for 2 min in 10 ml of Krebs-Ringer phosphate medium at 26° in air with 0·5 mM ACC or with 0·5 mM glycine with or without 5·6 mM glucose and inhibitors. The cells were preincubated for 25 min with inhibitors.

alone. It may be noted that sodium azide and the disodium salt of ethylenediamine tetraacetic acid (Versene) also inhibited ACC uptake. The presence of glucose in the medium did not appear to be necessary for the initial uptake, since the same amount of ACC was transported in the presence or absence of the inhibitors, whether glucose was added to the medium or not.

The effect of ACC on the apparent initial influx rate of natural amino acids was next studied. The data in Table 2 show the quantities of a series of amino acids trans-

TABLE 2. EFFECT OF ACC ON AMINO ACID UPTAKE INTO ASCITES TUMOR CELLS*

Amino acid	ACC	Amino acid uptake (μmole/ml H_2O/2 min)	Per cent of Control
DL-Lysine	—	0·169	
	+	0·127	75
L-Arginine	—	0·291	
	+	0·208	72
DL-Phenylalanine	—	0·158	
	+	0·048	30
Glycine	—	0·225	
	+	0·063	28
L-Isoleucine	—	0·232	
	+	0·061	26
DL-Leucine	—	0·293	
	+	0·064	22
DL-Serine	—	0·735	
	+	0·140	19
L-Histidine	—	0·681	
	+	0·120	18
L-Alanine	—	0·894	
	+	0·163	18
DL-Alanine	—	0·325	
	+	0·045	14
DL-Methionine	—	0·342	
	+	0·058	16
DL-Valine	—	0·116	
	+	0·017	15
L-Proline	—	0·076	
	+	0·007	9
α-Aminoisobutyric acid	—	0·210	
	+	0·023	11

* Tumor cells were incubated as described in Table 1 with 0·2 mM radioactive amino acids, with or without 10 mM ACC.

ported into ascites tumor cells in the presence and absence of ACC in the same ratios (50:1) that were used in the protein incorporation experiment (Fig. 2). These results were consistent with the results obtained with the incorporation of the amino acids into proteins—that ACC caused less of an effect on incorporation of lysine than on other amino acids.

Under these conditions, the degree of inhibition of transport did not appear to depend upon the stereoisomeric configuration of the natural amino acids. The uptake of DL-lysine and L-arginine was inhibited to approximately the same extent. In addition, in comparing the transport of the L-isomer of alanine with that of the racemic mixture, it may be noted that, although a greater quantity of the L-isomer was transported, both preparations were inhibited to the same extent.

The effect of ACC on the transport of several concentrations of glycine, valine, and leucine was determined and the results plotted according to the method of Lineweaver and Burk[23] (Fig. 8). It was found that the transport of DL-valine and DL-leucine was inhibited in a competitive manner, whereas the transport of glycine was inhibited noncompetitively.

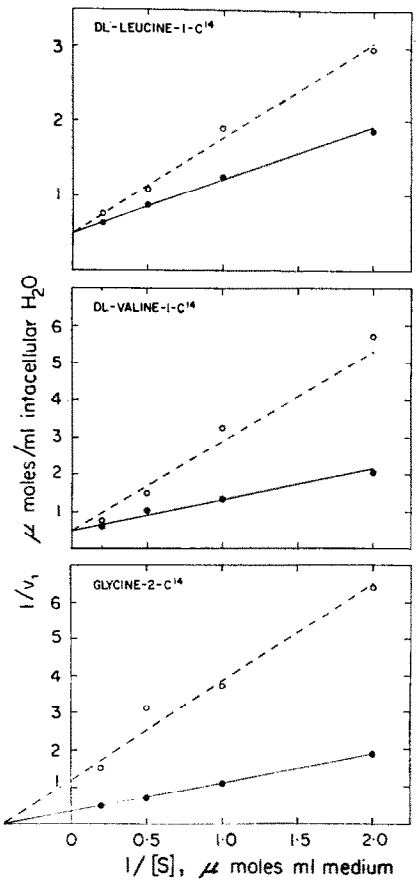

FIG. 8. Inhibition of amino acid uptake into 6C3HED ascites cells by ACC. Tumor cells were incubated as described in Fig. 6 with varying extracellular concentrations of radioactive amino acids with (○) and without (●) 5·0 mM ACC.

It was known from the investigations of Heinz and Walsh[24] that an "exchange diffusion" and a "heteroexchange diffusion" of amino acids may occur in Ehrlich ascites tumor cells. In order to determine whether ACC can participate in exchange diffusion, experiments were conducted in which cells "preloaded" with an amino acid were examined for their ability to transport ^{14}C-ACC.

Ascites tumor cells were preincubated with amino acids for 25 min to achieve equilibrium. The cell suspensions were then centrifuged and the supernatant removed, the packed cells were resuspended into a total volume of 10 ml of medium containing

^{14}C-ACC, and the influx of ^{14}C-ACC was measured. It may be noted from Table 3 that there was an increase in the uptake of ^{14}C-ACC into the cells that were preloaded with nonradioactive ACC, indicating that an exchange diffusion can occur. There was no increase in the uptake of ^{14}C-ACC into the cells preloaded with glycine. However,

TABLE 3. EFFECT OF PREINCUBATION WITH AMINO ACIDS ON ACC UPTAKE INTO ASCITES TUMOR CELLS*

Preincubated amino acid	ACC uptake (μmole/ml H_2O/2 min)
None	1·02
ACC	1·63
Glycine	1·03
DL-Valine	1·20
DL-Leucine	1·23

* Tumor cells were incubated for 25 min as described in Fig. 6 with or without 0·5 mM amino acids. The medium was removed after centrifugation; the cells were resuspended, added to medium containing 5·0 mM ^{14}C-ACC, and the uptake of ACC during 2 min was measured.

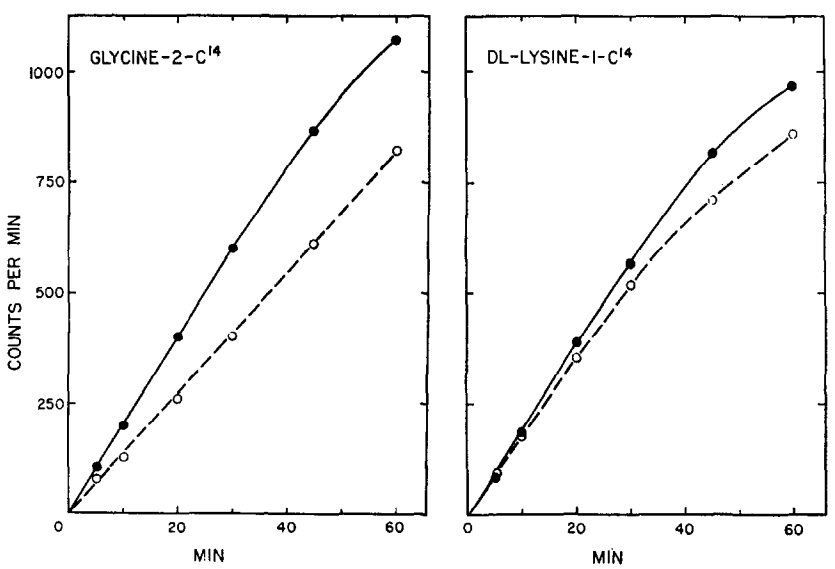

FIG. 9. Effect of α-aminoisobutyric acid (AIB) on the incorporation of amino acids into the acid-insoluble fraction of 6C3HED ascites cells *in vitro*. Tumor cells were incubated as described in Fig. 1 with 0·2 mM of radioactive amino acids with (○) and without (●) 10·0 mM AIB.

when the cells were preloaded with leucine or valine, there was evidence for hetero-exchange diffusion.

These results led to the conclusion that the inhibition of amino acid incorporation into protein caused by ACC was due to inhibition of the uptake of natural amino

acids into these cells. To test this conclusion, the effects of another nonmetabolized α-amino acid, α-aminoisobutyric acid (AIB), on the amino acid incorporation into protein were measured because this amino acid analog is known to inhibit the transport of certain amino acids.[25] Fig. 9 indicates that AIB inhibited amino acid incorporation into protein in a manner similar to that of ACC.

DISCUSSION

This study on the mechanism of action of ACC has demonstrated that this amino acid analog inhibited the incorporation of radiaoctivity from certain natural amino acids into tumor cell proteins. Because of the variation in the extent of inhibition of incorporation of different amino acids into protein, this did not appear to be a direct effect on protein synthesis.

The failure of ACC to be incorporated into the acid-insoluble fraction of ascites cell *in vitro* or in mice *in vivo*[7] indicated that ACC was not replacing any of the natural amino acids in the proteins and also indicated that it had not combined irreversibly with any proteins.

That ACC did not interfere with the endogenous synthesis or the intermediary metabolism of amino acids was suggested by its lack of inhibition of the incorporation of the metabolites of uniformly labeled glucose into the acid-insoluble fraction of ascites cells. Kit and Graham[18] investigated the biosynthesis of amino acids from glucose in 6C3HED ascites tumor cells and found that significant quantities of serine and glycine were synthesized from this monosaccharide. Berlinguet *et al.*[10] have also reported that ACC did not inhibit the oxidation of phenylalanine and did not interfere with the function of serum transaminases or with cellular respiration.

The results of this investigation have indicated that ACC is actively transported into 6C3HED ascites lymphosarcoma cells. This drug is not only transported into the ascites cells *in vitro* but also concentrated sixfold intracellularly. Christensen and Riggs, using Ehrlich ascites cells, showed that both natural amino acids[15] and unnatural amino acids[26] of the D and L configuration are strongly concentrated by these cells *in vitro*. The uptake of ACC is not linear with concentration but indicates the existence of a saturable process for entry. Such an effect was interpreted by Heinz[16] as an indication of a mediated transport by specific carriers in the membrane. The initial rate of uptake of ACC was decreased by metabolic inhibitors, and 2,4-DNP inhibited the uptake of ACC and of glycine to approximately the same extent. Under similar conditions, Heinz[16] has shown that in Ehrlich ascites cells, 2,4-DNP inhibited only the mediated influx of glycine without changing the efflux rate. As a final indication of active transport, it has been shown that ACC competed for the transport of the natural amino acids. This is a characteristic of amino acid transport that has been shown to occur among the natural amino acids both *in vivo*[27] and *in vitro*,[15] and between natural amino acids and many unnatural amino acids.[26] ACC inhibited the uptake of DL-leucine and DL-valine in a competitive manner and not only underwent autoexchange diffusion, but also heteroexchange diffusion with these amino acids. However, ACC inhibited the uptake of glycine in a noncompetitive manner and did not undergo heteroexchange diffusion with this amino acid. According to the extensive studies of Heinz,[16, 24, 28] these results indicate that ACC was probably transported by the same

carrier system that transports leucine and valine. The noncompetitive inhibition of glycine transport by ACC suggests that the analog may partially block the glycine carrier system even though it may not be transported by this system. Recent studies indicate that there is more than one carrier system involved in the transport of the monoaminomonocarboxylic amino acids.[29, 32]

The only biochemical effect of ACC found in this study is inhibition of amino acid transport. At the present time one cannot state that this is the mechanism of the carcinostatic action of ACC. Nevertheless, the work reported here, as well as that published by others[9, 33] is consistent with such a mechanism of action.

The present studies have shown that ACC can inhibit the uptake into tumor cells of a large number of amino acids *in vitro*. Although high ratios of the analog to the amino acids were required, this does not necessarily preclude the possible toxic effect of a small but constant degree of inhibition *in vivo*. The relative effects of the *in vitro* and *in vivo* environments on this inhibition are not known, but must also be considered. Such a mechanism of action could be effective only if the analog concentration remained relatively constant, and it has been shown that ACC remains in the blood and tissues for an unusually extended period.[7] The possibility also exists that ACC also displaces intracellular amino acids from the cells by exchange diffusion. In this regard, Berlinguet *et al.*[9] have found that rats fed ACC had higher serum levels of ornithine, lysine, glutamic acid, glycine, serine, taurine, threonine, and phenylalanine than did control animals.

The concept that amino acids which compete for transport in a specific tissue may affect the intracellular metabolism *in vivo* has recently been investigated by Guroff and Udenfriend[34] who found that phenylalanine and some other amino acids competed with tyrosine for uptake into the brain of rats. By this process the tyrosine level in the brain was reduced.

This type of mechanism of action has also been suggested by Wiseman and Ghadially[35] who found that elevated levels of diaminoamino acids reduced the uptake of the monoaminomonocarboxylic acid amino acids in suspended RD3 sarcoma cells; but these authors doubted that the inhibitory amino acid could be maintained at high enough levels to exert an effective action *in vivo*. ACC might, however, fulfill this requirement.

Several lines of evidence indicate that interference with the amino acid supply could have some degree of selectivity for tumors. Christensen and associates have shown that tumor cells concentrate amino acids to a greater extent than do normal tissues, both *in vivo*[36] and *in vitro*,[15] and it has been suggested that this gives the tumor a comparative advantage during growth. Net protein synthesis proceeds at a higher rate in Ehrlich ascites tumor cells than in other tissues of the mice bearing this tumor, and it is known that very little turnover of tumor protein occurs.[37] Roberts and Frankel[38] have shown that the free amino acid content of several tumors was relatively similar, even though the levels of free amino acids in normal tissues were characteristic for each tissue. Thus a drug that inhibits amino acid supply might have more uniform effects upon tumors than upon normal tissues.

Too little is known at the present time about the amino acid nutrition of both normal and neoplastic cells *in vivo*, and about the relative sensitivities of normal tissues and tumors to this type of interference with their amino acid supply to state that this is the mechanism of action of ACC. It appears that any inhibitor of this type would have

only limited use as a carcinostatic agent, since the degree of inhibition possible to achieve by such means could not be large, and probably would never lead to carcinolysis. Nevertheless, it is hoped that further investigations will be carried out to test the possibility that ACC does act by inhibition of amino acid transport.

During the preparation of this report, a study by Ahmed and Scholefield[33] has appeared which confirms the active uptake of ACC into tumor cells, and which also proposes competition with amino transport as the mechanism of action of this drug.

Acknowledgement—The authors wish to express their sincere appreciation to Dr. H. G. Mandel, in whose laboratory this work was done.

REFERENCES

1. R. B. ROSS, C. I. NOLL, W. C. J. ROSS, M. V. NADKARNI, B. H. MORRISON, JR. and H. W. BOND, *J. med. pharm. Chem.* **3**, 1 (1961).
2. A. GOLDIN, J. M. VENDITTI, I. KLINE and N. MANTEL, *Cancer Res.* **21**, 27 (1961).
3. T. A. CONNORS, L. A. ELISON and W. C. J. ROSS, *Biochem. Pharmacol.* **1**, 239 (1958).
4. Veterans Administration Cancer Chemotherapy Study Group, *Cancer Chemother. Repts.* **6**, 59 (1960).
5. W. W. BENEFIEL, J. T. HELSPER and G. S. SHARP, *Cancer Chemother. Repts.* **9**, 21 (1960).
6. D. E. BERGSAGEL, *Cancer Chemother. Repts.* **16**, 261 (1962).
7. W. R. STERLING, J. F. HENDERSON, H. G. MANDEL and P. K. SMITH, *Biochem. Pharmacol.* **11**, 135 (1962).
8. H. CHRISTENSEN and J. C. JONES, *J. biol. Chem.* **237**, 1203 (1962).
9. L. BERLINGUET, N. BÉGIN, L. M. BABINEAU, F. MARTEL, R. VALLÉE and R. O. LAFERTÉ, *Canad. J. Biochem.* **40**, 425 (1962).
10. L. BERLINGUET, N. BÉGIN, L. M. BABINEAU and R. O. LAFERTÉ, *Canad. J. Biochem.* **40**, 433 (1962).
11. L. BERLINGUET, N. BÉGIN and N. K. SARKER, *Nature, Lond.* **194**, 1082 (1962).
12. P. G. SCHOLEFIELD, *Canad. J. Biochem.* **39**, 1717 (1961).
13. F. U. BROWN, T. S. KANDASWAMY, M. KLEIN and H. G. MANDEL, *Cancer Res.* In press.
14. T. S. KANDASWAMY and J. F. HENDERSON, *Biochim biophys. Acta* **61**, 86 (1962).
15. H. N. CHRISTENSEN and T. R. RIGGS, *J. biol. Chem.* **194**, 57 (1952).
16. E. HEINZ, *J. biol. Chem.* **211**, 781 (1954).
17. E. HEINZ, *J. biol. Chem.* **225**, 305 (1957).
18. S. KIT and O. L. GRAHAM, *Cancer Res.* **16**, 117 (1956).
19. S. KIT and D. M. GREENBERG, *Cancer Res.* **11**, 495 (1951).
20. R. K. CRANE, R. A. FIELD and C. R. CORI, *J. biol. Chem.* **224**, 649 (1957).
21. E. HEINZ and H. A. MARIANI, *J. biol. Chem.* **228**, 97 (1957).
22. J. H. QUASTEL and I. J. BICKIS, *Nature, Lond.* **183**, 281 (1959).
23. H. LINEWEAVER and D. BURK, *J. Amer. chem. Soc.* **56**, 658 (1934).
24. E. HEINZ and P. M. WALSH, *J. biol. Chem.* **233**, 1488 (1958).
25. H. N. CHRISTENSEN and T. R. RIGGS, *J. biol. Chem.* **220**, 265 (1956).
26. H. N. CHRISTENSEN, T. R. RIGGS, H. FISCHER and I. M. PALATINE, *J. biol. Chem.* **198**, 1 (1952).
27. H. N. CHRISTENSEN, J. A. STREICHER and R. L. ELBINGER, *J. biol. Chem.* **172**, 515 (1948).
28. C. M. PAINE and E. HEINZ, *J. biol. Chem.* **235**, 1080 (1960).
29. R. M. JOHNSTONE and P. G. SCHOLEFIELD, *J. biol. Chem.* **236**, 1419 (1961).
30. A. TENENHOUSE and J. H. QUASTEL, *Canad. J. Biochem.* **38**, 1311 (1960).
31. J. A. JACQUEZ, *Amer. J. Physiol.* **200**, 1063 (1961).
32. D. L. OXENDER, *Fed. Proc.* **21**, 148 (1962).
33. K. AHMED and P. G. SCHOLEFIELD, *Canad. J. Biochem.* **40**, 101 (1962).
34. G. GUROFF and S. UDENFRIEND, *J. biol. Chem.* **237**, 803 (1962).
35. G. WISEMAN and F. N. GHADIALLY, *Brit. J. Cancer* **9**, 480 (1955).
36. H. N. CHRISTENSEN and M. E. HENDERSON, *Cancer Res.* **12**, 229 (1952).
37. A. L. LE PAGE, V. R. POTTER, H. BUSCH, C. HEIDELBERGER and R. B. HURLBERT, *Cancer Res.* **12**, 153 (1952).
38. E. ROBERTS and S. FRANKEL, *Cancer Res.* **9**, 645 (1949).