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INHIBITORY EFFECTS OF CHLORPROMAZINE AND DIETHYLAMINOETHYL DIPHENYLVALERATE (SKF 525-A) ON ALANINE INCORPORATION INTO PROTEIN AND α-AMINOISOBUTYRIC ACID UPTAKE IN RAT HEPATOMA CELLS IN CULTURE*

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Abstract—Chlorpromazine (CPZ) and SKF 525-A above 0·01 mM inhibit the incorporation of alanine into protein in cultures of a clonal strain of rat hepatoma cells. Fifty per cent inhibition was noted for both drugs at approximately 0·07 mM, and at CPZ 0·15 mM 97 per cent and at SKF 525-A 0·20 mM 96 per cent inhibition respectively was found. Brief washing partially restores normal incorporation rates. CPZ 0·15 mM reduces the amount of total intracellularly accumulated alanine to 11 per cent of controls. The non-metabolizable amino acid α-aminoisobutyric acid (AIB) is actively transported by these cells; CPZ and SKF 525-A inhibit the uptake of AIB, with 44 per cent reduction at a concentration of CPZ 0·15 mM and 55 per cent reduction at SKF 525-A 0·20 mM. The rate of AIB efflux from the cells was also reduced by CPZ 0·15 mM and SKF 525-A 0·20 mM. It is concluded that CPZ and SKF 525-A in a similar pattern inhibit the transport of amino acids in these hepatoma cells in culture.

CHLOPROMAZINE (CPZ) at low concentrations causes a so-called membrane stabilization in a variety of biological systems, viz. excitable cells,[1] erythrocytes,[2] lysosomes,[3] mitochondriae,[4] the heart.[5] The prolonging agent diethylaminoethyl diphenylvalerate (SKF 525-A) inhibits the biotransformation of a great number of drugs.[6,7] The exact molecular basis for the actions of SKF 525-A is not known with certainty. Interaction with the microsomal membrane leading to decreased permeability to drugs has been suggested as an explanation of some of the actions of SKF 525-A.[8] Stabilization of the erythrocytal membrane comparable to that of CPZ has been noted with SKF 525-A.[9]

Both CPZ[10] and SKF 525-A[11] inhibit the glucuronidation of *p*-aminophenol and *p*-nitrophenol by rat hepatoma cells in culture, presumably through interaction with the liver cell membrane leading to decreased uptake of substrates.

Previous studies have stated varying effects of CPZ at high concentrations on protein synthesis in other systems. CPZ has been found to inhibit protein synthesis in different brain areas.[12] At a concentration of 1·0 mM, CPZ reduced protein synthesis in mice liver slices by approximately 50 per cent.[13] Little or no effect of CPZ at 0·5 and 1·0 mM on the incorporation of phenylalanine into protein by a rat liver ribosomal system could be found.[14]

By the use of liver cell culture, the direct action of CPZ and SKF 525-A on amino acid transport and incorporation in living cells can be studied without the complex

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interactions taking place in whole animals. Alanine (ALA), not contained in the cell growth medium, and the non-metabolizable α-aminoisobutyric acid (AIB) were chosen for the incorporation and transport studies.

MATERIALS AND METHODS

Methods of cell culture. The clonal strain MH_1C_1 of rat hepatoma cells was grown in Dulbecco's modified Eagle's medium supplemented with 15% horse serum, 2·5% fetal calf serum and antibiotics as described.[15]

Alanine incorporation into cell protein. Replicate subcultures were preincubated with serum-containing medium without or with CPZ (or SKF 525-A) for 2 hr at 37°. [^{14}C]Alanine 0·5 μCi (0·005 mM) was then added to the cultures and incubated at 37° for various time intervals. Simultaneous addition of inhibitor and alanine did not alter the incorporation results compared to experiments with preincubation. At the end of the experiments, the cells were washed once with ice-cold barbital–NaCl buffer pH 7·4 and removed with ice-cold 0·02% EDTA–barbital–NaCl buffer pH 7·4 and precipitated with 10% (final) ice-cold TCA. The precipitate was washed three times with TCA, twice with ethanol–diethylether (2:1, v/v) and once in ether. The pellet was then dissolved in 1 N NaOH at 37° overnight. In some experiments total intracellular alanine was estimated after four washings with ice-cold barbital–NaCl buffer pH 7·4 and subsequent solubilization with 1 N NaOH for 2 hr at 37°. Aliquots in duplicate were counted in 15 ml BBOT scintillation fluid in a Packard TriCarb 3003 liquid scintillation spectrometer. To test the possible reversibility of the SKF 525-A effect on alanine incorporation, four subcultures without and eight with SKF 525-A 0·20 mM were preincubated for 2 hr at 37°. The medium was then poured off from half of the cultures with SKF 525-A, quickly washed three times with warm medium without inhibitor for a total of 30 sec and then supplied with fresh medium without any additions. All cultures were then incubated with 0·005 mM [^{14}C]alanine for 2 hr at 37°, and incorporated radioactivity was measured.

α-Aminoisobutyric acid uptake by the cells. Replicate subcultures without or with CPZ (or SKF 525-A) were incubated with [α-^{14}C]aminoisobutyric acid 0·5 μCi (AIB, 0·01 mM) in a serum-containing medium for different time intervals. AIB-uptake experiments were performed at 37° unless otherwise stated. At the end of the experiments the cells were quickly washed four times with ice-cold barbital–NaCl buffer pH 7·4, then solubilized for 2 hr at 37° with 1 N NaOH. Duplicate aliquots of the solubilized cells were counted in 15 ml BBOT in the liquid scintillation spectrometer. The last washings were always counted, the radioactivity in these samples were found to be between 1 and 2 per cent of the radioactivity in the medium at the start of the experiments, and approximately 5 per cent of the intracellular activity at 2 hr. The amount of radioactivity in the hydrolysate was thought to reflect the cellular uptake of AIB. No radioactivity appeared in cell proteins in the AIB-experiments. In the efflux experiments, the cells were first labelled with 0·5 μCi AIB in serum-containing medium for 2 hr, the medium was then poured off and the cells washed quickly four times with warm medium without any additions and finally incubated without or with CPZ 0·15 mM or with SKF 0·20 mM. Appearance of radioactivity in the different mediums at desired time intervals was then determined. Cell protein was measured by the method of Lowry *et al.*[16] with bovine albumin as standard.

Chemicals. Chlorpromazine–HCl was obtained from Dolder AG, Switzerland through Norsk Medisinaldepot. 2-Diethylaminoethyl 2,2-diphenylvalerate–HCl (SKF 525-A) was a gift from the Smith, Kline & French Laboratories. [U-^{14}C]alanine (Amersham, spec.act. 10 mCi/mmole) and [3-α-^{14}C]aminoisobutyric acid (New England Nuclear, spec.act. 5·3 mCi/mmole) were purchased through Norsk Atominstitutt, Kjeller.

RESULTS

A time course study of the alanine incorporation into protein in the MH_1C_1 cell cultures is shown in Fig. 1. This process runs at a constant velocity for the time intervals used. The addition of CPZ 0·05 mM or 0·10 mM to the incubation medium reduces the incorporation velocities, but the linearity of the reaction is maintained.

Figures 2a and 2b show a log–dose–response plot of the inhibition of alanine incorporation into cell protein by CPZ from 0·01 to 0·15 mM and SKF 525-A from 0·01 to 0·20 mM. Both drugs give 50 per cent inhibition at approximately 0·07 mM. At CPZ 0·15 mM only 3 per cent at SKF 0·20 mM 4 per cent of control values could be found.

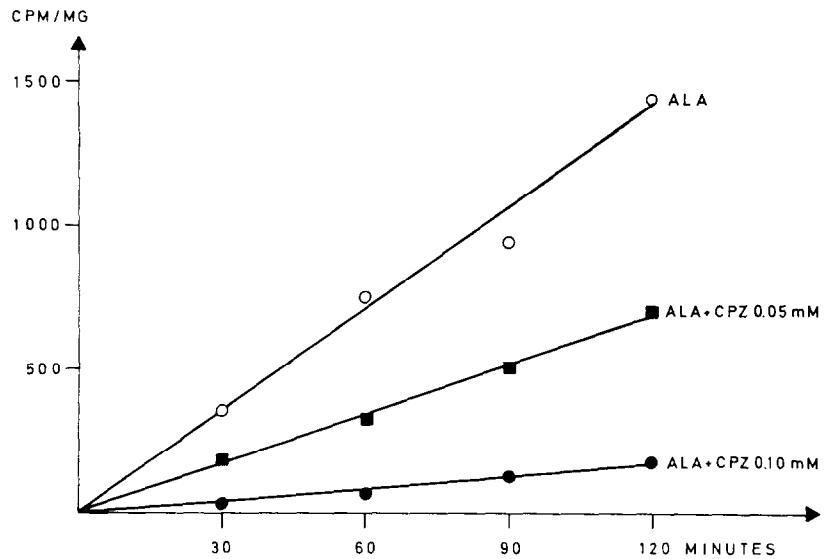

FIG. 1. Time course of alanine incorporation into protein in MH_1C_1 cell cultures with or without CPZ 0·05 or 0·10 mM. Replicate subcultures with or without CPZ were incubated with [^{14}C]alanine 0·005 mM in serum containing medium for 30, 60, 90 or 120 min at 37° (abscissa), ordinate counts/min and milligrams cell protein, two flasks for each of the time intervals.

Table 1 gives the values of total intracellular alanine found after incubating the cells with the amino acid for 2 hr without or in the presence of CPZ 0·15 mM. CPZ is seen to reduce the amount of alanine accumulated to 11 per cent of controls, so that the inhibitory effect seen on alanine incorporation is most probably a result of reduced net transport into the cells. Twenty-five per cent of total intracellular alanine in the controls was incorporated into protein after 2 hr.

Rapid washing of cell cultures that had been preincubated with SKF 525-A 0·20 mM for 2 hr, incorporated 64 per cent of alanine as compared to controls without inhibitor,

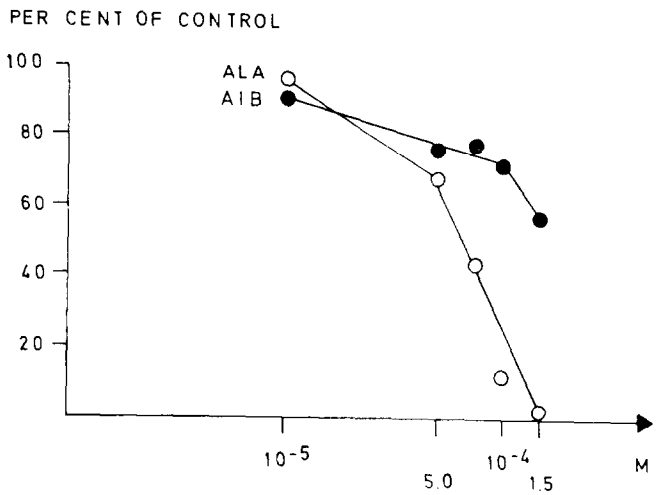

FIG. 2a.

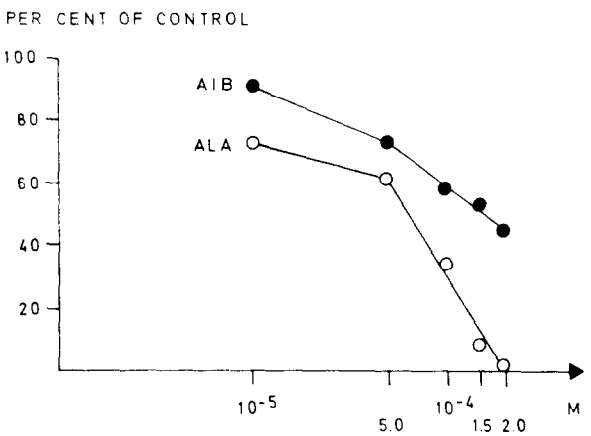

FIG. 2b.

FIGS. 2(a and b). Semi-logarithmic plot of inhibition of alanine incorporation into protein and AIB uptake by CPZ and SKF 525-A in MH_1C_1 cells. Replicate flasks were preincubated with or without varying concentrations of CPZ (2a) or SKF 525-A (2b) for 2 hr at 37°, then incubated with 0·005 mM [^{14}C]alanine for 2 hr or with 0·01 mM [^{14}C]AIB for 30 min at 37°; radioactivity measured as in Materials and Methods. Ordinate: Values (mean of duplicate flasks for each concentration of CPZ or SKF 525-A) in per cent of controls (three parallels for each of the set of experiments), abscissa: CPZ or SKF 525-A in moles/litre.

while cells with SKF 525-A 0·20 mM only incorporated 4 per cent (Table 2). Comparable results were found in washing experiments with CPZ. The washing procedure in itself did not reduce the amount of alanine incorporated in protein.

From Figs. 2a and b it can also be seen the effect of various concentrations of CPZ (2a) and SKF 525-A (2b) on the uptake of AIB by the hepatoma cells. Uptake of the non-metabolizable AIB is also inhibited by the two drugs, but not to the same extent as alanine incorporation. At a concentration of CPZ 0·15 mM 44 per cent inhibition was noted, and at SKF 525-A 0·20 mM 55 per cent reduction was seen.

TABLE 1. EFFECT OF CPZ ON TOTAL INTRACELLULAR ALANINE ACCUMULATION IN MH_1C_1 CELLS

Without CPZ	With CPZ 0·15 mM
6394·2 ± 913·4 (4)	718·8 ± 97·6 (4)

Four flasks without and four flasks with CPZ 0·15 mM were incubated with 0·005 mM [^{14}C]-alanine for 2 hr at 37° and total intracellular radioactivity was measured. Values are means ± S.D.

Figure 3 reveals the pattern of AIB uptake in the MH_1C_1 cells at 37° compared to 4°. The uptake process is seen to be temperature-dependent, a plateau in the radioactivity values was reached after 10 min at 4°, whereas the amino acid is transported at a fairly constant rate up to 30 min at 37°, suggesting that energy is required for the transport of AIB. Zero time values, measured after 10 sec both at 4° and 37°, were almost equal to the plateau-values seen at 4°. These could therefore at least partially be explained by cellular adsorption of AIB.

TABLE 2. EFFECT OF WASHING ON THE INHIBITION OF ALANINE INCORPORATION INTO PROTEIN BY SKF 525-A IN MH_1C_1 CELLS

Control (counts/min/mg protein)	Preincubation and continuous presence of SKF 525-A 0·20 mM (counts/min/mg protein)	Preincubation with SKF 525-A 0·20 mM and subsequent washing (counts/min/mg protein)
1243·9 ± 39·4 (4)	45·5 ± 3·9 (4)	789·6 ± 164·9 (4)

After 2 hr preincubation with SKF 525-A 0·20 mM, three flasks were quickly washed, supplemented with fresh medium, and incubated with 0·005 mM [^{14}C]alanine for 2 hr at 37°. Radioactivity in protein was compared to activities in parallels with SKF 525-A and in controls without SKF 525-A. Values are means of three flasks.

The cells continue to accumulate the non-metabolizable amino acid at the concentration used as measured at 2 hr (Figs. 4a and b). When CPZ 0·15 mM (4a) or SKF 525-A 0·20 mM (4b) is present in the incubation medium, the intracellular amounts of AIB were always lower at each time interval as compared to values without inhibitor, but a similar pattern in the time course of uptake was seen.

Lowered apparent uptake of amino acids could also be a result of increased efflux from the cells. Table 3 shows the effect of CPZ on AIB efflux after the cells have accumulated the amino acid for 2 hr. CPZ 0·15 mM also reduces this exit process, by approximately 32 per cent. A time course study of the efflux of AIB from the cells after loading the cells with AIB 0·01 mM for 2 hr (without SKF 525-A) is seen in Fig. 5. SKF 525-A 0·20 mM added after 2 hr loading clearly inhibits the egress of AIB, with approximately 35 per cent reduction as compared to efflux from cells without inhibitor after 1 hr.

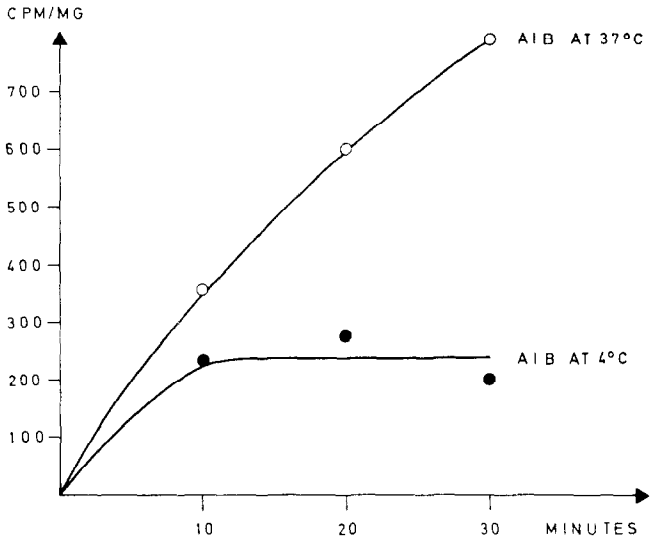

FIG. 3. Time course of AIB uptake by MH_1C_1 cells at 37° and 4°. Eighteen subcultures were incubated with [^{14}C]AIB 0·01 mM, intracellular radioactivity was measured after 10, 20 and 30 min for each of the incubation temperatures. Ordinate: Activity in counts/min and milligrams cell protein, abscissa: time in minutes. Each value represents mean of three flasks.

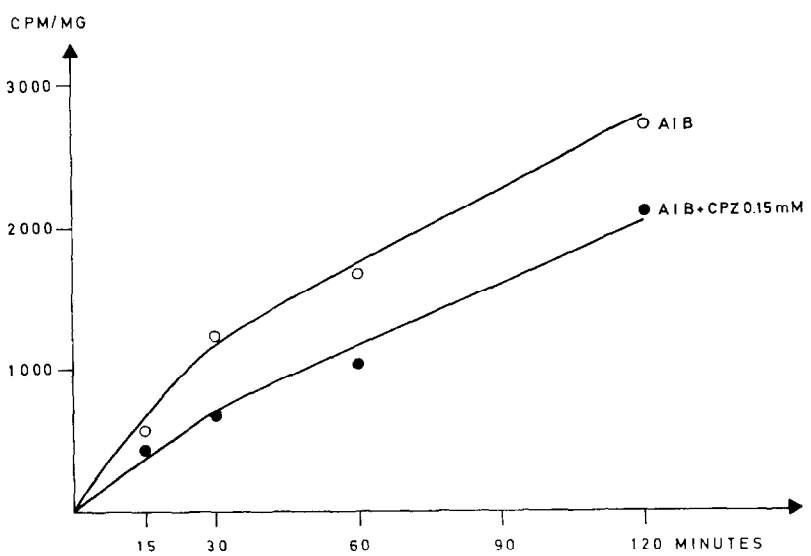

FIG. 4a.

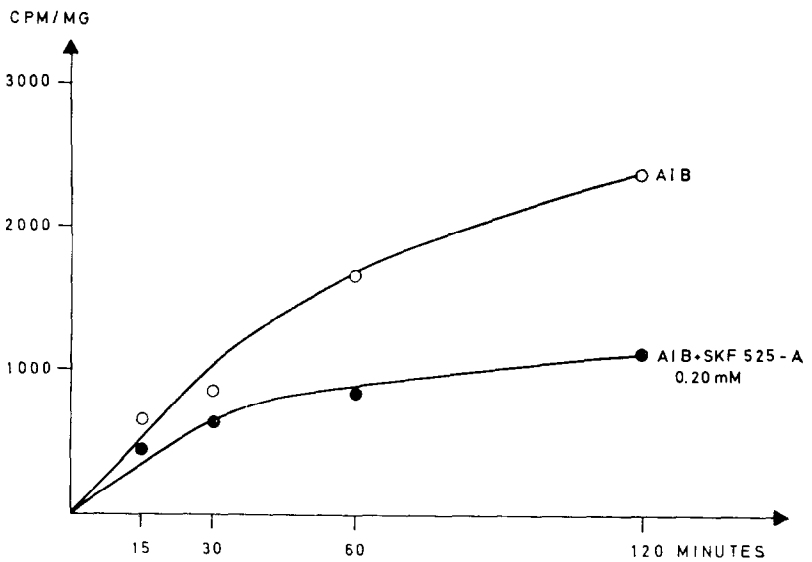

FIG. 4b.

FIGS. 4(a and b). Time course of AIB uptake by MH_1C_1 cells with or without CPZ 0·15 mM (4a) or SKF 525-A 0·20 mM (4b). Eight subcultures were incubated without and eight with CPZ (SKF 525-A) and then incubated with 0·01 mM [^{14}C]AIB. Intracellular radioactivity was measured as in Materials and Methods after 15, 30, 60 and 120 min in duplicate flasks from cells with or without CPZ or SKF 525-A. Ordinate: Activity in counts/min and milligrams cell protein, abscissa: time in minutes.

TABLE 3. EFFECT OF CPZ ON AIB-EFFLUX FROM MH_1C_1 CELLS

Without CPZ (counts/min/mg protein/hr)	With CPZ 0·15 mM (conts/min/mg protein/hr)
767·5 ± 16·6 (4)	520·6 ± 64·9 (4)

Eight flasks were incubated with medium containing 0·01 mM [^{14}C]AIB for 2 hr at 37°; after washing, four flasks were incubated without and four together with CPZ 0·15 mM for 1 hr at 37°, and appearance of radioactivity in the mediums was measured. Values are means ± S.D.

DISCUSSION

The clonal rat hepatoma cell line MH_1C_1 incorporates 0·005 mM alanine into protein at a constant rate, CPZ and SKF 525-A above 0·01 mM inhibits this process with 50 per cent reduction at approximately 0·07 mM. At CPZ 0·15 mM and SKF 525-A 0·20 mM virtually no radioactivity can be recovered in the cellular proteins. The inhibition of alanine incorporation by CPZ and SKF 525-A could be partially restored by rapid washing out of the inhibitors, pointing to a membrane location of action. Almost the same degree as the inhibition by CPZ of alanine incorporation into protein was noted when measuring total intracellular alanine reduction by CPZ.

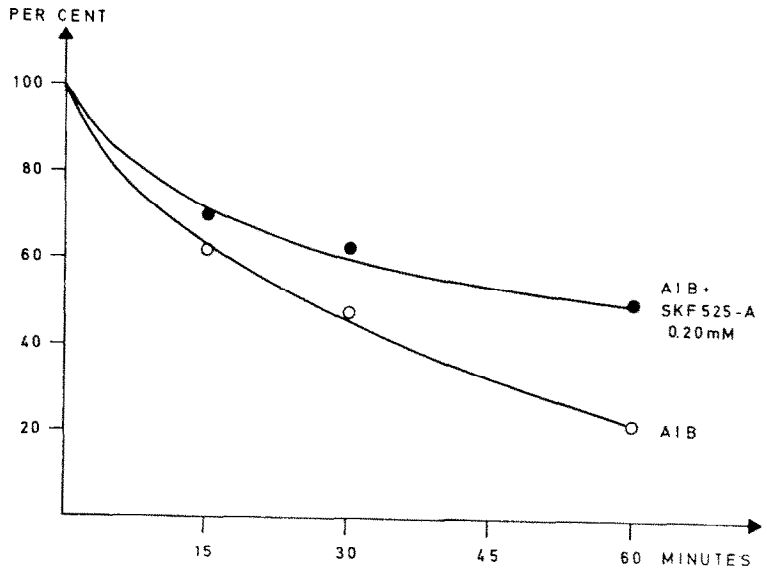

FIG. 5. Time course of SKF 525-A effect on AIB efflux from MH_1C_1 cells. Replicate subcultures were incubated with medium containing 0·01 mM [^{14}C]AIB for 2 hr at 37°; after washing, three flasks without and three with SKF 525-A 0·20 mM were incubated at 37°. Appearance of radioactivity in the mediums was measured after 15, 30 and 60 min (abscissa) and correlated in per cent to intracellular activity in three controls at zero time (ordinate).

Earlier reports have stated that CPZ inhibits amino acid incorporation into a rat liver homogenate system and cerebral proteins.[17] Overall utilization of lysine after injections of CPZ into mice was depressed; and the transport of the amino acid into liver and brain proteins, incorporation into hepatic and cerebral proteins, and the release of amino acids from the liver to plasma all seemed to be retarded by CPZ.[18] These CPZ effects were attributed to the well-established effect of phenothiazines on membrane permeability. Chou *et al.*[19] noted a similar degree of inhibition of amino acid incorporation and uptake in *Tetrahymena piriformis* by CPZ. Agents which directly blocked protein synthesis blocked uptake considerably less than incorporation, suggesting that the effect of CPZ on amino acid incorporation in this system was due to inhibition of uptake. Recently Peterson *et al.*[20] reported strong inhibition of amino acid uptake by isolated synaptosomal particles by low concentrations of CPZ and promazine. The incorporation of leucine was also inhibited by CPZ and promazine, with 50 per cent inhibition at CPZ 0·12 mM. It was suggested that the phenothiazines exert independent inhibitory effects on amino acid transport and incorporation. SKF 525-A has been reported to cause diminished incorporation of DL-alanine into proteins of rat liver microsomes.[21]

The non-metabolizable amino acid AIB is accumulated by the hepatoma cells through a temperature-dependent process indicated by the differences in uptake at 37 and 4°. Similar concentrative uptake of AIB has been found in other cellular systems.[22–26] CPZ inhibits the uptake of AIB with approximately 56 per cent of

control values at CPZ 0·15 mM, SKF 525-A 0·20 mM gives 45 per cent of controls. CPZ and SKF 525-A could affect either the AIB transport mechanisms in the membrane or the processes for supplying metabolic energy to move the amino acid against a concentration gradient or both. It remains to be explained, however, in which manner CPZ and SKF 525-A exert greater maximal effects on alanine uptake incorporation than on AIB transport. Should the widespread dichotomy in transport of neutral amino acids exist in this liver cell system, alanine could have greater affinity for one or both transport sites, as has been found in other cellular systems,[22] and thus alanine uptake might be inhibited to an appreciable greater extent than that of AIB. A direct action of CPZ and SKF 525-A on protein synthesis also must be taken into consideration.

Almost identical inhibitory actions of CPZ[10] and SKF 525-A[11] on glucuronidation of *p*-aminophenol by these cultured liver cells has been found earlier, it was suggested that the two drugs act through inhibition of substrate entrance into the cells. Inhibition of amino acid transport further strengthens the hypothesis that CPZ and SKF 525-A in this system interact with the liver cell membrane ("membrane stabilization"). The rapid reduction in inhibition of alanine incorporation by CPZ and SKF 525-A after brief washing also point to interference with membrane functions. CPZ and SKF 525-A have a biphasic effect on the cultured cells, concentrations above CPZ 0·15 mM and SKF 525-A 0·20 mM cause cellular detachment presumably due to lysis.[10,11] A biphasic action has also been found measuring erythrocytal stabilization against hypotonic hemolysis by SKF 525-A.[9] SKF 525-A has been shown to inhibit the transport of morphine into the renal tubular cells in chicken, presumably acting on the cationic transport system.[27]

CPZ inhibition of *p*-aminophenol glucuronidation was enhanced when serum was excluded from the incubations.[10] More than 90 per cent of CPZ is bound to human plasma protein within the therapeutic concentration range of 0·01–1·0 μg/ml.[28] Indications of metabolic alteration of CPZ by the MH_1C_1 cell cultures were not seen.[10]

CPZ and SKF 525-A inhibit both alanine uptake incorporation and AIB uptake, as well as glucuronidation, in a system consisting of intact, living cells most probably through interaction with the liver cell membrane. Effects of drugs on the liver cell membrane should thus also be taken into consideration when explaining drug action from results of experiments with subcellular fractions.

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