Br. J. Pharmac. (1976), **57**, 3–15

EFFECTS OF AMINO ACIDS AND CONVULSANTS ON SPONTANEOUS ACTION POTENTIALS IN CEREBELLAR CORTEX SLICES

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1 Picrotoxin selectively and reversibly suppressed the inhibitory action of γ-aminobutyric acid (GABA), but not that of glycine, taurine or β-alanine, on the frequency of spontaneous spike discharges in guinea-pig cerebellar slices. Strychnine reversibly suppressed the inhibitory action of glycine, taurine or β-alanine but had no effect on that of GABA.

2 GABA, glycine, taurine and β-alanine showed an early excitatory effect that was unaffected by picrotoxin or strychnine.

3 Studies of the dose-response relations indicated a competition between the amino acid and the convulsant at a common receptor site.

4 Kinetic analyses of the dose-response relations for the amino acids in the presence or absence of picrotoxin or strychnine indicated that the number of molecules of amino acid combining with the receptor site in order to produce a response (inhibition or excitation) was 3 for GABA, 2 for glycine, 3 for taurine and 4 for β-alanine. There appeared to be no evidence that the response was due to the cooperativity between the amino acid receptor complexes. The number of molecules of convulsant that combined with the receptor site was 1 for either strychnine or picrotoxin.

5 Mixtures of glycine with taurine or β-alanine, in contrast to those with GABA, appeared not to give additive inhibitory effects.

Introduction

It is now known that spontaneous action potentials are exhibited by isolated cerebellar slices of the frog (Hackett, 1972), and of the guinea-pig (Gardner-Medwin, 1972; Okamoto & Quastel, 1973; Yamamoto, 1973) and in cultured rat cerebellum (Hild & Tasaki, 1962; Gähwiler, Mamoon, Schlapfer & Tobias, 1972; Geller & Woodward, 1974). As the discharge frequency of spontaneous action potentials in the cat cerebellum *in situ* (Eccles, Ito & Szentágothai, 1967) is inhibited by iontophoretically applied γ-aminobutyric acid (GABA), glycine, taurine or β-alanine (Kawamura & Provini, 1970), we have carried out experiments to observe the effects of these amino acids on the spontaneous action potentials of guinea-pig cerebellar slices and to throw more light on the nature of the interactions between the amino acids and their respective receptor sites. Moreover, as the inhibitory actions of glycine, taurine or β-alanine on Purkinje cells and other CNS neurones have been reported to be antagonized by strychnine (e.g., Curtis, 1969; Curtis, Duggan, Felix & Johnston, 1970; Curtis, Duggan, Felix, Johnston & McLennan, 1971; Curtis, Duggan & Johnston, 1971), while that of GABA is antagonized by picrotoxin (e.g., Woodward, Hoffer, Siggins & Oliver, 1971; Hill & Simmonds, 1973; Hill, Simmonds & Straughan, 1973), we have also carried out experiments to observe quantitatively the relative effects of strychnine and picrotoxin on the responses of isolated cerebellar slices of the guinea-pig to amino acids. The results and conclusions of these experiments are described in this paper.

Methods

The preparation of cerebellar cortex slices and the methodology for the superfusion have been described previously (Okamoto & Quastel, 1973). Briefly, after the guinea-pig was killed by stunning, the slice was prepared (with a Stadie-Riggs slicer) from the superior vermis by cutting the isolated cerebellum parallel to its uppermost surface. The thickness of the slice was about 0.5 mm and care was taken to compress it as little as possible. The whole operation took about 1–2 minutes. The tissue was then promptly transferred to the superfusion chamber, where the slice was placed on a nylon mesh with its original surface upward. It was superfused with the oxygenated control solution

(kept at 37°C) for at least 15 min before the first insertion of the electrode. The level of the superfusion solution was maintained just high enough to cover the whole surface of the slice. The flow of the solution, sucked continuously through a small hole at the bottom of the chamber, created a downward force that kept the slice steady on the mesh.

All solutions, including the control solution and those containing substances to be tested, were applied to the slice by superfusion (flow rate $\simeq$ 1 ml/min) through a glass capillary tip (internal diameter of the tip $\simeq$ 0.3 mm) placed just above the surface of the slice and close ($\simeq$0.5 mm) to the recording glass microelectrode (1–2 MΩ, filled with 2.5 M NaCl). The solution around the electrode was exchanged for another by simply closing and opening the corresponding manifold tubes (see Cooke & Quastel, 1973). This took place within 1–2 seconds.

The control solution (pH = 7.4) consisted of (mM): NaCl 125, KCl 5, $CaCl_2$ 2, $MgCl_2$ 1, NaH_2PO_4 1, $NaHCO_3$ 24 and glucose 11. Amino acids or their salts or antagonists to be tested were dissolved in this solution, the pH being unchanged. For the series of experiments with raised K^+, NaCl was reduced by 25 mM and sucrose was added to solutions containing less than 30 mM K^+ to retain iso-osmosity. The solutions in the reservoirs were always bubbled with 95% O_2 and 5% CO_2 and warmed to 37°C by a water jacket placed near the superfusion tip.

Extracellular action potentials (spikes) were recorded on a Mingograf ink writer and magnetic tape recorder simultaneously. Spike discharge frequencies (spikes/s) were recorded also on the Mingograf after passing through a frequency counter. After the experiment the spike frequencies were counted from the magnetic tape and printed out using a PDP-12 computer programmed for spike frequency counting (Cooke, Okamoto & Quastel, 1973). Values of spike discharge rates (spikes/s), for drawing the dose-response curves and for quantitative assessment of the effects of mixed amino acids, were obtained from the printed values of spike frequencies. The spike frequencies were usually obtained by averaging as many as possible of the steady printed values during various periods, e.g., at the stage of the largest inhibition or at the steady stage when the frequency had partially returned towards the control value.

The cells that displayed steady continuous spontaneous discharges (20–100 spikes/s) of relatively large spikes (about 1 mV or greater) at a depth of 300–350 μm and that consistently responded to the amino acids tested, were selected for the present studies (see Okamoto & Quastel, 1973). The general character of the spontaneous spike discharge did not change significantly for several hours, and the responses of the spike frequency to the amino acids and to the antagonists tested were satisfactorily reproducible during a period of several hours.

In order to avoid possible desensitization due to prolonged exposure to the amino acids, that might cause the flattening of the dose-response curves (Feltz, 1973), the amino acids were only applied at increasing concentrations after a washing by superfusion of the control solution following each application.

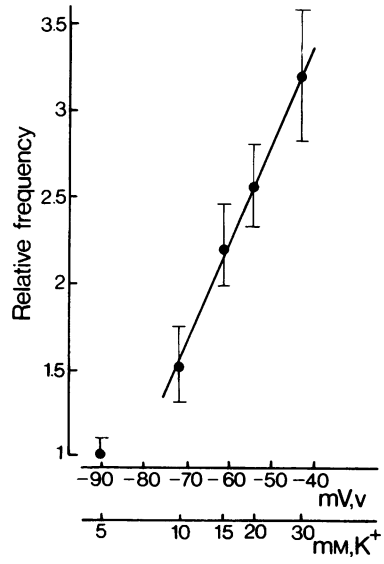

Figure 1 Effects of high-[K^+] on spike discharge frequency. Ordinate scale = frequency relative to that found in 5 mM K^+ solution. Abscissa scale = log [K^+] or calculated membrane potential (v). This was obtained from the Nernst equation, intraneuronal [K^+] being taken as 150 mM. Each point is mean of 3–5 values. Vertical lines show s.e. mean.

Effect of high-K^+ on spike discharge frequency

Experiments were also carried out, for purposes described in the discussion of the kinetic analysis, to examine the relation between the spike frequency and the membrane potential change brought about by the addition of high concentrations of K^+ to the superfusion medium.

It was found that a linear relationship existed between spike frequency and the logarithm of the external [K^+] (10–30 mM). There was an increase of the spike frequency by 5.6%, of the base-line level, for every 1 mV positive shift of the membrane potential calculated from the Nernst equation (Figure 1).

Kinetic analysis

If it is assumed (see Discussion section) that n molecules of amino acid, A, bind stepwise with a receptor, R, and that the response, p, the percentage

inhibition (or excitation) of spike discharge frequency by the amino acid, is linearly proportional to the concentration of the complex, A_nR, and also that the concentrations of the intermediate species, AR, ..., $A_{n-1}R$, are negligible compared with those of R and A_nR, then the dose-response curve may be given by eqn. (1) (see Discussion section).

$$p = p_{max}/[(1 + (K/[A])^n] \quad (1)$$

where K is the concentration of the amino acid giving 50% of the maximum response (p_{max}). The p_{max} is 100 for the inhibitory response.

Theoretical curves shown in Figures 4–7 have been calculated from eqn. (1), using various values of n, when no convulsant is present. Such curves, that fitted most closely to the observed values, were those in which the sum of the squares of deviations of the calculated percentage inhibitions from the observed values was minimal.

If m molecules of an antagonist, I, act on R competitively, the dose-response curve in the presence of the antagonist is given by eqn. (2) or (3), assuming that the antagonistic effect is proportional to $[I_mR]$ and that $[IR], \ldots, [I_{m-1}R]$ are negligible compared with [R] and $[I_mR]$.

$$p' = p_{max}/\{1 + (K/[A])^n[1 + ([I]/K_I)^m]\} \quad (2)$$

$$= p_{max}/[1 + (K'/[A])^n] \quad (3)$$

where p' = percentage inhibition of spike discharge frequency in the presence of the competitive antagonist, where K', the concentration of A to give 50% of p_{max}, is equal to $K[1 + ([I]/K_I)^m]^{1/n}$, and where K_I is the apparent dissociation constant $[=(K_{I_1} \cdot K_{I_2} \cdot \ldots \cdot K_{I_m})^{1/m}]$ of the processes: $[I][R] = K_{I_1}[IR]$; $[I][IR] = K_{I_2}[I_2R]$; ....; $[I][I_{m-1}R] = K_{I_m}[I_mR]$.

The value of m may be estimated by eqn. (4) which is derived from the combination of eqns. (1) and (2).

$$\ln[(p-p')/p'] = m\ln([I]/K_I) - \ln[p_{max}/(p_{max}-p)] \quad (4)$$

As $\ln[p_{max}/(p_{max}-p)] = \ln[1 + ([A]/K)^n]$ and is therefore constant when [A] is constant, then ln $[(p-p')/p']$ is linearly proportional to ln [I] and the plot of ln $[(p-p')/p']$ against ln [I] will give a slope equal to m. The calculated values of m have been obtained in this manner. Examples are shown in Figures 8 and 9.

The values of K_I were then estimated, using the above values of n and m, by means of the equal-response method (Gaddum, 1943; Hubbard, Llinàs & Quastel, 1969) using eqn. (5) which is derived from eqns. (1) and (2) when $p = p'$.

$$K_I = \{[I]^m/[([A_1]/[A_2])^n - 1]\}^{1/m} \quad (5)$$

where $[A_1]$ and $[A_2]$ are the concentrations of amino acid giving equal response in the presence and absence of the antagonist respectively. The values of n in eqn. (5) are the nearest higher integers to the non-integral n values that give the best fit of the observed data to the theoretical curves (Figures 4–7, see Discussion section).

It is evident from eqn. (5) that the value of $\ln[A_1] - \ln[A_2]$ is a constant depending only on the concentration of the antagonist. Thus, the effect of the antagonist is to shift the entire log dose-response curve to the right, in a parallel manner, when competitive inhibition takes place.

When the binding of I does not exclude binding of A, i.e. for non-competitive inhibition, eqn. (6) holds:

$$p'' = p_{max}/\{[1 + (K/[A])^n][1 + ([I]/K_I)^m]\} \quad (6)$$

where p'' = percentage inhibition in the presence of the non-competitive antagonist. Here the effect of the antagonist is to cause a non-parallel shift to the right of the log dose-response curve with a reduction of the maximum response.

Results

Effects of inhibitory amino acids on spike discharge frequencies in cerebellar slices

It has already been shown (Okamoto & Quastel, 1973) that the application, by superfusion, of GABA, glycine or taurine to incubated guinea-pig cerebellar slices greatly reduces the frequencies of spontaneous action potentials. Results of a similar nature have also been obtained with spontaneously firing neurones in cultures of neonatal rat cerebellum (Geller & Woodward, 1974).

The following changes in spike discharge frequencies took place during the superfusion of guinea-pig cerebellar slices with the inhibitory amino acids: (1) There occurred an early excitation followed by inhibition. This was observed with glycine (0.1 to 20 mM), with taurine (0.1 to 10 mM) and with β-alanine (0.2 to 1 mM). The early excitation occurred with GABA at concentrations lower than 0.1 mM but not at 0.2 mM (see Figure 2 or 3). This phenomenon of a phase of excitation followed by inhibition has also been reported by Geller & Woodward (1974) in cultured cells of rat cerebellum. The shortest onset time of the inhibition, i.e., the time between the peak of the early excitation and the time of maximal inhibition was 0.5–2 s with GABA, 1–2 s with β-alanine, 1–5 s with glycine and 5–15 s with taurine (Figure 2 or 3); (2) The onset time and the extent of inhibition were dependent on the amino acid concentration; the higher the concentration the shorter was the onset time and the greater was the extent of inhibition; (3) There was a partial recovery towards the initial frequency of spike discharge (see upward arrows in Figure 2), following the inhibition, although the amino acid was still present in the perfusion solution. The extent of the

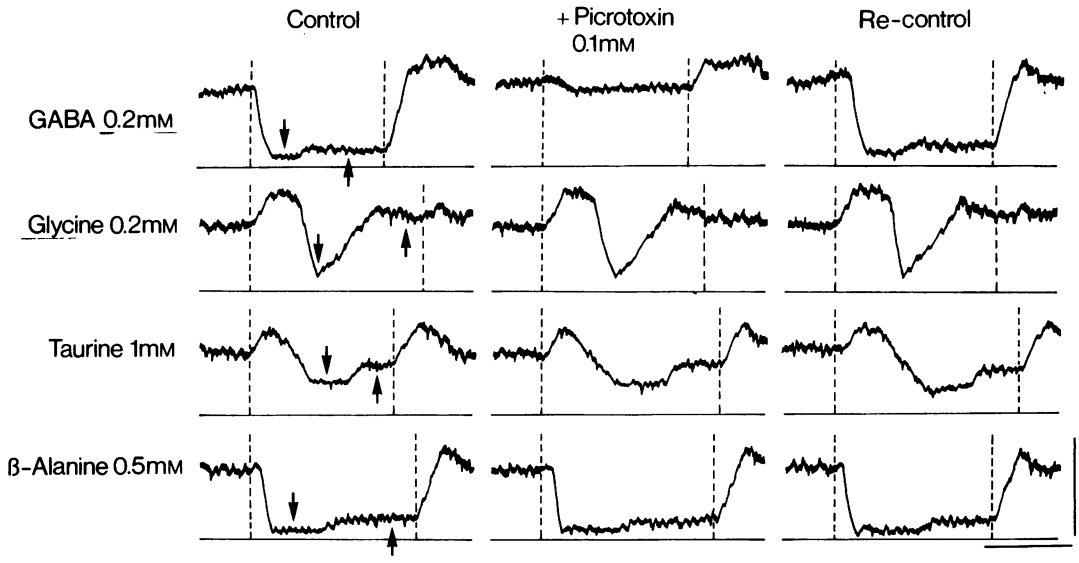

Figure 2 Effects of amino acids and picrotoxin on spike discharge frequencies. All records were obtained from same cell. Amino acids, at concentrations shown, were applied for a period shown by the two vertical broken lines. Picrotoxin (0.1 mM) was applied about 30 s before the application of the amino acids and its effects are given in the middle records. The left and right records are for the control and re-control (picrotoxin-free) conditions respectively. The latter were recorded about 3 min after the removal of the picrotoxin. Scales: vertical = 100 spikes/s; horizontal = 10 seconds. The downward arrows indicate examples of the largest inhibitions produced by the amino acids with which the dose-response plots (Figures 4–7) were obtained. The upward arrows indicate examples of the partially recovered spike frequency in the presence of the amino acids (see Results section).

recovery with GABA, taurine or β-alanine was dependent on the amino acid concentration present in the medium. There was, however, full recovery in the presence of glycine at all concentrations investigated. A similar recovery was reported by Hill *et al.* (1973) using cat cortical neurones *in situ* with microiontophoretic applications of glycine; (4) When the amino acids were removed, by superfusing with the control solution, there was an almost immediate increase of the spike frequency to a value above that of the control, followed by a return in about 10–20 s to the initial discharge rate (Figure 2 or 3). (See also Geller & Woodward, 1974.)

Effects of picrotoxin

Application of picrotoxin, at concentrations lower than 0.1 mM, caused little or no change in the spontaneous spike discharge frequency. Picrotoxin at 1 mM, however, caused an increase of spike discharge frequency that lasted about 20 s, after which the initial rate of spike discharge was resumed.

Application of picrotoxin (0.01–0.1 mM) to the cerebellar slices caused a block of the inhibitory action of GABA (0.05–1 mM, Figure 2). It had no such effect on the inhibitory behaviour of glycine, taurine or β-alanine (Figure 2). Removal of picrotoxin from the superfusion medium resulted, within a few minutes, in the restoration of the original inhibitory activity of GABA. The time of restoration depended on the concentration of picrotoxin used and the length of exposure of the slice to the drug.

The effects of glycine, taurine or β-alanine, on spike discharge rates, at concentrations that gave either just-maximum inhibitions (denoted by downward arrows in Figure 2), or partially-recovered steady states of inhibition (denoted by upward arrows in Figure 2), were unaffected by picrotoxin even at a concentration of 1 mM.

The early excitatory effects on spike discharge frequency exhibited by glycine, taurine or β-alanine were unaffected by picrotoxin (Figure 2), nor was the restoration of spike discharge frequency, on removal of any of these amino acids, affected by picrotoxin (Figure 2).

Effects of strychnine

Strychnine (0.005–0.1 mM) had no noticeable effect on the spike discharge frequency in the absence of

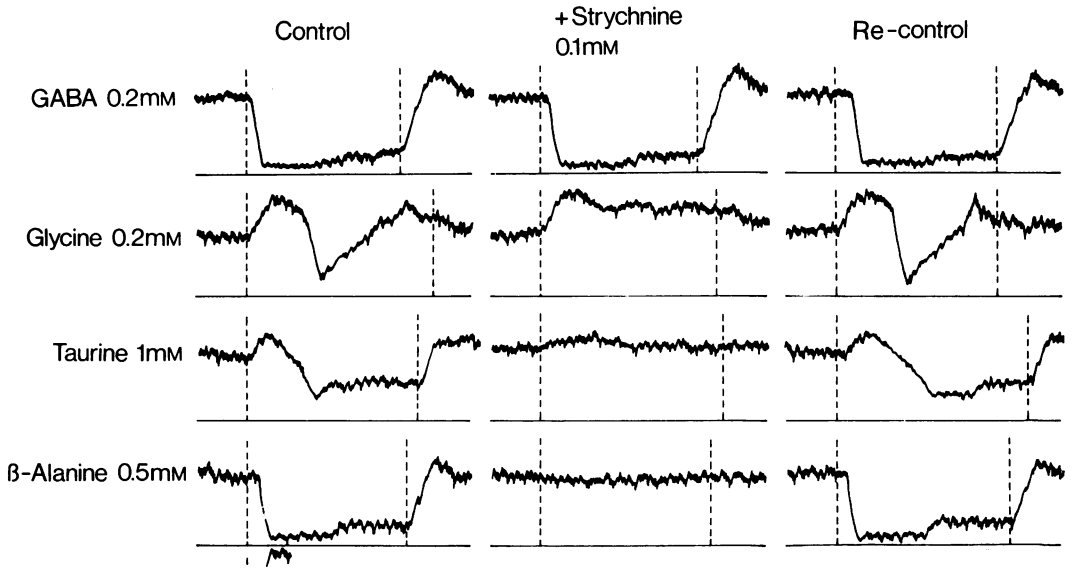

Figure 3 The antagonism by strychnine of inhibitory actions of glycine, taurine and β-alanine. All records were obtained from same cell. The amino acids were applied for a period shown by the two vertical broken lines. Strychnine (0.1 mM), whose effects are given in the middle records, was applied about 30 s before the application of the amino acids. The re-controls, given in the right records, were recorded about 3 min after the removal of strychnine. Scales: vertical = 100 spikes/s; horizontal = 10 seconds.

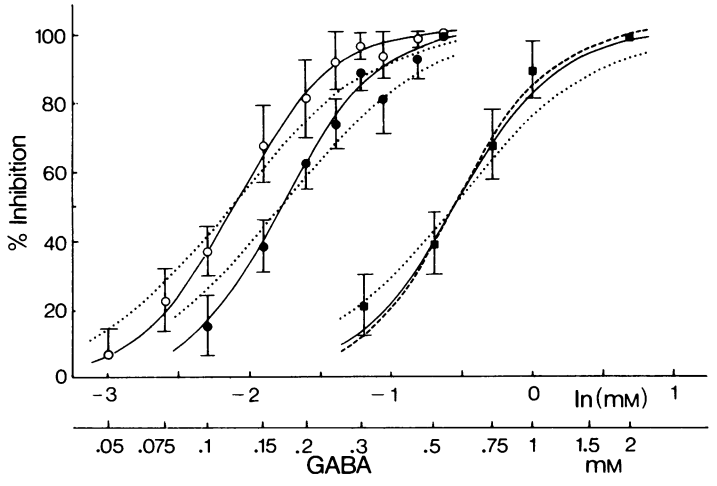

Figure 4 Log dose-response relations of γ-aminobutyric acid (GABA) and the effect of picrotoxin. Ordinate scale = percentage inhibitions of spike discharge frequency by GABA. Abscissa scale = log concentrations (mM) of GABA. Values plotted are means of observed values from 5–8 cells in several slices. Vertical lines show s.e. mean. (○): Control; (●): +10 μM picrotoxin; (■): +100 μM picrotoxin. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to the observed values. Values of n for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=2$ (· · · · · ·) and for $n=3$ (– – – –).

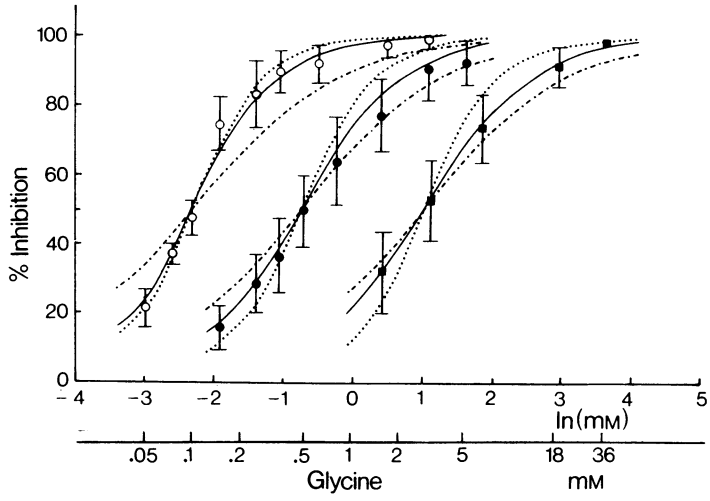

Figure 5 Log dose-response relations of glycine and the effect of strychnine. Ordinate scale = percentage inhibitions of spike discharge frequency by glycine. Abscissa scale = log concentrations (mM) of glycine. Values plotted are means of observed values from 5–9 cells in several slices. Vertical lines show s.e. mean. (○): Control; (●): +5 μM strychnine; (■): +50 μM strychnine. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to observed values. Values of n for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=1$ (– · – · – · –) and for $n=2$ (· · · · · ·).

added amino acids. Strychnine at high concentrations (e.g. 1 mM) caused a temporary increase of the spike discharge frequency.

The inhibitory action of glycine, taurine or β-alanine on spike discharge frequency was suppressed by strychnine (0.005–0.1 mM) which had no effect on the inhibitory action of GABA (Figure 3). Neither the early excitatory effects of the inhibitory amino acids nor the restoration of spike discharge frequency, on removal of the amino acids, was affected by strychnine (Figure 3). The antagonistic effect of strychnine disappeared within 2 min of removal of the drug (Figure 3).

Dose-response curves of the inhibitory amino acids

The effects of amino acids at different concentrations in the absence and presence of picrotoxin or strychnine on the spike discharge frequencies are shown in Figures 4–7.

The amino acid was applied at different concentrations to a single cell and the percentage inhibitions of the spike frequencies were calculated. The same procedure was repeated with a series of other cells. Averages were then taken of the percentage inhibitions found with a given concentration of this amino acid. As the amino acids under examination exhibited two phases of inhibitory action (see Figure 2, arrows), two types of dose-response curves could be obtained; one type when the largest percentage inhibition of the response was plotted against the dose (Figure 2, downward arrows) and the other when the percentage inhibition, observed when the depressed frequency had recovered to a steady state (Figure 2, upward arrows) was plotted. The first type of curve involved measurements made over a short period (3–5 s), whereas the second type involved measurements made over a period exceeding 12 seconds. Only curves of the first type are shown in Figures 4–7. Curves of the second type were found to be similar to those of the first but are not given because of the anomalous behaviour of glycine and because the reason for the partial recovery with the other amino acids is not yet clear.

Theoretical log dose-response curves are also given in Figures 4–7. They are derived from equation (1) or (3) given in the kinetic analysis using different values of n, the number of amino acid molecules combining at their specific receptor site(s). The values of n that gave the best fit of the theoretical curves to the observed values found in the absence or the presence of the convulsants are shown in Table 1. For the reasons given below the nearest higher integers to the non-integral value of n were assumed to be the correct n values. These were: $n=3$ for GABA, 2 for glycine, 3 for taurine and 4 for β-alanine.

The apparent dissociation constants of the amino acids at their receptor sites, both in the absence and

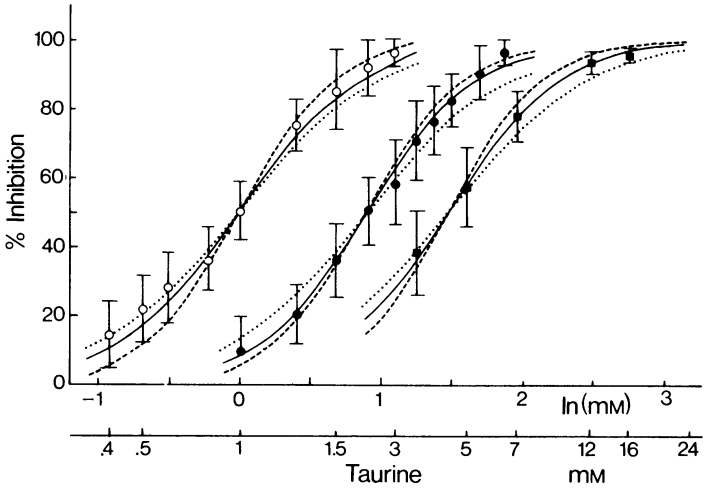

Figure 6 Log dose-response relations of taurine and the effect of strychnine. Ordinate scale = percentage inhibitions of spike discharge frequency by taurine. Abscissa scale = log concentrations (mM) of taurine. Values plotted are means of observed values from 7–19 cells in several slices. Vertical lines show s.e. mean. (○): Control; (●): + 10 μM strychnine; (■): + 50 μM strychnine. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to observed values. Values of n for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=2$ (· · · · · ·) and for $n=3$ (– – – –).

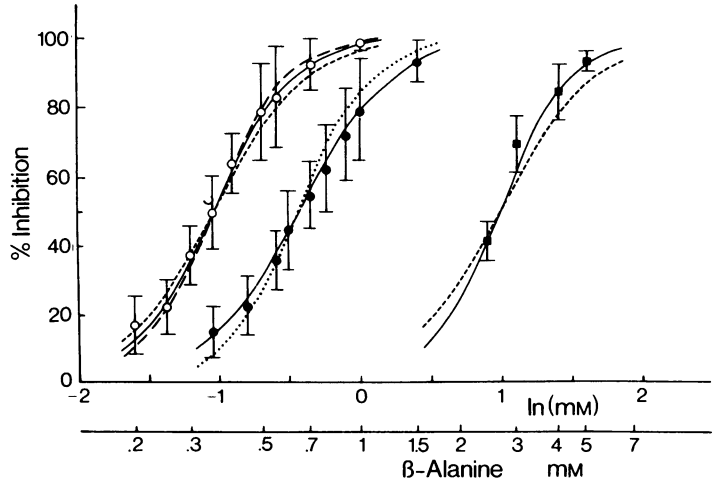

Figure 7 Log dose-response relations of β-alanine and the effect of strychnine. Ordinate scale = percentage inhibitions of spike discharge frequency by β-alanine. Abscissa scale = log concentrations (mM) of β-alanine. Values plotted are means of observed values from 5–6 cells in several slices. Vertical lines show s.e. mean. (○): Control; (●): + 5 μM strychnine; (■): + 50 μM strychnine. Solid lines are theoretical curves (eqn. 1 or 3) that give the best fit to observed values. Values of n for the best fit are given in Table 1. The broken lines are theoretical curves (eqn. 1 or 3) calculated for $n=2$ (· · · · · ·), for $n=3$ (– – – –) and for $n=4$ (— — —).

presence of the convulsants, are shown in Table 2. These are derived from equations (1) and (3) in the Kinetic analysis section.

Number (m) of molecules of the convulsant that combine with the receptor site

Picrotoxin brought about a parallel shift to the right of the dose-response curve due to GABA (Figure 4), whilst strychnine brought about a parallel shift to the right of the dose-response curves due to glycine, taurine or βalanine (Figures 5–7). Such parallel shifts by the convulsants indicate a competition between the amino acid and convulsant at a common receptor site (Van Maanen, 1950; Hubbard *et al.*, 1969).

Values of m for picrotoxin and strychnine (eqn. 4) were estimated from the slopes of the linear relations given in Figures 8 and 9 (see Kinetic analysis). The value for m for picrotoxin (1–100 μM), in the presence of 0.15 mM GABA, was 1.02 and that for strychnine (1–100 μM) was 0.96, 0.92 and 0.96 in the presence of 1 mM glycine, 3 mM taurine and 1 mM β-alanine respectively.

Linear relationships between the reciprocals of the percentage inhibitions in the presence of constant concentrations of the amino acid and the concentrations of the convulsants added (using the same data as those presented in Figures 8 and 9) were also obtained with either picrotoxin or strychnine. This is to be expected when m is equal to 1 (eqn. 4). These results indicated that only one molecule of picrotoxin or strychnine combined with the receptor site. Takeuchi & Takeuchi (1969) have indicated that one molecule of picrotoxin combines with its receptor site in the crayfish neuromuscular junction.

Table 1 Values of n giving theoretical dose-response curves having the best fit to the observed values

	n-*values*
GABA	3.0
GABA + picrotoxin (10 μM)	3.0
GABA + picrotoxin (100 μM)	2.8
Glycine	1.7
Glycine + strychnine (5 μM)	1.4
Glycine + strychnine (50 μM)	1.3
Taurine	2.3
Taurine + strychnine (10 μM)	2.6
Taurine + strychnine (50 μM)	2.5
β-Alanine	3.5
β-Alanine + strychnine (5 μM)	3.0
β-Alanine + strychnine (50 μM)	4.0

Values of n were calculated from data given in Figures 4–7 using equations (1) and (3) in the absence and presence of convulsants respectively

Apparent dissociation constants (K_I) of the convulsant-receptor complexes

The apparent dissociation constants (K_I) of the convulsant-receptor complexes were calculated from eqn. (5) using integral values of n (see Kinetic analysis) and with $m = 1$. These values were obtained from the magnitudes of the shifts, at 50% inhibition

Table 2 Apparent kinetic constants of the reactions of amino acids and convulsants

	K or K′ (mM)	*K_I (μM)*	n *values, for calculation of* K_I
GABA	0.12	—	—
GABA + picrotoxin (10 μM)	0.17	5.1	3
Glycine	0.10	—	—
Glycine + strychnine (5 μM)	0.39	0.25	2
Taurine	1.0	—	—
Taurine + strychnine (10 μM)	2.5	0.35	3
β-Alanine	0.35	—	—
β-Alanine + strychnine (5 μM)	0.65	0.48	4

Values of K or K′ and K_I (eqns. 1, 3 and 5), observed from the spike discharge frequency found at the largest depression (denoted by downward arrows in Figure 2), are given. Apparent dissociation constants, K_I, of the convulsant-receptor complexes were calculated from eqn. (5) using the values of the horizontal shifts of the best-fitted log dose-response curves (obtained with a convulsant concentrations of 5 or 10 μM) at 50% inhibition (Figures 4–7). The values of n in eqn. (5) used for the calculation of K_I were the nearest higher integers to the non-integral n values that gave the best fit of the observed data to the theoretical curves (see solid lines in Figures 4–7).

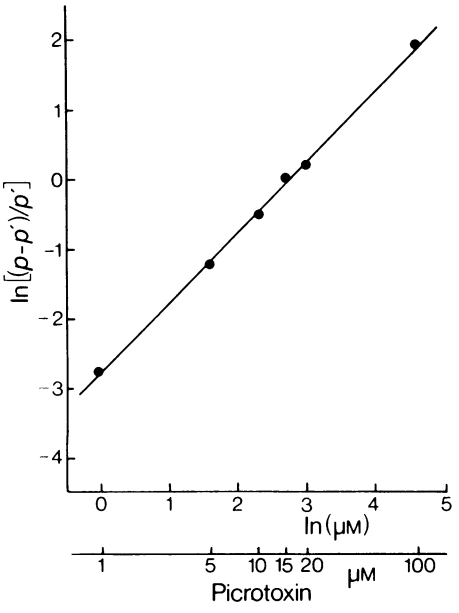

Figure 8 The relation between log[(p−p')/p'] and log concentrations of picrotoxin in the presence of constant concentration of γ-aminobutyric acid (GABA). Ordinate scale = ln[(p−p')/p'] where p and p' are percentage inhibitions by GABA in the abscence and presence of picrotoxin (eqn. 4). Abscissa scale=ln(μM) of picrotoxin. Values plotted are means of observed values from 4–6 cells in the presence of 0.15 mM GABA. The line, drawn by least mean squares method, gives a slope of 1.0 (see Kinetic analysis, eqn. 4).

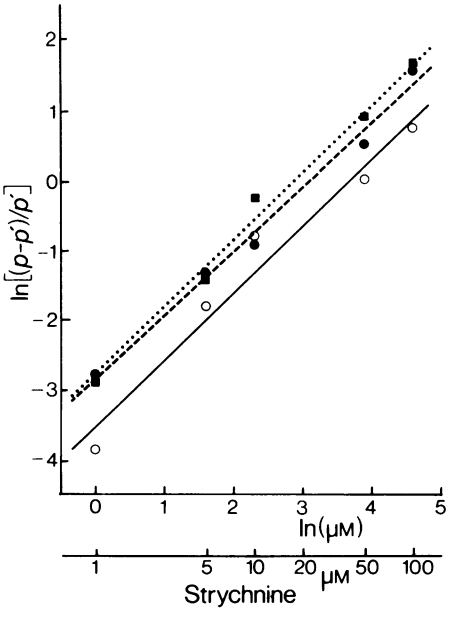

Figure 9 Relations between log[(p−p')/p'] and log concentrations of strychnine in the presence of constant concentration of glycine, taurine or β-alanine. Ordinate scale = ln[(p−p')/p'] due to the amino acids in the absence and presence of strychnine. Abscissa scale=ln(μM) of strychnine. Values plotted are means of observed values from 4–6 cells in the presence of 1 mM glycine (■ and dotted line), of 3 mM taurine (● and broken line) and of 1 mM β-alanine (○ and solid line). These lines, drawn by least mean squares method, give slopes of 0.96, 0.92 and 0.96 in the presence of glycine, taurine and β-alanine respectively (see eqn. 4).

brought about by the convulsants (5 or 10 μM), in the best-fitted log dose-response curves shown in Figures 4–7. The values of K_I obtained by this equal response method are given in Table 2.

Interrelations between the inhibitory amino acids

The possibility that glycine, taurine and β-alanine, whose inhibitory activities are similarly affected by strychnine, act at the same receptor site was examined. This was done by carrying out experiments with mixtures of the inhibitory amino acids.

Examples of the effects of mixing inhibitory amino acids on the spike discharge frequencies are shown in Table 3. With mixtures of GABA and glycine, taurine or β-alanine the net inhibition was equal to or slightly greater than the sum of the inhibitions produced by any of the amino acids alone at the concentrations tested. The same was true for a mixture of taurine and β-alanine. However, with mixtures of glycine and either taurine or β-alanine the net inhibition was less than the sum of inhibitions produced by the amino acids, i.e., the percentage inhibition by taurine or β-alanine was reduced in the presence of glycine. Data in Table 3 show the net inhibitions measured at the peaks of the inhibitory action. They indicated an affinity of glycine for receptors of taurine and β-alanine.

Discussion

Kinetic analyses of dose-response relations of amino acids and convulsants

Two assumptions were made in the analysis of the log dose-response curves of the amino acids in the presence and absence of the convulsants. The first assumption was that the spike discharge frequency

was directly proportional to the occupancy of the receptor site, i.e., to the amounts of the amino acid-receptor complex, A_nR (see Kinetic analysis). The second assumption was that the amounts of the intermediate amino acid-receptor complexes, AR, . . ., $A_{n-1}R$. (see Kinetic analysis) were negligible compared with those of R and A_nR.

The first assumption may be justified by the following considerations: the interaction between an amino acid, A, and a receptor, R, may be expressed by eqn. (7) (see e.g. Werman, 1969).

$$[A_nR]/R_t = 1/[1 + (K/[A])^n] \quad (7)$$

where R_t is the total number of receptors.

The membrane conductance change, Δg, is linearly related to the fraction of receptors occupied as given by eqn. (8) (see Werman, 1969).

$$\Delta g = \alpha([A_nR]/R_t) \quad (8)$$

where α is a proportionality constant.

The change of the membrane potential, v, caused by the membrane conductance change, Δg, is given by eqn. (9) (see Martin, 1955 and Hubbard *et al.*, 1969).

$$v = (E \cdot \Delta g)/(G + \Delta g) \quad (9)$$

where E is the difference between the resting membrane potential and the equilibrium potential of the ion concerned, and G is the resting membrane conductance.

On rearrangement of eqn. (9) by use of eqns. (7) and (8) we obtain eqn. (10).

$$v = [(\alpha \cdot E)/(\alpha + G)]/\{1 + [G/(\alpha + G)](K/[A])^n\}$$
$$= \beta/[1 + \gamma(K/[A])^n] \quad (10)$$

where $\beta = (\alpha \cdot E)/(\alpha + G)$ and $\gamma = G/(\alpha + G)$. Both β and γ are constants. The relation between v and [A] in eqn. (10) is similar to that between p, the spike frequency, and [A] in eqn. (1). As we have shown that a linear relation exists between spike frequencies and the membrane potentials due to varied external high-$[K^+]$ (Figure 1), it may be inferred that the spike frequency is also linearly proportional to the receptor occupancy.

Table 3 Effects of combinations of the amino acids on spike discharge frequency.

	% Inhibition		
GABA (mM)	*+Nil*	*+Glycine (0.05 mM)*	*+Taurine (1 mM)*
0	0	21 ± 6 (7)	50 ± 9 (17)
0.075	22 ± 10 (7)	45 ± 9 (5)	79 ± 7 (5)
0.1	37 ± 7 (8)	64 ± 7 (5)	93 ± 10 (4)
0.15	67 ± 11 (7)	86 ± 7 (5)	100 ± 0 (4)

	% Inhibition			
β-Alanine (mM)	*+Nil*	*+GABA (0.1 mM)*	*+Glycine (0.1 mM)*	*+Taurine (1 mM)*
0	0	37 ± 7 (8)	48 ± 5 (7)	50 ± 9 (17)
0.2	17 ± 10 (5)	56 ± 6 (6)	38 ± 8 (6)	69 ± 8 (5)
0.25	22 ± 9 (5)	71 ± 5 (7)	38 ± 11 (5)	78 ± 6 (5)
0.35	50 ± 10 (6)	—	52 ± 11 (5)	100 ± 0 (4)
0.4	64 ± 8 (5)	—	—	—

	% Inhibition	
Taurine (mM)	*+Nil*	*+Glycine (0.1 mM)*
0	0	48 ± 5 (7)
0.5	22 ± 18 (5)	45 ± 13 (5)
1	50 ± 9 (17)	53 ± 7 (6)
1.5	75 ± 8 (6)	63 ± 10 (5)

Values of percentage inhibitions (means ± s.e. mean) of spike discharge frequencies obtained at the stage of the largest inhibition, as denoted by downward arrows in Figure 2, are given. Number of the cells examined are shown in parentheses.

On the basis of the second assumption non-integral values of n (derived from eqn. (1) or (3)) were found to give curves that gave the best fit to the experimental results (Figures 4–7). As the true n value should be larger than the non-integral values (Cooke *et al.*, 1973) the nearest higher integers to the calculated non-integral values were considered to be the true n values (see Table 2).

It is evident, from eqn. (1), that the ratio (d log p)/(d log [A]) should approach the value of n with decrease in concentration of A, as indicated by eqn. (11).

$$(\text{d log } p)/(\text{d log } [\text{A}]) = n/[1 + ([\text{A}]/\text{K})^n] \quad (11)$$

The values of n given by this ratio are recorded in Table 4. There is no significant difference between the values of n given in Table 4, obtained from observations of the ratio (d log p)/(d log [A]) at low concentrations of A, and those given in Table 1, obtained from the best fits of the theoretical curves to the experimental values observed over a whole range of concentrations of A.

The ratios, (d log p)/(d log [A]), shown in Table 4 calculated from the values obtained from several individual cells (Table 4, column A), and those calculated from the averaged values obtained from a group of cells (Table 4, column B) are not significantly different. It is, therefore, reasonable to infer that the log dose-response curves shown in Figures 4–7 will represent the behaviour of an individual cell as well as that of a group of cells.

Table 4 Values of the ratio (d log p)/(d log [A])

	(d log p)/(d log [A])	
	A	*B*
GABA	2.9 ± 0.3 (6)	2.8 (6)
Glycine	1.2 ± 0.1 (5)	1.2 (5)
Taurine	2.5 ± 0.2 (5)	2.1 (8)
β-Alanine	3.4 ± 0.3 (5)	3.1 (6)

Values of (d log p)/(d log [A]) (see eqn. 11) were calculated from percentage inhibitions (p, given in Figures 4–7, in the absence of the convulsants, and in Table 3) due to the amino acid at two different concentrations of amino acids, corresponding to the lower portions of the log dose-response curves (Figures 4–7). The concentrations used were 0.05 mM and 0.075 mM for γ-aminobutyric acid (GABA); 0.05 mM and 0.1 mM for glycine; 0.4 mM and 0.5 mM for taurine, and 0.25 mM and 0.3 mM for β-alanine. The values shown were calculated in the following manner; e.g. $p = 22\%$ and 7% with 0.075 mM and 0.05 mM GABA respectively, therefore (d log p)/(d log [A]) = (Δ log p)/(Δ log [A]) = log (22/7)/log (0.075/0.05) = 2.8. Column A shows means (± s.e. mean) of the values calculated from values of p obtained from each of several separate cells. Column B shows values obtained from the averaged p values obtained from a group of cells. The number of cells tested are given in parentheses.

So far as convulsant binding is concerned only one molecule of picrotoxin or strychnine was found to combine with the amino acid receptor site (Figures 8 and 9). A Hill coefficient equal to 1 with strychnine has also been reported by Young & Snyder (1974) using suspensions of a brain fraction.

The parallel shift to the right of the entire log dose-response curves due to the convulsants (Figures 4–7) would indicate an apparently competitive inhibition by strychnine or picrotoxin (see Kinetic analysis). Curtis *et al.* (1971) have suggested that strychnine antagonism to glycine, with cat spinal neurones *in situ*, may be competitive, but Davidoff, Aprison & Werman (1969) have concluded that the antagonism between strychnine and glycine with spike discharges in cat spinal interneurones *in situ* is non-competitive. Takeuchi & Takeuchi (1969) have also reported that the antagonism between GABA and picrotoxin, in their studies of conductance changes, is non-competitive.

So far as combinations of amino acids and convulsants with receptor sites are concerned, Werman (1969) has given the value of n for glycine, with cat spinal interneurones, as at least 2. The value found by our results and listed in Table 2, gives $n = 2$. The number of GABA molecules combining with its receptor site has been reported to be 3 with crayfish muscle (Feltz, 1973) and with locust muscle (Werman & Brookes, 1969). The value for GABA obtained in our studies (Table 2) is $n = 3$. The concentration of GABA needed to give 50% of the maximum conductance increase in crayfish muscle has been quoted as 80 μM (Feltz, 1973) which may be compared with the value 120 μM given in Table 2. The dissociation constant for picrotoxin with crayfish muscle has been reported as 3.3 μM (Takeuchi & Takeuchi, 1969) which is about the same order as the value 5.1 μM that we have obtained (Table 2).

Co-operativity

It is possible to consider the response by inhibitory amino acids to be due either to a combination of the receptor (R) with n molecules of amino acid (A) to give A_nR whose amount determines the magnitude of the responses, as described in Kinetic analysis, or due to the co-operation of n molecules of AR, the complex A_nR_n controlling the amount of response. Under the latter circumstance the following equations apply:

$$\text{A} + \text{R} \rightleftharpoons \text{AR} \quad \text{where} \quad K_1 = [\text{A}] \cdot [\text{R}]/[\text{AR}],$$

$$n \cdot \text{AR} \rightleftharpoons \text{A}_n\text{R}_n \quad \text{where} \quad K_n = [\text{AR}]^n/[\text{A}_n\text{R}_n],$$

and

$$R_t = [\text{R}] + [\text{AR}] = [\text{AR}] \cdot [1 + (K_1/[\text{A}])]$$

where R_t is the total number of receptor sites. Therefore, $[AR] = R_t/[1 + (K_1/[A])]$. Then p, the percentage inhibition of spike frequency, is proportional to $[A_nR_n]$ and therefore:

$$p = C \cdot [A_nR_n] = C \cdot [AR]^n/K_n$$
$$= [[C \cdot (R_t)^n]/K_n]/[1 + (K_1/[A])]^n$$
$$= p_{max}/[1 + (K_1/[A])]^n \quad (12)$$

where C is a proportionality constant and p_{max} is the maximum percentage inhibition (equal to 100) obtained when [A] is large (see also Werman, 1969).

It is possible to decide whether equation (1) or eqn. (12) gives constant values of K or K_1 for various experimental values of p obtained with different amino acid concentrations. For non-co-operativity (eqn. 1), $p = 100/[1 + (K/[A])^n]$, i.e., $K = [A][(100/p) - 1]^{1/n}$; and for co-operativity (eqn. 12), $p = 100/[1 + (K_1/[A])]^n$, i.e., $K_1 = [A][(100/p)^{1/n} - 1]$. Values of p at different [A] used for the calculations of K or K_1 are taken from Figures 4–7.

Relevant values of K or K_1 for the response due to GABA are shown in Table 5. It is evident that constancy of K or K_1 only occurs when $n = 3$ for non-co-operativity. Similar results are obtained with glycine ($n = 2$), taurine ($n = 3$) and β-alanine ($n = 4$).

We infer, therefore, that co-operativity, in the sense used in this discussion, is not essential for an interpretation of the kinetics of the responses by inhibitory amino acids.

Receptor sites for the amino acids

It is evident that the receptor site for GABA must differ from that (or those) for glycine, taurine or β-alanine because of the specific antagonistic effects of picrotoxin and strychnine (Figures 4–7). The additive inhibitory effects observed with mixtures of GABA and taurine, glycine or β-alanine (Table 3) also suggest the existence of separate sites for GABA and the other amino acids. Additive inhibitory effects were also observed with mixtures of taurine and β-alanine (Table 3). More data are required, however, before it can be concluded that there are separate receptor sites for taurine and β-alanine, and possibly for glycine.

It seems to be clear that glycine affects the response to β-alanine or taurine (or *vice versa*) but not to GABA. It should be noted that in contrast to the other three inhibitory amino acids, the inhibitory action of glycine on spike frequency tends to disappear and the original frequency is quickly restored (Figure 2 or 3) (see also Hill *et al.*, 1973). This may indicate a possible desensitization by glycine of the receptor for glycine itself or that for taurine or β-alanine, leading to smaller inhibitory effects of taurine or β-alanine in the presence of glycine (Table 3).

We gratefully acknowledge financial assistance of the Medical Research Council of Canada and thank the Department of Pharmacology, University of British Columbia, for laboratory facilities for this work.

Table 5 Values of K or K_1 for response due to γ-aminobutyric acid (GABA) estimated from eqn. (12), based on co-operativity, and from eqn. (1), based on non-co-operativity

GABA [A] (mM)	n	$K_1 = [A][(100/p)^{1/n} - 1]$ *for co-operativity (eqn. (12))*	$K = [A][100/p) - 1]^{1/n}$ *for non-co-operativity (eqn. (1))*
0.1	2	0.065	0.132
0.15	2	0.033	0.105
0.2	2	0.023	0.100
0.25	2	0.012	0.078
0.1	3	0.040	0.120
0.15	3	0.021	0.119
0.2	3	0.015	0.126
0.25	3	0.009	0.115
0.1	4	0.029	0.115
0.15	4	0.016	0.126
0.2	4	0.011	0.141
0.25	4	0.006	0.140

Values of [A] and p are taken from Figure 4.

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(Received May 14, 1975. Revised November 8, 1975.)