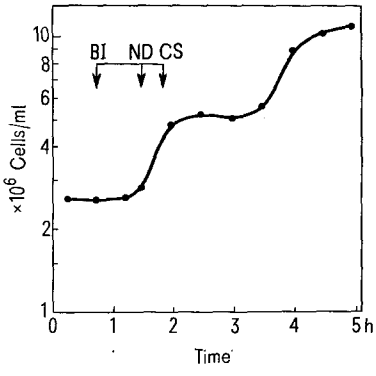
kinase and the presence of Mg^{2+} dependent ATPase[5] were made. An aliquot of the preparations was used to measure protein content[6] and radioactivity was measured in a Beckman LS-100 liquid scintillation counter.

The same experiment from growth of *Saccharomyces* to membrane isolation was repeated, except that cells were grown in the presence of $^{32}PO_4$ (10^5 cpm/ml) to label the lipid phase and collected after 90 min of incubation (in the first synchronized division cycle). Isolated membranes were extracted 3 times with chloroform-methanol, 2:1[7], and the extracts were assayed for lipid extractable phosphorus[8] and for radioactivity in D47 Nuclear Chicago flow counter.

The Figure shows the growth curve of a synchronous culture and the Table gives average data from 3 experiments. It is evident that the specific radioactivity for both leucine-^{3}H and $^{32}PO_4$ of membranes from buds and parent cells separated by sucrose gradient centrifugation is about half that of cells present in the inoculum. This fact suggests that macromolecules present in the membranes of inoculum cells are randomly diluted both in parent cells and buds. The results given in the Table (obtained by following the change of specific radioactivity in membranes purified from buds and mature cells at the indicated times) also show that there is no difference in mature cells and buds in rate of membrane growth.



Growth curve of synchronous cultures of *Saccharomyces cerevisiae*. CS, cell separation was determined by particle count in haemocytometers; ND, nuclear division was determined by HARTWELL[9], modification of the technique by ROBINOW and MARAK; BI, budding was determined by visual inspection in phase-contrast microscope.

Further, the behaviour of the change rate of specific activity in buds and mature cells excludes the possibility that what is transferred from parent cells to newly forming buds is a pool of membrane subunits. The experiments in which cells were incubated in the presence of $^{32}PO_4$ show that membrane lipid synthesis followed the same pattern (i.e. the dilution pattern) as membrane protein synthesis. The fact that the dilution effect is observed for both protein and lipid synthesis, strongly suggests that all the components of *Saccharomyces* membranes follow the same dilution pattern during cell division.

Riassunto. Cellule di *Saccharomyces cerevisiae* venivano coltivate in brodo centenente L-Leucina-^{3}H o $^{32}PO_4^{=}$ raccolte in fase log e quindi coltivate in modo sincrono in nuovo terreno privo di radioisotopi. Le cellule e le gemme venivano separate per centrifugazione su gradiente di saccarosio, quindi si isolavano le membrane. La radioattività specifica nelle membrane purificate risultava circa il 50% di quella presente inizialmente nelle cellule usate per la coltura sincronizzata.

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STUDIORUM PROGRESSUS

Localization of Ions in Cells of *Potamogeton lucens* L.[1]

Determination of ion content in various organelles and the relative distribution of ions within various cell-compartments is of great interest to biologists and has been the topic of numerous investigations (ARNOLD[2]; ARISZ[3]; OSMOND[4]; OSMOND et al.[5], LÄUCHLI and SCHWANDER[6], ZIEGLER and LÜTTGE[7]; LÄUCHLI and LÜTTGE[8], JENNINGS[9–11], LARKUM[12], NOBEL[13], WAISEL et al.[14], WAISEL and ESHEL[15], etc.) Undestanding of physiological phenomena like the ionic requirements for enzyme activity, into osmotic adaptation of cells, into symplast transport etc., depend very much on verification of the exact content of ions at the site of operation. Thus, such information is of great significance for the study of various processes which take place within the cells.

However, in spite of the use of several methods, i.e. autoradiography, specific dyes, electron microscopy, etc. (cf. JENSEN[16]; LÜTTGE[17,18]; LÄUCHLI[19,20]; VAN STEVENINCK and CHENOWETH[21] etc.), the precise localization and content of ions in subcellular compartments remains a matter of speculation.

Compartmental analysis had contributed a great deal to a better estimation of ion content inside various subcellular compartments. Nevertheless, being based on certain unmeasurable estimated values, its results are still open to criticism.

The recent application of X-ray microanalysis to biological material provides a better tool for determination of ion distribution within cells, and offers a direct method for localization of most of them in some subcellular compartments.

A stock of *Potamogeton lucens* plants was grown outdoors for several months in tap water. Leaves and branches were detached from those mother plants and transferred into tap water to which 100 m*M* NaCl were added. The material was kept there for 24 h under continuous light and controlled temperature (27° ± 2°C). Following such a period of adjustment, samples of 1 cm² each were taken from the midrib region of mature leaves for examination. The samples were placed on top of a cold (−70°C) microtome block-holder. Distilled water were used for

embedding and the blocks were frozen within a few sec. The blocks were then sectioned in a cryostat (−25 °C). The frozen cross-sections were placed on cold (−25 °C) aluminium plates which were previously coated with a thin layer of silicone grease. The mounted sections were then dried overnight under vacuum (10^{-3} Torr), in a lyophilizer. Strict care was taken to keep the material deeply frozen throughout the procedure. Following dehydration, the sections were coated with carbon, and then with gold-palladium (60:40). Distribution of calcium, potassium, sodium, chloride and magnesium was determined with a Jeolco modified JXA-3A X-ray microanalyzer. An acceleration voltage of 15 KeV and sample current of approximately 0.3×10^{-7} A, were used. Beam diameter was approximately 0.5 μm or lower. The spatial resolution of the X-ray signals was estimated to be 1 μm by measuring the distance between a peak and an adjacent deep along a line profile. Kα radiations of each of the elements were examined. Sodium and magnesium were analyzed with a KAP crystal, chloride with a mica crystal, and potassium and calcium were analyzed with a quartz crystal. The background levels were determined by lowering the spectrometer 1° off the specific Bragg's angle. This gives an over-estimation of the background at the peak and is thus safe for estimation of net specific signal levels. It was checked before for the absence of any specific radiation at that angle.

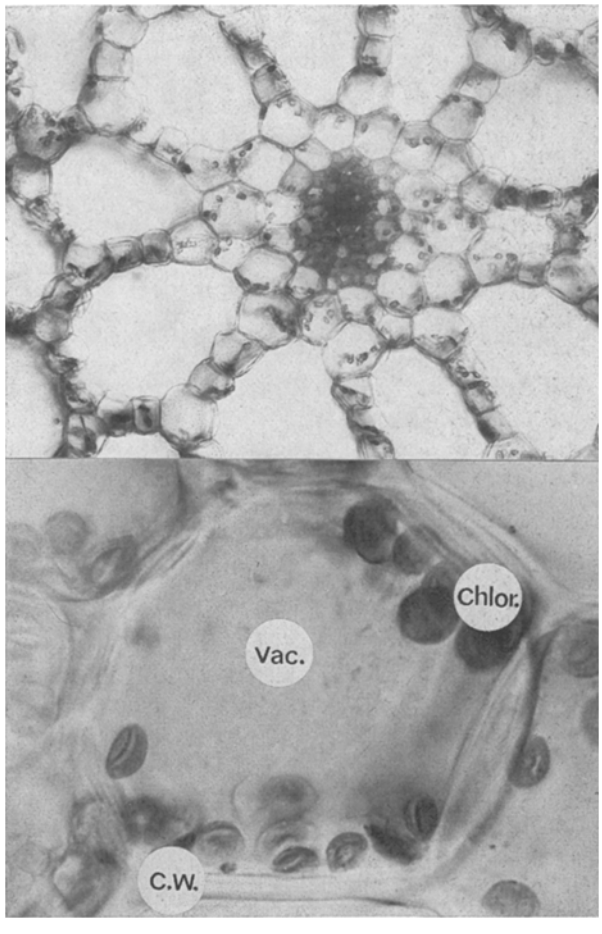

Fig. 1. A cross section of a leaf of *Potamogeton lucens* in the mid-rib region (upper; ×135) and one enlarged sheath cell (lower; ×1350) as seen in the light-microscope after staining with neutral-red. c.w., cell wall; chlor, chloroplast; vac, vacuole.

Total inorganic analysis of *P. lucens* chloroplasts in their natural position within cells was executed by fixing an electron beam of 25 KeV on one chloroplast at a time. Checking for the presence of elements from atomic No. 6 to atomic No. 92 was done by turning the spectrometer along its entire range.

Distribution patterns of sodium, chloride, potassium, calcium and magnesium were followed within 15 cells of *P. lucens*. Results of 3 representative cells are presented in Figure 3.

At the present state of resolution, the borders between various subcellular structures were not sharply distinct and certainly some overlapping of the X-ray signals occurred. Three compartments could be distinguished in the cells examined, i.e. the cell wall, the chloroplasts (including the embedding cytoplasm) and the vacuole (Figures 1 and 2). The first two constituents were clearly seen in the secondary electron image of the microprobe,

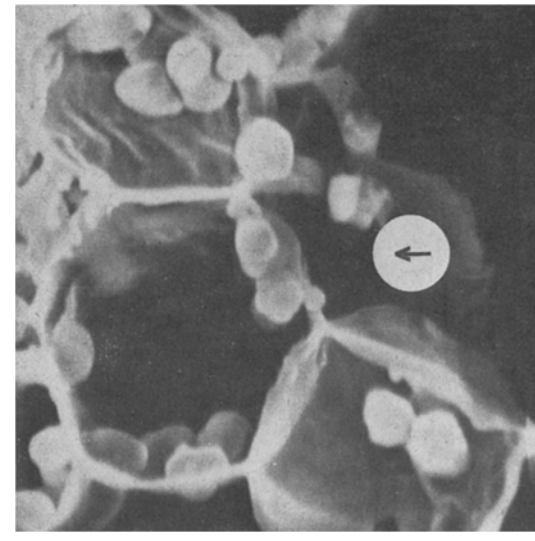

Fig. 2. Secondary electron image of one of the examined cells of *Potamogeton lucens*. ×700. Cell C of Figure 3. The arrow points to the analyzed site.

[1] This investigation was partly supported by a grant from the U.S. Department of Agriculture under No. P.L. 480.

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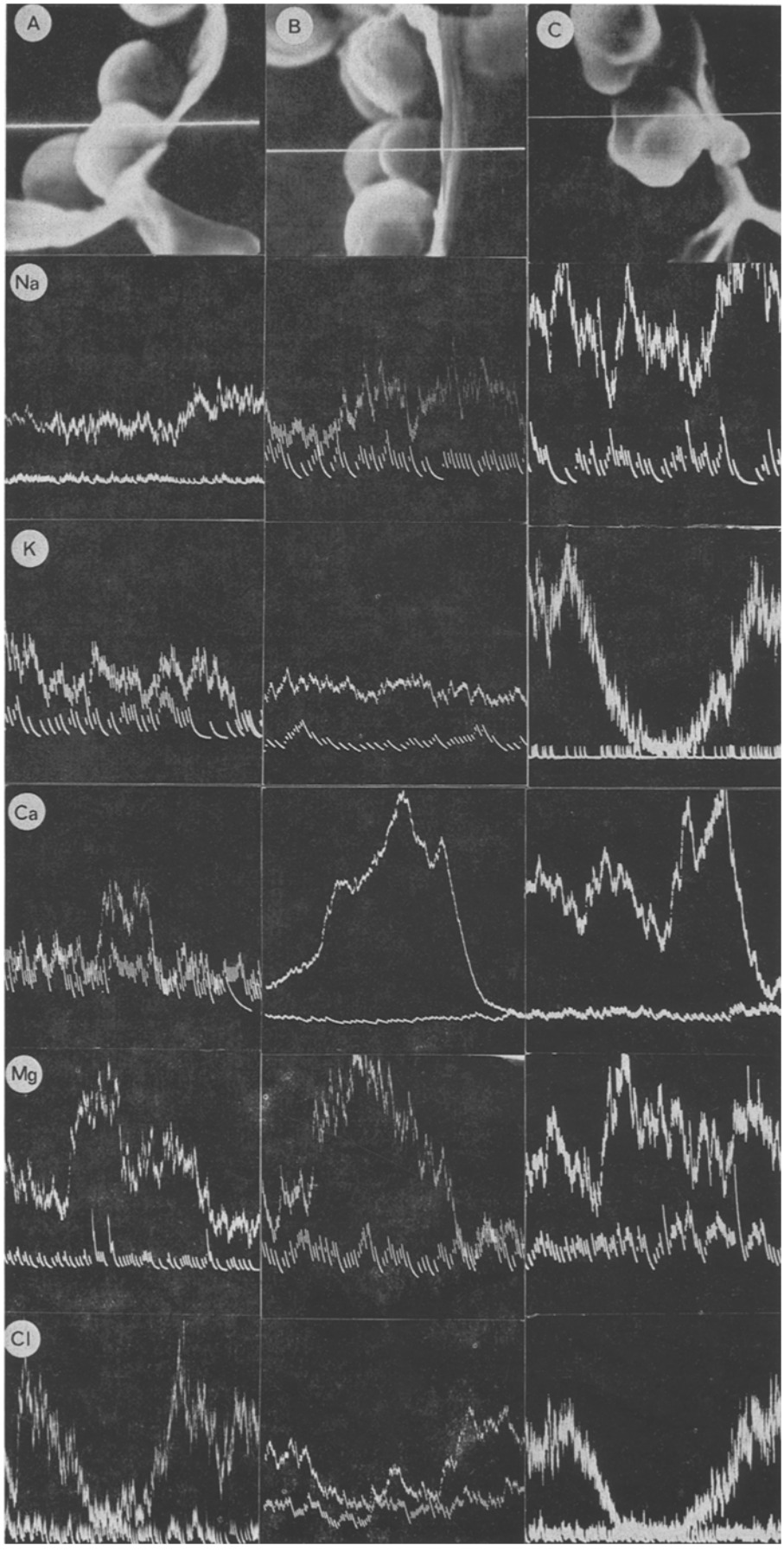

Fig. 3. Secondary electron images of sections of 3 leaf-cells (a, b, c; ×2750) showing a cross section through the cell-wall, cytoplasm, chloroplasts, vacuole, and line profiles for Sodium (Na), potassium (K), calcium (Ca), magnesium (Mg) and chloride (Cl) across the horizontal line.

whereas the site of the vacuole was determined by the lightmicroscope.

Cells were not uniform regarding ion localization. The line profile obtained for sodium had a constant level across all 3 compartments in 1 group of cells (Figures 3a and c), whereas in another group of cells (Figure 3b) it was high mainly in the cell-wall and chloroplast fractions.

The line profile for potassium was either of the same level within the whole cell (Figures 3a and b), or higher in the cell-wall and vacuole than in the chloroplasts (Figure 3c). Line scans of magnesium and calcium differed in their profile.

The signals for calcium were always more intensive in the chloroplasts (Figures 3a, b and c). In some cells calcium had a relatively intensive signal in the cell-wall (Figures 3b and c), whereas in others the line scan had the same level in all 3 compartments, i.e. in the cell-wall, the chloroplasts and the vacuole (Figure 3c). Magnesium appeared in all 3 cell compartments but most intensive signals were obtained inside the chloroplasts. High signals were recorded in the cell-wall fraction as well as inside the vacuole.

Line scans obtained for chloride always yielded higher signals in the vacuole than in any other compartment of the cells.

Total chloroplasts analysis indicated that only sodium potassium, calcium, magnesium, and sulfur yielded apparent signals. Chloride content of the chloroplast was low and approached the lower limits of detectability of the microprobe.

A quantitative interpretation of the observed scans is still difficult at the moment and suffers from several limitations. Dried sections made of plant tissues are not homogenous and vary in topography, composition and ion content. Under the conditions used (15 KeV) they are partially 'transparent' to the beam. Thus, such material cannot render to quantitative corrections which have been used in the analyses of uniform metallurgical or geological flat samples, of known composition and density (cf. HALL[22]). Considering the various methods available for localization of ions in biological tissues, we are inclined to believe that results obtained for frozen-dried sections by x-ray microanalysis are the closet to the actual situation in the undisturbed tissue. Other advantages of this method are that several ions can be directly measured on exactly the same sites and analyses can be repeated.

The biological meaning of the data obtained also needs some clarification. Localization of ions within the cell-wall may result either from their inclusion among the constituents of the wall (e.g. calcium in the middle lamellae) or from the fact that the free space resides at the cell-wall. Signals obtained can thus be attributed to both sources.

Another difficulty is confronted in the analysis of the chloroplasts. As previously noted, chloroplasts in their natural position inside the cell are embedded in the cytoplasm. A clear distinction between those two cell compartments is therefore impossible, unless a cross section through the chloroplast is obtained. Analyses of chloroplasts thus represent mainly the constituents of the chloroplasts themselves, but signals cannot be free of contamination of various cytoplasmatic inclusions.

Distribution of sodium in various cells of the submerged water plant *Potamogeton lucens* was uneven. Nevertheless, it is important to note that in all cases examined, high signals were obtained in the cytoplasm. Generally it was assumed that in terrestrial halophyte tissues sodium is mostly concentrated in the cavuole (STOCKER[23], WALTER and STEINER[24], STEINER[25], ADRIANI[26], JENNINGS[9–11] etc.). It was also suggested that sodium should be found in relatively low concentrations in the cytoplasm of succulent halophytes (JENNINGS[9–11]). However, using the microprobe, WAISEL and ESHEL[15] showed that the cytoplasm of mesophyll cells of the halophyte *Suaeda monoica* yielded higher signals for sodium than the vacuoles. The data for *Potamogeton* are thus the second case where sodium was shown to reside in the cytoplasm. Inspite of the ecological differences, similar patterns of distribution of sodium were observed, in the cells of the halophyte *Suaeda monoica* and the submerged water plant, *P. lucens*. Such a distribution of sodium might thus be of a general nature.

Calcium ions were reported as being located in the cell-wall, in the cytoplasm and in the vacuole (BRIGGS et al.[27], WINTER[28], SUTCLIFFE[29], DAINTY[30], LARKUM[12], NOBEL[13] etc.). However, practically no information concerning the relative distribution of calcium among those compartments was available.

Potamogeton lucens is known to absorb and assimilate bicarbonate ions from the water. The concomitant precipitation of calcium carbonate on the outer surface of the leaves was well described (cf. SCULTHROPE[31]). In view of the present data, where high signals for calcium were obtained inside the cells, it seems that calcium is also accumulated not only on the outer surface of the leaves of *Potamogeton* but also inside the cells and particularly in the chloroplasts.

Distribution of potassium inside plant cells is also an unclear case. Ptoassium was usually believed to be concentrated either in the chloroplasts and cytoplasm, or in the vacuoles, where it plays an important role in the cells' osmotic regulation (BERNSTEIN[32,33], DAINTY[30], SPANSWICK and WILLIAMS[34], SLATYER[35], LARKUM[12], JENNINGS[10], LATIES[36], WAISEL and ESHEL[15] etc.). Results obtained for *Potamogeton* in our experiments were not uniform. Signals for potassium were even across the cells of 1 group of cells, whereas in other cells they were higher in the vacuole and cell-wall (= free space) fractions. It is still unknown whether such distribution depends on cell types or on the cells physiological conditions.

Very little is known regarding localization of chloride inside tissues or cells. Different patterns of distribution of chloride were reported by various investigators. According to some of them, chloride is concentrated in the cytoplasm as well as in the vacuole of various plant-cells (EPSTEIN[37], BRIGGS et al.[27], DAINTY[30], HODGES and VAADIA[38], LARKUM[12], etc.). Support for this assumption

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was obtained also from the submerged water plant *Vallisneria spiralis* (ARISZ and SOL[39]). Accumulation of chloride in the cytoplasm as well as in the vacuole was also reported for *Nitella translucens* (MACROBBIE[40-42]). High chloride concentrations were also postulated by MACROBBIE[40] for the chloroplasts of *Nitella translucens*. Other reports claimed that in cells of halophytes the cytoplasm was rich in chloride and that chloride was maintained there as free ions (ARNOLD[2]; OSMOND et al.[5]).

Chloride distribution in *Potamogeton lucens* did not agree completely with either of the previously reported assumptions. In leaf-cells of *Potamogeton* the highest signals for chloride always appeared either in the vacuole or the cell wall (free space) fraction. Only very low signals of chloride were detected in the chloroplasts, and the connection between chloride uptake and electron transport in those chloroplasts would thus be questionable.

In summary, it is evident that individual cells of *Potamogeton* vary in their content as well as in their ion distribution, Thus, an investigation into the localization of ions inside subcellular organelles of plants must consider difficulties arizing from technical limitations, as well as from the high variability among cells, and tissues.

Zusammenfassung. Die Verteilung von Natrium, Kalium, Kalzium, Magnesium und Chlor wurde in Mesophyllzellen der Blätter von *Potamogeton lucens* mit Hilfe der Röntgen-Mikrosonde untersucht. Die Blattzellen dieses Wasserpflanze variierten beträchtlich in bezug auf Menge und Verteilung der Ionen. In den meisten Zellen wurden im Cytoplasma und in den Chloroplasten hohe Werte für Natrium und niedrige für Chlor beobachtet. In den Vakuolen wurden für sämtliche untersuchten Ionen hohe Werte gefunden.

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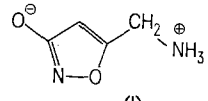
COGITATIONES

Molecular Orbital Studies on the Conformations of Bicuculline and β-Hydroxy GABA[1]

In a previous study we reported molecular orbital calculations of the preferred conformation of γ-aminobutyric acid (GABA) and muscimol (I)[2]. That study predicted an extended chain for the GABA zwitterion with free rotation for the carboxylate group. The distance separating the onium group from one carboxylate oxygen ranged from 5 to 6 Å. This was postulated to be a critical pattern or pharmacophore for GABA activity. In the same study the GABA agonist muscimol (I) was predicted to



(I)

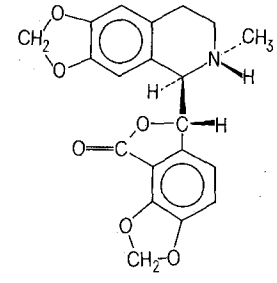
prefer a conformation in which the side chain is coplaner with the ring and bent toward the ring oxygen atom. The distance separating the exocyclic ring and onium group was predicted to be 5.8 Å, in good agreement with our postulated GABA pharmacophore[2].

A recent study of the crystal conformation of GABA has been reported using X-ray analysis[3]. This study reports a folded molecule with the onium group-oxygen separation 4.5 Å or less.

An X-ray analysis of the muscimol crystal reveals that the side chain is bent toward the ring oxygen atom but is 60° out of the ring plane[4]. The exocyclic oxygen to onium group distance was found to be 5.77 Å, in excellent agreement with our prediction.

Experimental support for our proposed GABA pharmacophore comes from evidence that 4-amino -2-butynoic acid is a GABA agonist[5]. Assuming that this rigid molecule exists in solution as a zwitterion, the separation of the onium group from a carboxylate oxygen atom is fixed between 5.2 and 5.8 Å, in good agreement with our postulated GABA pharmacophore.

In an effort to further examine the validity of our GABA hypothess we have periformed calculations on the GABA agonist β-hydroxy GABA[6] and the GABA competitive antogonist, bicuculline (II)[7]. A possible receptor equivalence has been speculated upon in which the carbonyl



(II)

oxygen to onium group distance in bicuculline may be comparable to the corresponding structural features in GABA[7].

The calculations were performed using extended Huckel theory[8] with parameters previously employed[9]. The dimensions used were the standard bond lengths and angles proposed for M.O. calculations and used extensively by us[10]. Angular increments of 30° were used.

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