# ELECTROPHYSIOLOGICAL OBSERVATIONS ON THE TELEOST OLFACTORY BULB

Neil Kenneth MacLeod

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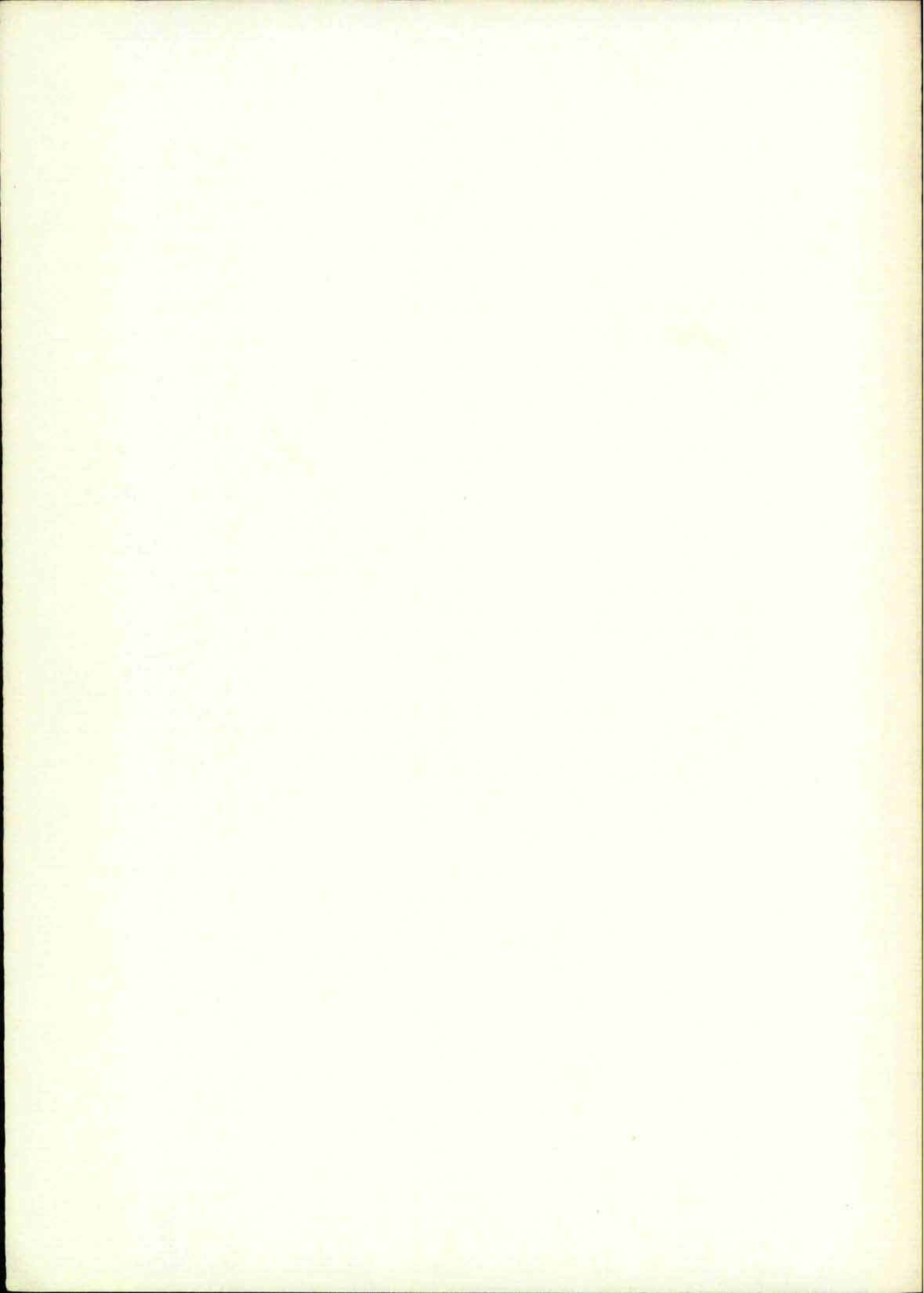

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# ELECTROPHYSIOLOGICAL OBSERVATIONS ON THE TELEOST OLFACTORY BULB

BY

Neil Kenneth MacLeod, B.Sc., M.Sc., D.I.C.

A DISSERTATION SUBMITTED FOR

THE DEGREE OF

DOCTOR OF PHILOSOPHY

OF THE

UNIVERSITY OF ST. ANDREWS

St. Andrews, 1975

Th 8415

F O R

J O Y C E

ABSTRACT

Previous electrophysiological research on the fish olfactory system is reviewed and the conclusion reached that present knowledge concerning, in particular, the fundamental physiology of neuronal connections within the olfactory bulb, was rudimentary and confused.

Field potentials were evoked in the olfactory bulb of teleost fish by electrical stimulation of the olfactory tract and nerve. The potential wave recorded at the bulbar surface consists of four components, $N_1$, $N_2$, $N_3$ and P, all of which appear to be of post-synaptic origin when the nerve is stimulated, whence they are usually preceded by a triphasic potential thought to represent the compound action potential of olfactory nerve fibres. The $N_1$ wave evoked by olfactory tract stimulation is not of synaptic origin. It probably represents the synchronous antidromic activation of secondary neurons.

The waves were analysed with respect to voltage and time and related to the underlying histology. The results indicate that the extracellular current flow around bulbar neuronal elements is essentially similar to that already described for mammals and is probably generated by similar pathways. This is surprising in view of fundamental anatomical dissimilarities, particularly regarding the dendritic field of mitral cells.

The field potentials proved to be useful in the identification of single units at the time of recording. The spontaneous and evoked activity of identified mitral and

granule cells could often be inhibited by stimulation of either the nerve or tract. The evoked field potentials could usually be similarly inhibited. Evidence has been obtained that this inhibition is mediated by GABA and that it may well take place via a recurrent pathway involving reciprocal dendrodendritic synapses as in the mammalian system. Evidence was also obtained that this inhibition may, in part, result from the activation of granule cells by adrenergic centrifugal fibres when the olfactory tract is stimulated.

Natural chemical stimulation of the olfactory mucosa with amino acid solutions produced a complex pattern of responses. Each odorant normally produced a unique pattern of excitatory and inhibitory responses across all units. Chi-square values were calculated for stimulatory effectiveness between forty-five pairs of odours. L-serine and L-alanine consistently showed a high degree of similarity with several other odours. The converse was true for GABA and L-histidine, although this pair had a high chi-square value when mutually compared. Enantiomeric pairs of amino acids were often found to have opposite stimulatory effects on bulbar units. These results are discussed in relation to the possible properties and configurations of odorant receptor sites for amino acids in the fish olfactory mucosa.

DECLARATION:

The work embodied in this thesis has been carried out by the author and has not been submitted in any previous application for a higher degree.

VITAE:

After graduating from the University of London as B.Sc., Zoology and Chemistry in 1969 and M.Sc., D.I.C., Biochemistry in 1970, I was a teacher of Biology until my admittance to the University of St. Andrews as a candidate for the degree of Doctor of Philosophy in October, 1971.

SUPERVISOR'S STATEMENT:

I confirm that the conditions of the Ordinance and Regulations have been fulfilled by the author in the presentation of this thesis.

(M. S. Laverack)

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# abbreviations

The following abbreviations have been used in this thesis:-

| | |
|---|---|
| AC | alternating current |
| DC | direct current |
| M | megohm(s) |
| k | kilohm(s) |
| nA | nanoampere(s) |
| V | volt(s) |
| mV | millivolt(s) |
| uV | microvolt(s) |
| S.I.U. | stimulus isolation unit |
| C.A.T. | computer of average transients |
| Hz | Hertz |
| sec. | second(s) |
| msec | millisecond(s) |
| m/sec | meters per second |
| mm | millimeter(s) |
| um | micrometer(s) |
| l | litre(s) |
| ul | microlitre |
| EEG | electroencephalogram |
| EKG | electrocardiogram |
| EOG | electro-olfactogram |
| MEC | microelectrode |
| EPSP | excitatory post-synaptic potential |
| IPSP | inhibitory post-synaptic potential |
| OB | olfactory bulb |
| ON | olfactory nerve |
| OT | olfactory tract |

| | |
|---|---|
| TC | telencephalon |
| MC | mesencephalon |
| C | cerebellum (also °C degrees centigrade) |
| M | medulla oblongata (also Molar) |
| VL | vagal lobe |
| GABA | $\gamma$ -amino butyric acid |
| ala | alanine |
| cys | cysteine |
| gln | glutamine |
| glu | glutamate |
| gly | glycine |
| his | histidine |
| phal | phenylalanine |
| ser | serine |
| val | valine |
| L | laevo rotatory |
| D | dextro rotatory |

# I

# introduction

## (i) A SURVEY OF PREVIOUS ELECTROPHYSIOLOGICAL STUDIES ON THE OLFACTORY SYSTEM OF FISH

For centuries man's imagination has been triggered by the barely credible feats of navigation accomplished by the salmon and the eel in their respective anadromous and catadromous migrations. Even today, however, our knowledge concerning the physiological mechanisms responsible for the uncanny accuracy with which these fish 'home' is rudimentary.

One particular theory is that the sense of smell is intimately involved, for at least the freshwater phase of the salmon's homeward migration. A start has been made in providing evidence to support this theory, but only when we have an infinitely clearer picture of fundamental olfactory physiology in fish, will it be possible to formulate a valid hypothesis.

The olfactory organs of fish lie in a pair of pits, usually situated on the dorsal side of the head. The organ itself is a folded sheet of epithelium consisting of mucous cells, supporting cells and receptor neurons, whose axons travel in the olfactory nerves to converge upon the secondary neurons in the olfactory bulb. The axons of the secondary neurons pass via the olfactory tracts to various regions of the telencephalon and diencephalon. This basic arrangement has remained unaltered throughout the vertebrate classes.

The most significant contributions to understanding the physiology of the nervous system have been made by exploring the activity of single neurons during controlled experimental conditions. Although all parts of the fish olfactory system are readily available for this kind of study it will become clear in

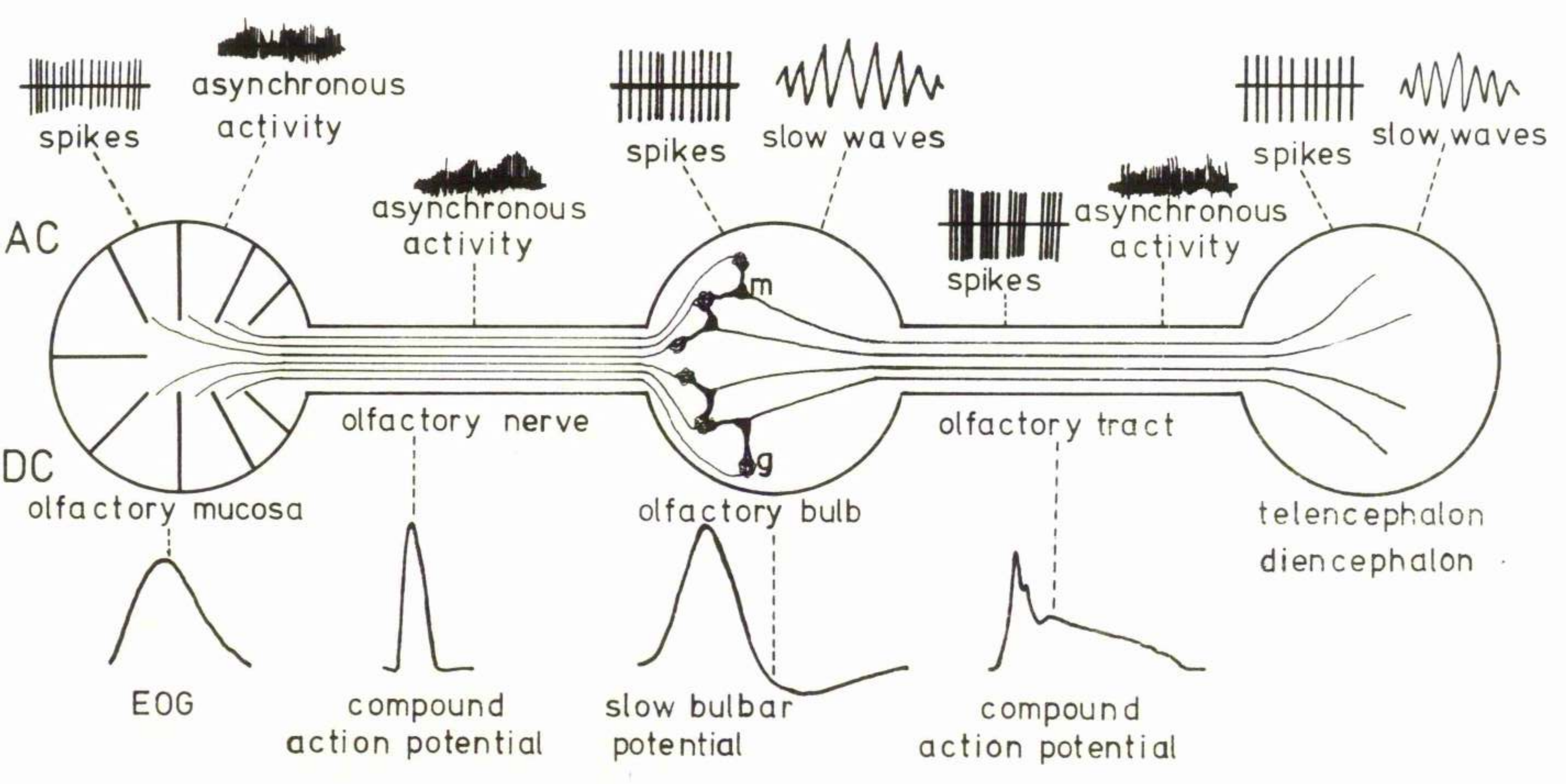
this review that such 'significant contributions' are few and far between.

Adrian and Ludwig (1938) were the first workers to use electrophysiological techniques to analyse the olfactory system of fish. They recorded a continuous discharge in the olfactory tracts of the decapitated carp (Carassius) and catfish (Amiurus) and tench (Tinca tinca). At a latency varying from 0.5 to 5 secs after placing a drop of stimulating fluid into the olfactory sac, the resting discharge shifted to a maximum and then declined slowly. This response, which could also be elicited by mechanical stimulation, was followed by a refractory period during which the organ was insensitive to a second stimulus.

## A. ELECTRICAL ACTIVITY OF THE OLFACTORY MUCOSA

Shibuya (1960) has described 'resting potentials' of between 4 and 18 mV, recorded on piercing the olfactory mucus, for six species of fish, Cyprinus carpio, Parasilurus asotus, Channa argus, Misgurnus anguillicaudatus, Entosphenus japonicus, and Anguilla japonica. He claims that this voltage represents the potential across the 'external limiting membrane', although he does not make clear exactly to what he is referring. On stimulation of the olfactory mucosa with odorous fluid, Shibuya observed "a slow action potential with a steep rise and a gradual exponential decline, recorded with macroelectrodes". This slow 'action potential', which obviously corresponds to the receptor potential designated the electro-olfactogram (EOG) by Ottoson (1956, 1971), had an amplitude between 0.3 and 3.0mV. The shape of the response was different for different fish. In Channa,

Figure I.1 Types of electrical activity recorded from the fish olfactory system




AC and **DC** refer to mode of recording; m - mitral cell; g - olfactory glomerulus.

*Cyprinus* and *Entosphenus* the EOG had a sharp rise and a slow decline, and a duration which increased with stimulus strength. In *Parasilurus* and *Anguilla* the EOG had a sharp rise, a rapid decline and a short duration, which was independent of stimulus strength. In addition, Shibuya sometimes observed an -off and an on-off response, which he attributed to two other types of receptor. He used decapitated preparations, deprived of eye-balls and facial muscles, and it is possible that this hardly credible hypothesis arose from inconsistent results from deteriorating preparations. Shibuya draws the interesting correlation between olfactory acuity, duration of EOG and colour of the olfactory mucosa. The eel, which has a highly sensitive olfactory sense, (see Page 25) had a very short duration EOG and darkly pigmented epithelium.

Suzuki and Tucker (1971) and Sutterlin and Sutterlin (1971) have used multi-unit recording with metal-filled glass microelectrodes to test the effectiveness of different odorous stimulants.

Sutterlin and Sutterlin found that spontaneous spike activity could be recorded between the secondary lamellae, but observed no activity at the edges of the primary or secondary lamella. This corresponds with the findings of Lowe and MacLeod (1975) that, in gadoid fish at least, the lamella edges are sparsely ciliated and lacking in receptor cells.

During chemical stimulation with low concentrations of amino acids, both Sutterlin and Sutterlin, and Suzuki and Tucker observed an increase in asynchronous spike activity. At very high concentrations of odorant a high amplitude oscillation developed (30-40Hz). Ottoson (1956) has seen a similar

oscillation superimposed on the EOG of the frog. The waves may arise as a result of intermittent synchronous activity in groups of receptors, or groups of nerve fibres, although transection of the ipsilateral olfactory nerve has no effect on the oscillation.

Suzuki and Tucker used only amino acids and small peptides as stimulants, but Sutterlin and Sutterlin employed a variety of simple and complex odours, including a series of amino acids. Amino acids as a class of compounds were highly stimulatory, but little or no response was seen to a variety of simple sugars, n-aliphatic acids, alcohols, or amino-substituted alcohols. The integrated responses of multi-unit activity increased linearly with the log of the concentration of the stimulating chemical. The most effective amino acids in these two studies were: L-alanine > DL-alanine > L-histidine > DL-serine > L-methionine > L-cystine, (Sutterlin and Sutterlin, 1971) and L-glutamine > L-methionine > L-alanine > L-aspartate > D-methionine > L-cysteine, (Suzuki and Tucker, 1971). No mention was made by Suzuki or Sutterlin of a slow mucosal potential. This can probably be attributed to their a.c. mode of recording, necessary to detect the fast asynchronous spike activity. Both Sutterlin and Suzuki stress that their reason for adopting the multi-unit approach was the difficulty of obtaining recordings from single units. The cells are extremely small and very closely packed, creating immense technical problems.

Shibuya (1960), and Sutterlin and Sutterlin (1971), have published records of single units recorded from the fish mucosa, which were both facilitated by olfactory stimulation. Sutterlin and Sutterlin observed also that spike amplitude was

increased by stimulation, although they may have recruited another cell closer to the recording electrode. Gesteland et al. (1963), have made a substantial advance by successfully recording from single receptors in the frog using specially constructed metal-filled microelectrodes. Thus so far there has been no significant physiological study on single receptors in the fish nose, and a study along the lines of Gesteland's is eagerly awaited.

B. ELECTRICAL ACTIVITY OF THE OLFACTORY NERVE

The extremely fine, unmyelinated fibres of the olfactory nerve have proved to be a stumbling block to successful electrophysiological studies of their activity, not only in fish, but in all vertebrates. Most studies have had to contend with recording asynchronous multi-unit activity (Beidler and Tucker, 1955; Mozell, 1962). Shibuya (1960) has presented the only report of spontaneous activity in the teleost olfactory nerve. Recording from *Channa*, he says this activity bears a relation to the slow mucosal potential. Shibuya also recorded a slow potential in the olfactory nerve which he claims represented the slow potential of the mucosa conducted by a passive electrotonus along the nerves. However, the poor cable properties of the olfactory nerve fibres together with evidence presented by Tucker (1963), using d.c. differential recording indicates that the slow nerve potential represents the summation of a lengthy barrage of many action potentials. The similarity with the EOG, which is generated by the cilia of the receptor cells, is coincidental (Ottoson, 1971).

The compound action potential of the fish olfactory nerve has been studied by Garten (1903), and Gasser (1956).

Using an in vitro, cocaine treated preparation of the pike (Esox lucius) olfactory nerve, Garten and Gasser recorded a negative monophasic wave with bipolar electrodes in response to a 1 msec. pulse. The duration of the compound potential was 30 msec., and the conduction velocity 0.2 m/sec. at 21°C. The conduction velocity in Raniceps ranius was found to be 0.12 m/sec. at 10°C by Doving (1967), and in the gar (Lepisosteus platyrhinous) 0.1 - 0.2 m/sec at room temperature by Easton (1965).

The lack of information on activity in the olfactory nerve is regrettable because it is at this level that comparisons with other sensory systems would be most useful, since the activity probably consists of entirely unmodulated impulses.

C. ELECTRICAL ACTIVITY OF THE OLFACTORY BULB

a) Slow bulbar potentials evoked by natural stimulation

A slow d.c. potential, resembling the EOG and slow nerve potential has been recorded from the olfactory bulb of the frog in response to chemical stimulation of the olfactory mucosa. (Leveteau and MacLeod, 1966); (Ottoson, 1954, 1959a, 1959b), Such a response, which is assumed to represent a pre-synaptic potential generated in the glomeruli, has not been observed in fish.

b) Slow bulbar potentials evoked by electrical stimulation

Evoked potentials, induced by stimulation of both afferent and efferent pathways have been recorded from the bulbar surface in several species of fish, Carassius auratus L. (Hara and Gorbman, 1967), Protopterus annectens O. (Orsini and Dupe, 1971), and Cyprinus carpio L. (Satou, 1971).

Afferent stimulation of either the olfactory nerve or olfactory mucosa evokes a negative-positive biphasic wave in Carassius, Protopterus and Cyprinus. Hara and Gorbman (1967) claim that the response is made up of two components. The first component, represented by a small inflection on the leading edge of the main wave, lacked a refractory period and summated to a sustained potential on repetitive stimulation. It was assumed to be of 'synaptic' origin. The second component had a refractory period of 30 msec. and was assumed to represent activity in second-order neurons. Transection of the ipsilateral olfactory tract removed the positive 'after potential', and caused a decline in threshold voltage. A conditioning shock applied to the ipsilateral nerve led to a reduction in amplitude of the main component, which did not return to normal until the two shocks were separated by 800 msec. Sectioning of the ipsilateral tract reduced this period to 400 msec. It was concluded that centrifugal tonic influences are normally exerted on the intrinsic excitability of the bulb via the medial bundle of the olfactory tract.

A negative-positive potential was evoked in the olfactory bulb of Cyprinus, (Satou 1971), by stimulation of the medial and lateral contralateral olfactory tracts and anterior commissure. The medial tract and anterior commissure potentials followed repetitive stimulation up to 50 Hz., whereas the potential evoked by stimulation of the contralateral lateral olfactory tract could only follow up to 10 Hz. Stimulation of the medial and lateral bundles of the ipsilateral olfactory tract evoked monophasic negative potentials, which could follow repetitive stimulation up to 50 Hz. The potential evoked by medial tract stimulation

consisted of two components. Stimulation of the contralateral nerve evoked a wave consisting of three positive components, the second and third of which vanished at frequencies as low as 1Hz.

Satou made no attempt to explain his findings and, as the quality of recording was so poor, direct comparisons with the wealth of literature on evoked potentials in the olfactory bulb of other vertebrates (Freeman, 1972d; Nicoll, 1969; Orrego, 1961; Ottoson, 1959; Phillips, Powell, Shepherd, 1963; Rall and Shepherd, 1968) will not be attempted at this stage.

c) Oscillatory potentials of the bulb

Spontaneous electroencephalographic (EEG) activity has been recorded from the olfactory bulb of several species of fish. Table I.1 summarizes the frequencies and amplitudes of this activity, together with the activity from other areas of the fish brain. The frequency of the intrinsic bulbar EEG varies from 2-16Hz., and the amplitude varies from 30-100µV.

When odorous substances are infused into the nasal chamber, the asynchronous EEG is replaced by a striking rhythmic oscillation of high amplitude (up to nearly 0.5mV). The frequency of this highly synchronous activity varies from 6-15Hz. This type of activity was first observed in the frog by Gerard and Young (1950), and named 'induced waves' by Adrian (1950), resulting from observations on the rabbit. The response is non-specific in that it can be induced by a remarkable variety of odorants varying from simple salts to highly complex mixtures, such as 'home-stream' water.

Oshima and Gorbman (1966) found three recognisable elements in the induced response to stimulation with 0.06M NaCl:

TABLE I.1a: Amplitudes and frequencies of spontaneous and evoked EEGs, recorded from the brains of fish

| SPECIES | REFERENCE | PREPARATION | SPONTANEOUS EEG FREQUENCY (Hz) | | | | | SPONTANEOUS EEG AMPLITUDE ($\mu V$) | | | | | EVOKED EEG (Olfactory Bulb) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | OB | TC | MC | C | M | OB | TC | MC | C | M | Frequency (Hz) | Amplitude ($\mu V$) |
| Carassius | Schadé | Decorporate | | 4-8<br>9-14 | 7-14<br>18-24 | 23-35<br>120-180 | 05-2<br>8-11<br>20-35 | | 40-70 | 60-180 | 20-50<br>5-15 | 10<br>10<br>10 | | |
| | Hara & Gorbman (1967) | Acute | 14-16 | | | | | 70-100 | | | | | 10-15 | 80-200 |
| | Adrian & Butendijk (1931) | in vitro | | | | | 1-3 | | | | | | | |
| Cyprinus carpio | Veda et al (1971) | Acute | | | | | | | | | | | 5.6-7 | |
| Gadus callarias | Enger (1959) | Chronic | | 8-13<br>4-6<br>2-3 | 8-13 | | 14-32 | | 160 | 20-160 | | 5-15 | | |
| Oncorhynchus nerka | Hara, Veda, Gorbman (1965) | Acute | 7-9 | | | 10-12 | | 35-65 | | | 25-40 | | | 80-250 |
| | Veda et al (1971) | Acute | 7-9 | | | | | 30-60 | | | | | 6-8 | 80-150 |
| Salmo gairdneri | Hara (1973) | Acute | 2-6 | | | | | 30-80 | | | | | 7-11 | 150-350 |
| | Veda et al (1971) | | | | | | | | | | | | 7-10 | |

TABLE I.1b

| SPECIES | REFERENCE | PREP-ARA-TION | SPONTANEOUS EEG FREQUENCY (Hz) | | | | | SPONTANEOUS EEG AMPLITUDE ($\mu V$) | | | | | EVOKED EEG (OLFACTORY BULB) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | OB | TC | MC | C | M | OB | TC | MC | C | M | Frequency (Hz) | Amplitude ($\mu V$) |
| Salmo salar | Sutterlin & Sutterlin (1971) | Acute | | | | | | | | | | | 8 - 12 | |
| Salmo alpinus | Døving et al (1973) | Acute | | | | | | | | | | | 7 | |
| Negaprion brevio-stris | Gilbert et al (1964) | Chronic | | 4-9 | 5-11 | | 1-2 | | 30-60 | 30-170 | 20-45 | 150-200 | ↑ | ↑ |
| Protop-terus | Dupé (1968) | Chronic | | 11-13<br>6-7 | | | | | 50-70 | | | | 12-13 | 150-200 |

Upward-pointing arrows indicate an increase in magnitude of the relevant parameter.

| | Frequency (Hz) | Duration (sec.) | Amplitude (µV) |
|---|---|---|---|
| (i) primary spindle-shaped burst | 10-15 | 2-6 | 80-200 |
| (ii) secondary brief phase | 4- 8 | - | 40- 90 |
| (iii) after-response | 9-13 | 40-120 | 50 |

(ii) and (iii) were abolished by cutting the olfactory tract. They found also that the response varied with the depth of the recording bipolar electrode, reaching a maximum amplitude between 100-300 µm below the surface.

Various authors have effectively used the 'induced waves', as a physical measure of the ability of migrating salmon to recognise their home-stream water, Hara (1965, 1970, 1971); Ueda, Hara and Gorbman (1967); Ueda, Hara, Satou and Kaji (1971), and Oshima, Hara and Gorbman (1969). The electrical response is specific in that it cannot be evoked by water from spawning sites of other groups of breeding salmon, and that the salmon respond clearly to water taken from places along their migratory routes below the spawning site. The nature of the substances responsible for this specificity is unknown, but an experiment by Oshima et al. (1967) suggests that the response must be partly elicited by a substance released from members of the same species already present at the spawning site. Døving et al. (1973), working with the char (Salmo alpinus), suggests that the response may be pheromone induced.

The magnitudes of these induced responses are usually measured by electronic integration. Hara (1973) has used this technique to compare the effectiveness of various amino acids as olfactory stimulants in Salmo gairdneri. The eight most effective amino acids were L-glutamine 〉 L-methionine 〉 L-leucine 〉 L-asparagine 〉 L-alanine 〉 L-cystine 〉 glycine 〉 L-serine. The

threshold concentrations were between $10^{-7}$ and $10^{-8}$ M for the most effective amino acids. Hara noticed that the D-isomer of an amino acid was always a less effective stimulant than its L-isomer. The relative effectiveness of amino acids as stimulants was shown to depend on the relative positions of the amino and carboxyl groups.

Hara (1973), has since adopted a more sophisticated approach to the analysis of induced waves. Spectral analysis of the frequency components in the response revealed that the spontaneous activity was predominated by low frequency components (2-6Hz) and that this shifted to high frequency components (7-11Hz) upon stimulation. Further, the peak frequency pattern was characteristic of each chemical tested and multipeak spectra were obtained for complex stimuli. It appears from Hara's work that the intrinsic frequencies of the induced wave may be of extreme importance in coding odour quality.

d) Unitary activity of the bulb

Table I.2 summarizes the available information on spontaneous activity of individual neurons in the teleost olfactory bulb. Although the authors have made guesses regarding the nature of the recorded units, there has been no attempt to accurately establish their identity. It is, however, probable that the large, biphasic action potentials reported at a depth of 300-400 μm by Hara (1967a), represent the spontaneous discharges of mitral cells. The mean firing rate of 'bulbar neurons' varies from 0.1 to 25Hz. About 10% of bulbar units appear to fire in bursts, whence the intraburst frequency may reach 50Hz (Døving, 1965).

**TABLE I.2: Spontaneous activity in bulbar neurons**

| SPECIES | REFERENCE | RECORDING DEPTH ($\mu m$) | AUTHOR'S NOMENCLATURE OF | | TYPE OF ACTIVITY | SPIKE FREQUENCY Hz | SPIKE AMPLITUDE |
|---|---|---|---|---|---|---|---|
| | | | Region | Cell Type | | | |
| Carassius auratus | Oshima & Gorbman (1966b) | 350-500 | Medullary | I) B) Mitral? | Irregular Bursting | 2.4 | low |
| | | 50-200 | Glomerular | G-Glomerular | Regular | 2.1 | high |
| | Hara (1967a) | 50-100 | Olfactory nerve | nerve fibres | Irregular | | low |
| | | 300-400 | External plexiform layer | short axon cells Mitral | Regular | 2-6 | high |
| Lota lota | Døving (1965) | - | - | Secondary neuron 10% | Irregular Bursting | 0.1 - 25 (mean 4.2) 50 | - |
| | Døving (1969) | - | - | Secondary neuron | Regular | 0.56 - 10 | - |
| Salmo alpinus | Døving et al (1973) | 600 | - | Secondary neuron | Regular | 0.1 - 10 | - |

Hara (1967a), showed that different individual neurons respond in different ways to chemical stimulation of the olfactory mucosa, finding: (1) Inhibition during or after the period of stimulation, (2) facilitation which could outlast the duration of stimulation for several seconds, (3) facilitation during the stimulus followed by a short inhibition when stimulation ceased, (4) facilitation at the beginning and inhibition at the end of the stimulation period, (5) a short inhibition at the onset of stimulation followed by facilitation, (6) no response. More than 60% of the neurons tested were of types (1) and (3), which represent opposite patterns of response. Analagously with studies on other vertebrates (Døving, 1965; Matthews, 1972a, 1972b; Walsh, 1956), Meredith (1974), has shown in a preliminary study that low concentration of odorant (amino acids in this case), facilitate, inhibit or produce no response in goldfish bulbar neurons. Units excited by low stimulus concentrations were often inhibited by higher concentrations (see also Boudreau, 1962). According to Meredith, his results suggest that across fibre temporal patterns may be important in coding odour quality (chemical structure), and odour quantity (concentration). However, in a statistical analysis of the firing pattern of bulbar units in response to chemical stimulation (Døving & Hyvärinen, 1969), individual units retained their activity patterns under a variety of experimental conditions, implying that temporal coding of specific impulse sequences is relatively unimportant. It is, however, difficult to draw any realistic conclusions from these experiments as the cells have not been identified. In any case, a wealth of experimental evidence from other vertebrates is against the idea of temporal

coding (MacLeod by personal communication).

Bulbar units can be antidromically driven at frequencies of up to 50Hz by electrical stimulation of the ipsilateral olfactory tract (Doving, 1965). A period of inhibition usually followed the stimulus; in fact inhibition was found to be the most common consequence of stimulating either the contralateral or ipsilateral olfactory tract (Doving, 1965; Hara, 1967a). Stimulation of the ipsilateral olfactory nerve (orthodromic) was generally excitatory, often producing a burst of spikes having an extremely variable latency. The induced afferent spike(s) could often be blocked by an efferent conditioning shock. This inhibition could result from either the activation of centrifugal fibres or the antidromic activation of the secondary neurons, or both. According to Eccles (1955), antidromic inhibition in motoneurons could be caused in a number of ways: (1) refractory period, (2) positive after potential, (3) synaptic feedback mechanisms or (4) direct collateral inhibition. Using chronically prepared rabbits in which the tract centrifugal fibres had degenerated, Green et al. (1962), postulated that mitral cell inhibition was mediated via recurrent collaterals acting directly. It is now generally concluded that for the mammalian olfactory bulb Eccles' third postulate is the correct one, the granule cells acting as inhibitory interneurons (Freeman, 1972d; Nicoll, 1969, 1972; Phillips, Powell, Shepherd, 1963; Shepherd, 1963b; Rall and Shepherd, 1968; Yamamoto et al. 1963). Hara (1967a), contends that the granule cell layer is poorly developed in teleosts and finds it difficult to accommodate his results with the existence of such an interneuronal system in fish. He postulates prolonged action of an

inhibitory transmitter substance to account for the long time-course of the observed inhibition. It is very difficult to accept the credibility of Hara's conclusion, particularly as in the same discussion he completely contradicts this idea, stating: "Coincident with this lower degree of segregation of glomerular transmission, the elaboration of intrabulbar associational system also seems to be less elaborately developed in fishes, which have relatively few or no granule cells and recurrent collaterals of the mitral cells. The complexity of the responses of the second-order neurons observed here can be explained on the basis of the various feedback patterns which are known in other vertebrates, (Kerr and Hagbarth, 1955: Baumgarten et al., 1962; and Yamamoto et al., 1962)". Døving (1965), while admitting the presence of granule cells in the fish olfactory bulb, observed no rhythmic firing in response to efferent stimulation (Yamamoto et al., 1963). He attributed the observed inhibition to the direct monosynaptic influence of centrifugal fibres on the secondary neurons.

In conclusion, all that can be said is that our knowledge concerning the activity and intrinsic connections of neuronal systems in the teleost olfactory bulb is very limited indeed. It is this lack of knowledge which provided the starting point for the present thesis.

## D. ELECTRICAL ACTIVITY OF THE OLFACTORY TRACT

In the majority of teleostean families, the olfactory bulbs are found closely apposed to the cerebral hemispheres. However, in the Gadidae and Cyprinidae the olfactory tracts are extremely long, forming discrete nerve bundles between the bulb

and telencephalon (Figure III.1). Furthermore, it is often possible to distinguish separate medial and lateral bundles. This latter type of anatomical arrangement has long been regarded as a very convenient preparation by electrophysiologists, since pioneered by Adrian and Ludwig in 1937. Analysis of afferent electrical activity in the tract, and comparison with activity in the nerve or mucosa, should provide an excellent indication of how the bulb processes the sensory information.

a) Multi-unit activity

The multi-unit approach has been applied by Adrian and Ludwig (1937), and Boudreau (1962). Boudreau, recording the electronically integrated activity, showed that increases in tract activity could be produced by dilute concentrations of various chemicals. The questionable thresholds for acetic acid and butanol were $10^{-13}$M and $10^{-15}$M respectively. Millimolar concentrations led to a decrease in tract activity. Both reports indicated that mechanical stimulation of the mucosa has an effect on tract activity.

b) Single unit activity

Successful recordings from single units have been documented by Døving (1966c), Døving and Gemne (1965), and Nanba et al. (1966). The frequency of spike discharge in afferent fibres of the burbot olfactory tract varied from 1-9Hz with a mean frequency of 3.9Hz (Døving, 1966c). In a later study Døving and Hyvärinen (1969) reported that the activity pattern of afferent tract fibres oscillated periodically, and that the cycle frequency (0.1 - 1.5 per sec.) was not related to the actual firing rate of the cell. The interval histograms for tract

fibres were often skewed and bimodal, indicating high frequency bursting activity, which was also observed by Nanba et al. It is interesting to note that interval histograms of bulbar units rarely indicated bursting activity.

The activity of single fibres is influenced by chemical stimulation of the olfactory mucosa (Døving, 1966c; Nanba et al. 1966): most of the chemicals tested evoked different responses from different units. 30% of Døving's stimuli produced increased activity; 20% caused inhibition, while about half the stimulations failed to produce any change in firing rate. Nanba et al., working on *Carassius auratus*, and *Abramis brama*, found that individual fibres showed different patterns of activity when different odours were employed, thus implying temporal as well as spatial coding of odour quality (chemical structure). By detailed statistical analysis of impulse sequence, Døving and Hyvärinen (1969), found that individual fibres retained essentially the same activity pattern under a variety of experimental conditions. This is in direct contradiction to Nanba's findings and suggests that odour quality is not coded temporally by alterations in the pattern of impulse sequence of individual cells. Thus it seems unlikely that temporal patterns of activity in tract fibres are of prime importance in quality coding.

c) The compound action potential of the olfactory tract

A careful analysis of electrically evoked waves in the olfactory tract of several teleosts has been reported by Døving and Gemne (1965) and Døving (1967). The compound action potential consists of three components with peak velocities in the ranges 0.8 - 5.5, 0.5 - 2.4, and 0.13 - 0.25 m/sec. at 10°C,

for the first, second and third components respectively. The first two components can be recorded from all portions of the tract and are associated with non-myelinated fibres. The third component was relatively large and present in the medial bundle of the tract. In Ostariophysians it was also present in the lateral bundle. The slow component is thought to be generated by non-myelinated fibres, which terminate on neurosecretory cells in the pre-optic nucleus (Jasinski et al., 1966: Kandel, 1964).

E. EFFERENT SYSTEMS TO THE BULB

Nervous activity ascending in certain sensory pathways can be directly controlled by influences originating in the central nervous system. One of the best established examples of such a 'centrifugal system' is the efferent gamma control of muscle spindles (Granit and Kaada, 1952). It is likely that the olfactory bulbs possess two such systems (Allison, 1953; Cajal, 1911; Sheldon, 1912), referred to by Cajal as the thin and the thick fibre systems. The former interconnects the two bulbs via the anterior commissure, and the latter projects bi-laterally to both bulbs from the rhinencephalic regions of both hemispheres.

The electrophysiological evidence is far less easily interpretable. In mammals, recurrent inhibition can be generated directly by antidromic stimulation of the mitral cell axons (Green et al., 1962; Nicoll, 1969; Phillips et al., 1963) and should not be excluded as a possible mechanism for the generation of inhibition in the fish olfactory bulb. The available evidence for efferent control comes from two separate approaches; namely, examining the effect on afferent inflow of

removing possible caudal influences by transecting the brain at various more posterior levels and, secondly, by examining the effect of electrical stimulation of various brain areas.

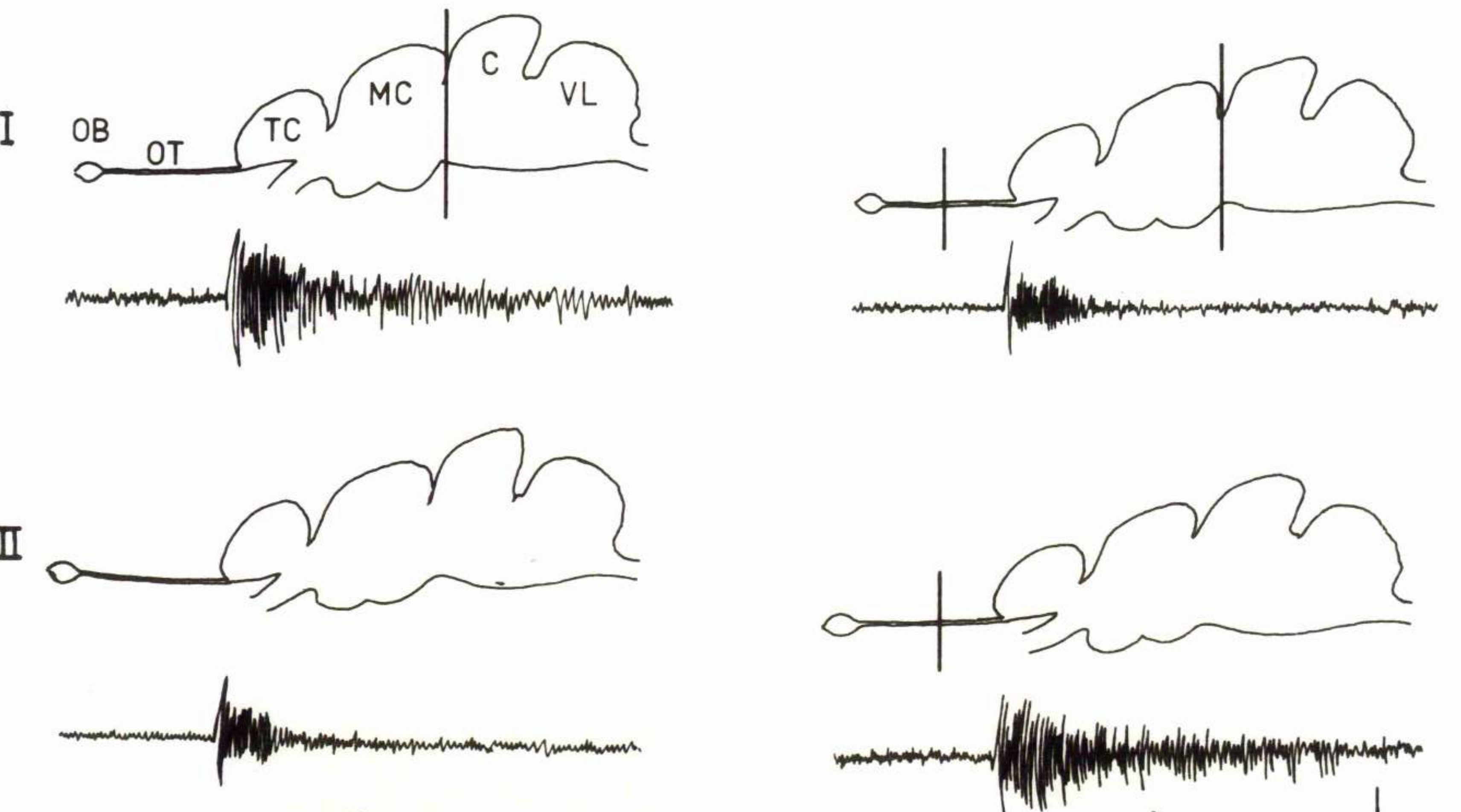
Hara and Gorbman (1967) have shown that electrical stimuli or strong chemical stimuli applied to the opposite bulb generally have a strong depressive influence on intrinsic and afferent induced activity in the ipsilateral bulb. Stimulation of the anterior commissure reduced these effects. It is assumed that, as in other vertebrates, the contralateral influence would be removed by transection of the anterior commissure (Callens, 1965), but this experiment does not appear to have been performed on fish.

Working with <u>cerveau isolé</u> goldfish, Oshima and Gorbman (1966a) found that transection of the ipsilateral olfactory tract led to an immediate reduction of the bulbar response induced by chemical stimulation of the olfactory mucosa with NaCl. They concluded that more posterior parts of the brain exert a facilitatory influence on the bulbar response. One year later, Gorbman, now working with Hara (Hara and Gorbman, 1967) on normal goldfish reported without quoting his earlier paper, that transection of the ipsilateral tract caused a marked augmentation of the bulbar response to NaCl, and concluded that the olfactory and centrifugal system exerts a tonic inhibitory influence on the olfactory bulb of the goldfish. The 1966 paper was similarly left unquoted by Bernstein (1970) in a review. Hara (1970), in another review, acknowledged Oshima's paper but makes no attempt to explain the obvious discrepancy. The two situations are summarized in Figure I.2; The extremely complimentary reciprocity is obvious. It is well known

(Mancia et al., 1962b) that in mammals the reticular formation exerts a complex influence on the olfactory bulb, such that the effects of afferent stimulation are often reversed. Oshima and Gorbman's data can possibly be explained, if it is assumed that the midbrain-hindbrain transection removes a strong reticular inhibitory influence. This would result in a 'normal response' of increased intensity, under the influence of a more rostral facilitatory input. Another partial explanation could lie in the observation made by Godet et al. (1964, 1965) that the sensitivity of the olfactory responses varies in different phases of the estivation cycle. Thus it may be important always to communicate the sexual state of experimental animals and the time of year when the experiments were performed.

Hara and Gorbman's result is more in line with the generally held dogma that the telencephalon exerts a bilateral tonic inhibitory influence on the olfactory bulbs. Transection of the olfactory tract also augmented the efferent induced activity and reduced the threshold for electrically evoked potentials in the bulb. Tract section also removed the positive 'after-potential' component of the evoked wave. In mammals the long duration surface positivity is thought to reflect summated IPSPs in the mitral cell population (Nicoll, 1972). Stimulation of the posterior telencephalon was found to be depressive, whilst stimulation of the preoptic area appeared to be slightly facilitatory (Hara and Gorbman, 1967). A slight diencephalic facilitatory influence on the frog's olfactory bulb was noted by Takagi (1962) but inhibition was by far the most important effect. No ascending diencephalic-bulbar pathway has been found in fish (Sheldon, 1912). It is possible that strong inhibition

Figure I.2 The effect of olfactory tract section on bulbar induced waves




I - taken from Oshima and Gorbman (1966a), II - taken from Hara and Gorbman (1967); a - before olfactory tract section, b - after olfactory tract section. OB - olfactory bulb; OT - olfactory tract; TC - telencephalon; MC - mesencephalon; C - cerebellum; VL - vagal lobe. Calibration - vertical 50 uV, horizontal 1 sec.

observed in the frog by Takagi resulted from the antidromic activation of mitral cell axons projecting to the hypothalamus. Slow fibres in the goldfish olfactory tract are known to orthodromically activate preoptic neurosecretory cells (Jasinski et al., 1968; Kandel, 1964).

Hara (1967a) and Døving (1966b) have shown that both facilitation and inhibition of bulbar neurons can be elicited by stimulating the olfactory tract but the most far-reaching and commonly occurring effect is inhibition. In conclusion, it appears that, in teleosts, centrifugal fibres in the olfactory tract, which arise from the telencephalon and possibly the reticular formation, exert strong inhibitory influences on the afferent induced activity of the olfactory bulb. Afferent activity is also modified by efferent fibres from the contralateral bulb and, to a lesser extent, by telencephalic-diencephalic facilitation. In this context it is interesting to note the observation by Døving and Gemne (1966) that efferent activity to the bulb can be inhibited by touching the skin.

## F. INFLUENCE OF HORMONES ON THE ELECTRICAL ACTIVITY OF THE OLFACTORY SYSTEM

A reciprocal relationship between the olfactory and endocrine system has been shown using electrophysiological techniques. Kandel (1964) was able to drive neurosecretory neurons in the goldfish preoptic nucleus orthodromically by stimulation of the olfactory tract. Jasinski et al. (1966) have demonstrated that stimulation of the goldfish olfactory tract also effectively depletes hypothalamic neurons of neurosecretory granules.

On the other hand, Hara (1967b), Oshima and Gorbman (1966) and Dupé (1968) have shown that sex steroids and thyroid hormones have a direct influence on both global and unitary electrical activity in the bulb. Oshima and Gorbman (1966) believe that thyroxine inhibits the telencephalic centrifugal (facilitatory) action upon the NaCl evoked bulbar responses, but has a local facilitatory effect on the bulb. In *Protopterus* (Dupé, 1968), EEG arousal in the primordium hippocampi to olfactory stimulation appears to be dependant upon the thyroid state of the animal. The response is lacking in thyroidectomized and encysted fish but can be induced by injections of thyroid hormone. Godet and Dupé (1964) believe that a thyroid sensitive site may exist in the diencephalon which arouses the hippocampal region to a threshold level of activity when stimulated by thyroxin. In *Protopterus* (Godet, 1964), the sensitivity of the olfactory response varies during the estivation cycle. He believes that arousal depends on the reactivity of telencephalic tissues as much as the bulb or receptor. The reactivity of the tissue may be under the direct influence of thyroid hormone on the dendritic fields of telencephalic and bulbar neurons (Godet, Personal Communication).

Hara and Gorbman (1967) observed a slight facilitation of the bulbar response by repetitive electrical stimulation of the preoptic area. Takagi has presented extensive evidence for a bilateral diencephalic-olfactory bulbar inhibitory centrifugal system.

These findings are of extreme importance. As a result of these connections, reproduction, growth, metabolic rate, glucose metabolism and ion balance and transport may be under

the direct influence of the olfactory system in fish.

## G. WHAT CAN FISH SMELL?

The primary information pattern in olfaction is the result of a physical interaction between a population of odorant molecules and a population of receptor sites of molecular dimensions, followed by summation of the resultant energy effects in the receptor cell. Table I.3 presents a list of generally complex substances which have been utilised in experiments on the olfactory sense of fish. However, all properties of a compound, physical, chemical and physiological are determined by the complete definition of its molecular structure. It is obvious that most of the compounds listed in Table I.3 represent ill-defined and unreproducible mixtures, which are of little use in a detailed analysis of primary olfactory interactions. These substances, however, remain of general and historic interest. An infinitely more useful approach is to compare the stimulatory effectiveness of pure single compounds. Thus, Table I.4 presents a list of compounds which have been used by various authors (see Table for references) as olfactory stimulants on fish.

The odorant molecules are presented to the receptor sites in a range of orientations and conformations (flexible molecules only), which are randomly distributed over the olfactory mucosa. The composition of this range will be mainly determined by the nature, position and environment of the functional group(s). The statistical composition of the resulting orientation pattern and steric characteristics of the predominating profiles are probably the major criteria for primary

TABLES I.3 and I.4: Responses of fish to complex and simple olfactory stimuli

SPECIES OF FISH REFERRED TO

a) Salmo gairdneri
b) Salmo salar
c) Salvelinus fontinalis
d) Oncorhynchus nerka
e) Oncorhynchus kisutch
f) Corregonus clupeaformis
g) Ictalurus cattus
h) Carassius auratus
i) Abramis brama
j) Lota lota
k) Cyprinus carpio
l) Phoxinus phoxinus
m) Anguilla anguilla
n) Rutilus rutilus
o) Hyborhynchus notatus
p) Negaprion brevirostris
q) Sphyrna tibura
r) Ginglymostoma cirratum
s) Salmo alpinus
t) Carcharhynus melanopterus
u) Carcharhynus menisorrah

Adrian & Ludwig (1939)
Boudreau (1962)
Døving (1966)
Døving et al (1973)
Hara (1972a, 1973)
Hara et al (1973)
Hasler (1957)
Marcström (1959)
Miesner & von Baumgarten (1966)
Nanba et al (1966)
Sutterlin & Sutterlin (1971)
Suzuki & Tucker (1971)
Tarrant (1966)
Teichmann (1959)
Tester (1963)

MOLECULAR VOLUME

$$MV = \frac{MW}{D \times N} \quad Å^3$$

MW = Molecular weight

D = Specific gravity

N = Avagadro's number

SOLUBILITIES

∞ - infinitely soluble

v - very soluble

s - soluble

d - slightly soluble

i - insoluble

RESPONSES

+ positive or negative response to odour

o no response

$[\alpha]_D$ = specific rotation

$\mu$ = Dipole moment (debyes)

THRESHOLDS

in molarities

TABLE I.3: Responses of Fish to Complex Olfactory Stimuli

| STIMULUS | FISH | PHYSIOLOGICAL RESPONSE | BEHAVIOURAL RESPONSE |
|---|---|---|---|
| Aqueous extract of fresh | | | |
| saliva | b,g | + | |
| frog muscle | g | o | |
| catfish muscle | g | o | |
| catfish skin | g | o | |
| earthworms | g | + | |
| liver | b,g | + | |
| blood | g,t,u | + | + |
| gelatine | g | + | |
| fish pellets | b | + | |
| Aqueous extract of decayed | | | |
| earthworms | g | + | |
| frog muscle | g | + | |
| liver | g | + | |
| head of alligator | g | + | |
| gelatine | g | + | |
| blood | g,t,u | + | + |
| Stagnant water | b | + | |
| Decoction of aniseed | g | + | |
| Decoction of ants' eggs | g | + | |
| Emulsion of oil of cloves | g | o | |
| " " oil of cedar | g | o | |
| " " oil of rhodian | g | o | |
| Tincture of asafoetida | g | o | |
| " " valerin | g | o | |
| Rosewater | g | o | |
| Starch solution | g | o | |
| Suspension of cheese | g | o | |
| Human hand-rinse | b,n,o,a | + | + |
| Crab extract | p,q | + | |
| Tuna extract | p,t,u | + | + |
| Water from tank containing same species | e,f,s | + | |
| Water from tank containing different species | e,f | + | |
| Home-stream water | e,f | + | |
| Aged eel extract | t,u | | + |
| Aged shark extract | t,u | | + |
| Human sweat (cf. 'hand-rinse') | t,u | | + |

FIGURE I. 4.a

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\;]_D$ μ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 1 | L-Glutamine | $H_2N.CO.CH_2.CH_2.CH(NH_2)COOH$ | 146.2 | | +6.3 | 2.17<br>9.13 | s | i | a,g. | | + | $10^{-8}-10^{-9}$ | | |
| 2 | L-Methionine | $CH_3.SCH_2.CH_2.\ CH(NH_2)COOH$ | 149.2 | 184.8 | -10.0 | 2.13<br>9.28 | v | i | a,b,c,d,<br>e,f,g. | | + | $10^{-8}-10^{-9}$ | | |
| 3 | L-Leucine | $(CH_3)_2CH.CH_2.CH(NH_2)COOH$ | 131.2 | 168.5 | -11.0 | 2.33<br>9.74 | s | i | a,b,c,f,g. | | + | | | |
| 4 | Homoserine | $HOCH_2.CH_2.CH(NH_2)COOH$ | 119.1 | | | 2.71<br>9.61 | v | i | a,c,f. | | + | | | |
| 5 | L-Asparagine | $H_2N.CO\ CH_2.CH(NH_2)COOH$ | 132.1 | 142.1 | -5.6 | 2.10<br>8.84 | s | i | a,g. | | + | | | |
| 6 | L-Alanine | $CH_3.CH(NH_2)COOH$ | 89.1 | 103.3 | +1.8<br>μ=13.3 | 2.35<br>9.87 | v | i | a,b,c,d,e,<br>f,g. | | + | $3.2x10^{-9}$<br>(b) | | |
| 7 | L-Cystine | $[-SCH_2.CH(NH_2)COOH]_2$ | 240.3 | 237.9 | -232<br>(HCl) | <1<br>2.10<br>8.02<br>8.71 | d | i | a,b,c,f,g. | | + | | | |
| 8 | L-Cysteine | $HS.CH_2.CH(NH_2)COOH$ | 121.2 | | -16.5 | 1.92<br>8.35<br>10.46 | v | i | a | | + | $10^{-7}$ | | |
| 9 | L-Glycine | $H_2N.CH_2.COOH$ | 75.1 | 150.6 | μ=17.5 | 2.35<br>9.78 | v | i | a,b,c,f,g. | | + | $10^{-7}$ | | |
| 10 | L-Serine | $HO.CH_2.CH(NH_2)COOH$ | 105.1 | 108.9 | -7.5 | 2.19<br>9.21 | s | i | a,b,c,d,<br>e,f,g. | | + | $10^{-7}$ | + | $8x10^{-10}$ |
| 11 | L-Histidine | (imidazole ring, HN N) $.\ CH_2.CH(NH_2)COOH$ | 155.2 | | -38.5 | 1.80<br>6.04<br>9.33 | s | i | a,b,c,f,g. | | + | $10^{-7}$ | | |
| 12 | L-Threonine | $CH_3.CH(OH).CH(NH_2)COOH$ | 119.1 | | -28.5 | 2.04<br>9.10 | s | i | a,b,c,f,g. | | + | $2.5x10^{-6}$<br>(b) | | |
| 13 | L-Valine | $(CH_3)_2.CH.CH(NH_2)COOH$ | 117.2 | 158.2 | +5.6 | 2.24<br>9.74 | s | d | a,b,c,f,g. | | + | | | |
| 14 | GABA | $H_2N.CH_2.CH_2.CH_2.COOH$ | 103.1 | | | 4.03<br>10.56 | v | i | a,b,c,f. | | + | | | |
| 15 | L-Glutamic acid | $HOOC.CH_2.CH_2.CH(NH_2)COOH$ | 147.1 | 158.7 | +12.0 | 2.10<br>4.07<br>9.47 | d | i | a,c,f,g,j. | | + | | | |

FIGURE I. 4.b

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\alpha]_D$ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 16 | L-Lysine | $H_2N.(CH_2)_4.CH(NH_2)COOH$ | 146.2 | | +13.5 | 2.16<br>9.18<br>10.79 | v | i | a,c,f. | | + | | | |
| 17 | D-Cysteine | $HS.CH_2.CH(NH_2)COOH$ | 121.2 | | +16.5 | 1.92<br>8.35<br>10.46 | v | i | a. | | + | | | |
| 18 | Isoleucine | $CH_3.CH_2.CH(CH_3).CH(NH_2)COOH$ | 131.2 | | 12.4 | 2.32<br>9.76 | | | a,b,c,f,g. | | + | | | |
| 19 | L-serine methyl ester | $HOCH_2.CH(NH_2)COOCH_3$ | 106.8 | | | 7.10 | | | a. | | + | | | |
| 20 | L-Arginine | $H_2N.C(=NH).NH(CH_2)_3.CH(NH_2)COOH$ | 174.2 | | +12.5 | 1.82<br>8.99<br>12.48 | s | i | a,b,c,f. | | + | | | |
| 21 | D-Serine | $HOCH_2.CH(NH_2)COOH$ | 105.1 | 108.9 | +1.5 | 2.19<br>9.21 | s | i | a,b,c,d,<br>e,f,g. | | + | | | |
| 22 | L-Phenyl-alanine | $C_6H_5.CH_2.CH(NH_2)COOH$ | 165.1 | | -34.5 | 2.16<br>9.18 | s | i | a,c,f. | | + | | | |
| 23 | L-Tyrosine | $HO.C_6H_4.CH_2.CH(NH_2)COOH$ | 181.2 | | -10.0<br>(HCl) | 2.20<br>9.11<br>10.13 | s | i | a,c,f. | | + | | | |
| 24 | L-Aspartic acid | $HOOC.CH_2.CH(NH_2)COOH$ | 133.1 | 133.0 | +5.1 | 1.99<br>3.90<br>9.90 | d | i | a,c,f,g. | | + | | | |
| 25 | D-Lysine | $H_2N.(CH_2)_4.CH(NH_2)COOH$ | 146.2 | | -13.5 | 2.16<br>9.18<br>10.79 | v | i | a. | | + | | | |
| 26 | N-Dichloro-acetyl, L-Serine | $HOCH_2.CH(NH.CO.CHCl_2)COOH$ | 203.9 | | | | | | a. | | + | | | |
| 27 | D-Cycloserine | $H_2N.$ (ring structure with O, =O) | 86.0 | | | | | | a. | | + | | | |
| 28 | Isoserine | $H_2N.CH_2.CH(OH)COOH$ | 105.1 | | -32.6 | 2.72<br>9.33 | s | i | a,c,f. | | + | | | |
| 29 | D-Alanine | $CH_3.CH(NH_2)COOH$ | 89.1 | 103.3 | -1.8 | 2.35<br>9.87 | v | i | a,b,c,d,<br>e,f,g.. | | + | | | |
| 30 | D-Methionine | $CH_3.SCH_2.CH_2.CH(NH_2)COOH$ | 149.1 | 184.8 | +10.0 | 2.13<br>9.28 | v | i | a,d,e,g. | | + | | | |

FIGURE I. 4.c

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\alpha]_D$ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 31 | β-Alanine | $H_2N.CH_2.CH_2.COOH$ | 89.1 | 109.6 | | 3.55<br>10.24 | s | i | a, c, f. | | + | | | |
| 32 | D-Phenyl-alanine | $C_6H_5$ ring $CH_2.CH(NH_2)COOH$ | 105.1 | | +34.5 | 2.16<br>9.18 | s | i | a | | + | | | |
| 33 | D-Histidine | [HN ring N] $\cdot CH_2.CH(NH_2)COOH$ | 155.2 | | +38.5 | 1.80<br>6.04<br>9.33 | s | i | a | | + | | | |
| 34 | D-Tyrosine | $CH_2.CH(NH_2)COOH$ | 181.2 | | +10.0<br>(HCl) | 2.20<br>9.11<br>10.13 | s | i | a | | + | | | |
| 35 | D-Arginine | $HN=C(NH_2).NH.(CH_2)_3CH(NH_2)COOH$ | 174.2 | | -12.5 | 1.82<br>8.99<br>12.48 | s | i | a | | + | | | |
| 36 | L-Trytophane | [ring NH] $CH_2CH(NH_2)COOH$ | 204.2 | | -33.7 | 2.43<br>9.44 | d | i | a | | + | | | |
| 37 | D-Asparagine | $H_2N.CO.CH_2.CH(NH_2)COOH$ | 132.1 | 142.1 | +5.6 | 2.1<br>8.84 | s | i | a, g. | | + | | | |
| 38 | D-Cystine | $[-S.CH_2.CH(NH_2)COOH]_2$ | 240.3 | 237.9 | +232<br>(HCl) | 1<br>2.1<br>8.02<br>8.71 | d | i | a | | + | | | |
| 39 | L-Proline | [ring N H] COOH | 115.1 | | -86.2 | 1.95<br>10.64 | v | i | a, b, g. | | + | | | |
| 40 | D-Glutamic acid | $HOOC.CH_2.CH_2.CH(NH_2)COOH$ | 147.1 | 158.7 | -12.0 | 2.10<br>4.07<br>9.47 | d | i | a | | + | | | |
| 41 | D-Glutamine | $H_2N.CO.CH_2.CH_2.CH(NH_2)COOH$ | 146.2 | | -6.3 | 2.17<br>9.13 | s | i | a, g. | | + | | | |
| 42 | D-Trypto-phane | [ring N H] $CH_2.CH(NH_2)COOH$ | 204.2 | | +33.7 | 2.43<br>9.44 | d | i | a | | + | | | |
| 43 | D-Leucine | $(CH_3)_2.CH.CH_2.CH(NH_2)COOH$ | 131.2 | 168.5 | +11.0 | 2.33<br>9.74 | s | i | a, g. | | + | | | |
| 44 | D-Threonine | $CH_3.CHOH.CH(NH_2)COOH$ | 119.1 | | +28.5 | 2.09<br>9.10 | s | i | a, b. | | + | | | |
| 45 | Hydroxy-L-Proline | [HO ring NH] COOH | 131.1 | | -76.0 | 1.82<br>9.66 | v | i | a, b. | | + | | | |

FIGURE I. 4.d

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\alpha]_D$ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 46 | D-Valine | $(CH_3)_2CH.CH(NH_2)COOH$ | 117.2 | 158.2 | -5.6 | 2.29<br>9.74 | s | d | a, b. | | + | | | |
| 47 | Taurine | $H_2N.CH_2.CH_2.SO_3H$ | 125.2 | | | 0.3<br>9.7 | s | i | a, b, j. | | + | | | |
| 48 | D-Alloiso-leucine | $CH_3.CH_2.CH(CH_3).CH(NH_2)COOH$ | 131.2 | | -15.9 | 2.32<br>9.76 | s | i | a | | + | | | |
| 49 | D-Aspartic acid | $HOOC.CH_2.CH(NH_2)COOH$ | 133.1 | 133.0 | -5.0 | 1.99<br>3.90<br>9.90 | | i | a | | + | | | |
| 50 | D-Proline | (ring N, H) COOH | 115.1 | | -86.2 | 1.95<br>10.64 | s | i | g | | + | | | |
| 51 | Glutathione | $CO.NH.CH.CO.NH.CH_2.COOH$<br>$(CH_2)_2$ $CH_2$<br>$CH.NH_2$ SH<br>COOH | 307.3 | | 21.3 | 2.12<br>3.59<br>8.75<br>9.65 | v | i | j | | + | | | |
| 52 | Glycyl-glycine | $H_2N.CH_2.CO.NH.CH_2.COOH$ | 132.1 | | | 3.14<br>8.25 | s[h] | i | a | | o | | | |
| 53 | Triglycine | $H_2N.CH_2CO$ $HN.CH_2COOH$<br>$HN.CH_2CO$ | 189.2 | | | 3.23<br>8.09 | s | i | a | | o | | | |
| 54 | Glycyl-L-serine | $H_2N.CH_2.CO.NH.CH.COOH$<br>$CH_2OH$ | 162 | | | 2.92<br>8.10 | v | i | a | | o | | | |
| 55 | Methylamine | $CH_3.NH_2$ | 31.1 | 77.9 | $\mu$=1.31 | 10.47 | v | ∞ | a | | o | | | |
| 56 | Ethylamine | $CH_3.CH_2.NH_2$ | 45.08 | 109.6 | $\mu$=1.22 | 10.81 | ∞ | ∞ | a | | o | | | |
| 57 | n-1,amino-butane | $CH_3.CH_2.CH_2.CH_2.NH_2$ | 73.1 | 160.6 | | 10.77 | ∞ | s | a, b. | | o | | | |
| 58 | 2,Amino-butane | $CH_3.CH_2.CH(NH_2).CH_3$ | 73.1 | 167.7 | 7.4 | 10.80 | s | ∞ | a, b. | | o | | | |
| 59 | Dimethyl-amine | $CH_3.NH.CH_3$ | 45.1 | 110.1 | | 10.63 | v | s | b | | o | | | |
| 60 | Diphenyl-amine | (benzene ring).NH.(benzene ring) | 169.22 | 242.4 | $\mu$=1.08 | .77 | d | v | j | | + | | | |

FIGURE I. 4.e

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | μ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 61 | Trimethyl amine | $(CH_3)_3N$ | 59.1 | 154.2 | .612 | 9.69 | v | s | b<br>j | | o<br>+ | | | |
| 62 | 1,4,-Diamino-butane | $H_2N.CH_2.CH_2.CH_2.CH_2.NH_2$ | 88.2 | 167.1 | | | s | | a | | o | | | |
| 63 | 1,5,-Diamino-pentane | $H_2N.CH_2.CH_2.CH_2.CH_2.CH_2NH_2$ | 102.2 | 195.8 | | 10.0<br>11.0 | s | d | j | | + | | | |
| 64 | Methanol | $CH_3OH$ | 32.0 | 40.0 | 1.69 | | ∞ | ∞ | b | | o | | | |
| 65 | Ethanol | $CH_3.CH_2OH$ | 46.1 | 56.3 | 1.69 | | ∞ | ∞ | b | | o | | | |
| 66 | Propanol | $CH_3.CH_2.CH_2OH$ | 60.1 | 124.2 | 1.68 | | ∞ | ∞ | a,b. | | o | | | |
| 67 | n-Butanol | $CH_3.CH_2.CH_2.CH_2OH$ | 74.1 | 151.9 | 1.66 | | s | ∞ | a,b<br>g | | o<br>+ | $10^{-15}$ | | |
| 68 | 1,-Pentanol | $CH_3.CH_2.CH_2.CH_2.CH_2OH$ | 88.1 | 179.8 | | | i | ∞ | a,b | | o | | | |
| 69 | 2,-Phenyl-ethanol | ⌬ $CH_2.CH_2OH$ | 122.2 | 198.9 | | | d | ∞ | a<br>j | a<br>l<br>m | o<br>+ | | + | $10^{-9}$<br>$1.5x10^{-8}$<br>$3.5x10^{-19}$ |
| 70 | 1,3,-Propane-diol | $HOCH_2.CH_2.CH_2OH$ | 76.1 | 119.2 | | | ∞ | v | j | | + | | | |
| 71 | 2,Amino-ethanol | $H_2N.CH_2.CH_2OH$ | 61.1 | 99.6 | | 9.50 | ∞ | d | b | | o | | | |
| 72 | L-2,Amino-1-propanol | $CH_3.CH(NH_2)CH_2OH$ | 75.1 | 127.2 | | 9.43 | v | v | b | | o | | | |
| 73 | 3,Amino-1-propanol | $H_2N.CH_2.CH_2.CH_2OH$ | 75.1 | 134.2 | | 9.96 | s | s | a,b. | | o | | | |
| 74 | L-Dimethyl-amino 2-propanol | $CH_3.CH(N(CH_3)_2).CH_2OH$ | 103.1 | | | | | | b. | | o | | | |
| 75 | 3-Dimethyl-amino-1-propanol | $(CH_3)_2.N.CH_2.CH_2.CH_2OH$ | 103.1 | | | | | | b. | | o | | | |

FIGURE I. 4.f

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\alpha]_D$ / $\mu$ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 76 | L-2-Amino-butanol | $CH_3.CH_2.CH(NH_2)CH_2OH$ | 89.14 | 156.2 | -9.8 | 9.52 | ∞ | ∞ | b | | o | | | |
| 77 | D-2-Amino-butanol | $CH_3.CH_2.CH(NH_2)CH_2OH$ | 89.14 | 156.7 | +9.8 | 9.52 | ∞ | ∞ | b | | o | | | |
| 78 | 5-Amino-pentanol | $H_2N.CH_2.CH_2.CH_2.CH_2.CH_2OH$ | 89.4 | 180.1 | | 10.46 | ∞ | s | b | | o | | | |
| 79 | 1-Pentyl-acetate | $CH_3(CH_2)_3CH_2O.CO.CH_3$ | 103.0 | 245.6 | $\mu$=1.75 | - | i | s | h,i. | k | + | | + | |
| 80 | Formic acid | H.COOH | 46.0 | 62.6 | $\mu$=1.82 | 3.75 | ∞ | ∞ | j | | + | | | |
| 81 | Acetic acid | $CH_3.COOH$ | 60.1 | 72.7 | $\mu$=1.74 | 4.76 | ∞ | ∞ | a | | o | | | |
| | | | | | | | | | g | | + | $10^{-13}$ | | |
| 82 | Propanoic acid | $CH_3.CH_2.COOH$ | 74.1 | 123.9 | $\mu$=1.75 | 4.87 | ∞ | ∞ | a,b | | o | | | |
| 83 | Butanoic acid | $CH_3.CH_2.CH_2.COOH$ | 88.1 | 152.7 | $\mu$=1.65 | 4.82 | ∞ | ∞ | a | | o | | | |
| 84 | 2,Methylpro-panoic acid | $(CH_3)_2CH.CH_2.COOH$ | 88.1 | 151.1 | | 4.86 | v | ∞ | a | | o | | | |
| 85 | Pentanoic acid | $CH_3(CH_2)_3.COOH$ | 102.1 | 180.5 | $\mu$=1.58 | 4.84 | s | s | a,b | | o | | | |
| 86 | 3,-Methyl-butanoic acid | $(CH_3)_2CH.CH_2.COOH$ | 102.1 | 182.5 | | 4.78 | s | ∞ | a | | o | | | |
| 87 | Hexanoic acid | $CH_3(CH_2)_4.COOH$ | 116.2 | 208.2 | | 4.87 | i | s | a,b | | o | | | |
| 88 | Heptanoic acid | $CH_3.(CH_2)_5.COOH$ | 130.2 | 234.9 | | 4.88 | d | s | a | | o | | | |
| 89 | Octanoic acid | $CH_3.(CH_2)_6.COOH$ | 144.2 | 236.5 | | 4.89 | $d^h$ | s | a | | o | | | |
| 90 | Thioglycollic acid | $HS.CH_2.COOH$ | 92.1 | | | | s | s | j | | + | | | |

FIGURE I. 4.g

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\alpha]_D$ / $\mu$ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 91 | Maleic acid | H.C.COOH ‖ H.C.COOH | 116.2 | 123.7 | | 1.97 6.24 | v | s | j | | + | | | |
| 92 | Fumaric acid | HOOC.C.H. ‖ H.C.COOH | 116.2 | 126.7 | | 3.02 4.38 | d | d | j | | + | | | |
| 93 | Benzene | | 78.1 | 147.5 | $\mu$=o | | i | ∞ | | h | | | + | $2x10^{-7}$ |
| 94 | Mesitylene | | 120.1 | 199.6 | $\mu$=o | | i | ∞ | j | | + | | | |
| 95 | Nitrobenzene | | 123.11 | 169.9 | $\mu$=4.2 | 3.98 | d | v | | o | | | + | $7x10^{-6}$ |
| 96 | 1,3-Dinitro-benzene | | 168.11 | 177.2 | | | d | s | | o | | | + | $7x10^{-6}$ |
| 97 | 1,3,5-Tri-nitro-benzene | | 213.11 | 239.5 | | | d | d | | o | | | + | $1.4x10^{-6}$ |
| 98 | Phenol | | 94.1 | 145.8 | $\mu$=1.4 | 10.0 | s | ∞ | | h, o | | | + | $9x10^{-6}$ |
| 99 | Resorcinol | | 110.11 | 143.8 | | 9.81 | s | s | | o | | | + | $5x10^{-7}$ |
| 100 | Phloro-glucinol | | 126.11 | 143.4 | $\mu$=o | | d | v | | o | | | + | $7x10^{-7}$ |
| 101 | o-Chloro-phenol | | 128.6 | 169.0 | | | d | s | j | | + | | | |
| 102 | p-Chloro-phenol | | 128.6 | 168.4 | $\mu$=2.11 | | d | s | j | o | + | | + | $10^{-4}$ |
| 103 | 2,4,6-Tri-nitro-phenol | | 229.11 | | | 0.38 | d | s | | o | | | + | $4x10^{-5}$ |
| 104 | 2,-Hydroxy-benzalde-hyde | | 122.1 | 173.7 | | | d | ∞ | j | | + | | | |
| 105 | Eugenol | | 164.2 | 256.0 | | | i | ∞ | j | d<br>l<br>m | + | | +<br>+<br>+ | $10^{-7}$<br>$6x10^{-8}$<br>$6x10^{-18}$ |

FIGURE I. 4.h

| CHEMICAL | | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\alpha]_D$ μ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 106 | Citral B | | 152.2 | 284.9 | | | i | ~ | j | m | + | | + | $6x10^{-18}$ |
| 107 | Citral A | | 152.2 | 284.6 | | | i | ~ | | m | | | + | $6x10^{-8}$ |
| 108 | Geraniol | | 154.3 | 288.2 | | | i | s | j | | + | | + | |
| 109 | Terpineol | | 154.3 | 274.1 | | | d | v | | m | | | + | $3.2x10^{-18}$ |
| 110 | L-Menthol | | 156.3 | 177.8 | -48.2 | | d | v | j | m | + | | + | $4x10^{-17}$ |
| 111 | α-Ionone | | 192.3 | 344.0 | +347 | | d | ~ | j | m | + | | + | $2x10^{-15}$ |
| 112 | β-Ionone | | 192.3 | 337.5 | | | d | ~ | j | m | + | | + | $1.5x10^{-6}$ |
| 113 | Morpholine | | 87.1 | 144.0 | | 8.33 | | s | j a,b. | e,g,h,i | + o | | + | $10^{-11}$ |
| 114 | Uracil | | 112.1 | | | -3.38 9.5 13 | d | v | j | | + | | | |
| 115 | 8,Hydroxy-quinoline | | 145.2 | 233.1 | | | i | i | j | | + | | | |
| 116 | Coumarin | | 146.1 | 259.5 | | | s | v | h,i,j,l a | h,k | + o | | + | $10^{-5}$ |

FIGURE I. 4.i

| CHEMICAL | | | | | | | | FISH | | PHYSIOLOGICAL | | BEHAVIOURAL | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| No. | Name | Structural Formula | Mol. wt. | Mol. vol. | $[\alpha]_D$ / $\mu$ | pKa | Solubility $H_2O$ | Solubility $Et_2O$ | Physiol. Expts. | Behav. Expts. | RESPONSE | THRESHOLD | RESPONSE | THRESHOLD |
| 117 | L-Camphor | [structure] | 153.2 | 255.4 | -44.3 $\mu$=2.95 | | i | v | j | | + | | | |
| 118 | α-Pinene | [structure] | 136.2 | 263.7 | | | i | ∞ | j | | + | | | |
| 119 | Diallyl-sulphide | $(CH_2:CH.CH_2)_2S$ | 114.2 | 213.6 | | | d | ∞ | j | | + | | | |
| 120 | Glucose (α) | [structure] | 180.2 | 191.8 | +52.7 | | v | i | b | | o | | | |
| 121 | Lactose | [structure] | 342.3 | 357.5 | +52.6 | | v | i | b | | o | | | |
| 122 | Sucrose | [structure] | 342.3 | 359.8 | +66.5 | | s | i | b,h. | | o | | | |

In addition the following ions have proved to be effective stimuli.

| Anions | |
|---|---|
| Citrate | $\left[\begin{matrix}CH_2COO \\ CH(OH)COO \\ CH_2COO\end{matrix}\right]^{3-}$ |
| Oxalate | $\left[\begin{matrix}COO \\ COO\end{matrix}\right]^{2-}$ |
| Phenylacetate | $C_6H_5.CH_2.COO^-$ |
| Hydrogenphosphate | $HPO_4^{2-}$ |
| Dihydrogenphosphate | $H_2PO_4^-$ |
| Sulphate | $SO_4^{2-}$ |
| Chloride | $Cl^-$ |

| Cations | |
|---|---|
| Lithium | $Li^+$ |
| Sodium | $Na^+$ |
| Magnesium | $Mg^{2+}$ |
| Potassium | $K^+$ |

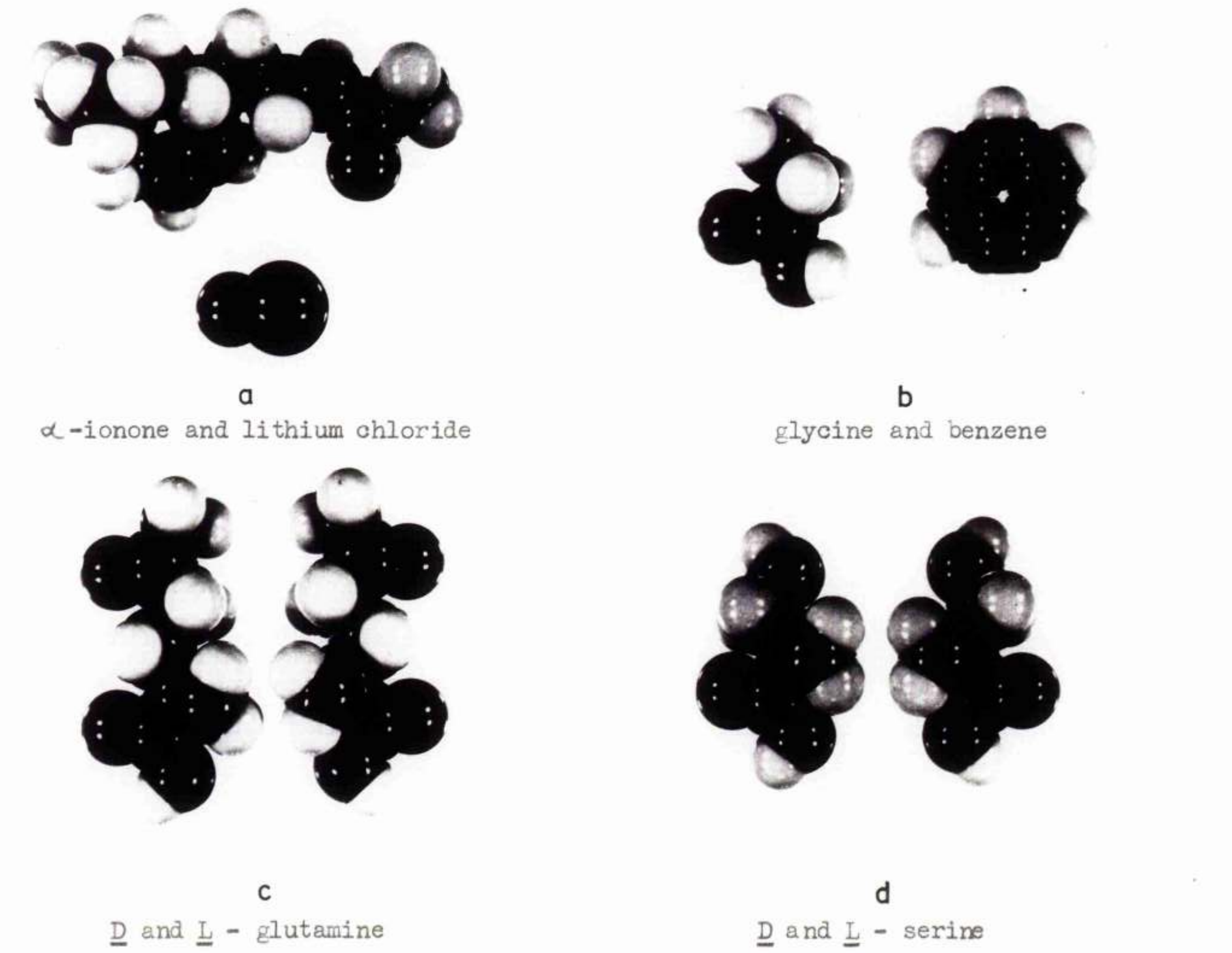
Erratum: Page 22, lines 24 - 26.

Glycine is highly soluble in water and insoluble in ether, while benzene is only sparingly soluble in water and infinitely soluble in ether.

odour quality. Thus, characteristic, identifiable odours will be associated with fairly homogeneous orientation patterns of rigid molecular structures containing sterically accessible functional group(s). Lack of odour and uncharacteristic odours should be associated with a random distribution of orientations, approximated by non-polar molecules with sterically inaccessible functional groups. The influence of the functional group on orientation pattern will be determined by the direction of the molecular dipole and its solvation tendency. Thus, in Table I.4, stimulatory effectiveness is presented alongside structural formulae and representative physical and chemical properties thought to be relevant to primary olfactory interactions.

The size of the receptor site must be expected to be a limiting factor. The range of molecular volumes shown suggests that the largest acceptable molecules are no bigger than about 340Å$^3$. Figure I.3a compares models of the largest and smallest effective stimulant molecules. Although dipole moment values were not available for the majority of compounds, it is obvious that the range of effective stimulants includes highly polarized as well as non-polar molecules. Figure I.3b compares models of glycine and benzene, representing the two extremes. The list contains few non-polar molecules, however, so direct comparison is difficult. The molecules shown in Figure I.3b raise another important point, namely that of solubility. Glycine is highly soluble in water and insoluble in ether, while benzene is only sparingly soluble. The stimulatory effectiveness of compounds such as benzene and mesitylene is of interest since they are known to be effective stimulants in terrestrial vertebrates, and thus, although their relative aqueous insolubility probably

Figure I.3 Molecular models of selected compounds known to stimulate the fish olfactory system



precludes them as natural stimulants for fish, it raises the question of common receptor sites for aquatic and terrestrial forms. Perhaps the only reason why man, for example, does not smell amino acids is by virtue of the unsuitable terrestrial transport medium. No evidence has arisen to indicate even partial lipophilicity as an important factor in receptor-odorant interaction.

The amino acids as a class of compounds are highly stimulatory, not only for fish but other aquatic organisms (Laverack, 1968; McLeese, 1970). The first 49 compounds listed in Table I.4 correspond to the relative stimulatory effectiveness found by Hara (1973) for the rainbow trout (Salmo gairdneri). From this data and the lack of response noted for several non-amino acids, Hara has attempted certain correlations between effectiveness and molecular structure. Replacement of the carboxyl or amino group by another functional group, altering the position of the $\alpha$-amino and $\alpha$-carboxylic groups, increasing the carbon chain length above 5, and the addition of an hydroxyl group reduced stimulatory effectiveness. The data obtained by Hara showed relatively good correlation ($r = 0.85$) when compared with the receptor responses of Salmo salar obtained by Sutterlin and Sutterlin (1971), but there are serious discrepancies when compared with data obtained from Ictalurus catus by Suzuki and Tucker (1971). Particularly obvious is the discrepancy between the stimulatory effectiveness of glycine and D-methionine. In both Salmo salar and Salmo gairdneri glycine is a potent stimulus but, out of 23 amino acids tested by Suzuki and Tucker, it is the least effective. In addition, many of the non-amino acids shown to be non-stimulatory by Hara have proved to be

highly potent in behavioural studies. Notably, Teichmann (1959) could condition Salmo gairdneri to respond to 2-phenylethanol in a solution 100 times weaker than the most potent amino acid.

Hara's conclusions are generally acceptable but he seems to have missed one or two obvious points. For example, the amidation of both enantiomers of glutamic and aspartic acid greatly increased their effectiveness.

$H_2N.CO.CH_2.CH_2.CH(NH_2)COOH$  $HOOC.CH_2.CH_2.CH(NH_2)COOH$

$H_2N.CO.CH_2.CH(NH_2)COOH$  $HOOC.CH_2.CH(NH_2)COOH$

Also, although he noticed that D-isomers of amino acids were less stimulatory than their antipodes, the relative effectiveness of enantiomers was not at all constant. Models of two extreme examples, Glutamine and Serine are shown in Figures I.3c and I.3d.

It is regrettable that more thought was not given to the selection of compounds. For example, 3-amino-1-butanoic acid (GABA) was shown to be stimulatory, whilst 1-aminobutane and 1-butanoic acid were not. The obvious compound to test the combined importance of both $\alpha$-amino and $\alpha$-carboxyl groups, namely 2-amino-1-butanoic acid, was not included in the experiment.

$H_2N.CH_2.CH_2.CH_2.COOH$ 3-amino-1-butanoic acid

$H_2N.CH_2.CH_2.CH_2.CH_3$ 1-aminobutane

$CH_3.CH_2.CH_2.COOH$ 1-butanoic acid

$CH_3.CH_2.CH(NH_2).COOH$ 2-amino-1-butanoic acid

Similarly, the chain length theory should have been more rigorously tested using the series glycine, alanine, 2-aminobutanoic acid, 2-aminopentanoic acid, 2-aminohexanoic acid, and 2-aminoheptanoic acid. Careful experimentation with groups of compounds such as these would be expected to yield valuable information regarding the nature of the amino acid receptor site(s).

Table I.4 indicates that several small inorganic ions are effective stimuli. Their effectiveness probably arises from a non-specific reaction of the receptor membranes.

The fish's nose is an extremely sensitive molecular detector. Teichmann (1959) managed to train eels (Anguilla anguilla) to respond to 2-phenylethanol at a concentration of $3.5 \times 10^{-19}$M, a formidable performance by any standard. This represents a molecular concentration of 210 per mm$^3$, which is about the volume of the eel's nasal chamber. The thresholds for the rainbow trout (Salmo gairdneri) and minnow (Phoxinus phoxinus) were $1.5 \times 10^{-8}$M and $1 \times 10^{-9}$M respectively. These figures correspond to molecular concentrations of $5.09 \times 10^{10}$ and $7.53 \times 10^{12}$ mols. mm$^3$. It should be remembered that, when working with such highly diluted solutions, local differences in concentration are inevitable. For example, soluted molecules at phase boundaries may well result in a million-fold increase in local concentration. Therefore, an open mind should be maintained when considering these results.

Thresholds obtained by electrophysiological methods are normally higher than those obtained using behavioural techniques (Prosser and Brown, 1961). Thus, pacific salmon (Oncorhynchus nerka) showed a marked avoidance response to

L-serine (a constituent of mammalian sweat), at a concentration of $1.9 \times 10^{-10}$M (Brett and MacKinnon, 1954). The electrophysiologically determined threshold for the same compound lies between $10^{-6}$ and $10^{-7}$M. (Hara, 1972).

The wide discrepancies in published data could easily be explained by the often wide variation in the purity of stimulus samples. If an eel can smell phenylethanol at $10^{-19}$M, then even the slightest trace of impurity can be expected to alter experimental results. As poignantly stated by Amoore (1970) "... to search for chemically specific elements in the olfactory nervous system using average pure organic chemicals as olfactory stimuli is about as ludicrous as trying to define the spectral sensitivity of retinal cones using Christmas tree lights as sources of monochromatic light ...". Truly meaningful results may perhaps only be obtained when the problem is tackled by teams which include a skilled organic chemist, particularly when studies relating to stereospecificity are envisaged.

Thus, fish are capable of smelling a wide variety of small and often highly polarized molecules. They seem unable to smell compounds with a molecular weight greater than 300 and many aliphatic alcohols and acids. The highly stimulatory nature of amino acids is probably related to the highly polarized region around the asymmetric carbon atom. The adjacent amino and carboxyl groups may enter into a reversible, but relatively stable, hydrogen bonded complex with an oppositely charged pair of moieties in a 'cavity' on the receptor protein. This is partly supported by the fact that naturally occurring proteins and thus the receptor proteins are constructed from L-amino acids,

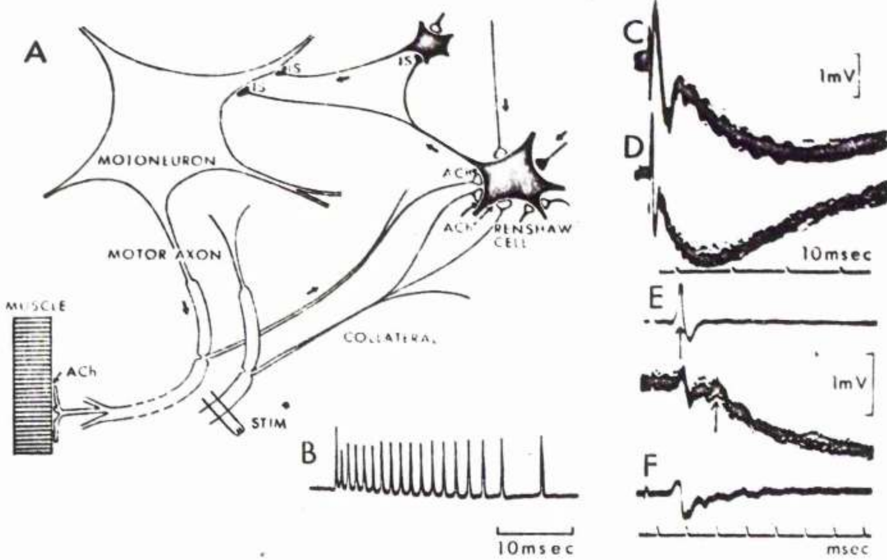
and that evidence from all available sources shows the L-amino acids to be more effective stimulants than their antipode.

## (ii) AETIOLOGY OF THE PRESENT STUDY

The above review reveals that there are extremely large gaps and occasionally flaws in our knowledge of the sense of smell in fish. This lack of knowledge extends from the nature and functioning of the nasal receptors, to the central integration of sensory information. Whilst this is also true of the other vertebrate classes, our understanding of olfaction in amphibians and mammals is far more extensive. In particular, many elegant biophysical, physiological and ultrastructural studies have led to a fairly thorough understanding of the basic neuronal circuitry in the mammalian olfactory bulb.

Almost all neurons in the central nervous system that have been adequately investigated are subjected to antagonistic excitatory and inhibitory post-synaptic actions. The neurons of the olfactory bulb are no exception to this. It has long been recognised that inhibition plays an important role in the processing of the excitatory afferent input on to mitral cells. An elegant intracellular study by Yamamoto, Yamamoto and Iwama (1963) revealed that the long-lasting inhibition of mitral cells following antidromic activation resulted from an inhibitory post-synaptic potential (IPSP). A concurrent rhythmic burst of impulses commonly occurred in granular layer cells, whose long radial dendrites make synaptic contacts with the secondary dendrites of mitral cells. By analogy with the well-established pathway, shown in Figure I.3a, for recurrent inhibition of spinal motoneurons (Renshaw, 1946: Eccles, Fatt and Koketsu, 1954)

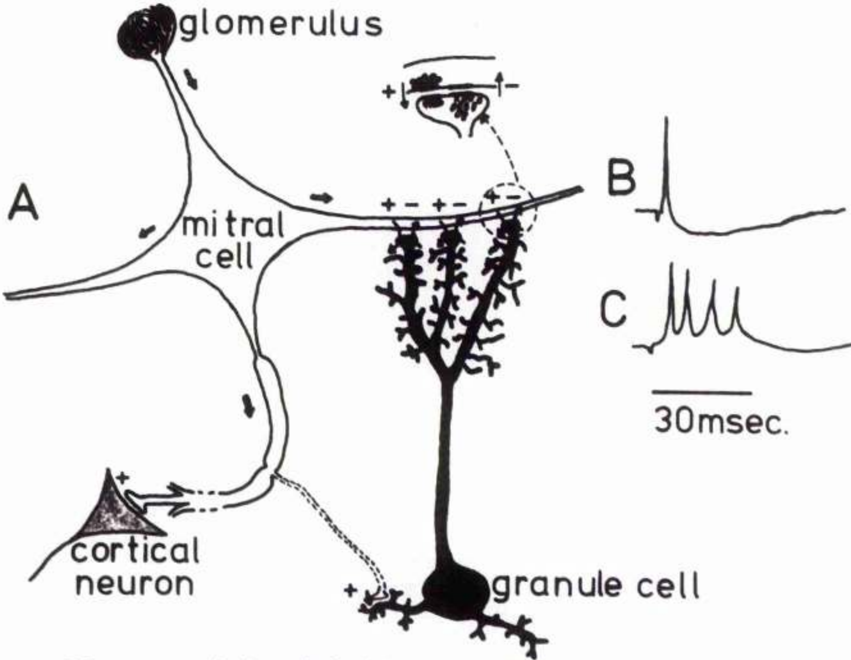
Figure I.4 Classical and dendrodendritic recurrent inhibition




a

FIG. 3. Diagram of the inhibitory pathways to motoneurons by their axon collaterals and Renshaw cells. *B* shows extracellular recording of a Renshaw cell being excited by an antidromic volley in the motor fibers of lateral gastrocnemius muscle, and *C* to *E* are intracellular responses evoked by an antidromic volley in the ventral root and are fully described in the text. *F* is extracellular control for *E*. Same time scale for *C*, *E* and *F*.

Taken from Eccles (1968)




b

Diagram of the inhibitory pathway on to mitral cells via the granule cell dendrites as found in the mammalian olfactory bulb. A, anatomical arrangement; inset shows detail of a reciprocal synapse. B, mitral cell spike followed by IPSP. C, simultaneous rhythmic burst in granule cell. Arrows show direction of transmission, + indicates an excitatory synapse and - indicates an inhibitory synapse. Recordings taken from Yamamoto et al., (1962).

it was concluded that the granule cells receive an excitatory input from mitral axon collaterals and, in turn, deliver synaptic inhibition to the mitral cells.

More recently Rall and Shepherd (1968), in a theoretical and experimental study, have indicated that, although the granule cells are probably responsible for mitral cell inhibition, they are not primarily activated by axon collaterals. From the analysis of extracellular field potentials they postulated a two-way dendrodendritic pathway in which excitation is delivered to granule dendrites by mitral dendrites which, in turn, receive inhibition from the same or adjacent granule dendrites. Subsequently, an independent electron microscopical study by Reese and Brightman (1965) revealed the presence of two-way 'reciprocal' synapses between these two types of dendrites. Furthermore the mitral - granule contact was morphologically similar to Type I synapses (Gray, 1969), thought to be excitatory and the granule - mitral contact was morphologically similar to Type II synapses, thought to be inhibitory, Figure I.3b. These findings, which are of fundamental neurobiological significance, are corroborated by Nicoll (1969), who has also presented evidence that the inhibitory transmitter released by granule cells is gamma-aminobutyric acid (Nicoll, 1970, 1971).

Although the organisation of the teleostean olfactory bulb is not tremendously different from other vertebrates, the existence of a physiologically similar 'wiring diagram' has been denied by several authors (see Pages 12-13). Hence, it was decided that, before any serious attempt could be made to investigate the mechanism of odour discrimination in

fish, a thorough study of neuronal events intrinsic to the bulb should be undertaken. Chapters IV and V present the results of research designed to test the hypothesis that a dendro-dendritic inhibitory pathway is present in the fish olfactory bulb. Some suggestions relating to the nature of the inhibitory neuro-transmitter are given in Chapter VII.

During the preliminary studies on extracellular field potentials, a method was developed for the identification of single units. The activity patterns of such identified units are described in Chapter VI, together with how this activity is modulated by olfactory stimuli.

It is hoped that the thesis here presented will provide essential groundwork for future workers in this field and ideas for all those interested in the comparative aspects of neurological science.

# II

# material and methods

In addition, a few comparative experiments were performed on the following species:

*Salmo trutta*

*Melanogrammus aeglefinus*

*Raniceps ranius*

*Pleuronectes platessa*

*Myoxocephalus scorpius*

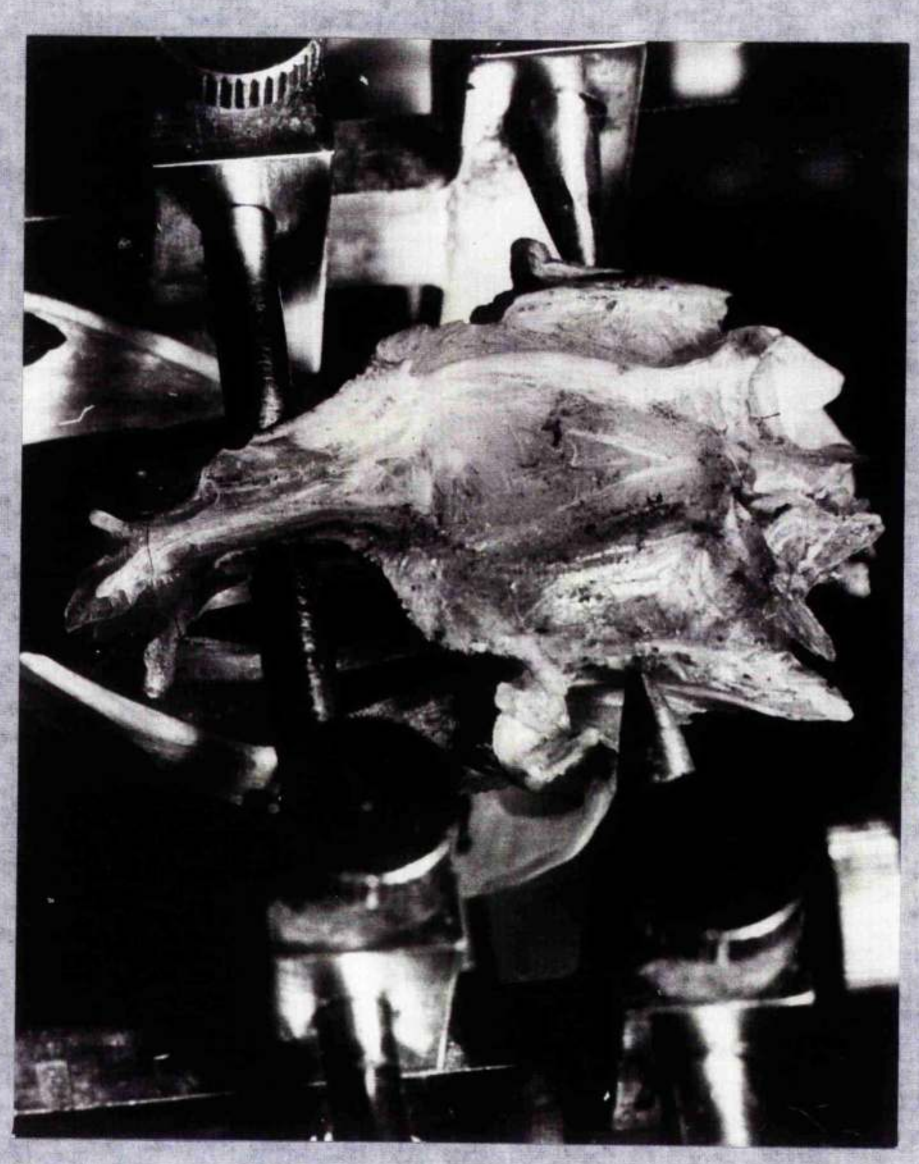
(i) PRELIMINARY REMARKS

The primary experimental animal throughout this project has been the rainbow trout, Salmo gairdneri. The trout has several advantages which make it an ideal experimental animal, in a way paralleling the ubiquitous white rat. Rainbow trout of a constant size are always readily available from local hatcheries and far more is known of its general biology and physiology than for any other fish (Frost, W.E. and Brown, M.E. 1970). Of particular importance are several studies concerning the bulbar response to odorants by Hara (1973a, b). The only major disadvantage of the trout proved to be the extremely short length of the olfactory tracts; a few comparative experiments have, therefore, been performed on the goldfish (Carassius auratus) and the cod (Gadus morhua) which possess long and easily accessible olfactory tracts. This study has used about one hundred rainbow trout (200-250g.), six goldfish (100-150g.) and twelve cod (750-1500g.)

(ii) PREPARATION OF THE ANIMAL

The fish were initially immersed in a solution of tricaine methanesulphonate (MS222, 50-100g/l) and left until Stage III anaesthesia was reached (MacFarland, 1959); this is characterized by slow opercular movements and by the fish rolling on to its back, usually after an exposure of 1-3 mins. An intramuscular injection of D-tubocurarine chloride was given and the fish placed in a specially designed stereotaxic head-holder, which is shown in Figures II.1 and II.2. This apparatus, which can accommodate any suitably sized fusiform fish was rigidly fixed to the holding aquarium and a heavy steel base to facilitate accurate and reproducible placement of electrodes. Essentially, the fish is held between two parallel sets of opposing steel bars which grip the

Figure II.1: Head of *Myoxocephalus scorpius* in head-holder.



a: Frame of head-holder, allowing lateral movement along supporting bar, which allows pitching movements.

b and c: Parallel sets of opposing bars for holding skull of fish.

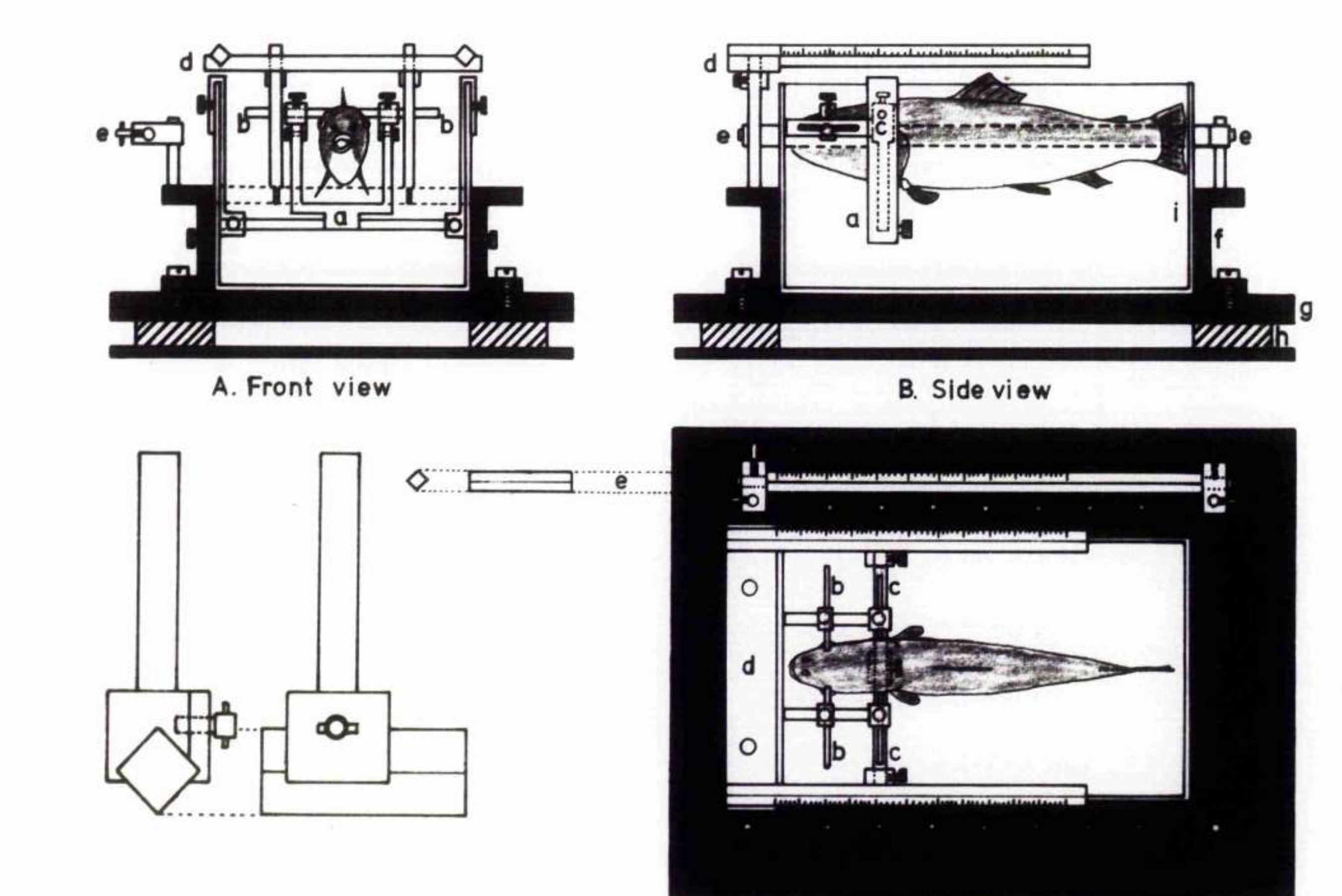
d and e: Horizontal bars for carrying micro-positioners.

f: Rectangular channel iron frame bolted to steel base plate.

g, h: Thick sponge shock absorbers.

i: Perspex aquarium fitting into iron frame.

FIGURE II.2: Plan of apparatus for holding fish and electrode micropositioners



supraorbital ridges and parietal bones, preventing movement in all planes. Support of the body was in general found to be unnecessary. A system of horizontal bars incorporating millimeter scales was constructed to provide a versatile framework for carrying micromanipulators (Figure II.2).

The fish was continuously perfused through the mouth with an air-saturated, dilute solution of MS222 at .5 l/min.; perfusion temperature was maintained at about 12°C by placing crushed ice in the constant head reservoir (Figure II.3). The depth of anaesthesia was monitored by reference to a continuous polygraph record of the electrocardiogram (EKG) and the tectal electroencephalogram (EEG) and regulated with a system of flowmeters modified from that used by Guthrie and Banks (Figures II.3 and II.4). During surgery the EEG amplitude was maintained between 25 and 40 µV but this was allowed to rise to about 85 µV during recording. The EKG provides an accurate monitor of the general condition of the animal. A deteriorating preparation was often heralded by an exaggerated P-wave and intermittent bradycardia. Increased depth of MS222 anaesthesia was usually accompanied by a marked cardiac acceleration.

Pressure and incision points were infused with a solution of lignocaine containing noradrenaline and a craniotomy was performed to expose the telencaphalon, olfactory bulbs and the more caudal part of the olfactory nerves. The craniotomy was usually performed by paring away the poorly ossified cranium with a scalpel but a dental drill had to be employed for large fish. The fatty tissue lining the cranium was removed by gentle sponging with cotton wool, great care being taken to avoid damaging the small vessel running between the olfactory nerves. After placement of the stimulating electrodes the craniotomy was flooded with mineral oil

Figure II.3 Perfusion and anaesthetic apparatus

Figure II.4 Example of routine stable EEG and EKG recording.

A

B

C

D

E

F

A- EEG (mesencephalon)

B-Stimulus duration

C-EEG (left olfactory bulb)

D-Time (sec)

E-EEG (right olfactory bulb)

F-ECG

100µv (EEG) 1mv (ECG)

2sec.

to prevent desiccation of the preparation. This procedure also greatly improved the visualization of the brain. The final preparation is shown in Figure II.5.

(iii) STIMULATION TECHNIQUES

(a) Electrical

Isolated square pulses of duration 1msec. and amplitude 2-8V were delivered to the olfactory nerves and tracts via concentric electrodes constructed from a 22 gauge hypodermic needle and 0.1mm insulated silver wire. The electrodes were normally positioned under visual observation with the aid of a dissecting microscope; however, the olfactory tract of the trout is fairly inaccessible and a rough stereotaxic atlas was constructed to facilitate the operation in this species. The output of the stimulator was arranged so that bursts of pulses or pairs of pulses having individually variable amplitudes could be delivered down the same or different electrodes. The central electrode of the concentric arrangement was always cathodal.

(b) Chemical

Amino acids of the highest purity available were obtained from Sigma Limited. Experimental solutions were diluted from 1mM stock solutions kept in a refrigerator. During experiments the test solutions were kept at the same temperature as the branchial perfusion water. They were administered by injection from disposable 1ml syringes via a short length of teflon tubing into a water stream gently perfusing the nasal mucosa (Figure II.3).

(iv) RECORDING TECHNIQUES

Figure II.6 is a diagrammatic representation of the recording and stimulating circuits used in most of the experiments to be described.

Figure II.5a: Overall view of the experimental preparation. This particular animal is a common dogfish, *Scyliorhinus caniculus*. The holding aquarium was later extended to more easily accommodate such large fish. Elasmobranchs in general were poor, short-lasting preparations due to extensive haemorrhaging from the edge of the operated area.

Figure II.5b: Detail of area around the craniotomy. This particular animal is a cod, *Gadus morhua*. In this photograph the left olfactory bulb is clearly visible in the bottom left-hand corner of the craniotomy. Note the bright pink colour, denoting a healthy preparation and the microelectrode penetrating the dorsal surface. E - electroencephalogram electrode; K - electrocardiogram electrodes; I - indifferent electrode (a self-tapping brass screw); M - microelectrode carrier; S - stimulating electrode; P - branchial perfusion tube; C - syringe connected to abdominal vein cannula; H - head holder; O - overflow; V - voltage follower. For the sake of clarity the stereotaxic frame was removed prior to taking these photographs.

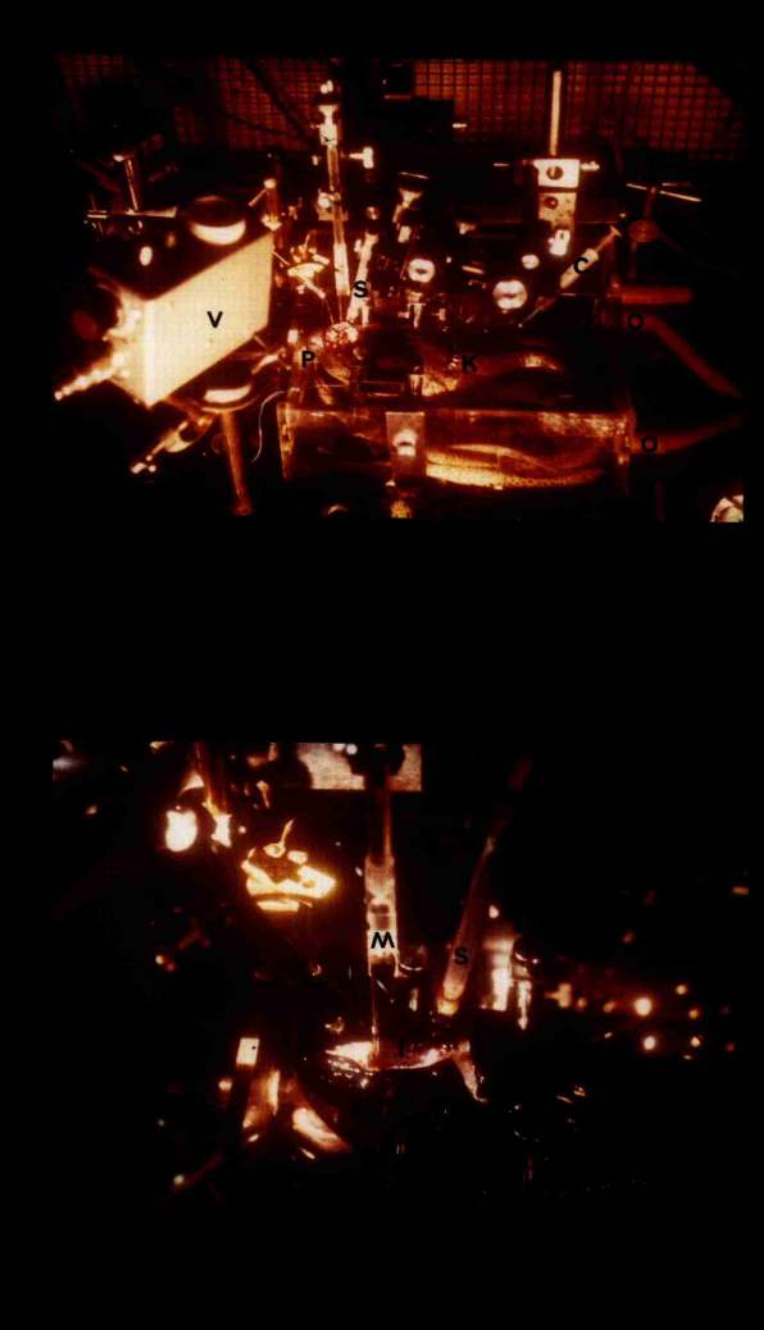

S
C
V
P
K
M
S

Figure II.6 Diagrammatic representation of recording and stimulating circuits

(a) Electrocardiogram

The EKG was recorded using bipolar leads, each consisting of a stainless steel hypodermic needle soldered to a short length of flexible, unscreened cable. The electrodes were fully inserted in the superficial muscles immediately behind the operculae on each side.

(b) Electroencephalogram

The EEG was recorded with a monopolar silver-silver chloride electrode insulated with polythene tubing except for the tip. This was forced carefully through a small burr-hole in the skull and fixed in place with dental cement. The indifferent electrode was a small self-tapping screw fixed in the skull.

Both the EKG and EEG were led from the Faraday cage via screened leads to a.c. amplifiers, the outputs of which were monitored continuously on a Devices M4 hot-wire recorder.

(c) Field Potentials and Unit Activity

Glass microelectrodes filled with 3M sodium chloride were used for recording field potentials and single units. The resistance of the electrodes was usually 2-5MΩ for field potential recording and 5-20MΩ for unit recording. The electrodes were pulled containing several strands of glass fibre and filled using a hypodermic syringe immediately before use.

Signals were led via a field effect transistor (FET) voltage follower to one side of a condenser coupled biological pre-amplifier (Roemmélé, 1973); the other side of the input was grounded. The output of the pre-amplifier was variously distributed according to the type of experiment in progress; the major connections are shown in Figure II.6. Field potentials were led to a Tektronix 561B oscilloscope and photographed with a Nihon-Koden oscillograph camera.

The potentials could also be led to a Biomac 500 computer for on-line averaging. The output from the Biomac was recorded on an X-Y plotter. During these experiments the trigger output of the Biomac was usually used to synchronize the stimulating and recording apparatus. A Tektronix storage oscilloscope proved to be extremely useful for the rapid visualization of potential depth profiles. Unit recordings were similarly led to Tektronix oscilloscopes; in addition, a window discriminator (Roemmélé, 1970) and pulse generator were used to provide controlled pulses for triggering the Biomac and an RC integrator. The output of the integrator was continuously written out by the Devices M4 recorder. An audio monitor was always used for 'listening in' to experiments. After familiarization this was found to be the most reliable way of recognising variations in recorded activity, particularly when searching for single units.

The microelectrode was carried in the electrode holder of a Narishige M20 micromanipulator. The vertical rack of this manipulator is fitted with a micron counter which, if set to zero at the brain surface, provides a direct indication of recording depth. The approximate position of the recording tip could also be estimated from the shape of the evoked field potential. Finally, a marking technique was used to give a more accurate confirmation of the recording position. A filtered 2% solution of Alcian Blue in 5% sodium acetate was used to fill the second barrel of a double barrelled electrode. This could be ejected iontophoretically to leave a blue spot which was easily detectable in histological sections stained with neutral red. The spot produced was rather diffused and was only useful when combined with the other methods to describe the approximate recording point.

(v) PHARMACOLOGICAL TECHNIQUES

(a) Drug Injections

Intravenous injections of drugs were occasionally given by cannulation of an abdominal vein but, frequently, it was more convenient and technically easier to inject directly into the caudal vein.

(b) Microiontophoresis

The introduction of drugs into the extracellular space around single recorded units was achieved by iontophoresis from drug-containing barrels of five barrelled microelectrodes. These were constructed and filled as shown in Figure II.7, from 1mm pyrex tubing pre-washed in chromic acid.

The recording barrel was filled with 4M sodium chloride and another barrel was filled with 3M sodium chloride for current balancing. The remaining three barrels were filled with aqueous solutions of the following drugs in various combinations: GABA (0.5M; pH 3.5; HCl), glycine (0.5M; pH 3.5; HCl), glutamate (0.5M; pH 7.5; NaOH), aspartate (0.5M; pH 7.5; NaOH), strychnine sulphate (1M), noradrenaline bitartrate (0.5M; pH 4.5; HCl), bicuculline (5mM in 0.165M NaCl; pH 3.7; HCl) and picrotoxin (5mM in 0.165 NaCl; pH 5.0; HCl). The electrode tips were broken to between 3 and 4 um under the high power of a compound microscope. The resulting resistance of the sodium chloride barrels was usually 2-5MΩ, the amine barrels 10-20MΩ, and the convulsant barrels 50-80MΩ.

Cationic or anionic current could be delivered to four barrels via individually screened leads from the specially constructed apparatus shown in Figure II.8. The essential part of the circuit comprises a potential divider and potentiometer to provide coarse and fine control of the output from three 90V batteries in series

Figure II.7 Construction and filling of multi-barrel microelectrodes

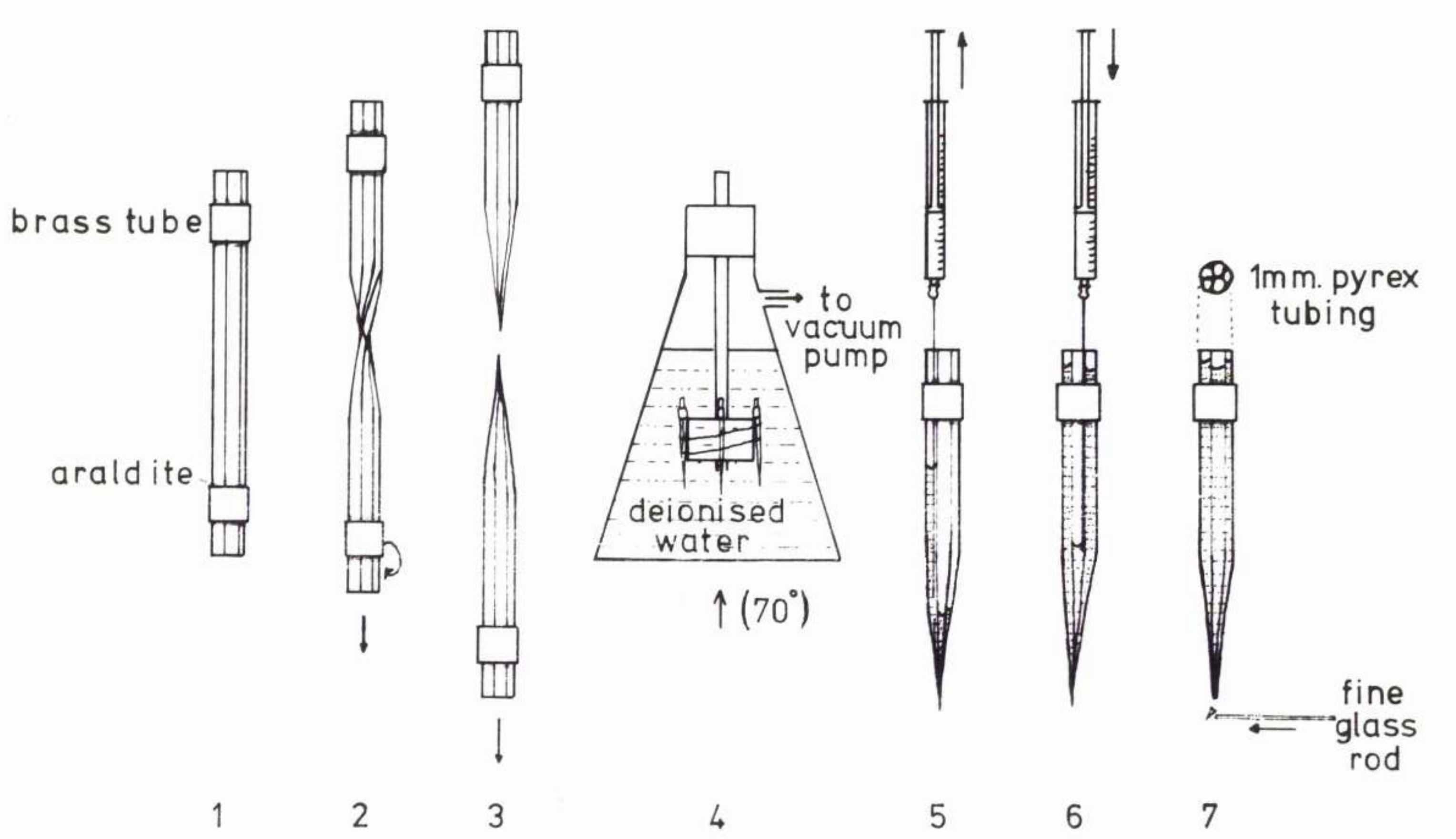
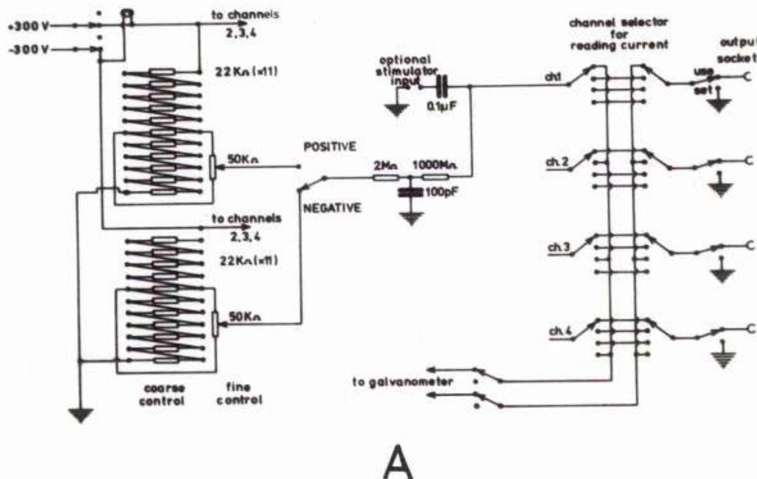
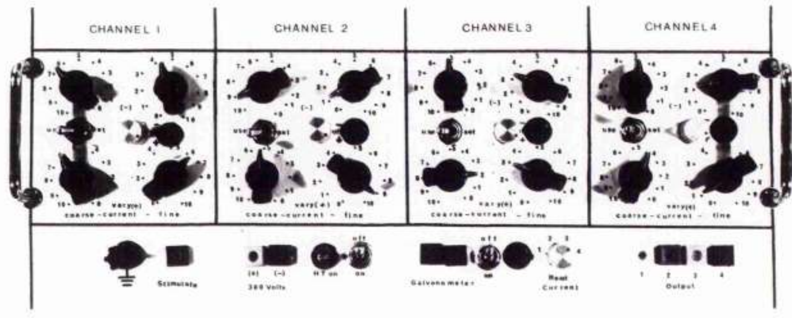


Figure II.8 Microiontophoresis apparatus




A

Circuit diagram of one channel and current reading switch




B

Front panel of apparatus

with 1000MΩ. This gives a maximum output of about 250nA per channel. A switching circuit is incorporated to allow the output from each channel to be read. A backing current of opposite polarity to the ejection current was applied to prevent spontaneous diffusion of drugs from the pippette tip. Both intensity and duration of the retaining current were kept constant (15nA, 1min.), since Bradshaw et al. (1973) have shown that a stable maximum or minimum firing rate cannot be obtained until the rate of release has reached an equilibrium value.

In studies on the spontaneous and induced activity of single neurons, an alteration in the firing rate of 20 per cent was taken as the standard for deciding whether a particular stimulus was excitatory or inhibitory.

(vi) HISTOLOGICAL TECHNIQUES

Fixation of neural tissue was normally carried out by cardiac perfusion with formol saline following initial perfusion with saline (Hubbard, Llinas and Quastel, 1969). Wax sections were usually stained with either cresyl violet or neutral red. Another useful technique sometimes employed, which differentiated both cells and fibres involved leaving the whole brain in a solution of formaline and thionine for at least one month, followed simply by embedding, sectioning and mounting (Chang, 1936). This technique was modified and improved in later experiments by prefixation with glutaraldehyde and the inclusion of gluteraldehyde in the staining solution. Golgi preparations were made according to the method of Ramon-Moliner (1970).

# III

# anatomy of

# the olfactory bulb

## (i) INTRODUCTION

A brief sketch of the basic organisation of the vertebrate olfactory system has already been given (See Page 1). It is the intention of this section to present a descriptive discussion of those aspects of olfactory bulbar anatomy relevant to the physiological study forming the main body of the present thesis. By way of introduction Figure III.1 shows the relationship of the bulb with the other parts of the olfactory system in two of the genera studied, *Salmo* and *Gadus*. In the Salmonidae and most other teleostean families the olfactory bulbs are sessile but, in the Mormyridae, Siluridae, Cyprinidae and Gadidae, the bulbs are connected to the rest of the forebrain by long, slender olfactory crura containing both centrifugal fibres and the axons of secondary olfactory neurons.

The olfactory bulbs of fish display a more or less distinct laminated structure. The layers, which are concentrically arranged around the bulbar ventricle are: (1) a layer of primary olfactory nerve fibres, (2) a glomerular layer, (3) an external layer containing large mitral cells and smaller elements, (4) a plexiform layer formed from the axons of mitral cells and the dendrites of deeper neurons and (5) an internal cellular region containing many small neurons. In most higher vertebrates there is a further plexiform layer between the mitral cells and the glomeruli. Figure III.2A shows these layers diagrammatically for a representative series of vertebrates. The general histological appearance of the bulb in sagittal sections is shown in Figure III.3, where the most prominent feature is the central core of granule cells. Another important feature is the near spherical symmetry of the neuronal elements. This, the five layered structure is equally visible in

Figure III. 1A: Dissection of the brain of *Salmo gairdneri* to show the olfactory system.

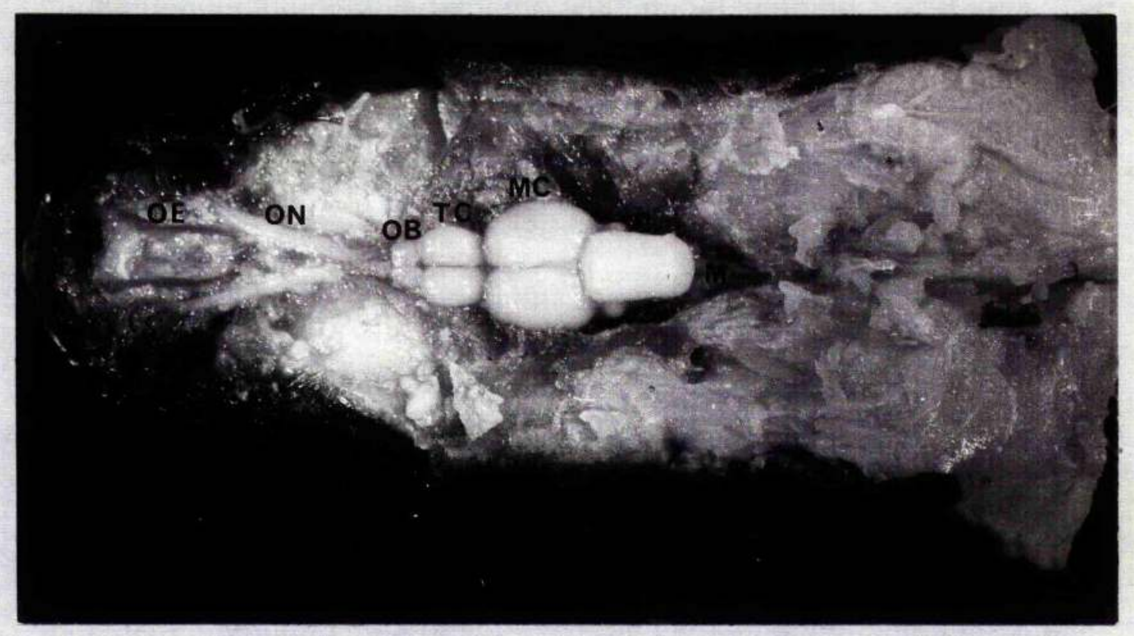
Figure III. 1B: Dissection of the brain of *Gadus morhua* to show the olfactory system.

OE - olfactory epithelium; ON - olfactory nerve;

OB - olfactory bulb; CT - olfactory tract;

TC - telencephalon; MC - mesencephalon;

C - cerebellum; M - medulla oblongata


OE
ON
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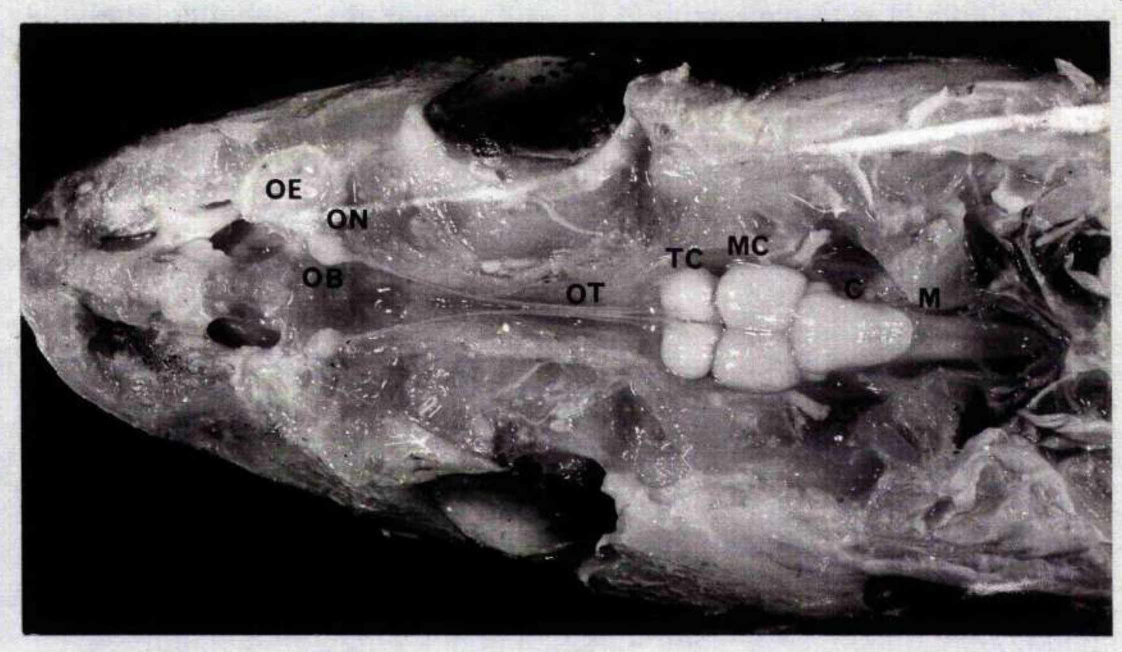
OE
ON
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TC
MC
C
M

Figure III.2. (A): Semi-diagrammatic representation of the fibres and cytoarchitecture of vertebrate olfactory bulbs. Perikarya, dendrites and axons of the mitral and tufted cells are distinguished. Granule cells are indicated by the oval outlines of their nuclei and their 'axons' are indicated by fine lines. Small, bold circles are cross-sections of myelinated fibres. A: lamprey, B: elasmobranch, C: amphibian, D: reptile, E: mammal. A and B have five layers: olfactory nerve layer (SN), glomerular layer (SG), mitral layer (M), plexiform layer (PL), periventricular layer (VL). Internal plexiform layer (IPL); external plexiform layer (EPL).

Figure III.2. (B): Diagram of synaptic connections in the olfactory bulb of fish. Left: hypothetical presentation of neuronal connections. Right: detailed drawings of types of synapses. Synapse between fila olfactoria and mitral cell dendrites (A); reciprocal granule cell ending on mitral cell dendrite ($B_1$), on the soma of a mitral cell ($B_2$), mitral cell collateral endings ($C_1$) and central bulbopetal fibre endings ($C_2$) on granule dendrites; synapses with interdigitated membrane complex in the periventricular zone, probably on stellate cells (D). $C_1$ and $C_2$ form morphologically similar symptoms. Granule cell (g), mitral cell (m), stellate cell (s), astrocyte (a), dendrite (d). The arrows indicate direction of transmission of the stimulus.

Figure III.2. (C): Cresyl violet stained section of cod olfactory bulb oriented in the same manner as B.

A and B taken from Andrés (1970).

Figure III.2

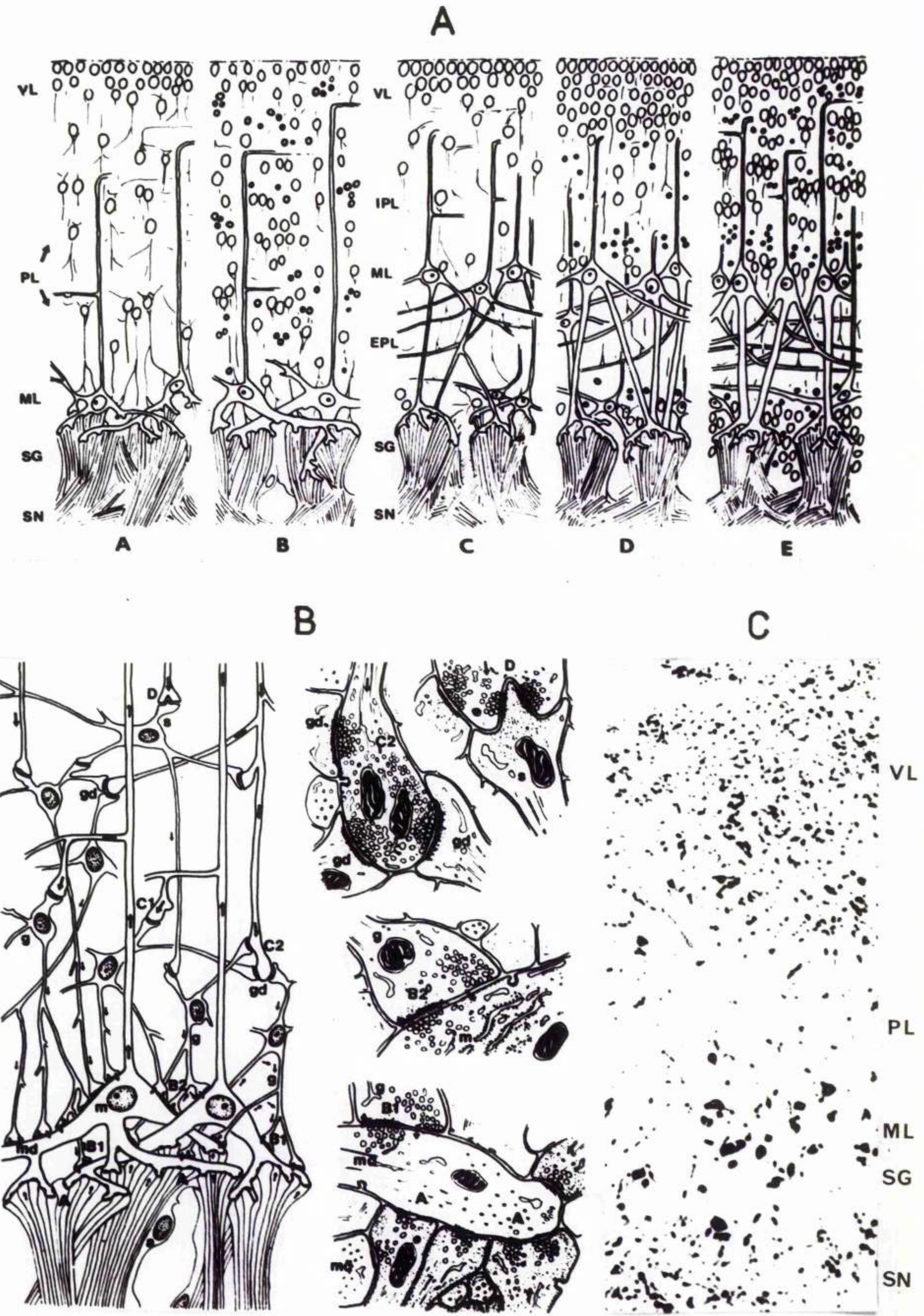
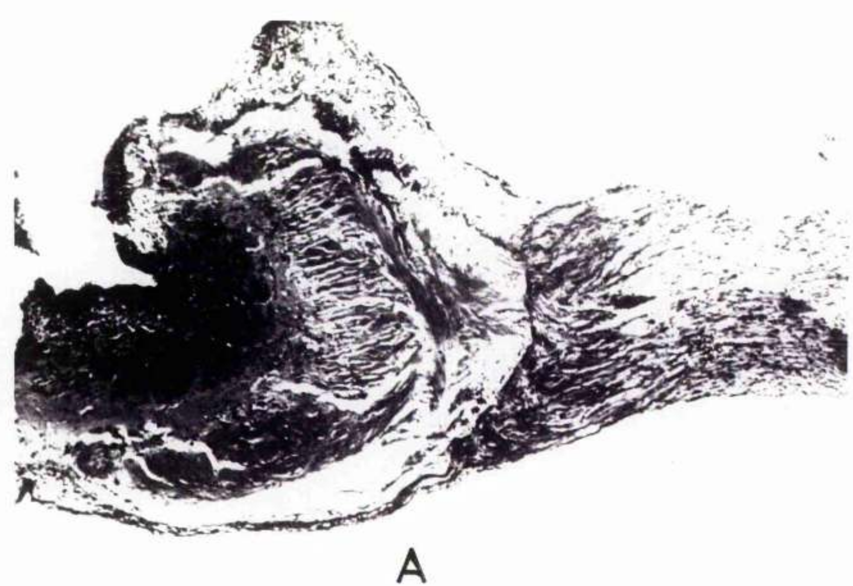
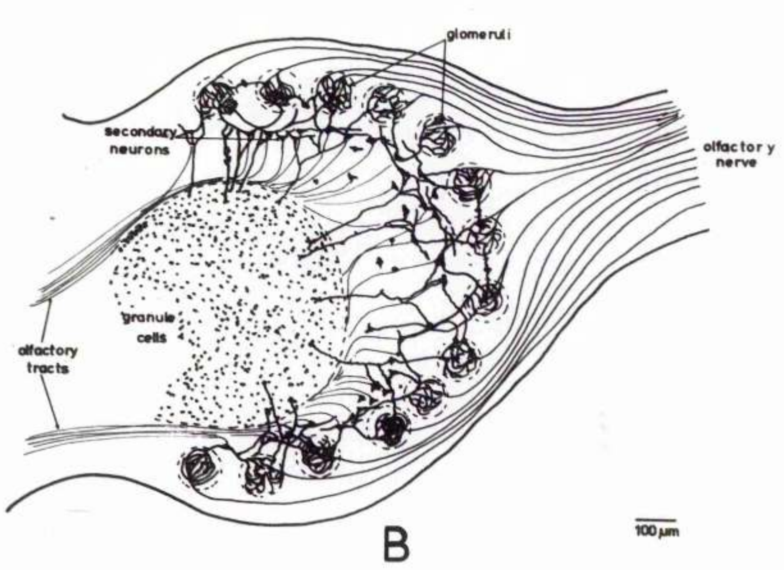


Figure III.3 Saggital sections of trout olfactory bulb



A

Thionin stained section




B

Drawing made from Golgi preparations

anterio-posterior and in dorso-ventral planes.

## (ii) DESCRIPTION OF NEURONAL ELEMENTS

### (a) Glomerular Layer

The olfactory nerve fibres end in well-defined spherical bodies, the glomeruli (Figure III.3B). The incoming olfactory nerve fibres do not divide before entering the glomeruli, although they branch freely within them according to Holmgren (1920) but Andres (1970) was unable to confirm this using the electron microscope. The axon of a receptor cell does not terminate in more than one glomerulus and each glomerulus receives impulses from a segregated and independent collection of olfactory receptors. Within the glomeruli the terminals of the olfactory receptor cells enter into synaptic contacts with the terminal dendritic tufts of mitral cells. The topological arrangement of the olfactory cell branches on the mitral dendrites is probably extremely important. Døving and Pinching (1973, 1974) have demonstrated that continued exposure of young rats to single odours leads to degeneration in specific anterio-posterior bands of the mitral cell layer.

Some relatively small cells are found between the glomeruli, often possessing thick dendrites which may enter glomeruli. Catois (1901) considered that these elements are equivalent to the periglomerular cells found in amphibians, reptiles and mammals.

### (b) Mitral Cells and a Discussion of Reciprocal Synapses

The large mitral cells (Figure III.3B) are found in a layer about 300 µm below the bulbar surface and their somata can be up to 30 µm in diameter, the normal range being between 15 and 25 µm. They possess from one to five thick dendrites, each of

which supplies one or more glomeruli; in addition, these dendrites receive synaptic contacts from the radially oriented dendrites of granule cells (Andrés, 1970; Cajal, 1894) as shown in Figures III.2B and III.3B. In mammals these synapses seem to be exclusively reciprocal in nature (Price and Powell, 1970b; Rall et al. 1966). Both sides of the contact are capable of transmitting and receiving information; the mitral - granule contact is thought to be excitatory and the granule - mitral contact is thought to be inhibitory, as discussed on Page 27. Andres (1970) has recently produced evidence for the existence of reciprocal synapses on the mitral dendrites of a wide cross-section of vertebrates including fish (Figure III.2). In fish and lower vertebrates both synaptic inputs to the mitral cells occur on the same dendrites but in higher vertebrates the reciprocal synapses occur predominantly on the accessory dendrites of the secondary neurons (mitral and tufted cells). The glomerular tufts are confined to radially oriented primary dendrites, the number of which undergoes a gradual reduction as the vertebrate series is ascended. In mammals there is only one primary dendrite, providing a very definite segregation of primary and accessory bulbar circuits. It is interesting to note here that reciprocal synapses have been demonstrated in various thalamic relay nuclei (Famiglietti, 1970; Harding, 1971; Morest, 1971; Wong, 1970) and Ralston (1971) has proposed a schema for their activation that bears a close resemblance to suggestions for the olfactory bulb. Reciprocal synapses in the retina between amacrine cells and bipolar terminals (Dowling and Boycott, 1966) may also be responsible for a similar control of receptor input. This analogy is particularly striking if it is remembered that the granule cells

are by definition 'amacrine cells'.

The axons of mitral cells join the medial or lateral olfactory tract and extend to the hemispheres (Cajal, 1894; Sheldon, 1902), but often emit several collaterals within the bulb which extend back to the glomerular zone and periventricular zone (Andres, 1970; Cajal, 1894; Johnston, 1898). Allison (1953) and Holmgren (1920) assert, however, that these axons have few or no collaterals within the bulb.

(c) Plexiform Layer

This is a dense neuropile formed from the axons of mitral cells and the dendrites of granular cells. In addition, this region contains stellate and spindle-shaped cells. The stellate cells are provided with long dendrites which extend fan-wise towards the periphery, where their branches end in the glomerular zone and shorter dendrites directed parallel to the bulbar surface (Nieuwenhuys, 1967). The spindle-shaped cells are bipolar elements which often send a dendrite towards the glomerular zone. The axons of the stellate and spindle-shaped cells run in the olfactory tracts and they often emit collaterals in the bulb which may reach the glomeruli (Johnston, 1898).

(d) Granular Cells

The granule cells (Figure III.2B) are by far the most numerous neuronal element present in the bulb but there is controversy in the literature as to whether the cells observed in fish correspond with the similarly situated cells in mammals. In a review, Nieuwenhuys (1967) reported that the periventricular bulbar grey matter of fish and cyclostomes contains numerous cells provided with distinct axons which contribute to the olfactory tracts. The dendrites of these cells often enter

into the formation of glomeruli and only in the dipnoans do elements occur which closely resemble the granules of higher vertebrates. However, Cajal had already recognised the existence of true granule cells in fish as early as 1894: "Finalemente, los granos se comportan como mi hermano ha reconocido en los batracios, es decir, que son celulas unipolares, y su unica expansion, dirigida hasia la periferia, se resuelve en un penacho de ramas espinosas." Recent electron-microscopical evidence (Andrés, 1970) suggests that, although a few granule cells send processes into the glomeruli, very many of them appear identical to the typical mammalian granule cells, thus agreeing with Cajal. Sheldon (1912) has also implied an associational function for the granule cells.

The true granule cells, which I have also found to be fairly numerous in fish, are small neurons with no morphologically identifiable axon. They possess several short, spiny basal dendrites and a long radially directed dendritic shaft, the spiny ends of which enter into the reciprocal synapses described above with the dendrites and somata of the mitral cells. In addition, the somata and dendrites of the granule cells receive synaptic contacts from mitral axon collaterals and from the terminals of centrifugal fibres (Andrés, 1970; Cajal, 1894; and Johnston, 1898). Thus, it appears that, as in mammals, the granule cells may be involved in intra-bulbar associational systems: the quotation by Hara cited on Page 13 seems, therefore, to be without foundation.

# IV

# olfactory bulb

# field potentials

INTRODUCTION

The complex structure of the central nervous system, with millions of active elements, makes it exceptionally difficult to interpret electrical records of brain activity. Potentials recorded with macroelectrodes are the vector sum of elementary electric fields from a large statistical assembly of neurons, often showing asynchronous, contrary changes. Recordings with microelectrodes usually represent the activity of individual neurons which cannot be representative of the entire population.

The field potentials produced by a group of cells reflect indirectly the membrane potential changes which the cells of a population undergo in unison, the membrane potential changes representing action and synaptic potentials within individual neurons. A study of such potentials will give valuable information about the average activity of the cells in the group and is a basic prerequisite to understanding the physiological characteristics of any neuronal population in the central nervous system. If the anatomy of the particular brain region is well understood, the activity of the pool can often be treated as if generated by a small number of ideal elements, each representing the average behaviour of the particular cells which are synchronously activated from specific sites of stimulation. For example, stimulation of the olfactory tract will result in the activation of the mitral cell axons and the subsequent antidromic invasion of their somata within the olfactory bulb. This, in turn, will lead to the activation of other cells in the bulb to which the mitral cells are synaptically connected. The extracellular action and synaptic currents thus generated can be recorded by the insertion into the bulb of a microelectrode connected to a

suitable amplification and display system.

The membrane potentials of individual neurons are the main source of electrical potentials recorded from the brain. During antidromic or synaptic activation, different parts of a neuron undergo asymmetric changes in membrane potential, resulting in electrical asymmetry of its component parts which, in a simplified form, appears as an electrical dipole. Current will flow in the extracellular space from the regions of high membrane potential (source) to regions of lower membrane potential (sink), accompanied by a simultaneous flow of intracellular current in the opposite direction. When recorded relative to a distant reference electrode, the source will be positive and the sink negative. However, the fact that the outside of a particular neuronal region is positive does not necessarily imply that the corresponding section of membrane is hyperpolarized. The membrane potential here may be normal but will be lower than that recorded at the sink.

To facilitate interpretation of recorded field potentials, it is assumed that the brain behaves as a perfect ohmic resistor and as an isotropic medium. The first implies that there are no capacitive or inductive elements present and that its resistance is independent of the current flowing through it. The second implies that the conducting properties of the medium are the same, independent of the direction of current flow. Obviously, neither of these assumptions can be true for the central nervous system but, by making them, the experimenter is relieved from having to perform numerous corrective vector analyses of his data. Thus, the voltage measured is a product of the integrated current density component and the specific

resistance of the medium which is assumed to be constant. If the sink is spatially smaller than the source, the current density in the area of the sink will be greater than at the source. The negativity will thus appear to be more pronounced than the positivity recorded at the source. In a volume conductor the current arising between two areas of a neuron spreads in far-reaching lines of current and, in distant parts of the field, the direction of current flow may be nearly reversed relative to the intrapolar sector. Lorente de No (1947) has shown that a monophasic nerve action potential changed to a triphasic one at certain points on a flat conductive medium. A positive wave may then be an expression of excitation approaching the electrode and a negative wave an expression of excitation reaching the area of the electrode.

As mentioned briefly above (Page 27), the field potentials in the mammalian olfactory bulb have been extensively and elegantly studied by several authors (Freeman, 1972b, c, d; Nicoll, 1969, 1972; Phillips, Powell and Shepherd, 1962; Rall, Shepherd, Reese and Brightman, 1966; Rall and Shepherd, 1968; Shepherd, 1970). In mammals, stimulation of the lateral olfactory tract generates a field potential which, at the bulbar surface, is compounded from two negative and two positive waves, $N_1$, $N_2$, $P_1$ and $P_2$ respectively. $P_1$ is believed to represent the current source during the antidromic passage of impulses in the mitral cell axons. $N_1$ is attributed to action currents around the dendrites and somata of mitral cells as a result of their antidromic invasion. $N_2$ is thought to reflect the depolarization of the granule cell primary dendritic terminals and $P_2$ the subsequent hyperpolarization of the mitral cell secondary

dendrites. Synchronous with the $N_2$ wave, but recorded in the granule cell region, is an intense positive wave. All authors agree that this dipolar field must be generated by the granule cells, whose particular anatomical features provide an intracellular return pathway for the current. The fact that the dipole is surface negative implies that the depolarization of granule cells takes place in the external plexiform layer. The $P_2$ wave is believed to represent synchronous hyperpolarization of the mitral cell population and, therefore, a reflection of the pronounced inhibition of mitral cells following olfactory tract stimulation. The hypothesis that this inhibition was delivered to mitral cells via the same population of synapses responsible for the excitation of granule cells found support in the discovery of reciprocal synaptic contacts between the two cell types in the external plexiform layer.

The waves evoked by stimulation of the mammalian olfactory nerve are very similar but are preceded by a triphasic wave, which is the compound action potential of the olfactory nerve (Nicoll, 1972). Stimulation of the reptilian (Orrego, 1961) and amphibian (Ottoson, 1959) olfactory nerve evokes fundamentally similar potentials and, although the analyses presented are relatively less sophisticated than the later mammalian studies, the conclusions are along similar lines. No suggestion of the dendro-dendritic pathway proposed for mammals was made in either of these two reports.

## RESULTS

### 1. Properties of the olfactory nerve compound action potential

The response of the olfactory nerve to single shock electrical stimulation is a positive - negative - positive

triphasic wave which could only be recorded within two millimeters of the stimulating electrode (Figures IV. 1A; IV. 2(i) and IV. 3). The duration of the action potential was found to be 10 msec. With increasing stimulus intensity, the amplitude of the compound action potential increased non-linearly, the maximum rate of increase occurring between 2.5 and 4x threshold (Figures IV. 1B and IV. 2(ii) ). Double shock stimulation showed that the absolute refractory period for the action potential was 20 $\pm$ 4 msec. (Figure IV. 1A).

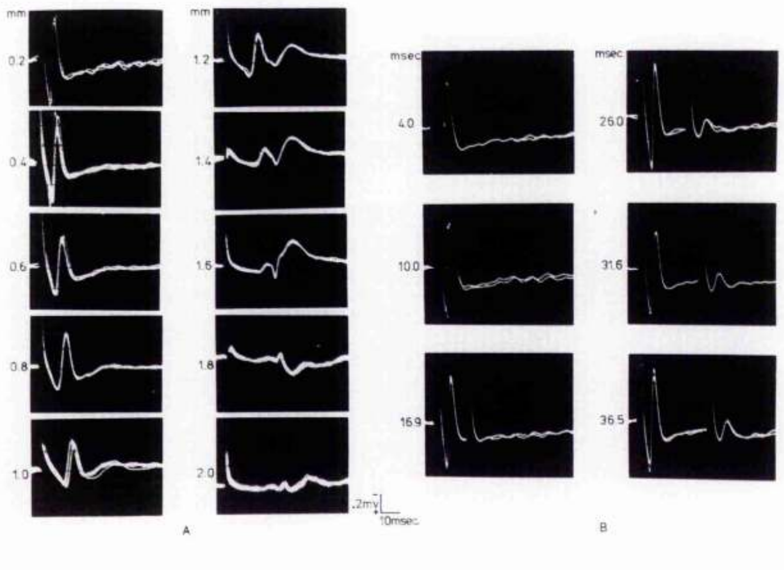
By trial and error, placing of the recording microelectrode along the nerve enabled a series of points of maximum response amplitude to be located along a narrow anterioposterior band which appeared to correspond to a single stria of the nerve. The response moved at a constant velocity but with decremental amplitude (Figures IV.2 (i) and IV.3). The mean conduction velocity of the wave was 0.13 $\pm$ 0.03 m/sec. which, if multiplied by the duration, gives a value of 1.3 mm for the wavelength of the action potential. This figure corresponds closely with the value obtained below by constructing a smoothed action potential for a section of the nerve (Figure IV.4). The action potential was shown to decrement similarly in the vertical direction by making a series of recordings perpendicular to a point on the horizontal axis (Figure IV. 3B). The amplitude distribution appears to be independent of stimulus intensity over the lower range but, at stimulus intensities of over 8x threshold, there was an increase in the width of the normal distribution. In one experiment (Figure IV. 5) an abnormal distribution of amplitudes occurred, appearing to peak at a point 1 mm from the stimulating electrode at all stimulation intensities.

Figure IV. 1A: The left-hand part of the figure shows the decrement of the olfactory nerve compound action potential as the recording electrode is moved away from the stimulating electrode in the direction of the olfactory bulb. The apparent abnormality of the record at 1.8 mm is due to the recording electrode penetrating the bulb. All other records are from the surface.

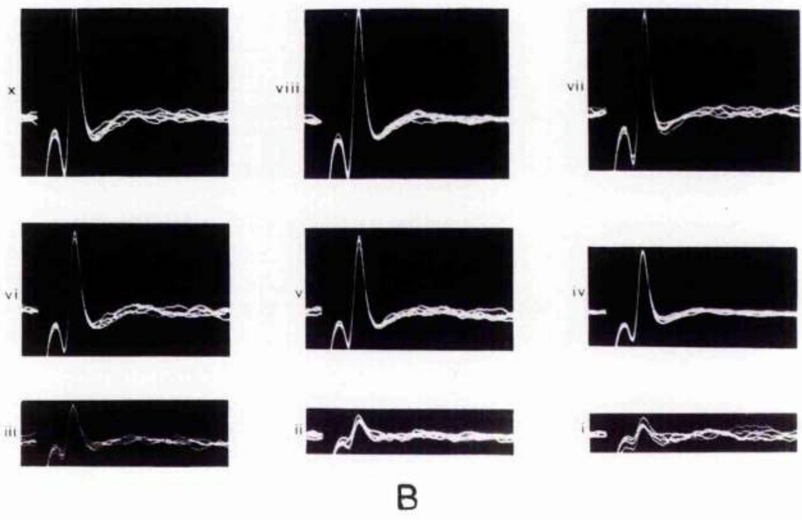
The right-hand part of the figure demonstrates the refractoriness of the olfactory nerve. The delay between a pair of shocks delivered to the nerve was gradually decreased until the test shock no longer produced a response.

Figure IV. 1B: This figure demonstrates the effect of increasing stimulus intensity on the olfactory nerve compound action potential from (i) to (ix).

Figure IV.1: Properties of olfactory nerve action potential




A

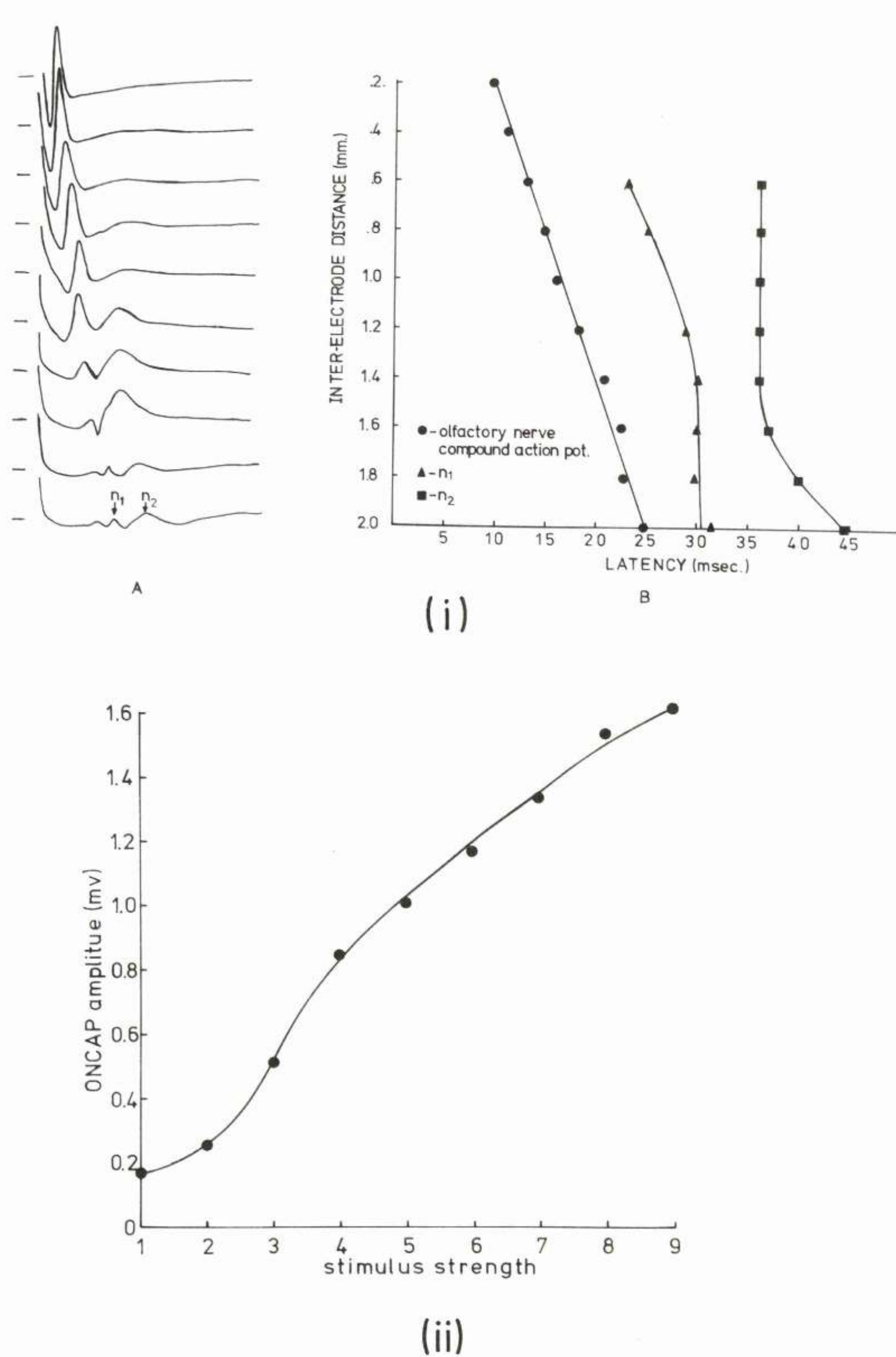



B

Figure IV.2(i): A shows a similar series of records to those in Figure IV. 1A taken from a different animal. B shows plots of the latency of the compound action potential, $N_1$, and $N_2$ as a function of the distance between the stimulating and recording electrodes.

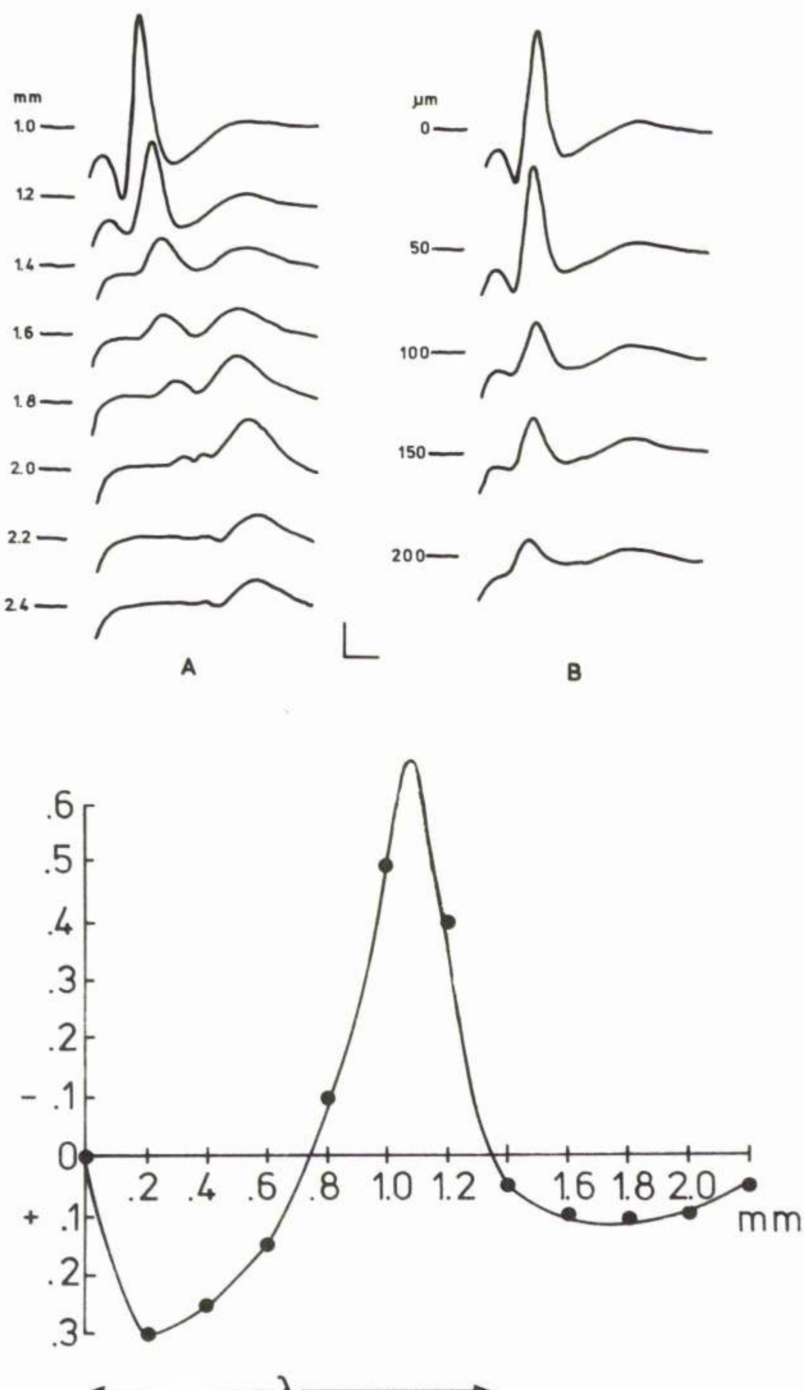
Figure IV. 2(ii): This is a plot of the experiment shown in Figure IV. 1B. Peak amplitude is shown as a function of stimulus strength. The units of intensity refer to the scale of a linear helipotentiometer controlling the output from a radio frequency isolation unit.

Figure IV.2: Properties of olfactory nerve action potential



<u>Figure IV.3</u>: A shows the decrement of the averaged compound action potential with distance horizontally along the nerve (x-axis). B shows the decrement of the averaged compound action potential along an electrode track perpendicular to the horizontal axis (y-axis). All records are on-line averages of 64 sweeps.
Calibration: vertical .2 mV; horizontal 5 msec.

<u>Figure IV.4</u>: This figure shows an example of a smoothed action potential for the nerve as a function of distance, constructed as described in the text. $\lambda$ refers to the wavelength of the action potential, which in this example is 1.34 mm.

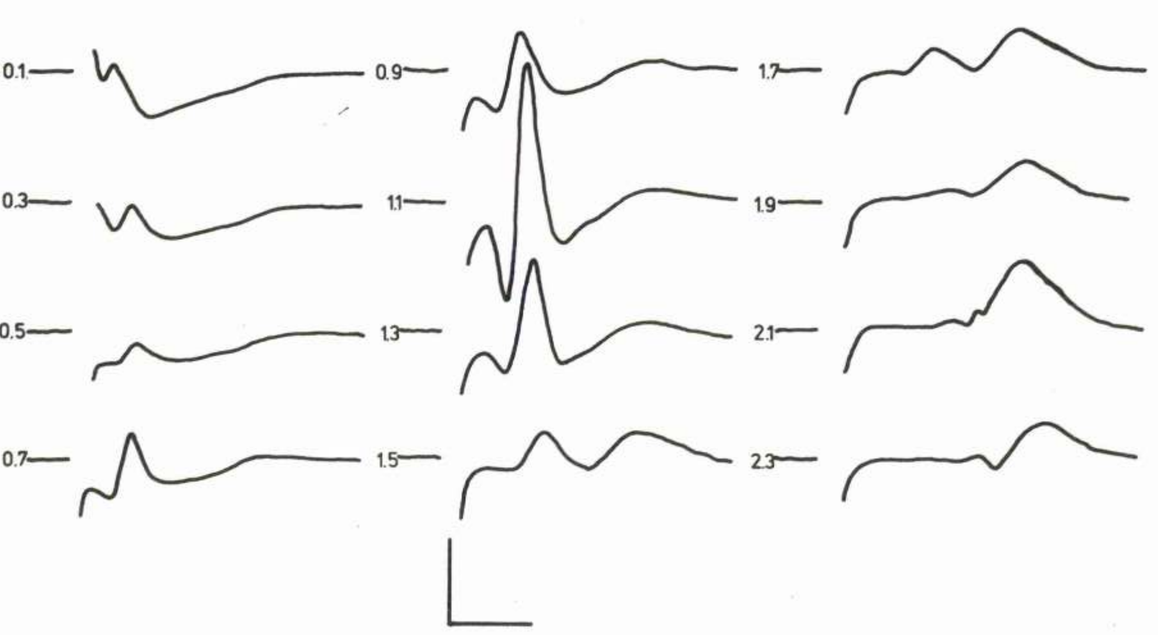


mm
1.0
1.2
1.4
1.6
1.8
2.0
2.2
2.4
A
µm
0
50
100
150
200
B
.6
.5
.4
.3
.2
- .1
0
+ .1
.2
.3
.2
.4
.6
.8
1.0
1.2
1.6
1.8
2.0
mm
λ

Figure IV.5: This figure shows a series of averaged compound action potentials recorded in the horizontal axis along the olfactory nerve, showing an unusual distribution of amplitudes. The peak amplitude is about 1 mm along the nerve from the stimulating electrode and not immediately adjacent to it, as would be expected.

Calibration: vertical .4 mV; horizontal 20 msec.

Figure IV.5: Abnormal distribution of compound action potential amplitude



To investigate further the distribution of potential within the nerve, the compound action potential was recorded and averaged on-line from a series of points of maximal response amplitude along its anterio-posterior trajectory (the x-axis) and from points along a dorsoventral line perpendicular to the trajectory at a distance of 1 mm from the stimulating electrode (the y-axis). The magnitudes of potential on both axes were measured at the point in time where the potential at the intersection of the x and y-axes reached its maximum amplitude on both sides of the x-y intersection. A smoothed action potential as a function of distance was then plotted by extrapolation. (Figure IV.4).

## 2. Description of field potentials evoked in the olfactory bulb

Moving the recording microelectrode nearer to the olfactory bulb leads to the appearance of three further negative waves and a later, slow positive wave accompanying the decrement of the compound action potential of the nerve. These waves, which will be referred to as $N_1$, $N_2$, $N_3$ and P, were usually clearly defined but, in surface records, $N_3$ was sometimes obscured by $N_2$.

The early negative waves could easily be distinguished from the compound action potential by their longer latency and their inability to follow repetitive stimulation. $N_1$ missed at 12 Hz; $N_2$ at 2 Hz; $N_3$ at about 10 Hz and P at about 2 Hz. Tetanic stimulation usually caused $N_1$ and $N_3$ to summate following initial inhibition (Figure IV.6). The amplitude of these waves was seen to vary over the bulbar surface (Figures IV.12, IV.13 and Appendix I). Generally, the majority of the bulb was surface negative in response to olfactory nerve stimulation but the caudal-most region was surface positive.

If a microelectrode is gradually lowered into the bulb while single shocks are applied to the nerve, the recorded potential transients undergo a series of changes in shape, amplitude and polarity (Figure IV. 7A, right). The degree of variation was related to the stereotaxic co-ordinates of the electrode penetration point but, for an electrode track near the centre of the bulb, the following changes were normally seen. The $N_1$ wave, if visible, reaches its maximum amplitude at the region of the mitral cell bodies. However, the wave recorded at the surface was usually synchronous with the leading edge of a

Figure IV.6: The effect of tetanic stimulation on field potentials evoked by stimulation of the olfactory nerve. Records are from a point near the focus of $N_2$. The numbers on record d refer to the sequence of the first three responses. Record a: three superimposed sweeps; all others ten superimposed sweeps.

Figure IV.6: Effect of repetitive stimulation on field potentials

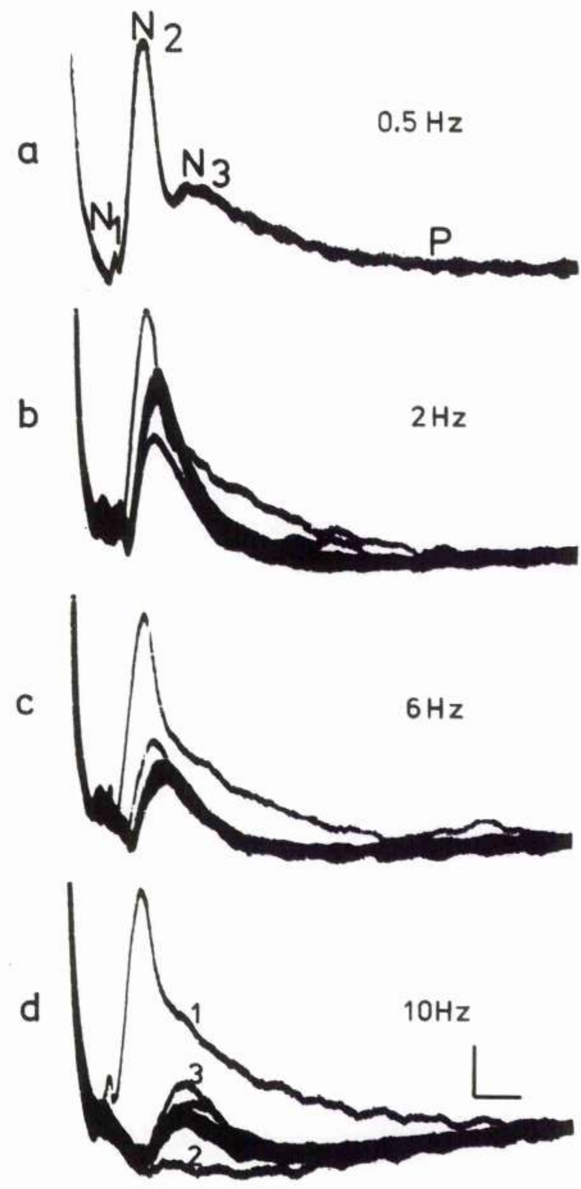
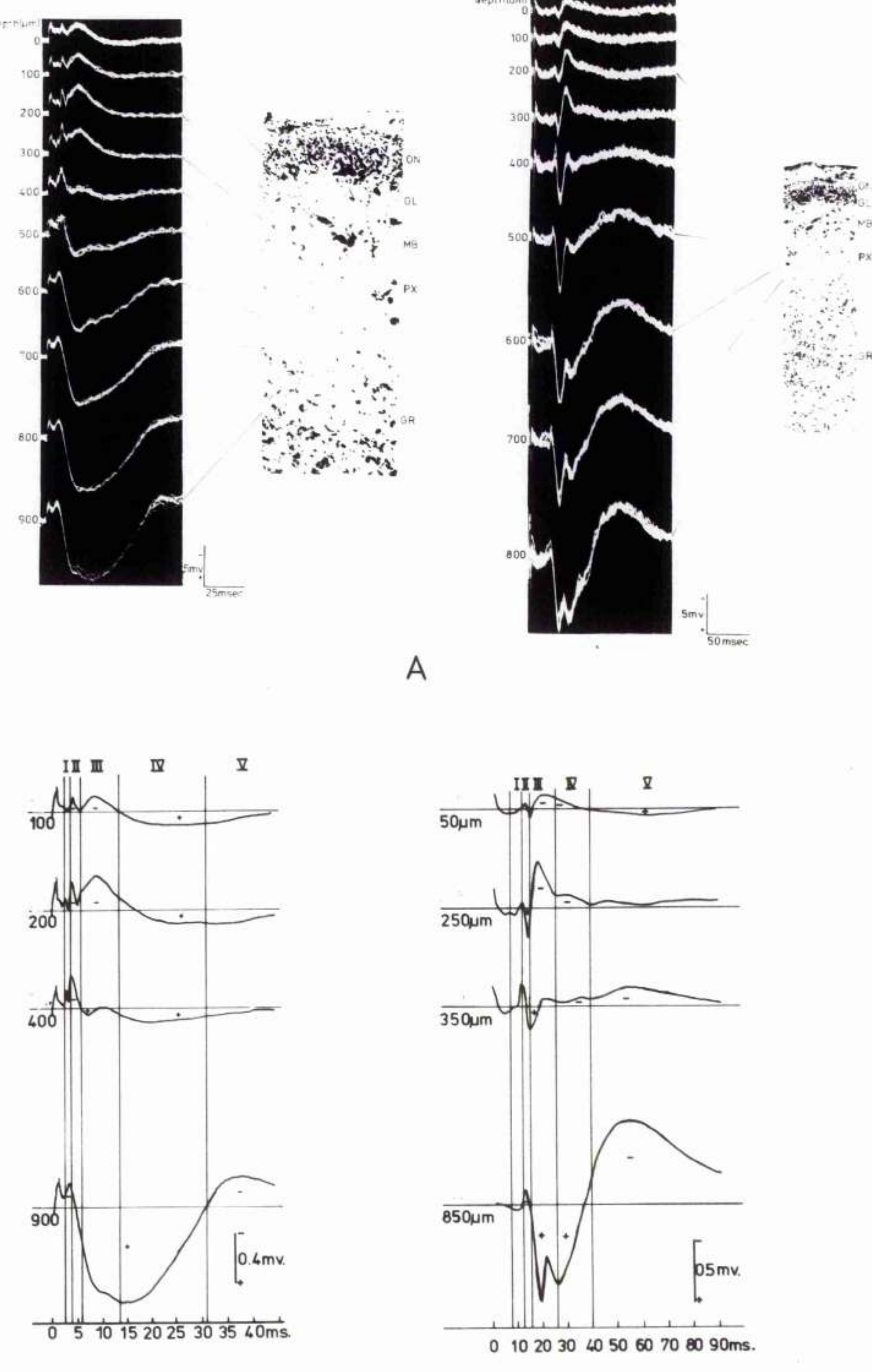


Figure IV. 7A: Field potentials recorded from the olfactory bulbs of *Gadus morhua* (left) and *Salmo gairdneri* (right), following single shock stimulation of the olfactory tract and nerve respectively. Each record represents ten superimposed sweeps of the oscilloscope beam. The histological sections shown alongside the physiological recordings are from points very close to the electrode tracks; they are stained with cresyl violet. ON, olfactory nerve layer; GL, glomerular layer; MB, mitral cell layer; PX, plexiform layer; GR, granule cell layer.

Figure IV. 7B: Line drawings of field potentials at the surface, glomerular layer, mitral cell layer and granule cell layer. Traced from the recordings shown above and divided into five periods, each corresponding to a particular surface wave. Left-hand side, olfactory tract stimulation, *Gadus morhua*; right-hand side, olfactory nerve stimulation, *Salmo gairdneri*.

Figure IV. 7: Field potentials from olfactory bulb of cod & trout



positive wave at the centre of the bulb. The $N_2$ wave reaches its maximum amplitude in the narrow region between the glomeruli and the mitral cell bodies, which contains the thick mitral dendrites and the terminals of granule cell dendrites. The wave reverses polarity just below the mitral cell bodies and is synchronous with an intense positivity at the centre of the bulb. The $N_3$ wave also reaches its maximum amplitude just above the mitral cells but it reverses polarity in the plexiform layer. It, too, is synchronous with a positivity at the centre of the bulb but of lower amplitude than that associated with $N_2$. The P wave is maximal in the region of the glomeruli; reverses polarity in the vicinity of the mitral cells and is synchronous with an intense negative wave at the centre of the bulb, being almost a mirror image of $N_2$.

Stimulation of the olfactory tract leads to the generation of a similar but not identical series of potentials. An initial positive wave often preceeds $N_1$ but $N_3$ is not often seen (Figure IV. 7A, left). As in the orthodromic situation, the $N_1$ wave always reached its maximum amplitude at the region of the mitral cell bodies, the surface wave being synchronous with a shallow positivity at the bulbar centre. $N_2$ reaches its maximum amplitude in the glomerular region and reverses polarity in the mitral cell layer. As in the case of the olfactory nerve induced $N_2$, the $N_2$ resulting from olfactory tract stimulation is also synchronous with an intense positive wave at the centre of the bulb. The P wave reaches its maximum amplitude in the glomerular layer and reverses polarity just below the mitral cell layer to become negative at the centre of the bulb. When seen, the initial positive wave appeared synchronously with the

$N_1$ wave in the centre of the bulb. (Figure IV. 10).

3. One dimensional analysis of field potentials

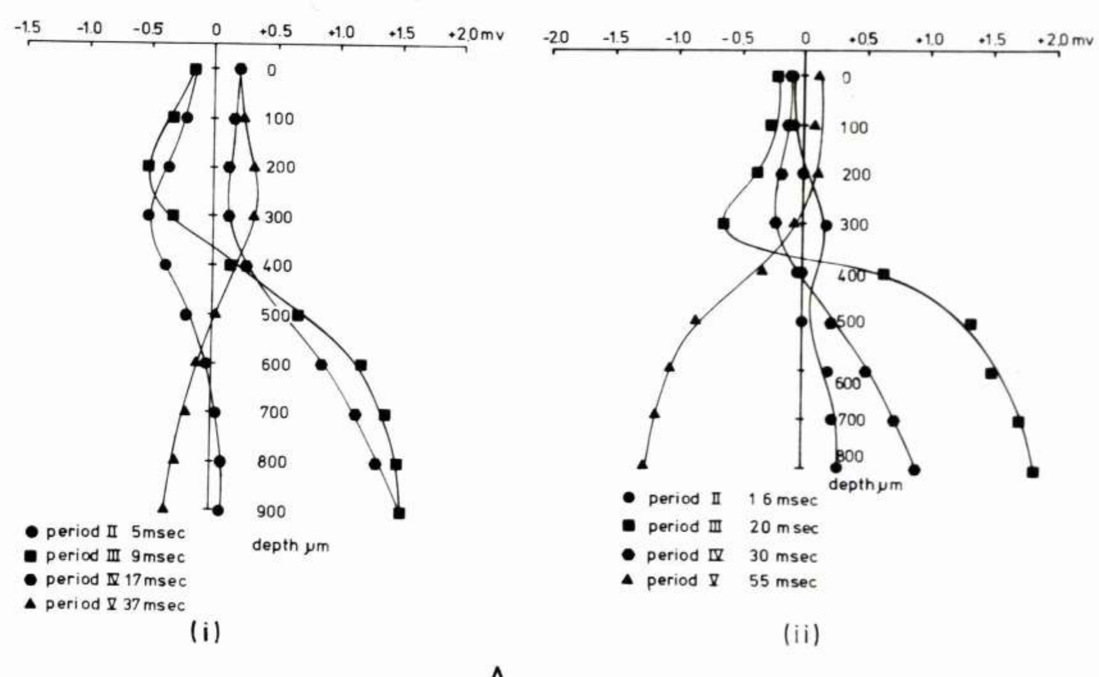
Line drawings of selected field potential records are shown in Figure IV. 8B for the two routes of stimulation. For convenience, the waveforms have been divided into five periods, I, II, III, IV and V, corresponding to the individual waves which form the complex field potentials. By measuring the magnitude of the recorded field potentials at particular latencies and plotting as a function of recording depth, it is possible to obtain a more accurate indication of the direction of current flow at the point of electrode penetration. Thus, Figure IV. 8A shows examples of such potential profiles for the sets of records in Figure IV. 7A. The potential profiles are for the times corresponding to periods II, III, IV and V of Figure IV. 7B, providing direct comparison of current flow in the bulb at these particular latencies. The most obvious feature revealed by these potential profiles is the intense flow of extracellular current across the mitral cell layer at the time of the $N_2$ wave, in both the orthodromic and antidromic situations, resulting in a dipolar field.

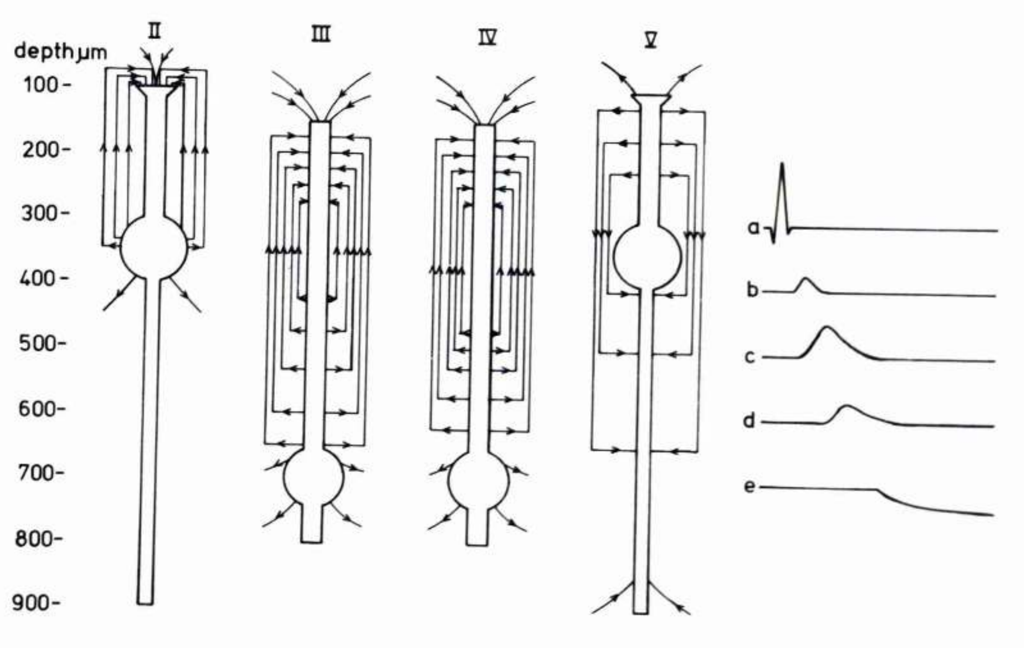
The data in Figures IV.7 and IV.8 was obtained from two different species, the reason being the relatively easy access to the relevant stimulation route in the species used. Figure IV.9, however, presents data from a single experiment using Gadus morhua. In this experiment the responses of the bulb to stimulation of both the olfactory nerve and olfactory tract were recorded consecutively along a single electrode track. The graph beside the recordings emphasizes the almost

Figure IV. 8A: Potential profiles of $N_1$, $N_2$, $N_3$ and P plotted at the latencies shown on the figure, from the records in Figure IV. 7A. (i) Olfactory tract stimulation, Gadus morhua: (ii) Olfactory nerve stimulation, Salmo gairdneri.

Figure IV. 8B: Diagram showing the current flow around bulbar neurons following a single shock to the olfactory nerve. II, III, IV and V correspond to the equivalent periods in Figures IV. 7B and IV. 8A. This figure is presented here for ease of comparison with the preceeding figures. A more complete legend and a full text explanation is given in the discussion at the end of this chapter.

Figure IV. 8: Analysis of bulbar potential fields



<u>Figure IV. 9</u>: Field potentials evoked by stimulation of both the olfactory tract and nerve in <u>Gadus morhua</u>. Each pair of records was recorded at the same point. On the left is a histological section from a point close to the electrode track, stained with cresyl violet. On the right is plotted the potential profile of the $N_2$ wave evoked by both routes of stimulation. ON, olfactory nerve; OT, olfactory tract; GL, glomerular layer; MB, mitral cell body layer; PX, plexiform layer; GR, granular layer; RP, reversal point of field potential.

Figure IV. 9: Field potentials evoked in the olfactory bulb of Gadus morhua by olfactory tract & nerve stimulation

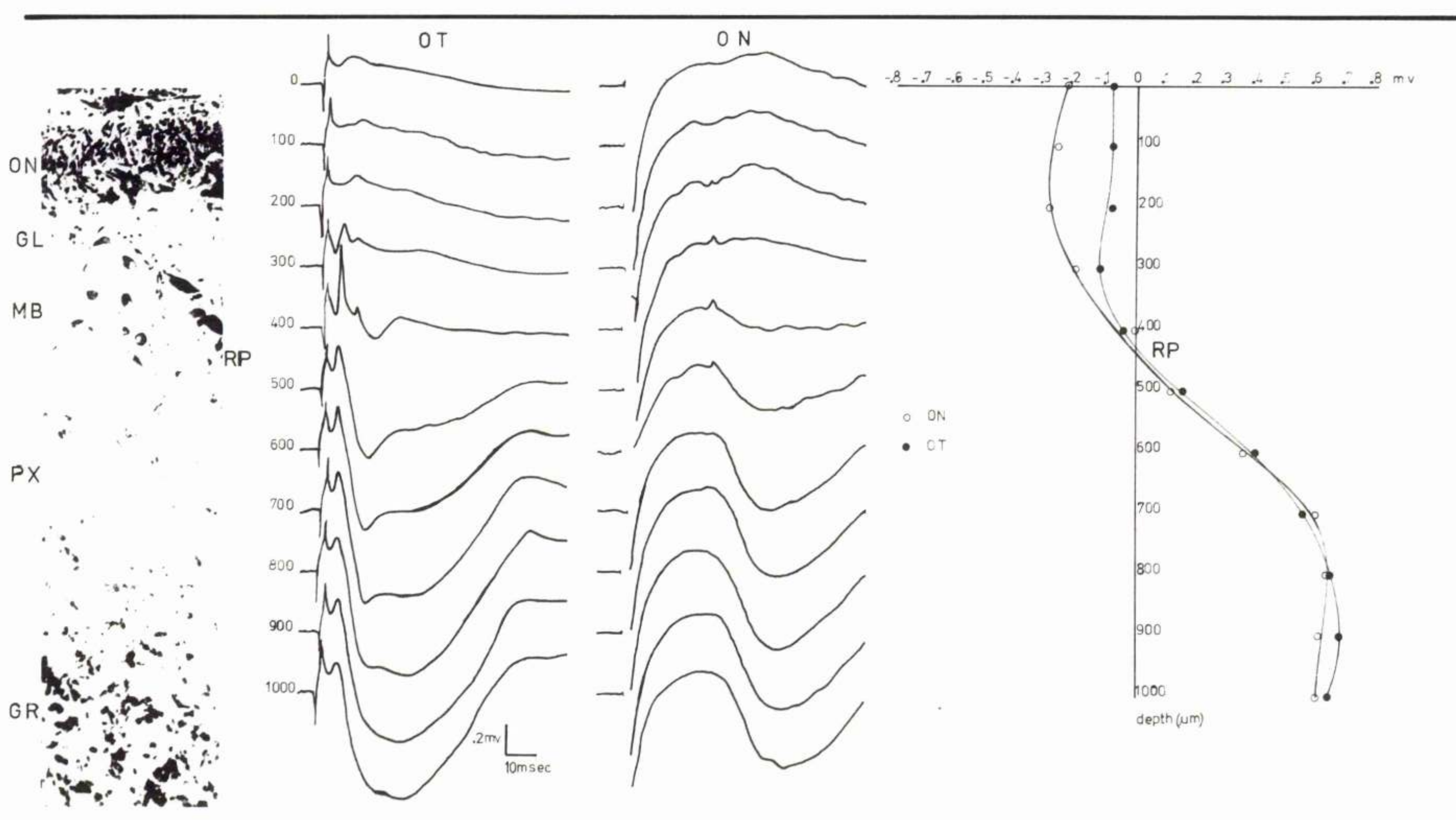


Figure IV. 10: Field potentials evoked by olfactory tract stimulation in *Gadus morhua*. Each record in A represents a single sweep; all eight traces were photographed together from a storage oscilloscope. In B the peak latency of $N_1$ is shown as a function of recording depth. Note the initial positive deflection ($P_1$) in the more superficial records shown in A.

Figure IV. 10: Field potentials evoked by olfactory tract stimulation

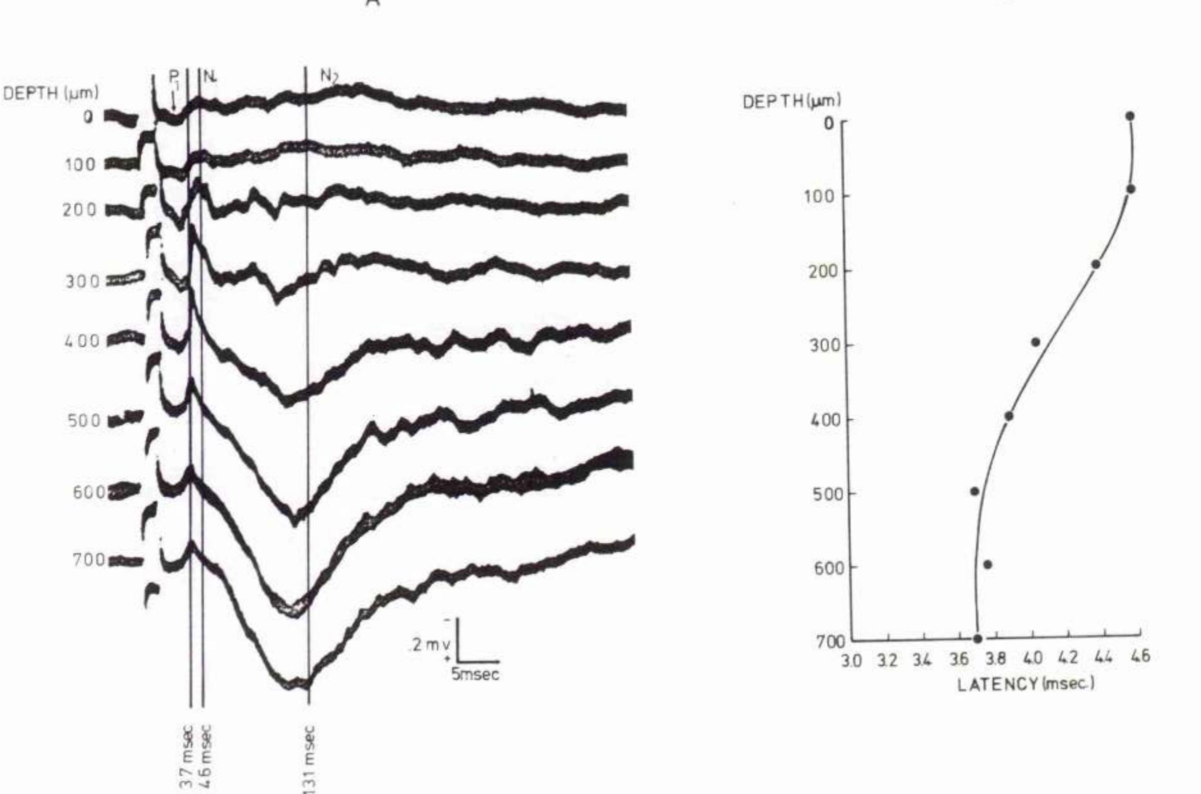


Figure IV. 11: Averaged evoked potentials from the olfactory bulb of *Salmo gairdneri*. The records in A are averages of 32 sweeps; recordings were made at intervals of 25 μm right across the bulb. In B the magnitudes of the field potentials at 15 and 20 msec. are plotted as a function of recording depth. The abnormal distribution of potential was associated with an abnormal ventral bias in the distribution of cellular elements in this particular olfactory bulb.

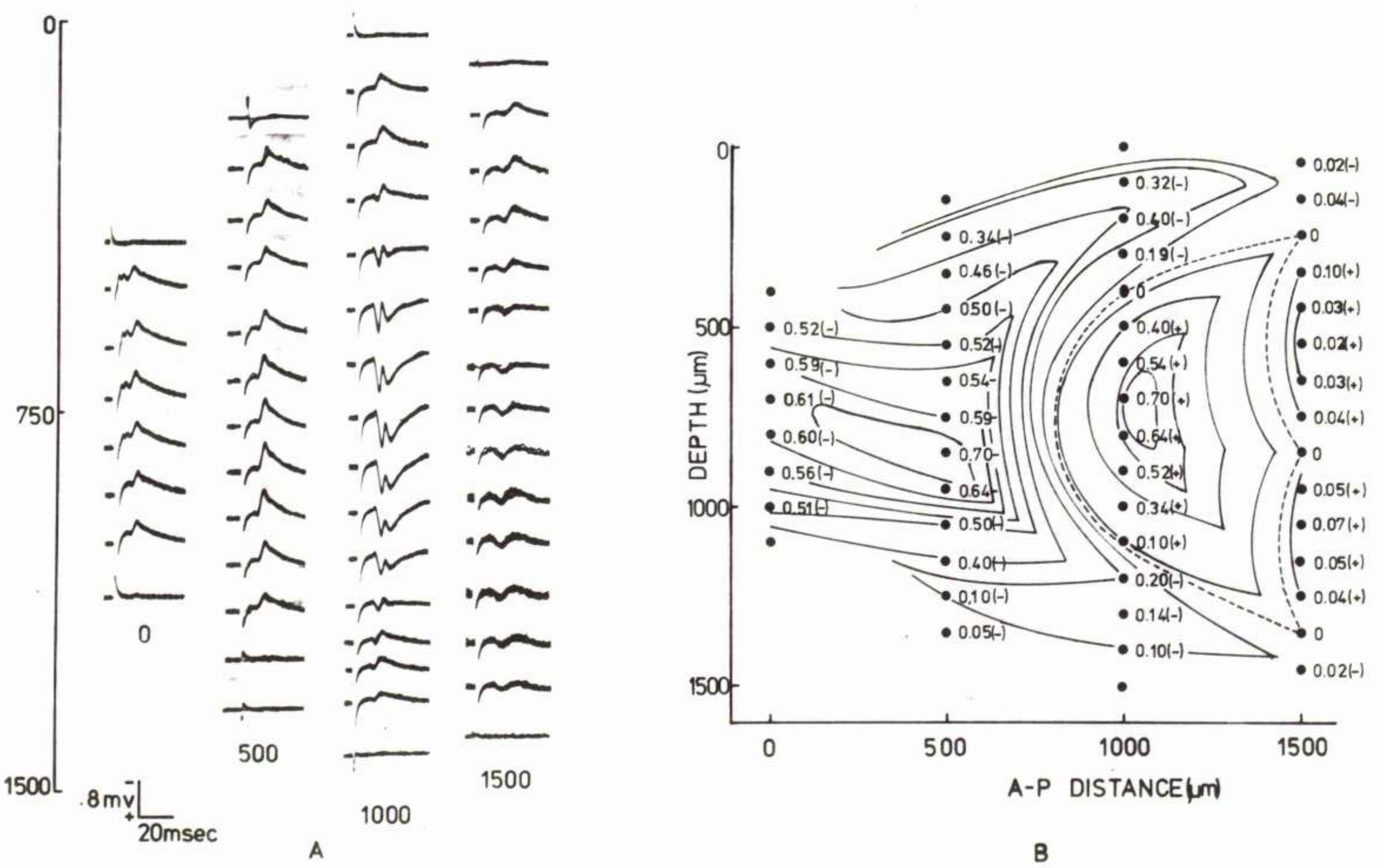
Figure IV. 11: Averaged olfactory nerve evoked field potentials

A

B

Figure IV. 12: A simple isopotential plot showing the bulbar distribution of potential at the time of the $N_2$ wave. This map is simple in that the number of recording points is relatively few. A is the array of recordings from which the map was drawn; they are arranged relative to the series labelled 1000, which represents the point of maximum diameter in this plane. The first record in each group was recorded at the bulbar surface. The map B was hand-drawn by the method of approximate linear interpolation.

Figure IV. 12: Isopotential diagram for the time of the $N_2$ wave and original recordings



identical potential profiles of the $N_2$ wave evoked by both routes of stimulation. The two waves reverse polarity at exactly the same depth, just below the layer of mitral cells.

In this type of experiment, recordings were routinely made at intervals of 100 µm and measurements of potential from the records were then plotted as a function of depth. In the experiment shown in Figure IV. 11, recordings were made at 25 µm intervals and averaged on-line. Measurements from the resulting averaged evoked potentials were plotted against depth for latencies corresponding to $N_1$ and $N_2$. As a result of the short distance between individual recordings, the small variations from the smoothed profile can be accounted for by the activity of individual neurons close to the electrode track. The unusual distribution of potential in this experiment was associated with an abnormal histological appearance of the bulb. The recordings were made from the anterior part of the bulb, where a ventral bias was shown in the distribution of the cellular elements.

4. Two dimensional analysis of field potentials

The above type of analysis is limited in the amount of information it can provide concerning the activity of the whole area under investigation. Gross extrapolation is needed to explain the results on a wider basis. A more complete picture can be formed by making recordings along a series of pre-determined stereotaxic co-ordinates, forming either a two or a three dimensional array of points. By measuring the magnitude of the potentials recorded, again always at the same latency, and writing these on a graph corresponding to the recording co-ordinates, isopotential

contour maps can be constructed by joining together points of equal potential. The examples given in this section (Figures IV. 12 and 13) are two dimensional analyses of potential distribution in the sagittal plane resulting from olfactory nerve stimulation. Appendix I contains the results of a comprehensive computer analysis of potential distribution within a three dimensional array of recording points.

An array of oscillographic records of field potentials arranged relative to the largest dorsoventral series is shown in Figure IV. 12. The isopotential map to the right of this array was drawn by visual linear interpolation for the time corresponding to the peak of the $N_2$ wave. From this map it can be seen that the negative focus of the dipolar field associated with $N_2$ is located in the anterior rather than circumjacent parts of the bulb. In other words, the intrapolar axis lies in the anterio-posterior plane of the bulb. The zero isopotential contour approximates to a parabola and corresponds to the layer of mitral cell bodies.

A more sophisticated approach is shown in Figure IV. 13. The recorded waveforms are on-line averages of 32 sweeps, arranged as in Figure IV. 12. The magnitude of these potential waves was measured at four different latencies to construct the isopotential maps below. The latencies correspond to the surface peaks of the $N_1$, $N_2$, $N_3$ and P waves. These plots confirm and extend the results obtained from the type of experiment shown in Figure IV. 12. The negative pole in I corresponds to the region of mitral cells in this

_Figure IV. 13_: An array of averaged evoked potentials recorded from the olfactory bulb of _Salmo gairdneri_, following single shock stimulation of the olfactory nerve at 0.5 Hz and a series of isopotential contour maps constructed from them. Each of the potential transients shown is an average of 32 sweeps and all the records are arranged relative to the series labelled 1700, which was recorded at the point of greatest bulbar diameter in this plane. The isopotential maps were drawn for the latencies shown and the numbers I, II, III and IV correspond to the numbers at the top of each series of records. In each map the zero isopotential contour is drawn as a heavy line. In this obviously long experiment, there was no visible deterioration of the preparation.

Figure IV. 13: Averaged evoked potentials & isopotential maps constructed from them

particular sagittal plane; it also corresponds with the zero isopotential contour in II and IV. At these two latencies the two poles of the respective dipolar fields are mirror images of one another. The distribution of potential in III is quite different from the other three examples. The overall pattern is of an asymmetrical dipole having its positive focus in the centre of the bulb and a circumjacently oriented, shallow negative pole. Thus, there is an intense negative focus associated with the $N_2$ wave at the anterior end of the bulb. Moving caudalwards, we see the development of the $N_1$ wave, followed by a sudden change in polarity towards the centre of the bulb (Figure IV. 21). Similarly, the slow positive wave seen at the anterior end of the bulb becomes zero in the region of the $N_1$ peak and develops into an intense, slow negative wave at the centre. It is interesting to note that this negativity appears to be compounded from two separate components (See also Figure IV. 18).

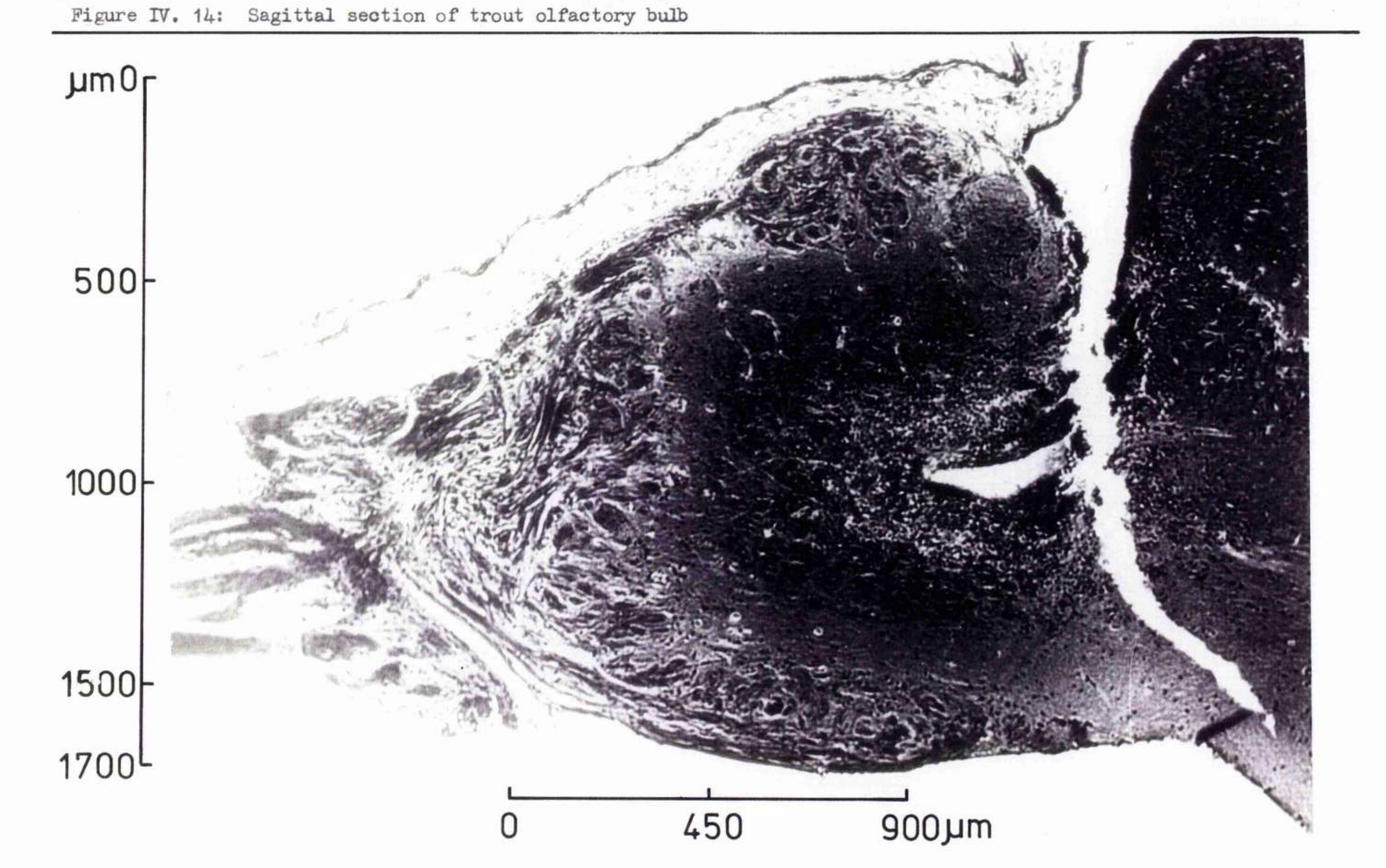
The anterio-posterior axes shown by most of the intrinsic dipolar fields appear to be associated with a bias of cellular elements, particularly mitral cells, towards the anterior end of the bulb. This can clearly be seen in some of the figures presented in the previous chapter and by reference to Figure IV. 14, a histological section taken from the brain used for the averaged evoked potential experiment described above.

Field potentials have been evoked in the olfactory bulbs of nearly all the experimental animals used in this study, the waveforms described above being entirely

<u>Figure IV. 14:</u> Low power light micrograph of entire olfactory bulb in sagittal section. The recordings of averaged evoked potentials in Figure IV. 13 were made from this particular olfactory bulb.

(*Salmo gairdneri*; thionin stain)

Figure IV. 14: Sagittal section of trout olfactory bulb



reproducible in several species: (*Salmo gairdneri*, *Salmo trutta*, *Gadus morhua*, *Melanogrammus aeglefinus*, *Raniceps ranius*, *Pleuronectes platessa*, *Myoxocephalus scorpius* and *Carassius auratus*).

5. Response to double and repetitive stimulation

It has been found that the most consistent and overwhelming effect of a conditioning shock applied to the olfactory nerve or tract is suppression of all or part of the field potential evoked by a subsequent test stimulus. This is well-illustrated in Figures IV. 15, 16, 17 and 18. All components of the field potential have been shown to be inhibited to varying extents. Figures IV. 16 and 17 show that $N_1$ is inhibited by a testing shock given to the olfactory nerve but a facilitation was seen at intensities slightly lower than those needed to cause inhibition. Figures IV. 15 and 16 show the pronounced inhibition of $N_2$ and $N_3$, following a conditioning shock to the olfactory nerve, this inhibition being also reflected by the positive waves at the bulbar centre synchronous with $N_2$ and $N_3$. At certain stimulus intensities and conditioning - test shock intervals a facilitation was shown by $N_2$ and the deep negative wave synchronous with P (Figures IV. 16b and 18), the latter facilitation being accompanied by suppression of the positive reflection of $N_3$. The facilitation was always followed by inhibition at shorter conditioning - test intervals or at greater conditioning shock intensities. The pronounced inhibitory effect of a conditioning shock to the olfactory tract is shown in Figure IV. 19.

The effects of repetitive stimulation on the orthodromically induced field potentials are shown in Figures IV.

Figure IV. 15: Inhibition of field potentials at both poles of the $N_2$ dipolar field, following a conditioning shock to the olfactory nerve. A, inhibition at the negative pole; B, inhibition at the positive pole. Each graph is a plot of test response amplitude as a percentage of the conditioning response amplitude against the C-T interval. a and b are sample records from this experiment, each point on the graphs being the mean of measurements made from ten such records. Circles represent measurements made at the time of $N_2$; triangles at the time of $N_3$ and squares at the time of the late deep negative wave synchronous with the surface P wave.

Figure IV. 15: Inhibition produced by a conditioning ON shock

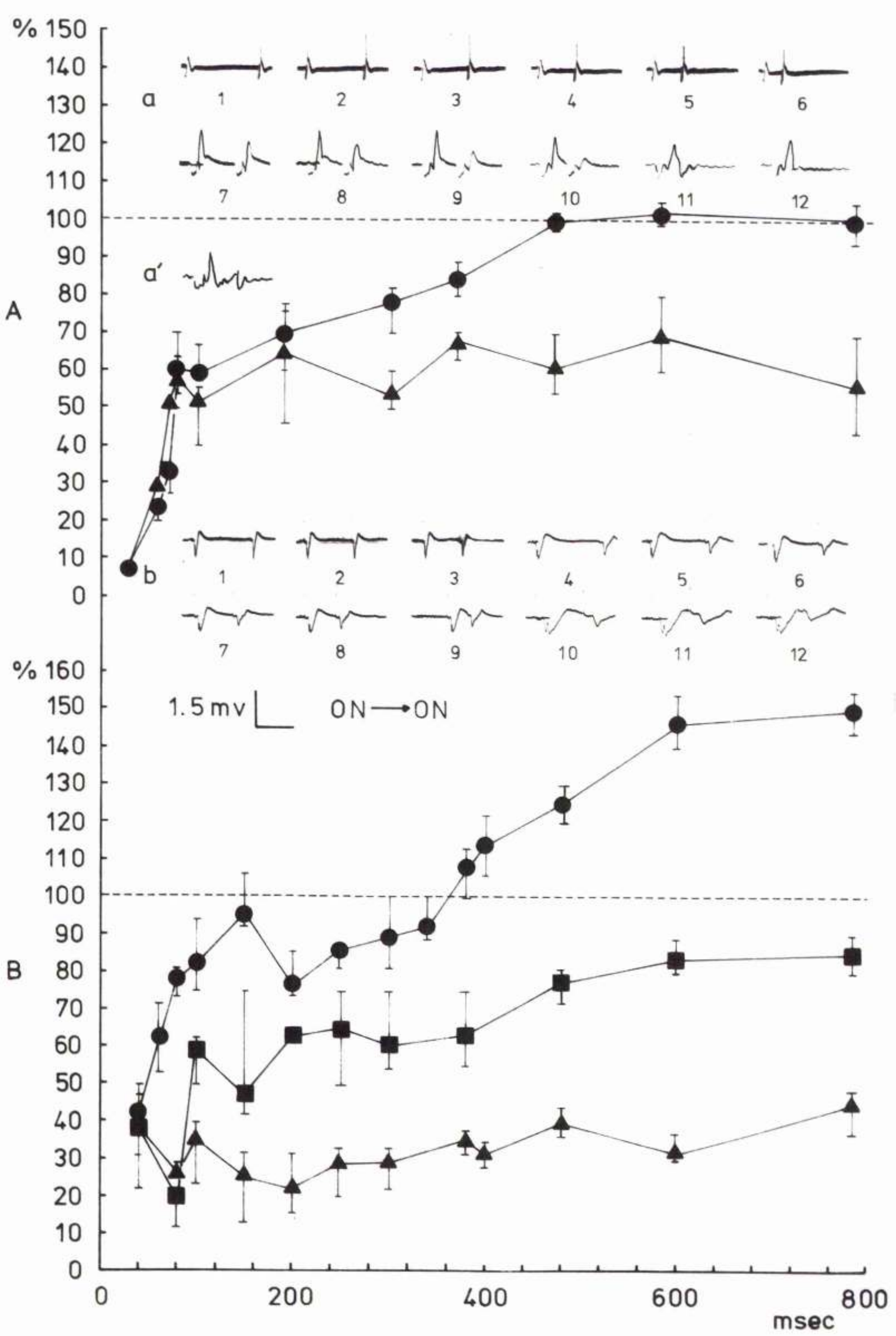


Figure IV. 16: The effect of an olfactory nerve conditioning shock on the field potential recorded in the region of the mitral cell bodies. Note the high amplitude of the $N_1$ wave and the relatively small $N_2$ wave. a shows the effect of reducing the time interval between conditioning and test shocks maintained at constant intensity. b, c, d and e show the effect of increasing the intensity of the conditioning stimulus while the intensity of the test stimulus is kept constant for differing C-T intervals. f is a series of responses to single olfactory nerve shocks; recordings were made at 100 μm intervals from the surface to show the relationship of the waves in a-e to the fields in adjacent bulbar strata. g shows detail of the field potential at 900 μm, the depth at which the double shock study was made.

Figure IV. 16: Effect of CN conditioning shock on $N_1$ wave.

ms: 18, 40, 50, 70, 84, 98, 115, 120, 140, 150 — a

130: 1–13 — b

90: 1–13 — c

50: 1–13 — d

30 msec: 1–13 — e

μm: 0, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200 — f

$N_1$, $N_2$, C — g

Figure IV. 17: Graphs plotted from the experimental recordings shown in Figure IV. 16. b, c, d and e are plots of the amplitude of the test response as a function of conditioning stimulus intensity. f is a potential profile plot of the corresponding series of potentials in Figure IV.16. g is a plot of the test response amplitude in Figure IV. 16a as a function of the conditioning - test interval; note the exaggerated voltage scale in this plot. Filled circles correspond to measurements at the latency of $N_2$; filled squares to the latency of $N_1$ and open circles to the latency of $N_3$.

Figure IV. 17: Graphic representation of the records in Figure IV. 16

Figure IV. 18: The effect of an olfactory nerve conditioning shock on the field potential recorded in the granule cell region. All records in this figure are responses to conditioning and test shocks of equal amplitude. The C-T interval gets progressively shorter from top left to bottom right. Note the overall facilitation at long intervals, which is replaced by inhibition as the shock interval decreases. These records clearly show that the negative reflection of the surface P wave actually consists of two distinct components. At long C-T intervals the first component shows distinct potentiation, whilst the second component is inhibited. This is accompanied by a potentiation of the first deep positive wave and complete inhibition of the second. At shorter test intervals, when the overall effect is one of suppression, the second positive and negative components remain inhibited, accompanied by suppression of the first positive and negative components.

Calibration: Vertical - .8mV; Horizontal - 1, 160 msec., 2, 50 msec., 3(5), 25 msec.

Figure IV. 18: Effect of ON conditioning shock in granule cell region

1

2

3

6 and 20. Repetitive stimulation clearly distinguishes the compound action potential of the olfactory nerve from the waves generated within the bulb. As shown here, relatively low frequency tetanic bursts often cause an increase in the amplitude of the compound action potential. The $N_1$ wave is sustained up until about 12 Hz, whilst $N_2$ and P fail to respond to every shock at frequencies in excess of 2 Hz. Except at very low stimulation frequencies, $N_3$ is often masked by $N_2$. As the frequency of stimulation is increased, the latency and duration of $N_2$ appear to increase but close observation reveals that the $N_2$ wave is rapidly lost and its apparent broadening is due to summation with $N_3$. The wave becomes smaller as fewer cells in the population responsible for generating $N_2$ are activated until, at frequencies around 10 Hz, the only negative synaptic component remaining, other than $N_1$, appears to closely resemble the $N_3$ wave, which is clearly visible at very low frequencies and is probably identical with it. $N_1$ follows olfactory tract stimulation at frequencies well over 100 Hz but $N_2$ and P fail to respond to every shock at frequencies as low as 2 - 5 Hz.

## DISCUSSION

The most plausible explanation for the decrement of the olfactory nerve action potential with distance is temporal dispersion. In other words, the volley of action potentials is distributed in time, owing to differing rates and distances of axonal conduction. Although the individual fibres are generating action potentials, they are not 'seen' by an extracellular recording electrode placed further than a certain critical distance away from the point of stimulation.

Figure IV. 19: Inhibition of olfactory nerve induced field potential by conditioning shock to the olfactory tract in *Myoxocephalus scorpius*. In A, 1-12, the interval between shocks of equal intensity was gradually reduced. In B and C, 1-12, the conditioning shock intensity was gradually raised, while the testing shock intensity was kept constant. Shock intervals: B, 55 msec., C, 110 msec. Calibration: vertical 1mV; horizontal 40 msec.

Figure IV. 19: Inhibition of O.N. field potential by O.T. stimulation

A B C

1
2
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10
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14

Figure IV. 20: The effect of increasing stimulation frequency on the averaged potential evoked by olfactory nerve stimulation. Note the broadening and loss in amplitude of $N_1$ and $N_2$, also the latency increase of $N_2$. Comparison with Figure IV. 6 reveals that this apparent latency increase may actually be the unmasking of $N_3$ by the simultaneous loss of $N_2$. Also of interest is the increase in amplitude of the compound action potential (CAP) with increasing stimulation frequency. Calibration: Vertical - .2 mV; Horizontal - A, 5 msec., B, 10 msec.

Each record is an average of 64 sweeps.

Figure IV. 20: Effect of increasing stimulation frequency on averaged evoked potential

A similar but altogether more sophisticated biophysical investigation of the properties of the primary olfactory nerve in the cat (Freeman, 1972a) has shown that the disappearance of this compound action potential from extracellular records could indeed be accounted for by variations in axon diameter and, therefore, conduction velocity in the nerve. Freeman also concluded that, owing to divergence of the afferent axons in the olfactory nerve layer, receptors excited electrically in any small part of the olfactory mucosa would deliver impulses to only about twenty per cent of the bulbar surface. From the data presented above, and work in progress concerning the distribution of axon diameters in the fish olfactory nerve (MacLeod and Lowe, unpublished observations), it seems reasonable to conclude that similar properties of spatial divergence and temporal dispersion are common to the olfactory nerve of teleost fish, in which case the abnormal result in Figure IV. 5 can be attributed to damage of superficial fibres near the stimulating electrode. Work in progress on the African lungfish Protopterus annectens (MacLeod and Godet, unpublished observations) has revealed a similar decremental process and Ottoson (1959) has published similar observations obtained from the frog. It, therefore, seems likely that these properties are shared by the olfactory nerves of all vertebrate classes. It should be pointed out here that the values observed in the present study for conduction, velocity, duration and refractoriness for the compound action potential agree well with values obtained from other species of fish and from other vertebrate classes, when allowances are made for temperature differences (Table IV. 1).

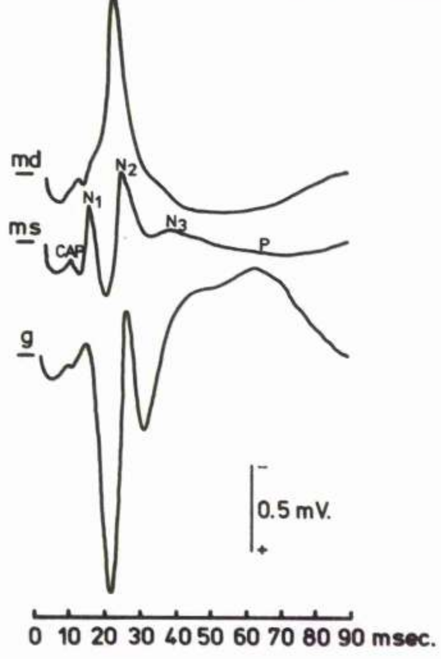
Table IV.1: Physiological properties of various olfactory nerves

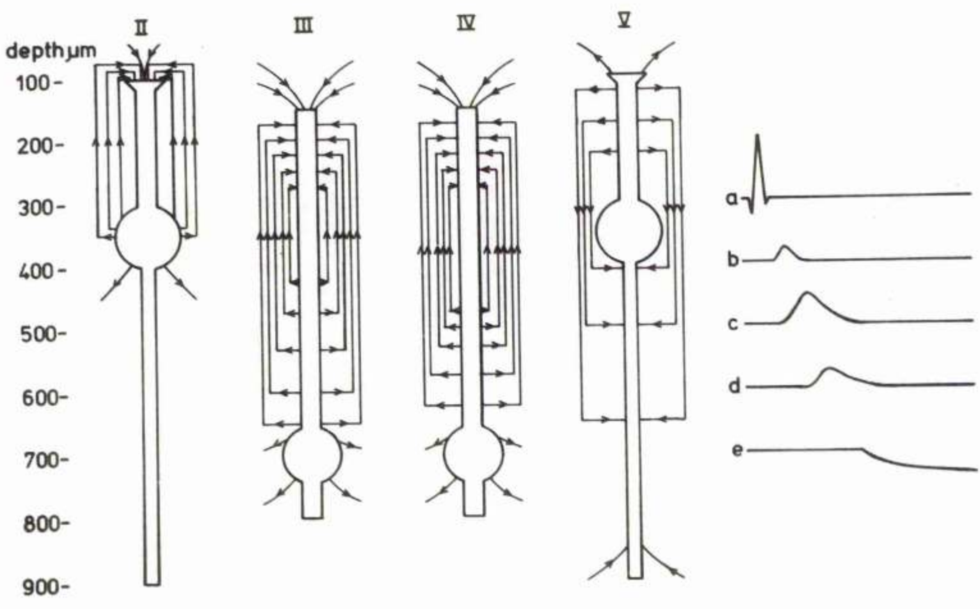
| | Authur | Conduction Velocity (m/sec) | Duration (msec) | Wave-length (mm) | Refractory period (msec) | Temp. °C. |
|---|---|---|---|---|---|---|
| Cat | Freeman (1972a) | 0.42 ± .05 | 2.5 | 1.0 | | 37 |
| Rabbit | Nicoll (1972) | 0.34 | 2 | 0.68 | 2.7 | 37 |
| Frog | Ottoson (1959) | 0.14 | | | 30 | |
| Lungfish | MacLeod Godet (unpubd.) | 0.2 | | | | 15 |
| Pike | Gasser (1956) | 0.2 | 30 | 6.0 | 30 | 21 |
| <u>Raniceps ranius</u> | Doving (1967) | 1.2 | | | | 10 |
| <u>Salmo gairdneri</u> | MacLeod (This thesis) | 0.13 | 10 | 1.3 | 20 | 12 |

Figure IV. 21: Averaged evoked potentials from trout olfactory bulb. Each record is an average of 64 sweeps following olfactory nerve stimulation at 0.5 Hz. The upper trace (md) is from the negative focus of the granule cell dipolar field and the lower trace (g) is from the positive focus. Thus, they represent the extremes of polarity which can be recorded from the bulb under these conditions. The middle trace (ms) is from the region of the mitral cell bodies in the anterior part of the bulb. All three were recorded sequentially in the rostro-caudal plane along the intrapolar axis.

Figure IV. 22: Diagram showing the current flow around bulbar neurons at various latencies following a shock to the olfactory nerve. II, III, IV and V refer to the equivalent periods shown in Figure IV. 7B. Mitral cells are represented in II and V, whilst 'granular' cells are represented in III and IV. On the right is a diagrammatic breakdown of the components contributing to the complex field potential recorded at the bulbar surface. The compound action potential of the olfactory nerve is represented by a, while $N_1$, $N_2$, $N_3$ and P are represented by b, c, d and e respectively. To describe the antidromic situation, another mitral cell would have to be inserted at the beginning of the cycle with the two poles of the potential field inverted relative to the field around the cell in II. To account for the second deep negative component, another mitral cell could be added at the end of the cycle, with the direction of current flow identical to that around the cell in V.

Figures IV. 21 and 22



The waves recorded in the olfactory bulb following stimulation of the olfactory nerve are all thought to be generated by synchronous post-synaptic events in bulbar neurons. The potential profile of the $N_1$ wave and the fact that the majority of single units encountered in the mitral cell layer (see next chapter) fire at the same latency, indicates that this wave represents synchronous synaptic depolarization of the mitral cell dendritic tufts in the glomerular layer by afferent impulses travelling in the olfactory nerve fibres. At the time of the surface $N_1$ wave, the somata and axons of the mitral cells are supplying current to their depolarizing dendrites (Figure IV. 22.II). The most consistent and striking fact to have emerged from this study of olfactory bulb field potentials is the large dipolar field at the time of the $N_2$ wave (Figure IV. 21). This field reflects an intense flow of extracellular current across the mitral cell layer and indicates extensive depolarization of a neuronal population in the glomerular layer, which is anatomically suited to providing an intracellular return pathway. Such an anatomical arrangement is provided by the granule cells; thus, the $N_2$ wave is thought to be generated by massive synaptic depolarization of the granule cell dendrites just beneath the glomerular layer. This region is far less easy to define in fish than in mammals since, in the fish olfactory bulb, as already explained above, there is no clear separation of the mitral cell dendritic field into primary and secondary components. The synapses between the mitral and granule dendrites are thus scattered along the mitral dendritic shafts from the glomeruli to their somata. However, it appears that, at the time of the $N_2$ wave, the

peripheral extremities of the granule dendrites in this ill-defined locus are drawing current from their repolarizing somata lying at the centre of the bulb (Figure IV. 22.III). The surface P wave is thought to represent the synchronous synaptic hyperpolarization of the mitral cell dendrites by the 'just depolarized' dendrites of the granule cells. In this case, the mitral dendrites are supplying current to their somata and axons, thus accounting for the intense sink at the centre of the bulb (Figure IV. 22.V). The origin of the $N_3$ wave is less easily defined. It appears to follow repetitive stimulation in a way roughly equivalent to $N_1$ but its depth profile more closely resembles that of $N_2$. It could result from the monosynaptic activation of cells in the granular layer, which send processes directly into the glomeruli. This anatomical connection has been demonstrated (Johnston, 1898, 1901) but the long latency involved makes this a rather unlikely explanation. A more plausible explanation is that there are two distinct populations of interneurons within the granular layer which are differentially activated by the mitral cells. Both populations could be activated via the dendrodendritic pathway, or perhaps the radially directed collaterals of mitral cell axons are responsible for activating a different population of interneurons in a stratum slightly deeper than that of the mitral dendrites. The idea of a second population of inhibiting interneurons is well-supported by the finding that the deep negative wave synchronous with P consists of two components. The first could be the effect of the $N_2$ population on mitral cells and the second could be the effect of the $N_3$ population on the mitral cells.

Although an attractive possibility, the idea of a population of quaternery neurons being depolarized in the region of the mitral cells must be ruled out unless Dale's Law is to be contravened. It is interesting to note here that, although the $N_3$ wave is not mentioned as a separate component in any of the relevant mammalian literature, recordings of averaged evoked potentials given by Freeman (1972b) are strikingly similar to the records presented in this thesis, including the wave referred to here as $N_3$. The group of cells most likely to be responsible for generating the $N_3$ wave is the band of small neurons lining the ventricle. A more detailed anatomical understanding is needed before coming to any more precise conclusion.

In the antidromic situation, resulting from stimulation of the olfactory tract, the $N_1$ wave must be attributed to compound action currents generated during synchronous antidromic invasion of the mitral cell somata and dendrites following stimulation of their axons. The rate of antidromic invasion of the mitral dendrites, given by the slope of the graph in Figure IV.11, is 0.5 m/sec., which compares favourably with the value of 0.7 m/sec. for the rabbit (Nicoll, 1972). The $N_2$ and P waves appear to be generated by mechanisms identical with those postulated for the orthodromic route of activation. The $N_3$ wave does not often appear in records of field potentials evoked by olfactory tract stimulation, which would tend to support a monosynaptic pathway for its generation following olfactory nerve stimulation. However, the emphasis in this thesis has been on the afferent input into the bulb and it may be that a more thorough study using antidromic

stimulation may consistently reveal such a wave.

If a dendrodendritic pathway is responsible for the inhibition of mitral cells following olfactory tract stimulation, then the inhibitory pathway would be blocked by a conditioning stimulus to the tract, since the pathway through the mitral cell dendrites would be inhibited. Alternatively, if the inhibition of mitral cells is generated by a recurrent collateral pathway, then the inhibitory pathway should not be blocked by a conditioning shock to the tract, as the recurrent collateral pathway by-passes the mitral cell dendrites. A similar situation would be expected to arise following a conditioning shock to the olfactory nerve. It was found that the $N_2$ and P waves of the antidromic and orthodromic field potentials were inhibited by a conditioning shock applied to the olfactory tract or nerve. This supports the postulate of a dendrodendritic pathway, since the conditioning IPSP in mitral cells would block the $N_2$ wave if it were due to the depolarization of granule cell dendrites by mitral cell dendrites, because the excitatory pathway through the mitral cells would be blocked.

The results presented in this section indicate that the pattern of extracellular current flow around the neuronal elements in the teleost olfactory bulb, following electrical stimulation of the major afferent and efferent pathways, is similar to that previously observed in mammals. Furthermore, the results are in very close agreement with similar studies on the lungfish (MacLeod and Godet, unpublished observations); frog (Ottoson, 1959) and turtle (Orrego, 1961) and thus lend support to an hypothesis that a dendrodendritic inhibitory feed-back mechanism is common to the olfactory bulb of all vertebrates.

# V

# identification and physiological properties of bulbar neurons

## INTRODUCTION

The identification of single neurons in the vertebrate central nervous system, particularly at the time of recording in physiological experiments, has always been a difficult problem. This is undoubtedly why the authors of hitherto published works concerning the activity of single cells in the teleost olfactory bulb have been forced to refer to all recorded units as "bulbar neurons". The advent of intracellular dye injections has begun to make the unequivocal identification of recorded units a reality. However, the paucity of stable intracellular recordings obtainable in most parts of the central nervous system precludes this technique from many experimenters. The far easier extracellular ejection of dyes is of use in identifying the approximate location of the recording pipette tip but the spot produced is normally too diffuse to allow precise cellular identification.

At the time of recording, the position of the recording tip can be estimated from the stereotaxic co-ordinates. Another and far more reliable method is to monitor the extracellular field potentials evoked by pathways related to the structure under investigation. For example, the paired Mauthner neurons in the fish medulla can be antidromically activated by electrical stimulation of the spinal cord. In response to this stimulation, the Mauthner cells generate an intense negative extracellular field potential, which reaches a maximum amplitude of about 40mV in the region of the axon cap. By careful exploration with a microelectrode, it is possible to localize different regions of the cell (Furshpan &

Furukawa, 1962). The field potentials evoked in the mammalian olfactory bulb have also proved to be extremely useful in determining the position of the electrode tip at the time of recording since, as previously described, the waveform undergoes a series of changes in amplitude and polarity as the various histological layers are traversed. Of particular importance was the finding that the $N_2$ wave reversed its polarity at or near the layer of mitral cells. Single units encountered at this level, and which could be antidromically driven by olfactory tract stimulation, were considered to be mitral cells by Phillips, Powell and Shepherd (1963).

The olfactory bulb field potentials are generated by synchronous activity in bulbar neurons. An exploratory microelectrode in the vicinity of an active cell should, therefore, record its activity superimposed upon the particular region of the wave-form generated by the neuronal population of which the recorded unit is a part. Thus, the monitoring of extracellular field potentials provides information concerning the approximate location of the pipette tip and also provides a method for the identification of specific neuron types.

Several authors have used this technique to identify single units during physiological studies of neuronal circuitry in the mammalian olfactory bulb (Nicoll, 1969; Shepherd, 1963a & b; Yamamoto, Yamamoto & Iwama, 1962). Stimulation of both afferent and efferent pathways generates an IPSP in mitral cells and it is generally accepted by the above authors that these two modes of stimulation lead to the activation of tertiary interneurons (granule cells) by a similar route. A subsequent rhythmic discharge of the granule cells then feeds

back inhibition on to the secondary dendrites of the mitral cells.

RESULTS

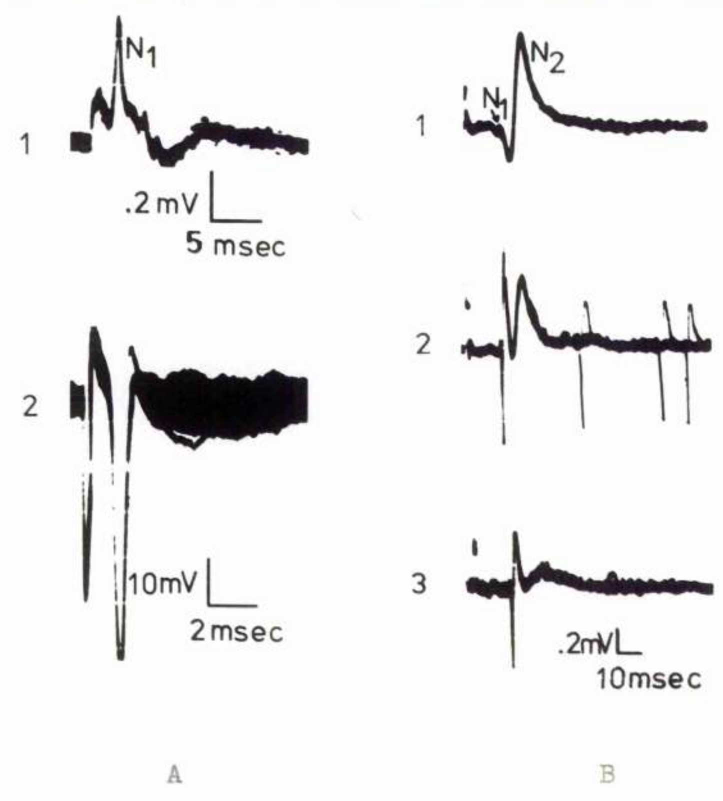
The physiological identification of recorded units

Mitral Cells

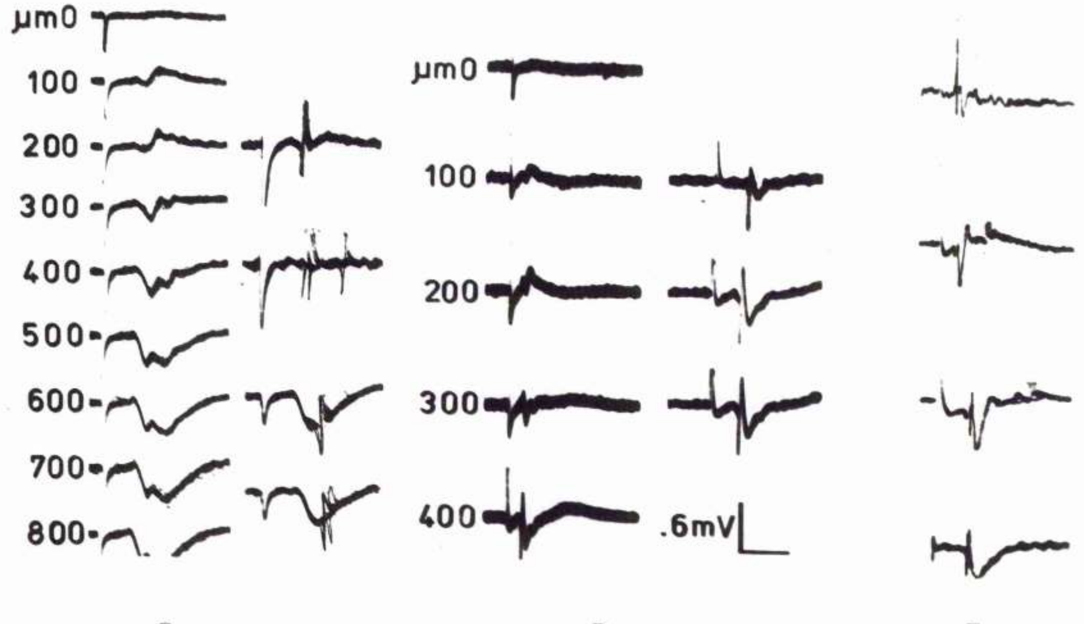
In both the antidromic and orthodromic situations, the mitral cell layer could be identified by a polarity reversal of the $N_2$ wave. In the previous chapter, this reversal was clearly shown to occur at or slightly central to the mitral cells and was often associated with a high amplitude $N_1$ wave, attributed to synchronous depolarization of the mitral cell population. Single units encountered in this layer almost invariably fire action potentials, superimposed upon this wave, following either olfactory tract or olfactory nerve stimulation (Figure V. 1). At threshold stimulus intensities, the units normally respond with a single spike but, as the shock strength is raised, up to three spikes can usually be evoked, particularly following orthodromic stimulation (Figure V. 5A). These units can be antidromically driven to frequencies of over 200 Hz.

Action potentials recorded from cells in the mitral cell layer are usually of long duration (1.7 - 2.8 msec.) and are considered to be recorded from the somata and proximal dendrites of mitral cells. However, units are frequently encountered in deeper regions of the bulb which also fire synchronously with the $N_1$ wave and which easily follow antidromic stimulation. Unlike the more superficial units, these action potentials are of short duration (0.5 - 1.6 msec.) and are considered to be recorded from the axons of mitral cells (Figure V. 1E).

Figure V. 1: The identification of recorded mitral cells




A B




C D E

FIGURE V.1: The physiological identification of mitral cells at the time of recording. A.1 shows the olfactory tract evoked field potential in the mitral cell layer. A.2 is an antidromically driven unit recorded intracellularly at the same co-ordinates as A.1. B.1 shows the olfactory nerve evoked field potential just above the mitral layer. An orthodromically driven unit is shown in B.2 and B.3, firing synchronously with the N.1 wave. C shows a typical field potential profile from the central region of the bulb with two units encountered during the experiment. The upper unit is a mitral cell; the lower one a granule cell. D shows a similarly identified mitral cell. The units shown in E were recorded from mitral cell axons.

Granule Cells

The field potential in central regions of the bulb normally consists of two high amplitude positive deflections followed by a long duration negative wave. Single units encountered in this region usually fire at least two spikes in response to a threshold shock delivered to the olfactory tract or nerve (Figures V.2 and V.3). The spikes were always of variable latency and could not follow repetitive stimulation above 10 Hz. Increasing the stimulation intensity usually resulted in a decrease in latency of the first spike and an increase in the overall number of spikes in the burst (Figure V.3). The first spike of the evoked burst always fired synchronously with one or other of the two waves. It is likely that these represent two distinct populations of tertiary neurons and these two responses have tentatively been labelled $G_1$ and $G_2$ respectively.

Periglomerular Cells

Rhythmically firing cells were sometimes encountered in the superficial part of the bulb. By analogy with the mammalian system (Shepherd, 1963b), these are considered to be periglomerular neurons (Figure V.3D).

Some physiological properties of identified neurons:

Mitral cells

Throughout this study it has proved extremely difficult to obtain stable intracellular recordings from mitral cells. In all, five successful penetrations were held for over five minutes. During these recordings the intracellular spike amplitudes rarely exceeded 50mV. Resting potentials ranged

Figure V.2: The physiological identification of granular layer cells at the time of recording. A shows the identification of the cell in Figure V.3A. To the left is a series of field potentials recorded at the depths shown. The cell was located at 800 μm and the approach to the cell is shown on the right. This cell was classified as a type I granule cell. B is a similar identification of a type II granule cell. C is another type I granule cell and D another type II granule cell.

Calibration: vertical 1mV; horizontal 50msec.

Figure V.2: The identification of recorded granule cells

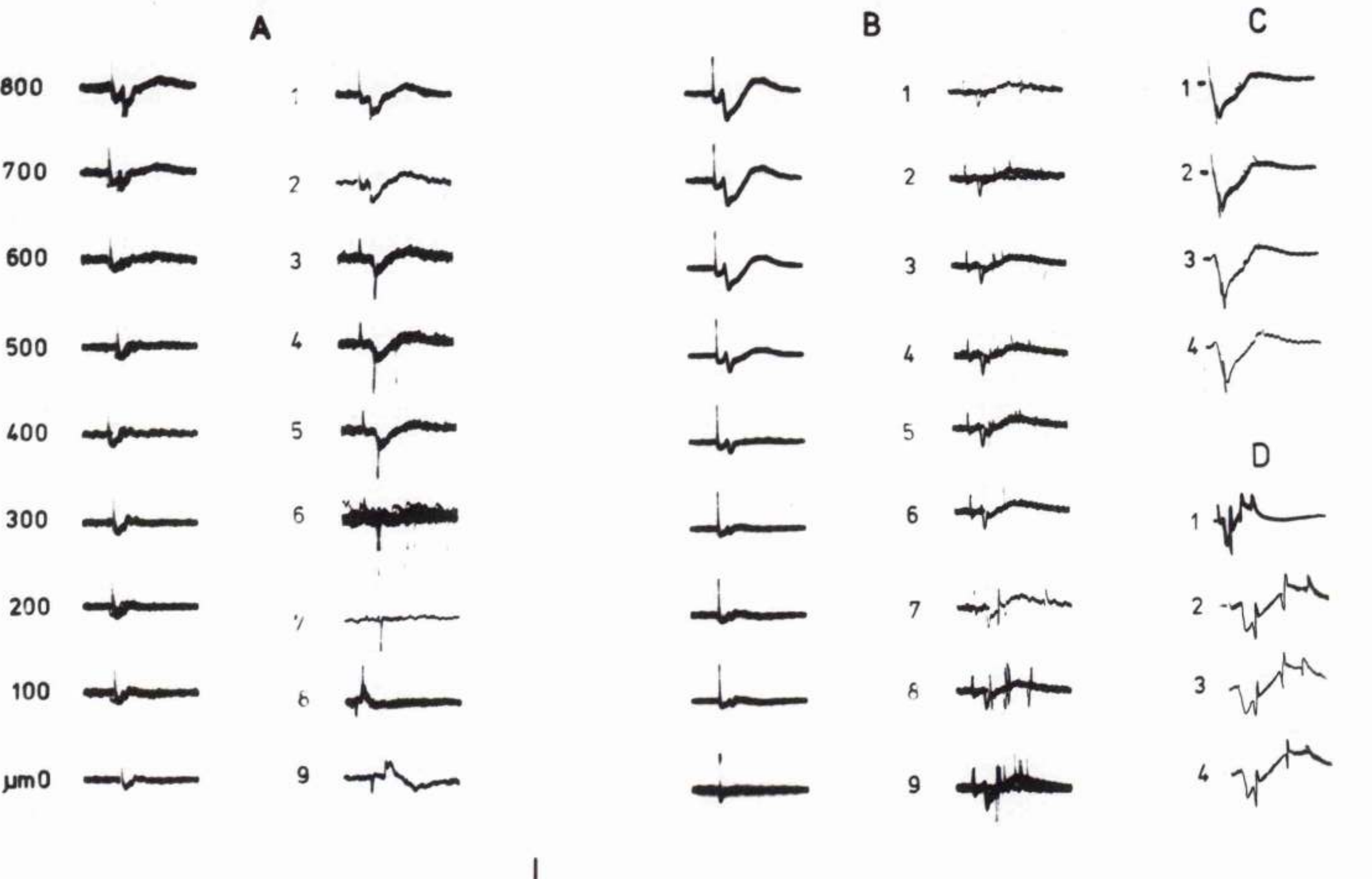
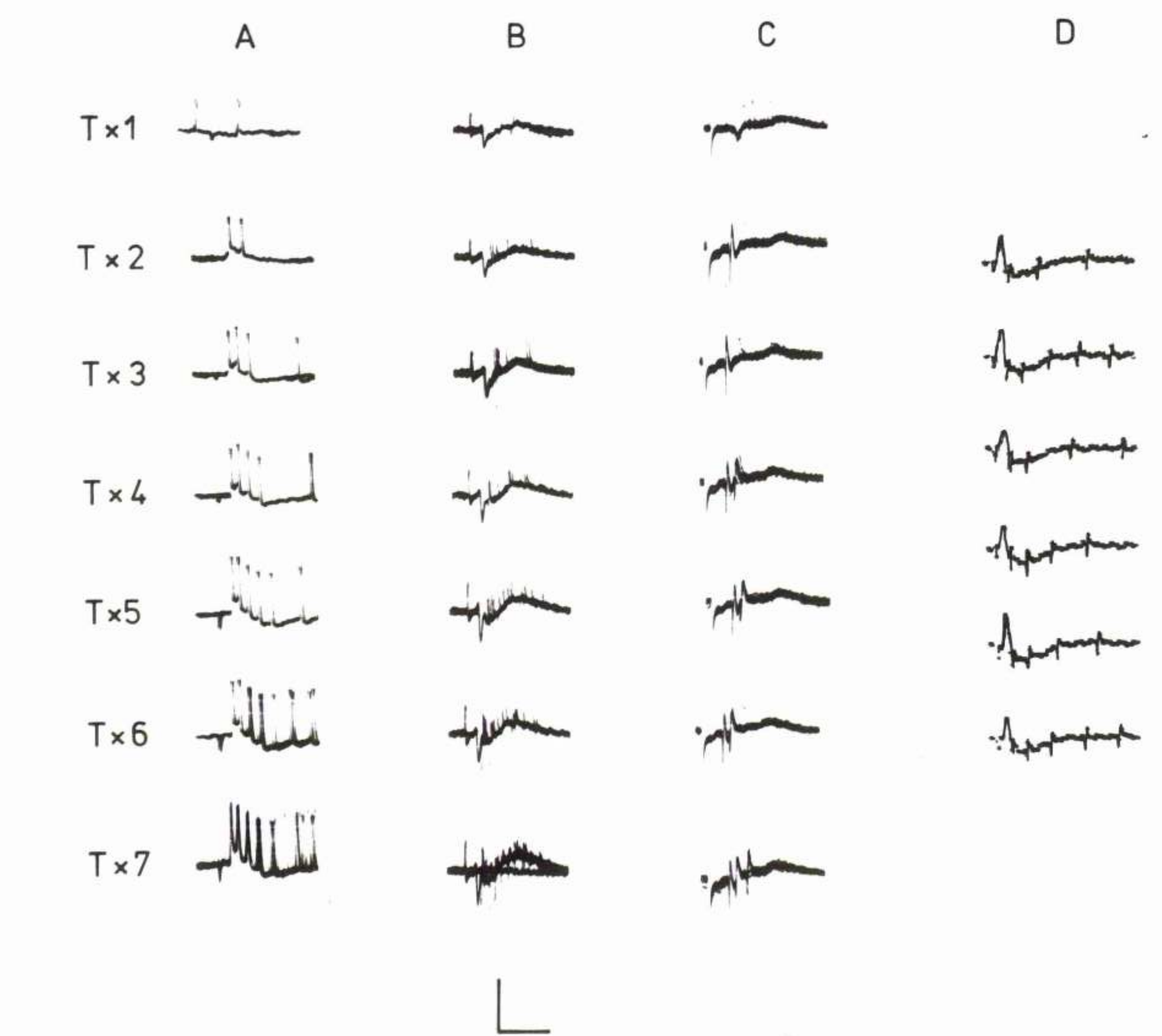


Figure V.3: Rhythmic responses of bulbar neurons to olfactory nerve stimulation. A, B & C responses of a type I granule cell, a type II granule cell and a mitral cell to shocks of increasing intensity. Note in A particularly the linear relationship between spike number and stimulus intensity. D shows consecutive responses of a periglomerular cell to olfactory nerve stimulation. T = threshold stimulus intensity.

Calibration: Vertical A 50mV; B,C,D, .5mV: horizontal 50msec.

Figure V.3: Rhythmic responses of bulbar neurons to olfactory nerve stimulation



from -50 to -65mV. The action potentials elicited by both antidromic and orthodromic stimulation were followed by irregular, long duration hyperpolarizations in four out of five cells studied. The fact that the hyperpolarization could be evoked by a sub-threshold shock (Figure V.4B), indicates that this hyperpolarization is a true IPSP and not an after hyperpolarization. Its latency (4-5msec) and long, irregular time course (50 - 150msec) suggests that one or more interneurons are probably present in the pathway responsible for its generation.

Stable positive - negative extracellular action potentials of up to 2mV in amplitude were easily obtained from mitral cells and could be held for periods in excess of one hour. Three spikes could be obtained at high stimulation intensities but threshold shocks (normally 1-4V) always produced a single spike. It was found that a just suprathreshold conditioning shock applied to either the olfactory tract or nerve could block the second and third but not the first spike evoked by a high intensity test shock. However, at shock intervals of 40-150msec, a suprathreshold antidromic or orthodromic test spike could be blocked by a sub-threshold conditioning shock applied to either route. Since the test spike is blocked by a conditioning stimulus of insufficient strength to discharge the cell, the blockage cannot be due to post-spike refractoriness of the mitral cell membrane, but must result from true inhibition (Figure V.5C). It is significant that the inhibition is greatest at shock intervals of 40-50msec, since it corresponds with the peak latency of the surface P wave of the field potential. The total duration of the inhibition varies from

Figure V.4: Inhibitory post-synaptic potentials recorded from mitral cells. A. 1-4 shows the orthodromic activation of a mitral cell with increasing stimulus intensity. Note the EPSP developing into a full spike and the hyperpolarization visible in 2 and 4. B shows the antidromic activation of a mitral cell, at threshold and sub-threshold stimulus intensities. The IPSP amplitude is 7.5 mV.

Figure V.4: Inhibitory post-synaptic potentials in mitral cells

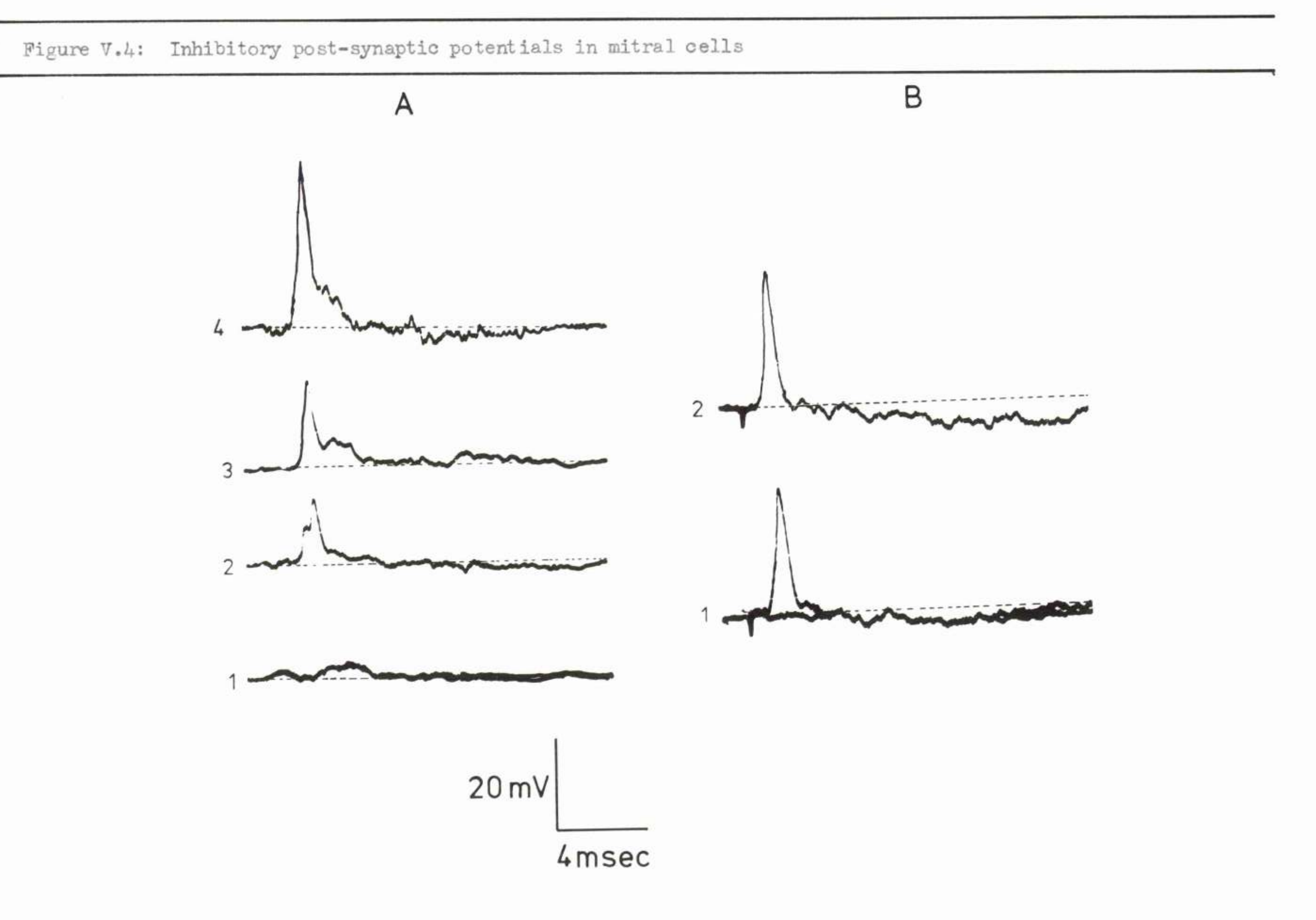


Figure V.5: Inhibition of evoked mitral cell responses by a prior conditioning shock. A, the effect of increasing stimulus intensity on mitral evoked response. B shows the effect of increasing intensity conditioning stimuli on the response evoked by a testing shock strong enough to elicit three spikes. C, both conditioning shock intensity and shock interval were varied in this experiment to produce inhibition of a just supra-threshold test spike by a sub-threshold conditioning shock. All records are from the same neuron in response to olfactory nerve stimulation. T = threshold shock intensity.
Calibration: vertical 1mV; horizontal 50msec.

Figure V.5: Inhibition of evoked mitral cell responses

100-150msec.

Granule cells

Although the granule cells are particularly small (ca. 10 $\mu$m), twelve stable intracellular recordings were possible during the course of this study, one of which was held for over an hour. This greater success is probably due to the extremely large number of cells in the granular region. Eight of the recordings were from type $G_1$ cells.

Granule cells respond to a single shock with one or several spikes, often superimposed on a high amplitude (16.52 $\pm$ 2.5 mV), long duration (24.87 $\pm$ 4.51msec) depolarization (Figure V.3A, V.6, V.7 and V.8). As shown in Figure V.3A, the number of spikes in an evoked burst could be increased by raising the intensity of stimulation. The maximum number of spikes obtained in response to a single shock in the olfactory nerve was eleven, the total burst duration being around 200msec. As observed by Yamamoto et al (1963) in the rabbit, the granule responses often consist of a single spike, followed by a burst of attenuated spikes on a large depolarization (Figure V.6). Raising the stimulus intensity often changed these into full-sized action potentials. This large membrane depolarization is assumed to be an EPSP, the characteristics of which are shown in Figures V.7B and V.8. It is of particular interest to note that the time course of this EPSP, recorded from a type I granule cell, corresponds almost exactly with a time course of the field potential $N_2$ wave. This lends support to the proposal that this wave is generated by synchronous synaptic depolarization of granular region cells.

Figure V.6: Recordings from granule cells to illustrate the miniature spike (or EPSP) activity following stimulation of the olfactory nerve. Note in A how the full spikes are related to the underlying pattern of depolarization. Also of interest in A.7 is the sinusoidal oscillation of the membrane potential. B.1-3 and 4-6 show intra and juxtacellular recordings from two type I granule cells, together with the extracellular field potential at the recording point. In C are shown further responses of the cell in A.

Calibration: vertical A; B.2, 3; C; 50 mV:

B.1, 4, 5, 6; 1mV:

horizontal A.1-4; B; C; 50msec.,

A.5-8; 100msec.

Figure V.6: Granule cell recordings to illustrate miniature spike activity

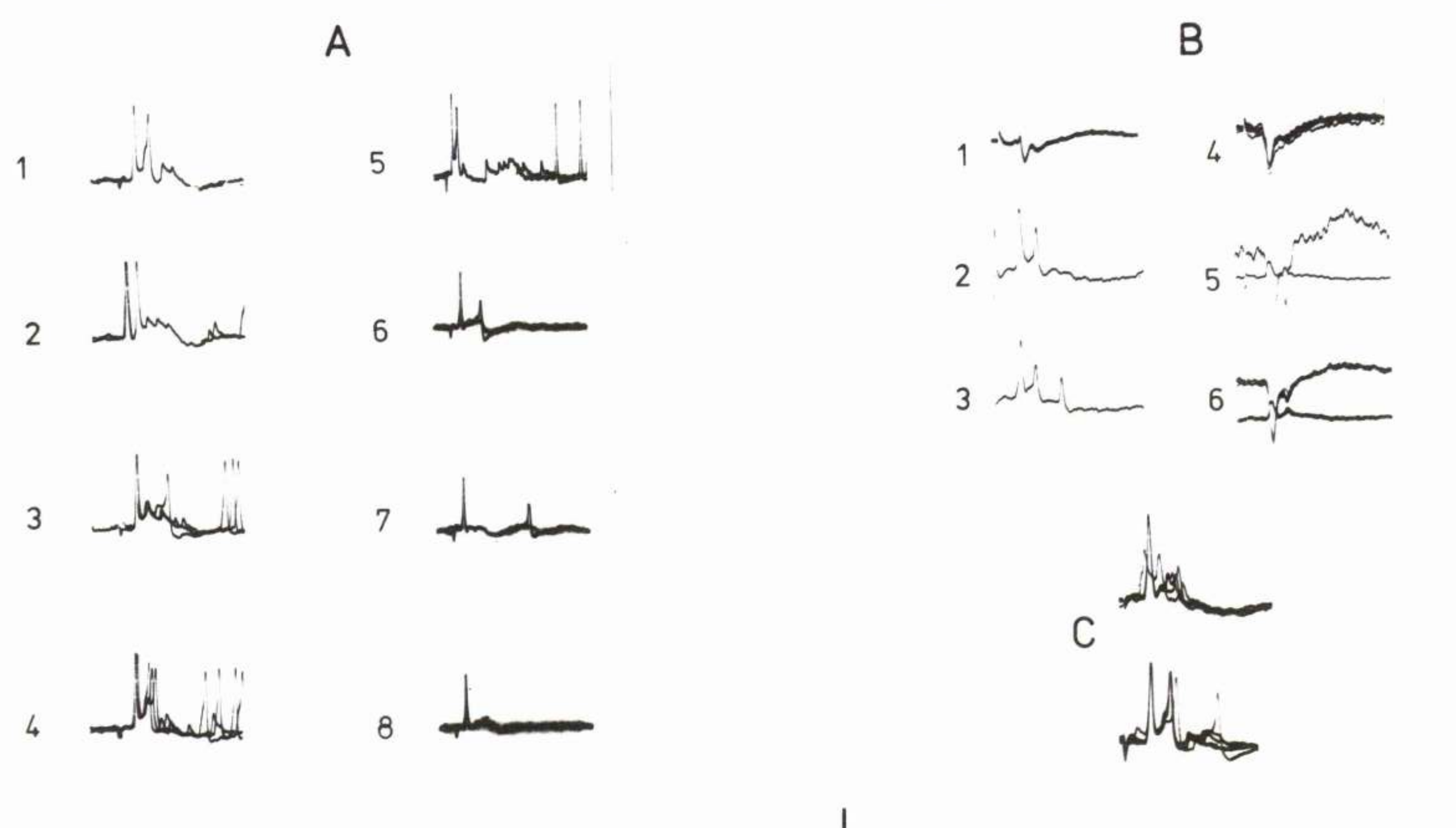


Figure V.7: Orthodromic responses of bulbar neurons to stimuli of increasing intensity. (i) A, response of an unidentified cell encountered in the caudal region of the bulb. Stimulus intensity was increased from 1 through 4; in 4 another unit appears to have been recruited. (i) B shows the response of a mitral cell encountered close to the unit shown in A. Note the increasing variation in latency as the shock intensity was lowered (1-3). Stimulation was at 5 Hz. (ii) intracellular responses of type I granule cells to olfactory nerve stimulation. A & B show the granule EPSP produced by sub-threshold stimulation and spike generation at just supra-threshold shock intensities. The records in C are from an adjacent cell; stimulus intensity was increased from a to c. The response was almost graded in nature.

Figure V.7: Orthodromic responses of bulbar neurons

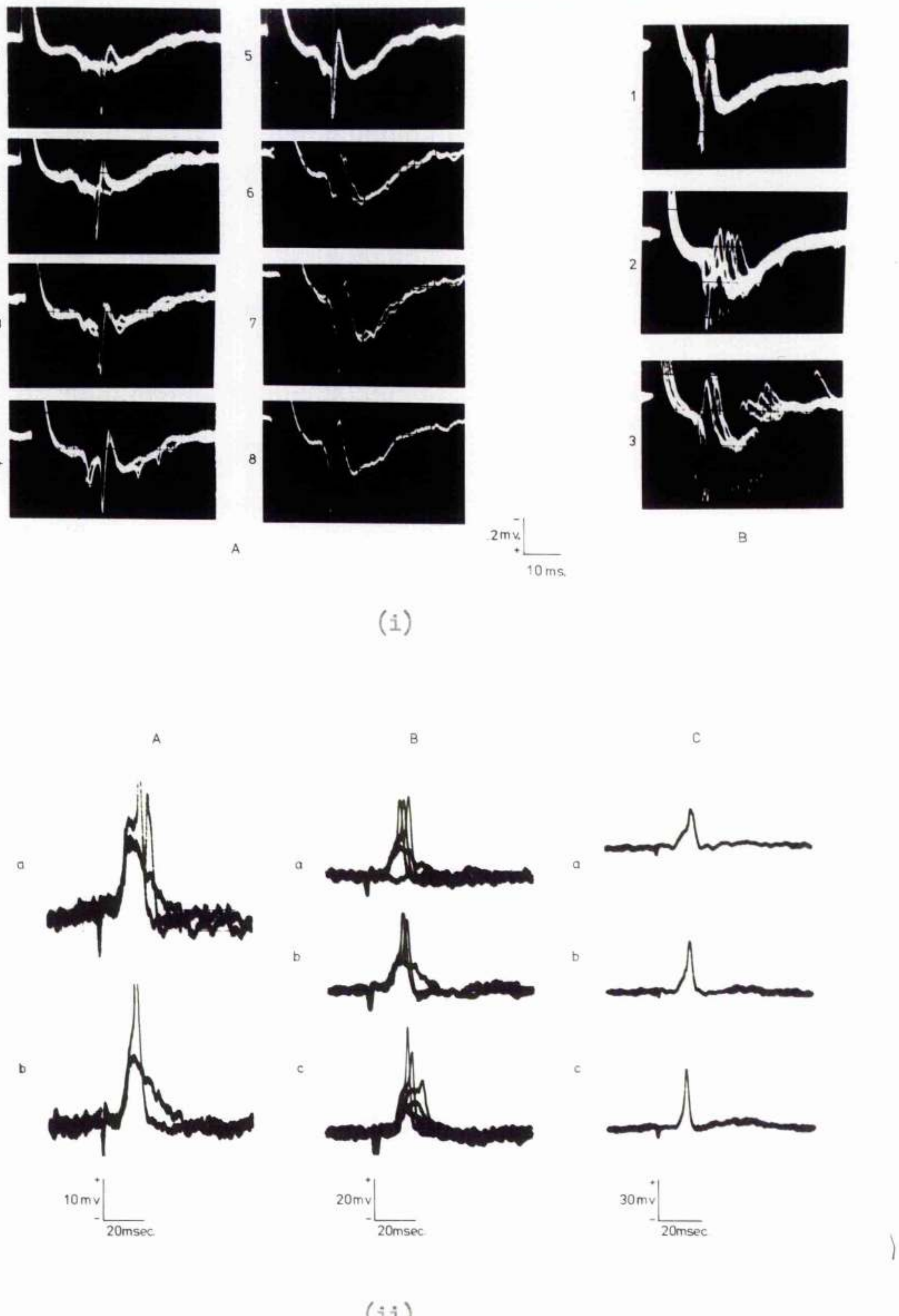


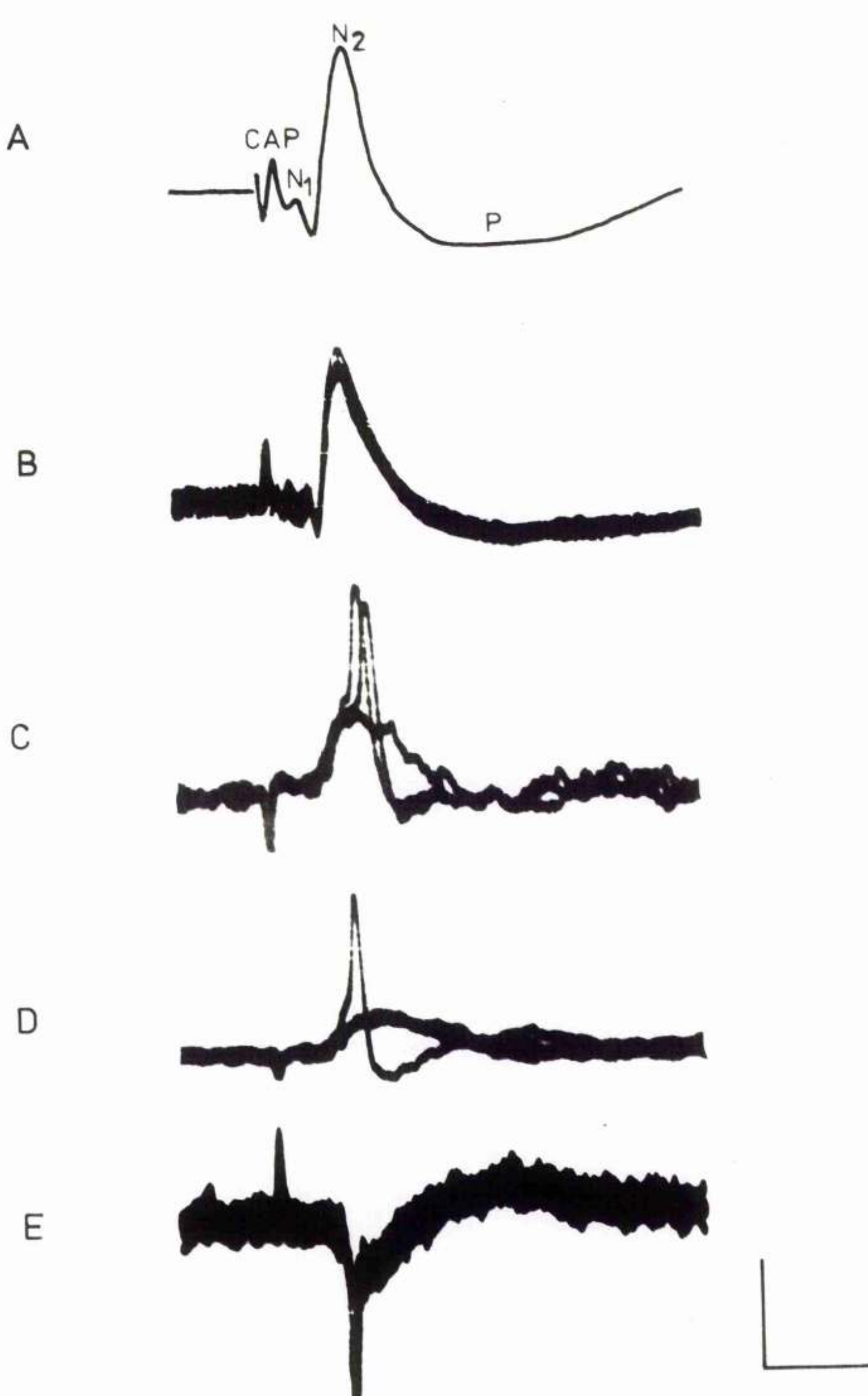
Figure V.8: Relation of the $N_2$ wave to the granule cell EPSP. A, averaged evoked potential from region of mitral cell dendrites. B, field potential recorded from same region. C, intracellular recording from a granule cell, showing an EPSP and two spikes evoked in 3 consecutive superimposed sweeps. The spikes were evoked by slightly raising the stimulus intensity. This cell was encountered along the same electrode track as the field potentials in A & B. D & E, intracellular and extracellular records from a type I granule cell. All records are arranged to show the similarity between the extracellular fields in the 'external plexiform' region and the intracellulary recorded synaptic potentials. CAP, compound action potential, $N_1$, $N_2$ & P refer to components of the field potential.

Calibration: vertical, intracellular 25mV;

extracellular 0.5mV.

horizontal, 20msec.

Figure V.8: Relation of $N_2$ wave to granule cell EPSP



Extracellular recording has shown that conditioning shocks applied to the olfactory nerve or tract can suppress the spike generating ability of granule cells for up to 300msec. Figure V.9 illustrates this suppression when both conditioning and test shocks are delivered to the olfactory nerve. This cell maximally fired three spikes, the second and third of which were blocked by a single conditioning spike delivered 50msec before the testing shock. Three conditioning spikes, however, were needed to block the initial spike. In many granule cells the control number of spikes was not evoked by a testing stimulus until the field potential had returned to its control configuration. At brief shock intervals there is often a facilitation of the test response as shown in Figure V.9C.

## DISCUSSION

The most frequently observed result of olfactory tract stimulation on the spontaneous activity of bulbar neurons is a long-lasting depression (Døving, 1966b; Hara, 1967), which both authors attribute to the presence of centrifugal fibres in the olfactory tract. However, the evidence presented above, together with preliminary observations in the present study that ipsilateral nerve stimulation can suppress spontaneous firing in bulbar neurons, indicates that this inhibition is, in part at least, generated by intrinsic bulbar circuits. It is tentatively suggested that the mitral cell IPSP, evoked by both antidromic and orthodromic activation, is generated by the dendrodendritic pathway proposed in Chapter IV. If this postulated pathway is correct, then a

Figure V.9: Effects of conditioning shocks on unit responses from a granule layer cell. Records show the effects of varying the shock interval and conditioning amplitude on the test response. Conditioning shock strengths were raised by $\frac{1}{2}$ V intervals until the control response was surpassed.

T3 - threshold for three test spikes

T2 - threshold for two test spikes

T1 - threshold for one test spike

Shock interval in A, 50msec; in B, 35msec; in C, 20msec.

All shocks delivered to olfactory nerve.

Calibration: Vertical 1mV; horizontal 50msec.

Figure V.9 (i): Effect of conditioning shocks on granule cell responses

A T 3

1 2 3 4 5 6 7 8 9 10

11 12 13 14 15 16 17 18 19 20

T 2

1 2 3 4 5 6 7 8 9 10

11

T 1

1 2 3 4 5 6 7

C T 3

1 2 3 4 5 6 7 8 9 10

11 12 13 14 15 16 17 18

Figure V.9 (ii): Effect of conditioning shocks on granule cell responses

conditioning stimulus to the olfactory tract or nerve should suppress the mitral IPSP. Unfortunately, no sufficiently stable intracellular penetration has so far been made to allow confirmation of this. The theory is supported, however, by the double-shock studies with field potentials and unit responses in the granular layer. The inhibitory effect of conditioning olfactory nerve and tract shocks on a test olfactory nerve response is expected, since olfactory nerve impulses must pass via the mitral cells to excite the granule cells. Inhibition of the mitral cells following a conditioning shock to the nerve or tract would suppress the response of granule cells to olfactory nerve stimulation by blocking the excitatory pathway. The inhibition of the test olfactory tract response of granule cells would not be expected if the cells are activated by recurrent collaterals, since inhibition of the mitral somata should not block conduction through collaterals of their axons.

This type of feed-back circuit could be of use in limiting the natural discharge frequency of secondary neurons and could easily be responsible for generating the highly synchronous oscillatory potentials evoked by natural chemical stimulation of the olfactory mucosa. Shepherd (1972) has drawn parallels between this type of lateral inhibition in the mammalian olfactory bulb and the retina as a physiological mechanism for providing contrast between odours. In view of the evidence presented here, there is no reason to suppose why this type of information is not also conveyed to higher integrative centres in the fish brain.

The influence of centrifugal fibres on these intrinsic circuits remains an open question. One possibility is that they

may be responsible for exerting fine control on the mechanisms described above. The question can only be answered by the use of chronically decentralized preparations in which the centrifugal fibres and their endings have degenerated.

# VI

# spontaneous activity of bulbar neurons and its modulation by olfactory stimulation

## INTRODUCTION

In non-piscean vertebrates, the coding of olfactory information at the receptor level is relatively straightforward. Odour intensity is temporally coded by variations in the firing rate of the primary neurons. The more concentrated the odour, the faster the firing rate of the excited cell. Odour quality is spatially coded, some receptor cells being insensitive to particular odours at any concentration (Mozell, 1971). There have been no studies on olfactory discrimination at this level in fish but the close anatomical relationship with primary olfactory neurons in other vertebrates leads one to suspect a similar method of odour coding by this class of vertebrates.

As shown in a multitude of studies on other vertebrates (Adrian, 1950; Døving, 1965, 1966a, b, c & d; Mathews, 1972a & b; Mozell, 1971), differential spatio-temporal patterning is also well developed at the bulbar level. However, as shown in the previous section, the secondary neurons do not purely reflect the crude sensory information coming from the receptor cells; they also display the influence of several neural feed-back loops, intrinsic and extrinsic to the bulb. Our present state of knowledge concerning odour coding at this level is best summed up by a quotation from a recent review by P. MacLeod (1974): "First order neurons give a first approximation of a topographical coding of odours. This fuzzy picture is worked out by mitral cells to give a well-defined and well-contrasted image which is kept almost constant throughout the intensity range". It is the purpose of this section to demonstrate that the patterns

of secondary neuronal responses to odours are as complex in the fish brain as in the brains of other vertebrate classes and that olfactory discrimination is, therefore, also particularly well-developed.

## RESULTS

### Spontaneous activity in bulbar neurons

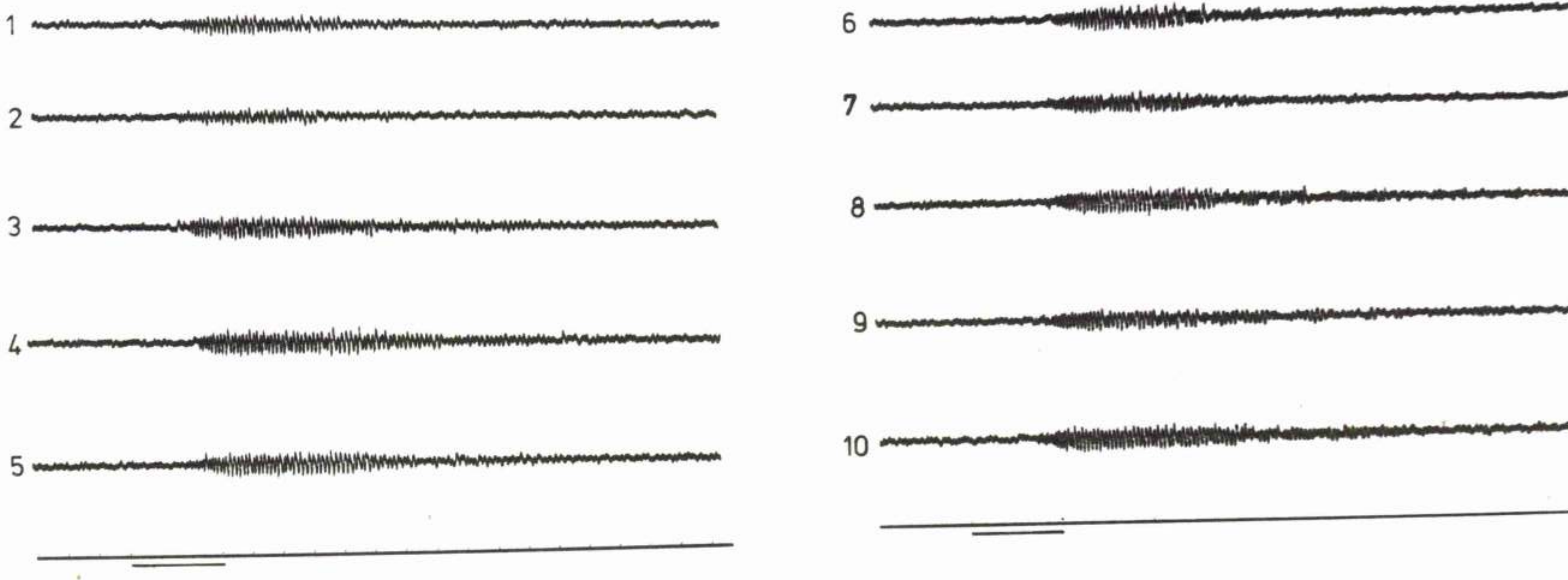
The spontaneous firing frequencies have been measured for one hundred and twenty-six olfactory bulb neurons from twenty-three rainbow trout (*Salmo gairdneri*). The mean frequency of discharge was found to be 3.5 Hz, which compares favourably with the value of 4.2 Hz obtained by Døving (1966b) during his studies on the burbot (*Lota lota*). Approximately one half of the bulbar units encountered during these experiments exhibited bursting activity (Table V1.1; Figure V1.2). On-line interval histograms of such units were bi-modal, with peaks occurring at approximately 75 msec. and 2000 msec. Several interval histograms from bursting cells are shown in Figure V1.7, with the low frequency peak omitted in order to show minute variations in the position of the high frequency peak. Døving (1966b) found that only 10% of bulbar neurons in the burbot exhibited bursting activity. In a later study, Døving found a proportionately higher number of bursting cells when recording from olfactory tract fibres. As shown in Table V1.1, bursting activity was more commonly associated with periglomerular and granule cells than with mitral cells. This agrees well with the response patterns of these cells to electrical stimulation described in Chapter V.

Table V1. 1: Firing Rates of bulbar neurons

| | Total No. | Mean Cell Firing Rate | No. of Bursting Cells as % of Total | Mean Burst Frequency | Spike Frequency Within Bursts |
|---|---|---|---|---|---|
| Periglomerular cells | 11 | 3.51 ± 1.8 (0.8 - 6.3) | 81.82 | 0.36 ± 0.14 (0.1 - 0.55) | 11.8 ± 5.1 (5.0 - 15.5) |
| Mitral cells | 70 | 3.59 ± 0.25 (0.2 - 10.1) | 34.29 | 0.37 ± 0.18 (0.08-0.61) | 13.47 ± 3.78 (5.76 -21.17) |
| Granule cells | 27 | 3.75 ± 2.14 (0.5 - 11.5) | 88.9 | 0.41 ± 0.13 (0.18-0.33) | 16.85 ± 8.0 (6.2 - 34.0) |
| Unidentified cells | 11 | 3.4 ± 3.6 (0.6 - 13.1) | 18.18 | 0.49 ± 0.27 | 30.03 ± 0.4 |
| All units | 126 | 3.5 ± 2.34 (0 - 13.1) | 52.38 | 0.38 ± 0.16 (0.08-0.83) | 15.12 ± .70 |

Values are means ± standard deviation.

Figure V1.1: Bulbar electroencephalographic responses to olfactory stimulation




Responses to 100 μl pulses of the following odorants at a concentration of $10^{-4}$ M.

1. L-glutamine, 2. L-alanine, 3. L-cysteine, 4. glycine, 5. L-serine,

6. L-histidine, 7. L-valine, 8. GABA, 9. L-glutamate, 10. L-phenylalanine.

These odours are arranged in order of stimulatory effectiveness according to Hara (1972b). The period of stimulation is shown by the black line beneath the time scale, which is marked at 1sec. intervals.

Figure VI. 2(i): Responses of two bulbar neurons to olfactory stimulation. In all records the upper trace is the EEG activity and the middle trace the unit activity, recorded by the same electrode. The two signals were separated with a system of band-pass filters. The time marks in the lower traces are 1 and 9 secs. for the short and long periods respectively. The stimulus period is indicated by the short pulses on the time trace.
A: Granule cell, 1 & 2 inhibitory responses to alanine. Note the greater inhibition in 2 following a longer stimulus. B: Mitral cell, 1, inhibitory response to L-phenylalanine. 2, more pronounced inhibitory response to L-glutamine. 3, excitatory response to L-histidine.

Figure V1. 2(i): Responses of bulbar neurons to olfactory stimulation

<u>Figure V1. 2(ii)</u>: Responses of two bulbar neurons to olfactory stimulation. C: 1 & 2, inhibitory responses of two adjacent mitral cells to L-glutamine. The upper trace is the EOG recorded from the ipsilateral olfactory mucosa. Each time mark is 1sec. D: Granule cell, 1, inhibitory response to glycine; 2, excitatory response to L-glutamine, followed by a long period of inhibition. 3, excitatory response to L-histidine. The EEG was recorded with a separate microelectrode, deeper in the granule layer. The short time marks are 1sec.

Figure VI. 2(ii): Responses of bulbar neurons to olfactory stimulation

C

1

2

D

1

2

3

## Summated responses to olfactory stimulation

The overall electrical response of the olfactory bulb to chemical stimulation of the ipsilateral olfactory mucosa is a high amplitude oscillatory potential (7-9 Hz). Figure VI. 1 shows the response of the trout olfactory bulb to ten of the odours used in this study. The odours are arranged according to Hara's classification of stimulatory effectiveness (Hara, 1972b). Although this will not be dealt with further in the present report, it is clear that the responses shown, which were highly reproducible, are not in agreement with Hara's classification. This result tends to throw some doubt on the significance of Hara's work, particularly as he was using the same species.

The EEG was initially employed to confirm the efficacy of the odours to be used in the unitary studies. It was found, however, that the responses of single units were often difficult to categorize. For example, an inhibitory effect is often followed by a post-inhibition burst of spikes. In studying the effects of various substances, it is essential to be able to distinguish between these and other similarly confusing effects. In this study, the bulbar EEG and/or the mucosal EOG were recorded simultaneously with the unit activity, The EOG (Ottoson, 1971) gives information about the onset and duration of the effect on the receptors (Figure VI. 2c). The EEG gives information about the onset and duration of the effect on the whole olfactory bulb. Thus, excitatory effects on bulbar units could easily be distinguished by comparing the time-course of the unitary response with either the EEG or EOG (Figure VI. 2).

There often appeared to be a relationship between the spontaneous and evoked EEG activity and the spontaneous and evoked unitary activity. Spontaneous unitary discharges seem to coincide with negative going EEG waves. During inhibitory and excitatory evoked responses, a fast burst of spikes occurs synchronously with the second overall negativity of the evoked EEG response. Similar relationships have been observed in the mammalian olfactory bulb (Baumgarten, von et al 1962) and the mammalian cerebral cortex (Fromm et al, 1964).

Responses of bulbar units to olfactory stimulation

The spontaneous activity of single olfactory bulb neurons was affected in various ways by chemical stimulation of the olfactory mucosa with amino acid solutions at a concentration of $10^{-4}$ M. The most commonly observed responses were:-

(i) Excitation, i.e. increase in firing rate

(ii) Inhibition, i.e. decrease in firing rate

(iii) Excitation followed by inhibition

(iv) Inhibition followed by an excitatory burst

(v) No response at all

For simplicity of analysis, types (iii) and (iv) were classified as excitatory and inhibitory by comparing the response time course with the evoked EEG/EOG response. Thus, responses have been divided into three classes: excitation, inhibition and unaffected, (Figures VI. 2 and VI. 3).

The olfactory stimulants used in this study were the sixteen amino acids shown in Table VI. 2. They were chosen from the most readily available amino acids classified as highly stimulatory by Hara (1972b). Table VI.2 shows the

Figure VI. 3: Dwell histograms of the responses of two mitral cells to 200 ul pulses of various odorants. Histograms were compiled on-line with a Biomac 500 computer fed with standardized pulses from a pulse generator triggered by action potentials from the recorded unit via a window discriminator. Each histogram is for a total of 500 events, each bin storing the time interval between consecutive events. Excitation is implied by a decrease in inter-spike interval; inhibition by an increase in inter-spike interval and thus a greater pen deflection.
A. Histograms from a cell showing three different response patterns; excitation, excitation followed by inhibition and pure inhibition to alanine, glutamine and cysteine respectively. B. Histograms from a cell which showed purely excitatory responses to all stimulants.

Figure VI. 3: Dwell histograms of mitral cell responses to odorants.

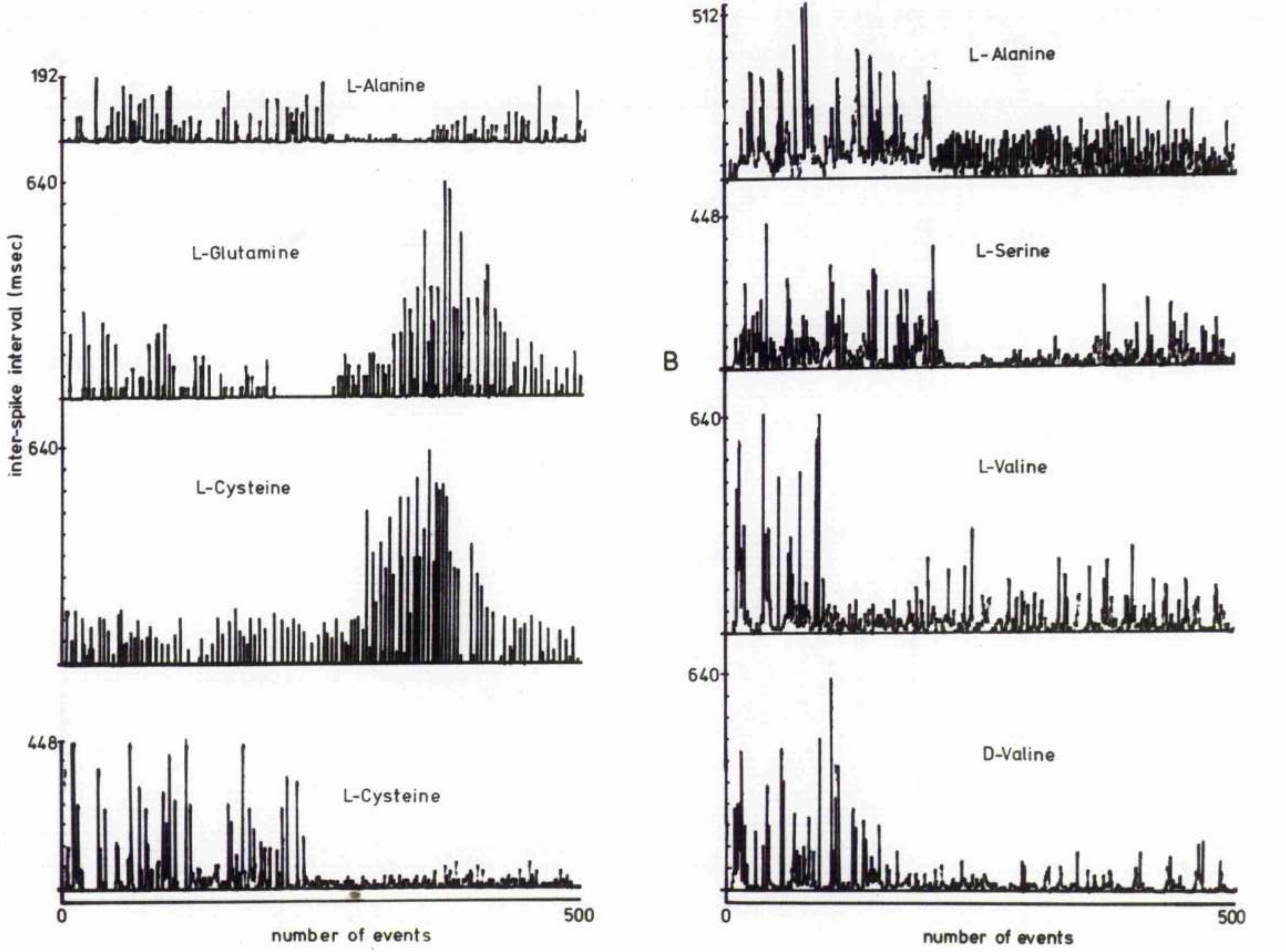


**TABLE V1.2:** The number of units excited, inhibited or unaffected by the different odours.

| ODORANT | Excited (+) | | | | Inhibited (-) | | | | Unaffected (o) | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | T | PG | M | G | T | PG | M | G | T | PG | M | G |
| L-glutamine | 32 | 8 | 20 | 4 | 10 | 4 | 4 | 2 | 2 | 0 | 2 | 0 |
| D-glutamine | 12 | 2 | 8 | 2 | 14 | 1 | 10 | 3 | 4 | 0 | 4 | 0 |
| L-serine | 24 | 4 | 15 | 5 | 18 | 2 | 12 | 4 | 8 | 2 | 6 | 0 |
| D-serine | 18 | 4 | 12 | 2 | 12 | 0 | 8 | 4 | 8 | 2 | 6 | 0 |
| L-histidine | 30 | 4 | 22 | 4 | 12 | 2 | 10 | 0 | 2 | 0 | 2 | 0 |
| D-histidine | 4 | 0 | 4 | 0 | 12 | 3 | 8 | 1 | 4 | 0 | 4 | 0 |
| L-valine | 18 | 6 | 6 | 6 | 10 | 2 | 8 | 0 | 10 | 2 | 8 | 0 |
| D-valine | 8 | 0 | 6 | 2 | 8 | 2 | 4 | 2 | 6 | 0 | 6 | 0 |
| L-phenylalanine | 14 | 2 | 8 | 4 | 16 | 3 | 12 | 1 | 4 | 0 | 4 | 0 |
| D-phenylalanine | 4 | 2 | 2 | 0 | 14 | 2 | 8 | 4 | 2 | 0 | 2 | 0 |
| L-glutamate | 16 | 6 | 8 | 2 | 16 | 2 | 12 | 2 | 4 | 0 | 4 | 0 |
| D-glutamate | 0 | 0 | 0 | 0 | 10 | 0 | 8 | 2 | 2 | 0 | 2 | 0 |
| L-alanine | 20 | 4 | 10 | 6 | 26 | 4 | 20 | 2 | 4 | 0 | 4 | 0 |
| L-cysteine | 23 | 6 | 12 | 5 | 26 | 4 | 18 | 4 | 2 | 0 | 2 | 0 |
| glycine | 12 | 2 | 6 | 4 | 24 | 4 | 18 | 2 | 8 | 4 | 4 | 0 |
| GABA | 18 | 4 | 10 | 4 | 4 | 0 | 4 | 0 | 8 | 4 | 4 | 0 |
| **Totals:** | 285 | 54 | 149 | 50 | 232 | 35 | 164 | 33 | 78 | 14 | 64 | 0 |

**TABLE V1.3:** Matrix of mean values extracted from Table V1.2.

| Cell Type | Total no. of responses | % excited | % inhibited | % unaffected |
|---|---|---|---|---|
| periglomerular cells | 103 | 52.4 | 34.0 | 13.6 |
| mitral cells | 377 | 39.5 | 43.5 | 17.0 |
| granule cells | 83 | 60.3 | 39.7 | 0. |
| unidentified cells | 38 | 57.9 | 42.1 | 0. |
| all units | 601 | 47.4 | 38.6 | 14.0 |

numbers of various bulbar units influenced by them during the course of this study. Over all units encountered, there were slightly more excitatory responses than inhibitory ones. This trend is reflected in the response patterns of periglomerular and granule cells. Mitral cells consistently showed a higher proportion of inhibitory responses (Table V1.3),

Figure V1.4 shows a matrix of responses by units and odorants. Only units held sufficiently long enough for testing five or more odours are included. Units responding to four or less odours have been included in Tables V1.2 and V1.3. Units were occasionally encountered which responded with exclusively excitation or exclusively inhibition to all odorants tested. Most units encountered responded with excitation to some odours and inhibition to others. Only one unit was found to be totally specific, in that it was normally silent but responded with excitation to only a limited number of odours (Unit 13, Figure V1.4). Each odorant normally produced a unique pattern of excitatory and inhibitory responses across the units. Also, the odorants to which a unit was sensitive produced responses of different magnitudes. Results from other vertebrates are normally similar to those obtained here. Mathews (1972a), however, working with the tortoise (Gopherus polyphemus), found no units that responded exclusively with inhibition. Since he only recorded from nineteen units, this discrepancy is probably due to insufficient data.

Comparison between odour pairs

The three-way classification of neural responses to olfactory stimulation makes the data suitable for enumeration

Figure VI. 4: Matrix of responses across units and odorants

stimulus | P.G. Cells | Mitral Cells | G. Cells | Others

L-gln
D-gln
L-ser
D-ser
L-his
D-his
L-val
D-val
L-phal
D-phal
L-glu
D-glu
L-ala
L-cys
gly
GABA

cell number 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28 29 30

excitation inhibition no response not tested

statistics. If the responses of a sufficiently large number of units to stimulation with a given number of odorants are observed, the chi-square value (with four degrees of freedom) between pairs of odours can be calculated from a 3 x 3 response matrix. The chi-square value indicates whether or not a particular pair of odorants have similar stimulating properties (Døving, 1965). A low chi-square value indicates that the stimulating properties of a pair of odorants are mutually independent. The first column in Table V1.4 gives the chi-square values with four degrees of freedom for forty-five odour pairs. The ten odours compared are those listed in Table V1.2, excluding the D-isomers. The chi-square value for the pair L-alanine/L-cysteine is 22.78. With four degrees of freedom at P = 0.001, the chi-square value is 18.47. Therefore, the hypothesis of independence is rejected at the 0.1% level of significance. This indicates that the two odours have similar stimulating properties.

The chi-square test is less helpful if the number of entries in one or more cells of the contingency table is below five. This was often the case for categories representing units unaffected by one or both members of a pair. If these are omitted, the data fit a 2 x 2 contingency table and the chi-square value can be calculated by Yate's formula for one degree of freedom.

$$\chi^2 = \frac{n(|ad-bc| - \frac{1}{2}n)^2}{(a+b)(c+d)(a+c)(b+d)}$$

(the vertical lines in $|ad-bc|$ mean that the absolute, i.e. positive, value of the difference between ad and bc is taken. The difference is, therefore, always reduced in size by

Table V1.4: The chi-square values obtained for 45 pairs of odours with one and four degrees of freedom (D.F.). The pairs are arranged according to the chi-square values with one degree of freedom.

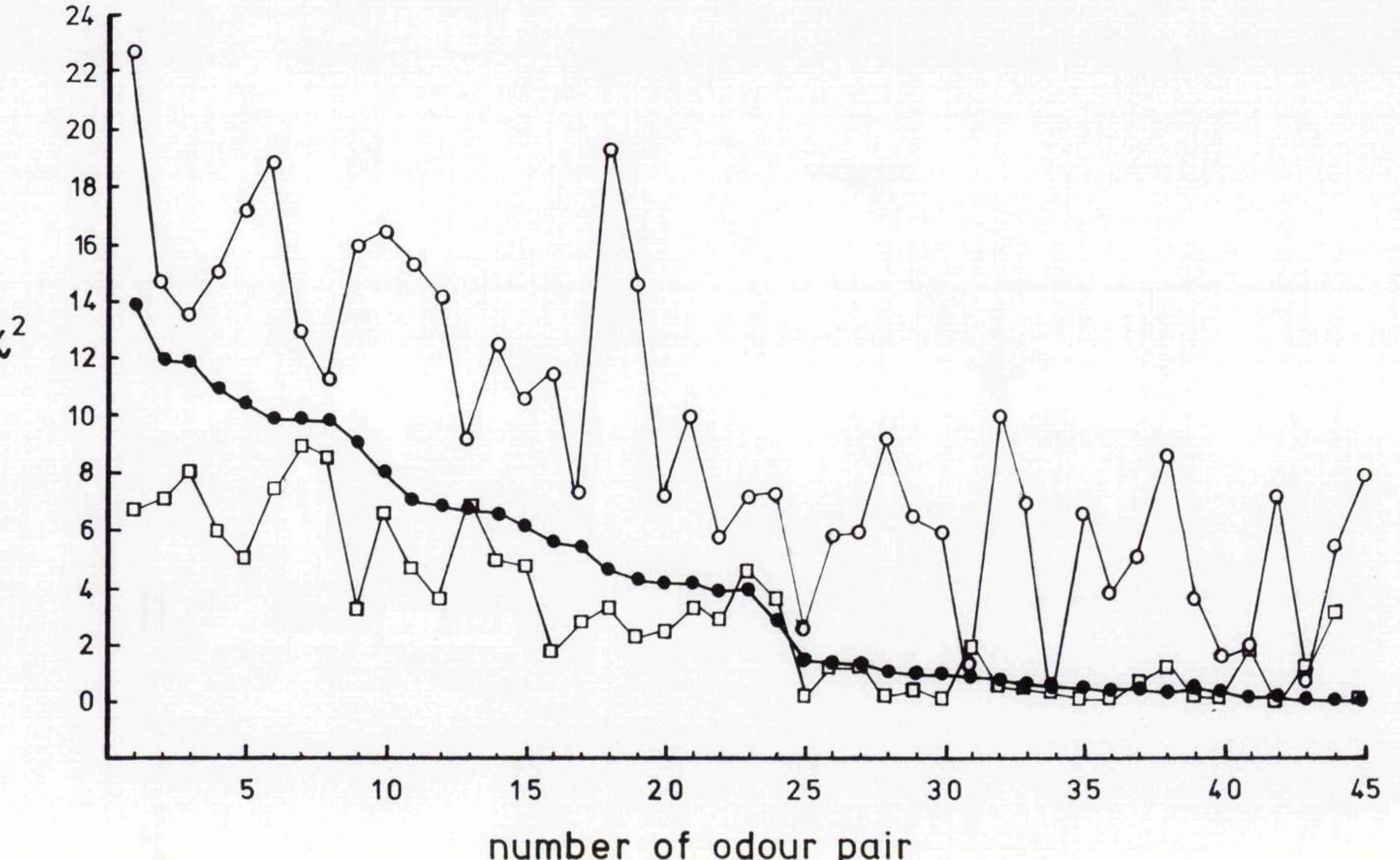
***: significant at 0.1%; ** significant at 1%;
*: significant at 5%.

S.L. = significance level

TABLE V1.4: The chi-square values obtained for 45 pairs of odours.

| No. | Pair of Odours | | All Units | | | | MitralCells | |
|---|---|---|---|---|---|---|---|---|
| | | | 4 D.F. | | 1 D.F. | | 1 D.F. | |
| | | | $\chi^2$ | S.L | $\chi^2$ | S.L | $\chi^2$ | S.L |
| 1 | L-alanine | L-cysteine | 22.78 | *** | 13.91 | *** | 6.62 | * |
| 2 | L-serine | L-phenylalanine | 14.76 | ** | 12.00 | *** | 6.96 | ** |
| 3 | L-serine | L-glutamate | 13.48 | ** | 12.00 | *** | 8.00 | ** |
| 4 | L-valine | L-glutamate | 15.04 | ** | 11.02 | *** | 6.00 | * |
| 5 | L-serine | L-valine | 17.20 | ** | 10.50 | ** | 5.00 | * |
| 6 | L-serine | L-alanine | 18.93 | *** | 10.06 | ** | 7.53 | ** |
| 7 | L-histidine | GABA | 12.90 | * | 10.00 | ** | 8.77 | ** |
| 8 | L-serine | L-cysteine | 11.36 | ** | 9.90 | ** | 8.57 | ** |
| 9 | L-glutamate | L-alanine | 16.02 | ** | 9.11 | ** | 3.21 | |
| 10 | L-glutamine | L-serine | 16.49 | *** | 8.13 | ** | 6.43 | * |
| 11 | L-phenylalanine | L-cysteine | 15.37 | ** | 7.14 | ** | 5.65 | * |
| 12 | L-valine | L-alanine | 14.07 | ** | 6.98 | ** | 3.71 | |
| 13 | L-glutamine | L-glutamate | 9.34 | | 6.72 | ** | 6.96 | ** |
| 14 | L-valine | L-phenylalanine | 12.42 | * | 6.67 | * | 5.00 | * |
| 15 | L-phenylalanine | L-glutamate | 10.62 | * | 6.19 | * | 4.80 | * |
| 16 | L-phenylalanine | L-alanine | 11.51 | * | 5.82 | * | 1.89 | |
| 17 | L-glutamine | L-cysteine | 7.38 | | 5.53 | * | 2.86 | |
| 18 | L-glutamate | L-cysteine | 19.30 | *** | 4.67 | * | 3.40 | |
| 19 | L-glutamine | L-alanine | 14.48 | ** | 4.39 | * | 2.36 | |
| 20 | L-glutamine | L-phenylalanine | 7.29 | | 4.30 | * | 2.47 | |
| 21 | L-serine | glycine | 10.02 | * | 4.27 | * | 3.21 | |
| 22 | L-glutamine | L-valine | 5.84 | | 3.99 | * | 3.00 | |
| 23 | L-alanine | glycine | 7.38 | | 3.99 | * | 6.45 | * |
| 24 | L-valine | L-cysteine | 7.27 | | 2.94 | | 3.71 | |
| 25 | L-histidine | L-alanine | 2.45 | | 1.75 | | 0.00 | |
| 26 | L-glutamine | GABA | 5.86 | | 1.49 | | 1.20 | |
| 27 | L-glutamate | GABA | 5.90 | | 1.41 | | 1.12 | |
| 28 | L-phenylalanine | GABA | 9.31 | | 1.08 | | 0.31 | |
| 29 | L-serine | GABA | 6.47 | | 1.06 | | 0.49 | |
| 30 | L-phenylalanine | glycine | 5.97 | | 1.03 | | 0.00 | |
| 31 | L-histidine | L-cysteine | 1.36 | | 1.01 | | 2.00 | |
| 32 | L-valine | GABA | 10.02 | * | 0.89 | | 0.83 | |
| 33 | L-alanine | GABA | 7.03 | | 0.62 | | 0.48 | |
| 34 | L-histidine | glycine | 0.65 | | 0.61 | | 0.63 | |
| 35 | glycine | GABA | 6.64 | | 0.59 | | 0.00 | |
| 36 | L-cysteine | GABA | 3.87 | | 0.55 | | 0.25 | |
| 37 | L-histidine | L-valine | 5.18 | | 0.48 | | 0.97 | |
| 38 | L-cysteine | glycine | 8.67 | | 0.47 | | 1.41 | |
| 39 | L-valine | glycine | 3.76 | | 0.44 | | 0.00 | |
| 40 | L-serine | L-histidine | 1.69 | | 0.41 | | 0.02 | |
| 41 | L-histidine | L-phenylalanine | 1.96 | | 0.25 | | 2.06 | |
| 42 | L-glutamate | glycine | 7.20 | | 0.21 | | 0.00 | |
| 43 | L-glutamine | L-histidine | 0.81 | | 0.19 | | 1.41 | |
| 44 | L-glutamine | glycine | 5.54 | | 0.13 | | 3.35 | |
| 45 | L-histidine | L-glutamate | 8.03 | | 0.03 | | 0.08 | |

Figure VI. 5: Plot of chi-square against odour pairs, taken from Table VI. 4.



subtracting the correction $\frac{1}{2}n$ and the squared quantity in the numerator is less than the corresponding quantity in the numerator of a 'normal' chi-square calculation).

The chi-square values for all units and for mitral cells, with one degree of freedom, are given in the second and third columns of Table V1.4. No separation of periglomerular and granule cell responses was possible, owing to insufficient data. All three groups of chi-square values are plotted in Figure V1.5. It can be seen that general agreement exists between the results obtained by the two methods but there are several exceptions. For example, the pair L-glutamate/L-cysteine have a relatively much higher chi-square value when calculated with four degrees of freedom. This is probably due to the small number of units unaffected by these two odours. Other than the overall reduction in chi-square values, the only major difference between the values obtained for mitral cells and for all units is the higher degree of independence between L-alanine and L-serine and the other odours when considering the mitral cell responses.

The amount of information in Table V1.4 is far too great to be easily assimilated by visual observation. Thus, all ten odours have been listed in Table V1.5 with their corresponding numbers of responses rejecting independence with one degree of freedom. Across all units tested L-serine was the compound that most consistently showed stimulating properties in common with the other odours. L-alanine also showed a low degree of independence when compared with the other odours but it evoked fewer significantly similar responses than L-serine at the 1% level. It is particularly

TABLE V1.5: Odorants which show similar stimulatory properties to at least one other odorant, arranged in order of number of variations from independence across all units. Significance levels are for one degree of freedom. T = total.

| Odorant | All Units | | | | Mitral Cells | | |
|---|---|---|---|---|---|---|---|
| | * | ** | *** | T | * | ** | T |
| L-serine | 1 | 4 | 2 | 7 | 1 | 4 | 5 |
| L-alanine | 3 | 3 | 1 | 7 | 2 | 0 | 2 |
| L-glutamate | 2 | 2 | 2 | 6 | 2 | 2 | 4 |
| L-glutamine | 4 | 2 | 0 | 6 | 1 | 1 | 2 |
| L-phenylalanine | 4 | 1 | 1 | 6 | 3 | 1 | 4 |
| L-cysteine | 2 | 2 | 1 | 5 | 2 | 1 | 3 |
| L-valine | 2 | 2 | 1 | 5 | 3 | 0 | 3 |
| glycine | 2 | 0 | 0 | 2 | 1 | 0 | 1 |
| L-histidine | 0 | 1 | 0 | 1 | 0 | 1 | 1 |
| GABA | 0 | 1 | 0 | 1 | 0 | 1 | 1 |

TABLE V1.6: Chi-square values obtained for five enantiomeric pairs of odours with one and four degrees of freedom (D.F.) The pairs are arranged according to the chi-square values with four degrees of freedom. ***: significant at 0.1%; **: significant at 1%; * significant at 5%.
S.L. = significance level

| No. | Pair of odours | 4 D.F. | | 1 D.F. | |
|---|---|---|---|---|---|
| | | | S.L. | | S.L. |
| 1 | L- and D-serine | 29.82 | *** | 10.37 | ** |
| 2 | L- and D-phenylalanine | 10.1 | * | 2.19 | |
| 3 | L- and D-valine | 8.0 | | 2.68 | |
| 4 | L- and D-histidine | 4.63 | | 1.6 | |
| 5 | L- and D-glutamine | 2.41 | | 0.5 | |

interesting to note that the two odours found to be independent of serine and alanine were histidine and glycine in both cases. The structural formulae of these compounds are given in Figure V1.6. In addition, the structural formulae of histidine and phenylalanine are compared, showing that their molecular profiles are almost identical but they show a high degree of independence.

One of the most intriguing problems in olfaction research is the search for molecular properties which confer a particular smell upon a particular compound. Correlations have been attempted with molecular weight, molecular volume and pKa. The ratios between these parameters were calculated for each pair of odorants and the rank order compared with the experimental chi-square values. The correlation coefficient for molecular weight is 0.46, which for forty-four degrees of freedom is significant at the 1% level. Correlations with molecular volume and pKa showed no significance. It is not surprising that no correlation was obtained with molecular volume, since the density values used to calculate the volumes were mainly derived from measurements on crystalline solids, exhibiting close molecular packing. It is particularly surprising that no correlation was observed for the second pKa values. The stimulant solutions were all made up with samples of the same tapwater used to perfuse thegills and no attempt was made to control pH. It is probable that significant correlation could be found if all solutions were at neutral pH. When the first pKa value was compared with the compounds showing similarity with serine and alanine, a slight upward trend was noticed with decreasing similarity in stimulating effectiveness.

Figure VI. 6: To show the structural relationship between selected odorant molecules.

A. Molecules showing olfactory stimulating properties in common with L-serine, arranged in order of decreasing similarity.

B. As in A but for molecules which have stimulating properties in common with L-alanine.

C. Two molecules which had stimulating properties in common but which were both significantly different from L-serine and L-alanine. The formulae are drawn to demonstrate their similar molecular profiles.

D. Two molecules with almost identical molecular shapes but having widely dissimilar olfactory stimulating properties.

Figure VI. 6: Structural formulae of selected odorant amino acids

**A.**

ser: COOH — H—C—$CH_2OH$ — $NH_2$

phal (1): COOH — H—C—$CH_2$.$C_6H_5$ — $NH_2$

glu (2): COOH — H—C—$CH_2$.$CH_2$.COOH — $NH_2$

val (3): COOH — H—C—$CH(CH_3)_2$ — $NH_2$

ala (4): COOH — H—C—$CH_3$ — $NH_2$

cys (5): COOH — H—C—$CH_2SH$ — $NH_2$

gln (6): COOH — H—C—$CH_2$.$CH_2$.$CO.NH_2$ — $NH_2$

**B.**

ala: COOH — H—C—$CH_3$ — $NH_2$

cys (1): COOH — H—C—$CH_2SH$ — $NH_2$

ser (2): COOH — H—C—$CH_2OH$ — $NH_2$

glu (3): COOH — H—C—$CH_2$.$CH_2$.COOH — $NH_2$

val (4): COOH — H—C—$CH(CH_3)_2$ — $NH_2$

**C.**

GABA: COOH — H—C—H — H—C—H — $NH_2$

his: COOH — H—C—$NH_2$ — H—C—H — imidazole (HN, N)

**D.**

phal: COOH — H—C—$CH_2$.$C_6H_5$ — $NH_2$

his: COOH — H—C—$CH_2$.imidazole (N, NH) — $NH_2$

Comparison between enantiomeric pairs

It has been repeatedly reported in the literature that the olfactory efficacy of the D-isomer of an optically active molecule is less than the corresponding L-isomer (Hara, 1972b; Hara, Law and Hobden, 1972; Sutterlin & Sutterlin, 1971; Suzuki & Tucker, 1971). All of the above reports were based upon multi-unit or EEG responses but, nevertheless, it was surprising to find in the present study several single units giving opposite responses to enantiomeric pairs of amino acids. Table V1.6 shows that only between L- and D-serine was there any significant degree of similarity at the 1% level. Even serine gave opposite responses in a number of trials and it was during an experiment with D- and L-serine that this effect was discovered. It is usually the naturally occurring L-isomer that is excitatory and the D-isomer inhibitory but all possible combinations have been observed during recordings from different units. In cases where the responses were in the same direction, the L-isomer was always the most effective (Figure V1.7). It is interesting that the L-isomer of serine is the odorant showing independence with the least number of the other compounds tested. Døving and Hara (separate personal communications) have both confirmed that they have observed units which respond differentially to enantiomers.

DISCUSSION

The results presented above appear to agree well with similar studies on other vertebrate species. It is unfortunate that no comparable study has yet been attempted with

Figure V1. 7: Interval histograms showing the response patterns of four bulbar neurons to olfactory stimulation with 200 µl pulses of amino acid enantiomers. Histograms were compiled on-line with a Biomac 500 computer fed with standardized pulses. All four neurons showed bursting spontaneous activity, not shown by these histograms. The time scale has been expanded to more clearly show the difference between enantiomers for the high frequency components.

Figure V1. 7: Interval histograms single unit responses to D and L amino acids

other species of fish. The fact that Doving could find only 1% of a large sample of units showing bursting activity in the burbot is significant. It points to a fundamental difference in the activity pattern of olfactory neurons and, thus, bulbar circuitry in the two species. No olfactory discrimination experiments were performed using the other main species employed in earlier sections of this thesis, Gadus morhua. It is to be expected that slight differences may have resulted. One must certainly take care in making generalized statements concerning piscean olfaction. In the rainbow trout, at least, the neurons of the olfactory bulb appear to be capable of discriminating between a variety of chemical stimuli, with only slightly dissimilar molecular structures and conformations. This indicates a particularly well-developed mechanism for odour quality discrimination.

Some points raised by the section on the comparison of odours are worthy of comment. L-serine and L-alanine each showed similar stimulating properties with the same six compounds. They also showed independence with the same two compounds, namely L-histidine and GABA. The second pKa value of histidine is 6.04 and so, in approximately neutral aqueous solution, the imidazole ring system will lose a proton and have one unit of negative charge delocalised around the ring. It will be more negative than the amino group and will, therefore, be electrostatically repelled from the also negative carboxyl group. The molecule will then possess a profile not unlike that of GABA, with two methylene (or equivalent) groups interposed between its two negatively charged ends. These two compounds will thus present a molecular profile, larger by one

tetrahedral carbon atom, than the other molecules considered here. This is presumably also the reason for the dissimilarity between histidine and phenylalanine; two molecules which superficially at least appear to have almost identical molecular shapes. On the basis of this evidence one can postulate the existence of at least three separate amino acid receptor sites. Two of them will possess single positive and negative charges but in one of these the two charges must be separated by a greater distance, this distance being equivalent to the dimensions of a single methylene group. The third receptor site should contain two positive charges, separated by two tetrahedral carbons in order to accommodate the doubly ionized histidine. The results indicate that the second and third of these sites are coded similarly by bulbar neurons.

The experiments with enantiomeric pairs also allow speculation about receptor sites. The different response patterns obtained for L- and D- amino acids immediately points out the stereospecificity of receptor - odorant interactions. There is, presumably, one type of receptor for L-isomers and another receptor type for D-isomers. The lower amplitude EEG responses commonly observed with D-isomers can now probably be explained by the fact that many neurons will be inhibited by the D-isomer and will not, therefore, contribute to the evoked wave. The oscillation produced will, therefore, be of lower amplitude and shorter duration.

The high number of inhibitory responses observed, particularly by mitral cells, is in line with the studies on other vertebrates already mentioned. This, together with the fact that a far higher degree of bursting activity is found in

granular and periglomerular cells, supports earlier statements that the granule cells are inhibitory interneurons. They are responsible for inhibiting the recently active mitral cell, together with its inactive neighbours, to provide a well-defined and highly contrasted olfactory image for processing by higher forebrain centres.

Obviously, much work is still necessary to confirm these speculations but at least the door has been opened.

# VII

# some pharmacological properties of bulbar neurons

## INTRODUCTION

Neuropharmacological investigations of the fish central nervous system are few in number; the only notable exception to this is the work on the Mauthner cell by Diamond and his colleagues (1968, 1973a & b). GABA, glycine and L-glutamate were shown to cause an increase in membrane conductance when applied iontophoretically to the cell surface in a region known to receive inhibitory synapses. Their work, however, provided no clear evidence for these compounds as neurotransmitters, since the pharmacologically evoked inhibition appeared to be only distantly related to that evoked physiologically.

On the other hand, there have been numerous neuropharmacological investigations of many regions within the mammalian central nervous system and the olfactory bulb is no exception. Salmoiraghi and co-workers have obtained evidence for an adrenergic synapse in the olfactory bulb which appears to be activated during the olfactory tract evoked inhibition of mitral cells (Salmoiraghi, Bloom & Costa, 1964; Salmoiraghi & Nicoll, 1968). Nicoll (1970, 1971) has produced compelling evidence that GABA is the neurotransmitter substance responsible for mediating the granule cell induced inhibition of mitral cells. In addition, he believes that this synapse forms part of the dendrodendritic feed-back loop proposed by Rall, Shepherd, Reese and Brightman (1966). Nicoll's work is well supported by McLennan, who further suggested that the granule cells receive excitatory adrenergic and inhibitory GABA-mediated synapses. The amino acids, aspartate and glutamate are thought to be likely candidates for the excitatory

transmitter released from mitral cells on to granule cells (Nicoll, 1971).

This section presents the results of experiments designed to test the hypothesis that similar mechanisms of chemical transmission exist in the olfactory bulb of teleost fish.

## RESULTS

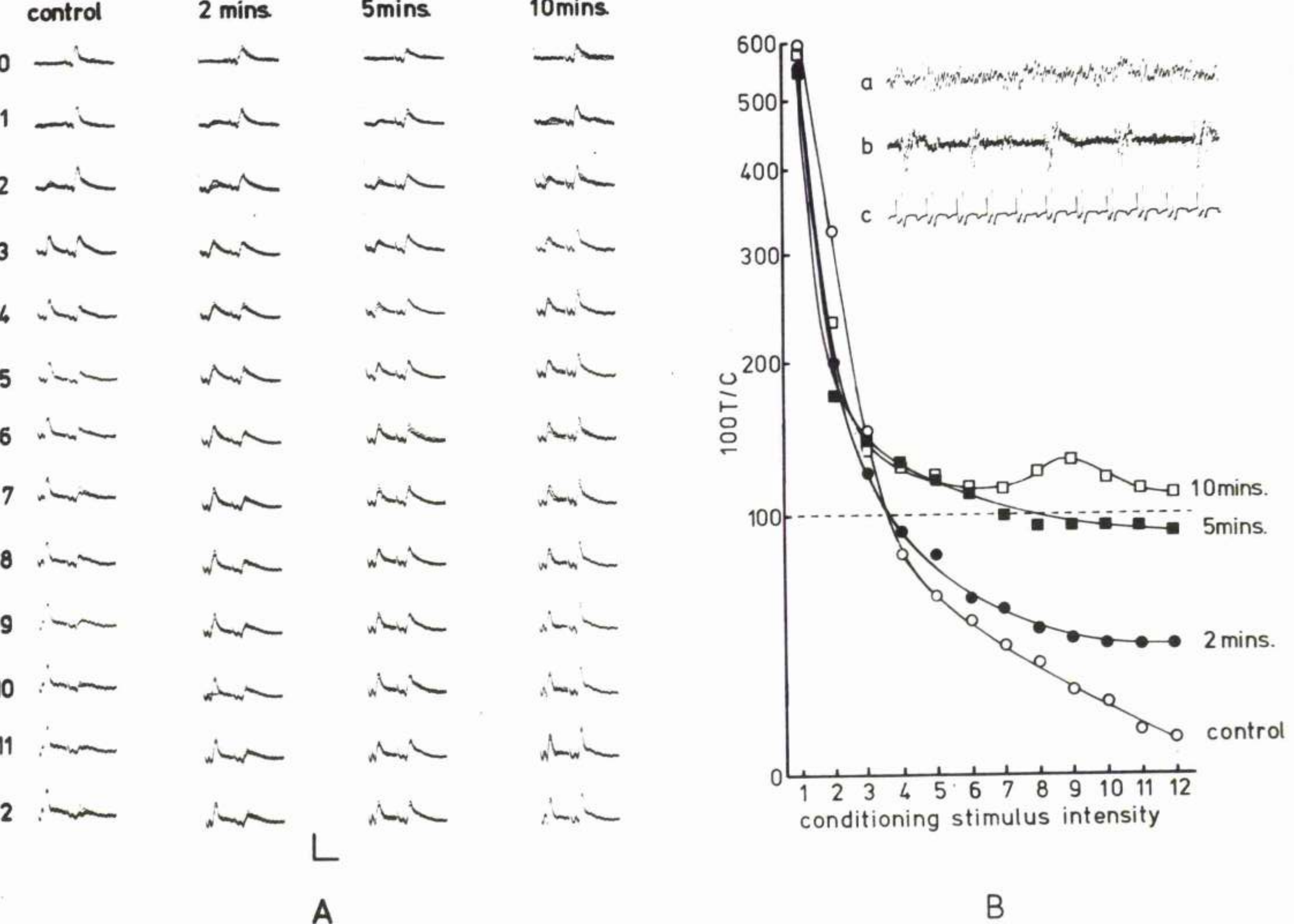
### Responses of evoked field potentials to antagonists of suspected inhibitory neurotransmitters

In Chapter IV it was demonstrated that a conditioning stimulus applied to the olfactory nerve or tract caused a substantial reduction of the field potential evoked by a testing stimulus delivered to either pathway. If this inhibition is mediated by GABA, then drugs which antagonize its action should remove the inhibition and possibly cause a potentiation of the test response. Bicuculline and picrotoxin were used as GABA antagonists (Curtis, Duggan, Felix & Johnston, 1971; Curtis, Duggan, Felix, Johnston & McLennan, 1971; McLennan, 1970) and strychnine as an antagonist of glycine (Curtis, Hösli, Johnston and Johnston, 1968; Roper & Diamond, 1969). These convulsants were administered intravenously since it proved impossible to obtain any significant alteration in the evoked potential field by the iontophoretic method of application. This was presumably due to the low drug concentrations released from the pipette and also the large population of neurons responsible for generating the field.

Figure VII.1 shows an experiment in which conditioning

Figure V11.1: The blocking action of picrotoxin on inhibition of the $N_2$ wave by a prior conditioning stimulus to the olfactory nerve. The records in A were obtained by delivering paired shocks to the olfactory nerve at a constant interval. The numbers 0 - 12 refer to the increasing strength of the conditioning shock in arbitrary units. Inhibition is well developed in the control situation but is gradually replaced by facilitation following an intravenous injection (4 mg/kg) of picrotoxin. Calibration: vertical 1mV., horizontal: 50msec. B is a graph showing the testing response as a percentage of the conditioning response, plotted on a log scale, against conditioning shock intensity. The inset shows the mesencephalic EEG before (a) and after (b), repeated injections of picrotoxin; c is the EKG before drug injections. The time interval between successive p-waves is about 750msec.

Figure V11.1: The blocking action of picrotoxin on inhibition of the $N_2$ wave.



and testing shocks were delivered to the olfactory tract at a constant interval. Inhibition was generated in the control situation (i.e. prior to the injection of convulsant) by gradually increasing the intensity of the conditioning stimulus. Following an intravenous injection of picrotoxin (4 mg/kg), there was a gradual reduction in the degree of inhibition until, after about ten minutes, it was replaced by a marked potentiation. In similar experiments bicuculline was equally effective at doses of about .25 mg/kg, whereas strychnine could not block the inhibition at any concentration tested. An identical result is achieved when both shocks are delivered to the olfactory tract. Figure VII.2 shows such an experiment. Shocks of equal amplitude were paired at different intervals, and following an intravenous injection of bicuculline, the inhibition observed at short shock intervals was replaced by a slight facilitation. Picrotoxin, but not strychnine, was also effective in blocking this inhibition.

The overall effect of these convulsant alkaloids on the electrical activity of the brain is shown in the inset of Figure VII.1B. The electroencephalographic convulsions shown here were recorded from the optic tectum, after repeated injections of picrotoxin. Superficially at least, their effect on the fish brain appears very similar to their well-known epileptogenic action on mammals.

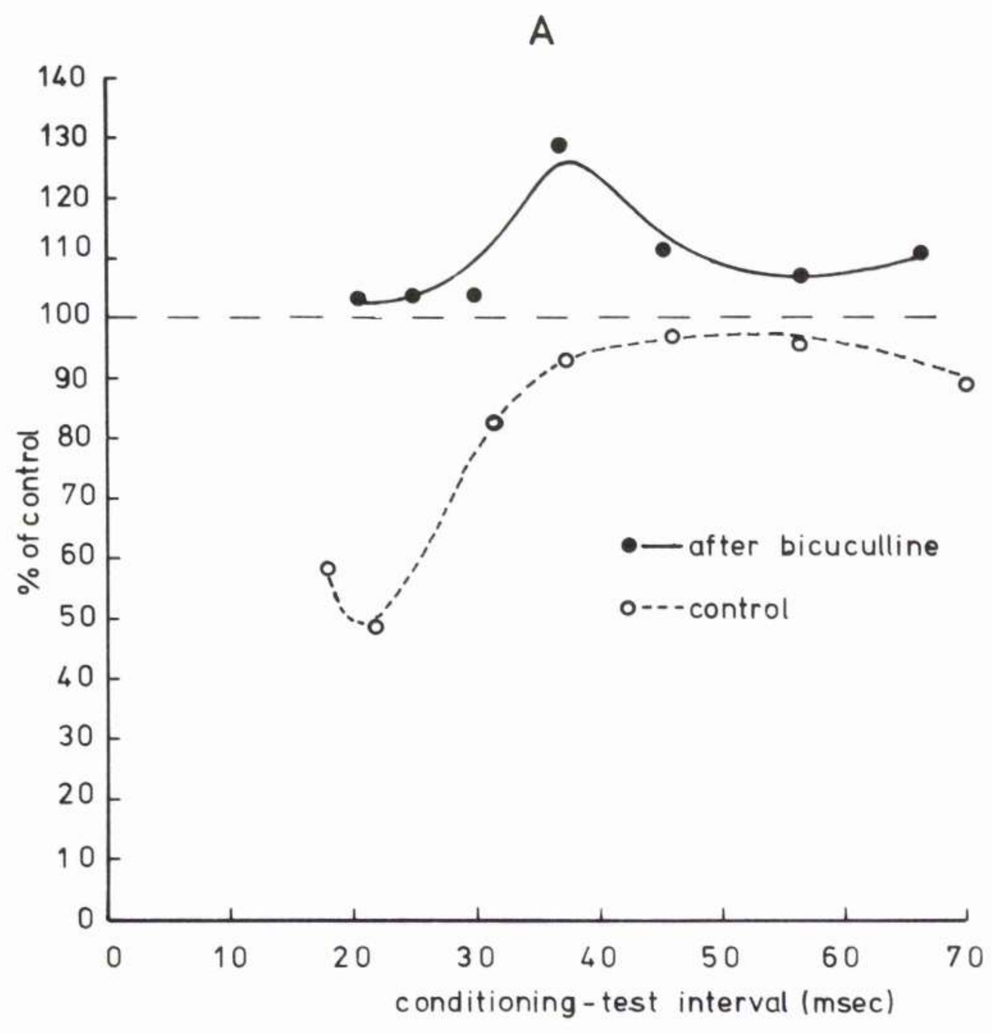
Unitary responses to suspected neurotransmitters

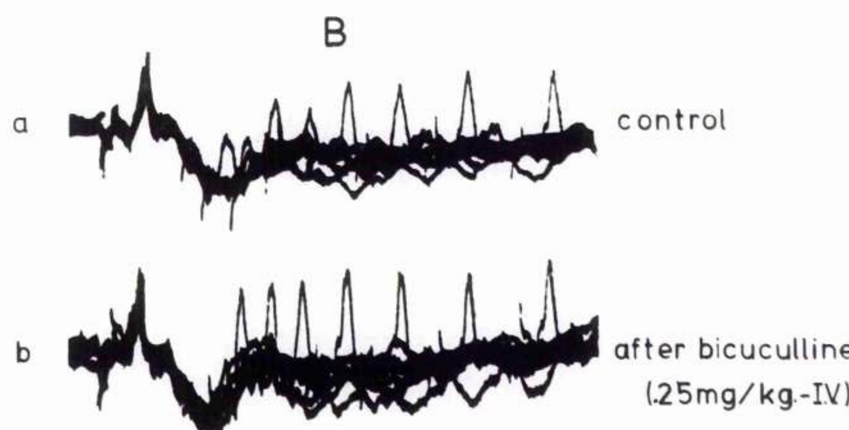
Mitral cell layer

Spontaneously active units identified as mitral cells were invariably inhibited by the iontophoretic application of

Figure V11.2: The effect of bicuculline on the inhibition of the $N_1$ wave following a prior conditioning shock. In this experiment both shocks were delivered to the olfactory tract. Paired shocks of the same amplitude were delivered at decreasing intervals. Responses to a whole series of paired shocks were superimposed on a storage oscilloscope. B shows such records obtained before (a) and after (b) an intravenous injection (.25 mg/kg) of bicuculline. The amplitudes of the test responses as a percentage of the conditioning response are plotted in A, against the conditioning-test interval.

Figure V11.2: Effect of bicuculline on $N_1$ inhibition



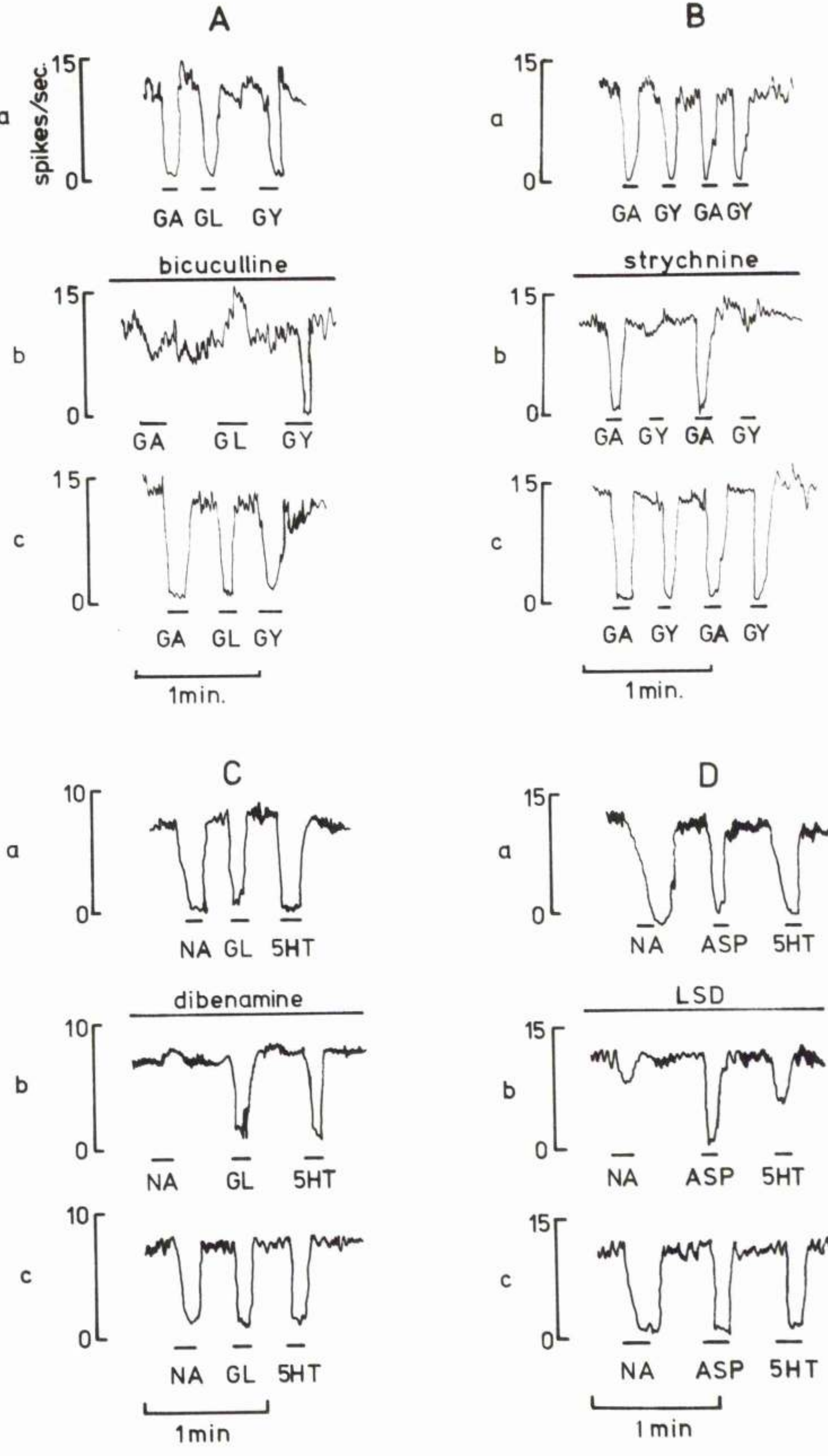
many compounds suspected of having a transmitter function in the central nervous system (Figure VII. 3). The amino acids, L-glutamate and L-aspartate, have been found to be powerful excitants of virtually all central nervous neurons studied, except in the mammalian olfactory bulb (Baumgarten, Bloom, Oliver & Salmoiraghi, 1963; Curtis & Watkins, 1960; Krnjević & Phillis, 1963).

The spontaneous firing of teleost mitral cells was likewise often depressed by these compounds. The iontophoretic application of glycine and GABA also suppressed the spontaneous activity of mitral cells. To totally suppress mitral cell activity it was necessary to employ far higher ejection currents for glycine than for GABA. The inhibitory effect of GABA was nearly always antagonized by the simultaneous ejection of bicuculline or picrotoxin from another barrel of the electrode (Figure VII. 3A). The inhibitory effect of glycine was occasionally blocked by picrotoxin and always by strychnine. Strychnine, however, was never shown to antagonize the action of GABA (Figure VII. 3B). It is interesting to note here that, on the few occasions when a sufficiently stable antidromically or orthodromically driven unit was located in this area, with a multi-barrelled pipette, the type of inhibition shown in Figure V.5C could be blocked by picrotoxin but not strychnine. Bicuculline was not tested on this kind of response.

The amines noradrenaline and serotonin (5HT) were also effective inhibitors of spontaneous activity in mitral cells (Figure VII. 3). The inhibitory effect of noradrenaline was readily antagonized by the $\alpha$-blocker, dibenamine and by

Figure V11.3: Responses of mitral cells to iontophoretically applied drugs. A & B show the effects of bicuculline and strychnine respectively on the inhibitory responses produced by GABA (GA), glutamate (GL) and glycine (GY). Ejection currents were: GABA, 10nA; glutamate 50nA; glycine 20nA; bicuculline, 100nA; strychnine 50nA.
C & D show the effects of dibenamine and LSD on the inhibitory responses produced by noradrenaline (NA), glutamate (GL) and 5HT. Ejection currents were: noradrenaline, 40nA; glutamate 45nA; 5HT, 40nA; dibenamine, 50nA; LSD, 50nA.

Figure VII. 3: Mitral cell responses to iontophoresed drugs



LSD, which is normally a 5HT antagonist. LSD did antagonize 5HT but, on nearly all mitral cells tested, it was a more effective antagonist of noradrenaline. This anomalous finding is in agreement with the studies by Bloom on the olfactory bulb of the rabbit (Bloom, Costa & Salmoiraghi, 1964).

During the studies with mitral cells, it was occasionally possible to locate neighbouring units by a slight movement of the electrode, which responded with excitation to many of the compounds used. None of these units could be antidromically driven by stimulating the olfactory tract.

Granule cell region

With the exception of glycine and GABA, all drugs found to cause a depression of spontaneous mitral cell activity were usually excitatory when applied iontophoretically to cells in the granular region (Figure VII. 4). The inhibitory effect of GABA and, to a lesser extent, glycine could be antagonized by the simultaneous ejection of bicuculline or picrotoxin. These alkaloids produced no detectable alteration in the response of granule cells to noradrenaline, 5HT, glutamate, aspartate or acetyl choline. Occasionally bicuculline caused a slight increase in the firing rate of both mitral cells and granule cells. The inhibitory action of glycine was antagonized by strychnine.

The amines 5HT and noradrenaline were nearly always excitatory when iontophoretically applied to granular cells. In the responses of mitral cells, LSD antagonized noradrenaline to a greater extent than 5HT but, in the case of granule cells,

<u>Figure V11.4:</u> Responses of granule cells to iontophoretically applied drugs. A. shows how the inhibitory effect of GABA (GA) but not the facilitatory effects of noradrenaline (NA), 5HT and glutamate (GL) is blocked by picrotoxin. B. shows the antagonism of the excitatory response to noradrenaline by dibenamine and LSD. Note that 5HT is only partially antagonized by LSD and not at all by dibenamine.
Ejecting currents were: noradrenaline 30nA; 5HT, 25nA; glutamate, 30nA; GABA 15 nA; dibenamine 50nA; LSD 50nA; picrotoxin 150nA.

Figure V11.4: Responses of granule cells to iontophoretically applied drugs.

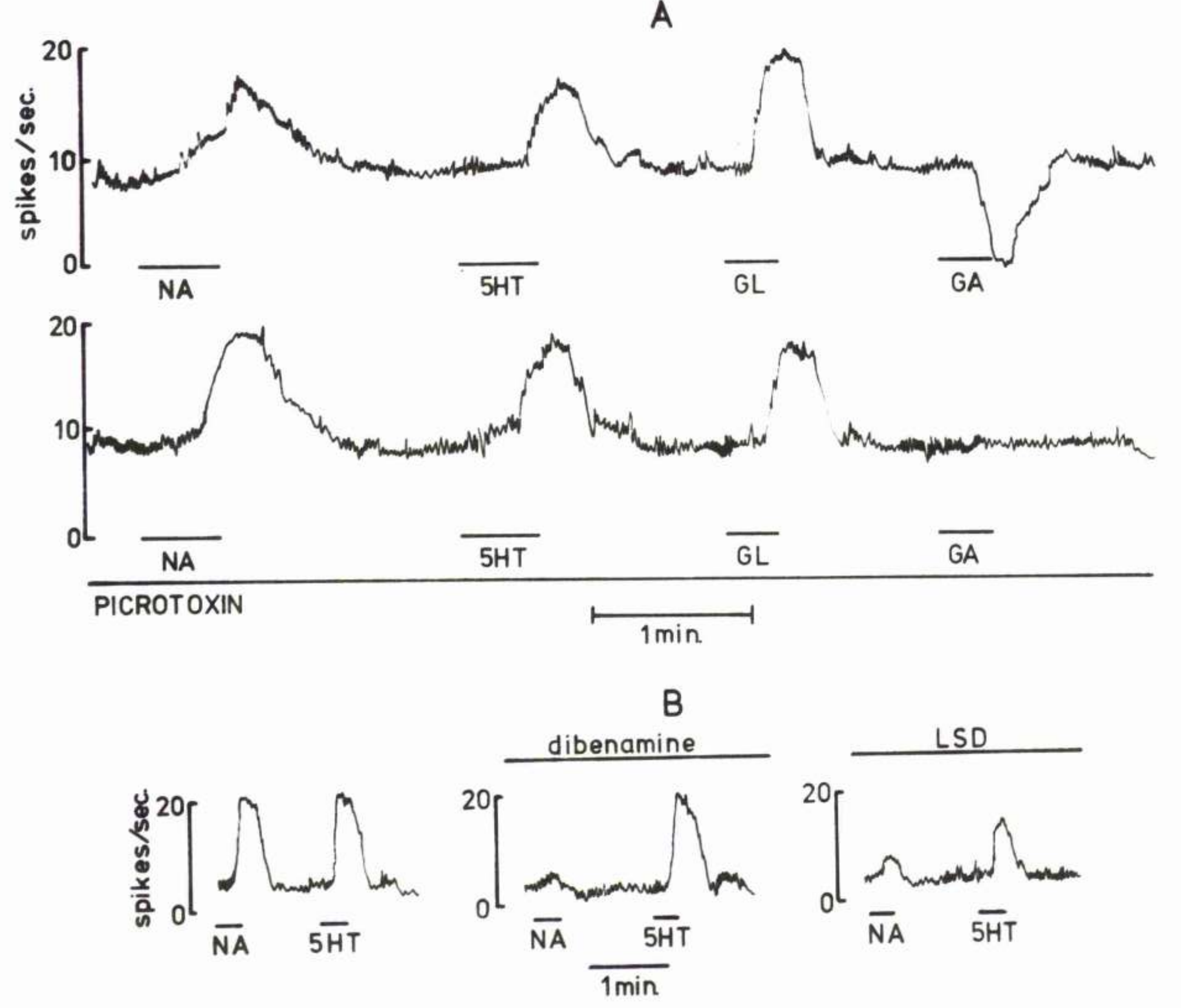


Table VII. 1: Responses of individual olfactory bulb neurons to iontophoretically applied suspected neurotransmitters. Responses classified as: excited (+); inhibited (-) and unaffected (o).

| Drug | Type of Response | Mitral Cells | | Granule Cells | |
|---|---|---|---|---|---|
| | | Total No. Tested | Responses | Total No. Tested | Responses |
| glycine | + | | 0 | | 0 |
| | - | 20 | 12 | 23 | 17 |
| | o | | 8 | | 6 |
| GABA | + | | 0 | | 0 |
| | - | 24 | 20 | 28 | 19 |
| | o | | 4 | | 9 |
| glutamate | + | | 2 | | 30 |
| | - | 21 | 17 | 39 | 2 |
| | o | | 2 | | 7 |
| aspartate | + | | 4 | | 15 |
| | - | 27 | 13 | 24 | 2 |
| | o | | 10 | | 7 |
| noradrenaline | + | | 3 | | 16 |
| | - | 19 | 10 | 30 | 6 |
| | o | | 6 | | 8 |
| 5HT | + | | 3 | | 13 |
| | - | 19 | 10 | 26 | 3 |
| | o | | 6 | | 10 |
| acetylcholine | + | | 1 | | 3 |
| | - | 10 | 4 | 15 | 2 |
| | o | | 5 | | 10 |

the antagonism led to a reduction in the excitatory response. Dibenamine removed the excitatory response of noradrenaline but not of 5HT.

## DISCUSSION

Findings from other parts of the vertebrate central nervous system, particularly the mammalian olfactory bulb, are in good agreement with the pharmacological interactions described above between GABA, glycine, bicuculline, picrotoxin and strychnine (Curtis, Duggan & Felix, 1970; Curtis, Duggan, Felix & Johnston, 1970; Curtis, Duggan, Felix, Johnston & McLennan, 1970; Duggan & McLennan, 1971, Engberg & Thaller, 1970; Nicoll, 1970, 1971). The results are essentially similar for cells in both the granule and mitral cell regions and are indicative of both cell types possessing receptors sensitive to glycine and GABA.

The inhibition of the $N_2$ wave by a prior conditioning stimulus was previously used as an argument for the existence of a dendrodendritic inhibitory pathway. The fact that this inhibition is effectively blocked by bicuculline and picrotoxin indicates that the inhibitory transmitter responsible for mediating the inhibition may very well be GABA. The blockage of mitral cell inhibition and the other pharmacological evidence described above support this hypothesis. Since strychnine was unable to block the physiologically induced inhibition, it is unlikely that glycine is the inhibitory transmitter at this synapse.

Since the amino acids, glutamate and aspartate, have been shown to be excitatory on so many other neurons in

the central nervous system of vertebrates, it seems unlikely that their inhibitory effect on mitral cells is due to their direct activation of inhibitory synapses. In view of the observation that granule cells were almost invariably facilitated by these compounds, a more plausible explanation for the above phenomenan is that the mitral cell inhibition is delivered via the granule cells, which are themselves facilitated by the excitatory transmitters glutamate and aspartate. The inhibitory effects of acetyl choline, noradrenaline and 5HT can also possibly be explained by the same argument.

Thus, the nature of the excitatory transmitter at the reciprocal synapses is much less definite. It has proved impossible to block the physiologically induced inhibition with cholinergic blockers and only to a limited extent with adrenergic blockers following an olfactory tract conditioning shock. Unfortunately, no reliable antagonists exist for either glutamate or aspartate and, therefore, the possibility that one of them is the excitatory transmitter involved must go unchallenged.

In a histochemical study on the rabbit olfactory bulb, Dahlström et al (1965) have shown the presence of noradrenaline containing nerve endings in the region of the granule cell bodies. A few preliminary observations on the olfactory bulb of the trout have revealed intensely green and yellow fluorescing endings in the same region, suggesting both adrenergic and serotonergic inputs. No experimental evidence has been obtained regarding the origin of these fibres in fish but, in the rabbit, they appear to have a suprabulbar

origin (Dahlström et al, 1965). If these fluorescent endings represent the terminations of centrifugal fibres, the pharmacological evidence given above is more easily explained. The excitatory action of noradrenaline on granule cells and its inhibitory action on mitral cells, could possibly be occurring via the same group of synapses, namely post-synaptic receptors on the granule cells which are activated by noradrenaline and 5HT. The granule cells then deliver inhibition to the mitral cells via the GABA mediated side of the granule - mitral reciprocal synapse. A summary of these possibilities is given in Figure VII. 5.

All of these tentative explanations are possible. The iontophoretic technique leaves much to be desired but, at the time of writing, it is the best available tool for studying the pharmacological properties of individual neurons within the central nervous system (Salmoiraghi & Stefanis, 1967). It is hoped that biochemical and further histochemical evidence will soon be available to shed further light on the complex interactions described. It may then be possible to draw some firmer conclusions concerning the nature of the neurotransmitters involved in the complex synapses of the olfactory bulb. The evidence presented here supports well the hypothesis of similar mechanisms of chemical transmission in the mammalian and piscean olfactory bulb.

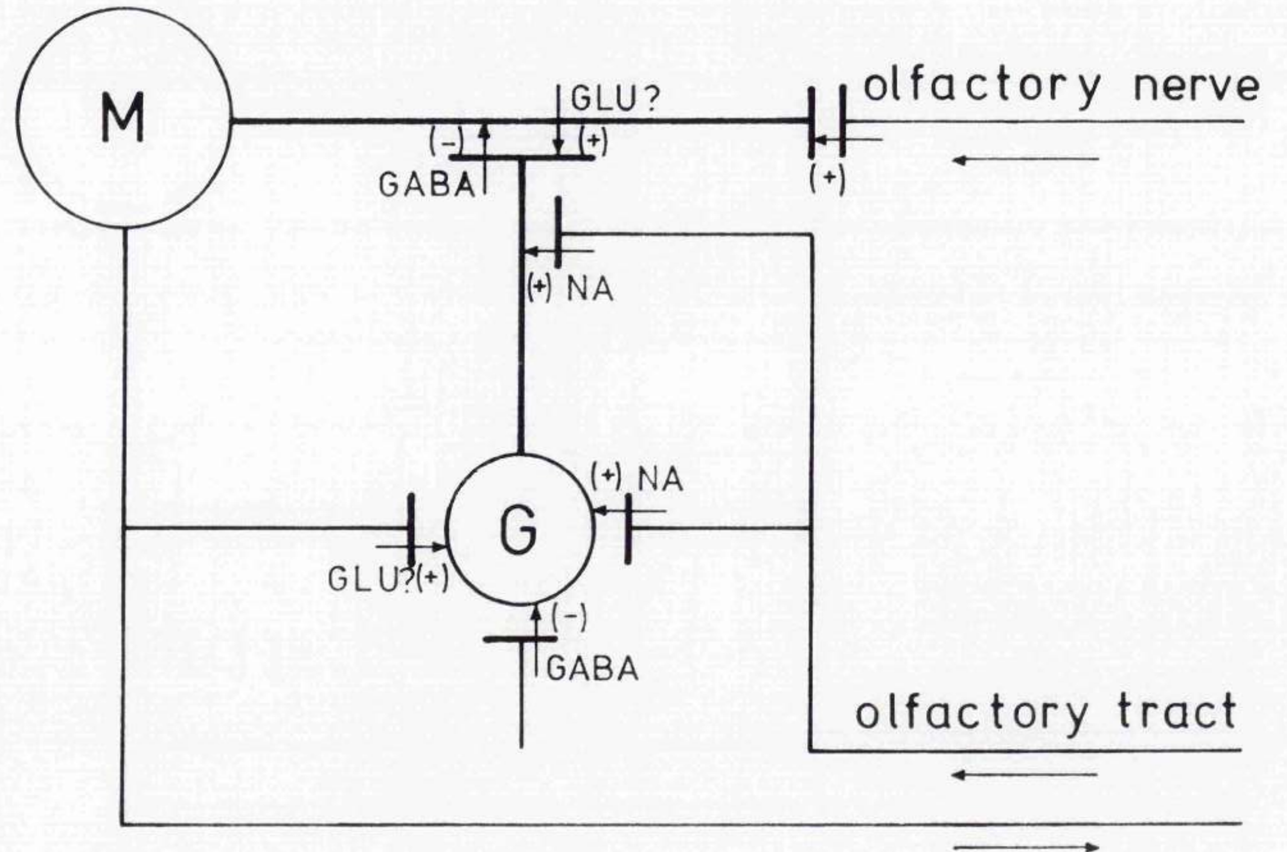
Figure VII. 5: Arrows indicate direction of transmission.

M = mitral cell; G = granule cell;

(+) indicates an exitatory synapse;

(-) indicates an inhibitory synapse

Figure VII. 5: Schematic wiring diagram to explain pharmacological results



# VIII

# reflections

In any study of this magnitude, it is inevitable that one should develop a particular soft spot for the experimental subject. Sentimentality aside, however, it has become increasingly obvious during the course of this study that fish are particularly well-suited experimental subjects for physiological studies of olfaction.

At the mucosal level, fish are indeed ideal subjects. The very fact that olfactory stimulants reaching piscean olfactory receptors are water-borne, implies that experimental odorants can be easily applied and their concentrations easily controlled. The use of radioactively labelled odorants immediately springs to mind as a method of investigating the spatial distribution of receptors.

Biochemical studies of primary transduction mechanisms are commonly performed on bovine olfactory mucosae (Dodd, Personal Communication) but this preparation involves a painstaking separation of olfactory mucosa from the inaccessible nasal bones. The olfactory organs of the larger elasmobranchs are enormous and highly accessible, therefore they would be ideal preparations for membrane fractionation studies. Consequently, the separated olfactory cilia, possibly from different mucosal regions, could be used in conjunction with radioactive odorants to study the kinetics of receptor - odorant interactions.

By the use of several distinct electrophysiological techniques, it has been shown that the fundamental physiology of the fish olfactory system, at least to the level of the olfactory bulb, differs very little from the equivalent system in amphibians, reptiles and mammals.

Any major difference in bulbar physiology should have been revealed by the extensive studies of bulbar field potentials. However, the evoked fields possess almost identical properties to those reported in the literature for mammals. Although more precise comparative studies are needed on representative amphibians and reptiles, it appears that the same fundamental patterns of current flow are preserved independently of any segregation of mitral primary and secondary dendritic fields. One is led to suspect, therefore, that this segregation merely represents a gradual modification of an already sophisticated fine tuning system. In phylogenetic terms, this increased convergence could be thought of as an attempt to maintain an effective analytical olfactory system with relatively fewer neuronal elements as the rhinencephalon gave way to the cerebral cortex.

The studies of unitary responses to olfactory stimulation indicate that the fish olfactory bulb is capable of discriminating between closely related compounds. The across units response patterns generated are certainly as complex as similar patterns reported in the literature for the rat and the tortoise (Mathews, 1972a, b). Whether or not the output of the mammalian bulb is more or less ordered is a question awaiting considerable further research.

The studies comparing odour molecular properties and olfactory efficacy also need to be considerably extended. It is hoped that this will be along similar lines to the results already presented but employing a set of odours including molecules other than amino acids and whose physical properties are extremely well known. The technique of "fishing"

for single units obviously leaves a great deal to be desired but the preliminary results obtained suggest that it is at least as useful as Hara's method based upon induced waves (Hara, 1972b). In view of the interesting relationships noticed between evoked unitary activity and the induced waves, a fruitful line of research might possibly be to make a thorough investigation of this relationship during stimulation with different odours. A possibly useful technique would be the simultaneous on-line computation of the averaged induced wave and the unitary post-stimulus time histogram.

One factor that seems to have passed without consideration in the olfaction literature is the possible influence of current fields on the activity of bulbar neurons. The large extracellular field potential generated by the Mauthner cell is responsible for the electrical inhibition of a neighbouring population of interneurons (Faber & Korn, 1973). Although the maximum amplitude of the bulbar evoked field is only about 3mV, it would be surprising if this did not influence the excitability levels of bulbar neurons to some extent.

It is genuinely hoped that the research embodied in this thesis will provide the background material for future research on the olfactory system of fish and that, one day, we will know a great deal more about what the fish's nose tells its brain.

# appendix

A Computer analysis of olfactory bulb field potentials

The results presented here are an extension of the two-dimensional study presented in Chapter IV. Recordings were made of the evoked field potential from a total of 1000 points forming a 1000 um cubic array. Each point was 100um distant from its nearest neighbour.

The series of field potential recordings labelled A42/10 - 100 were evoked by stimulation of the ipsilateral olfactory nerve with just suprathreshold shocks at 0.5 Hz. Each record consists of five superimposed sweeps. The arrays of recordings in each set from A42/10 through 100 represent ten consecutive frontal sections 100 um apart, passing caudalwards. Recordings were made during electrode withdrawal to avoid unwanted unitary activity and analysed using a film reading device designed by Mr. P. Balch and Mr. R.E. Young.

The voltage was measured from each record at 13.6msec., 19.5msec., 24.4msec., 29.0msec., 35.8msec. and 53.9msec., together with a zero measurement from before the stimulus artifact. The values were punched on to paper tape and subsequently fed into an IBM 360 computer (Young, 1973). After updating, the data was transferred to cards and isopotential contour maps and three-dimensional graphs were constructed, using Fortran IV programmes written by Mr. P. Balch for the IBM 360. A digital plotter was used for the final drawing of contour maps and three-dimensional graphs.

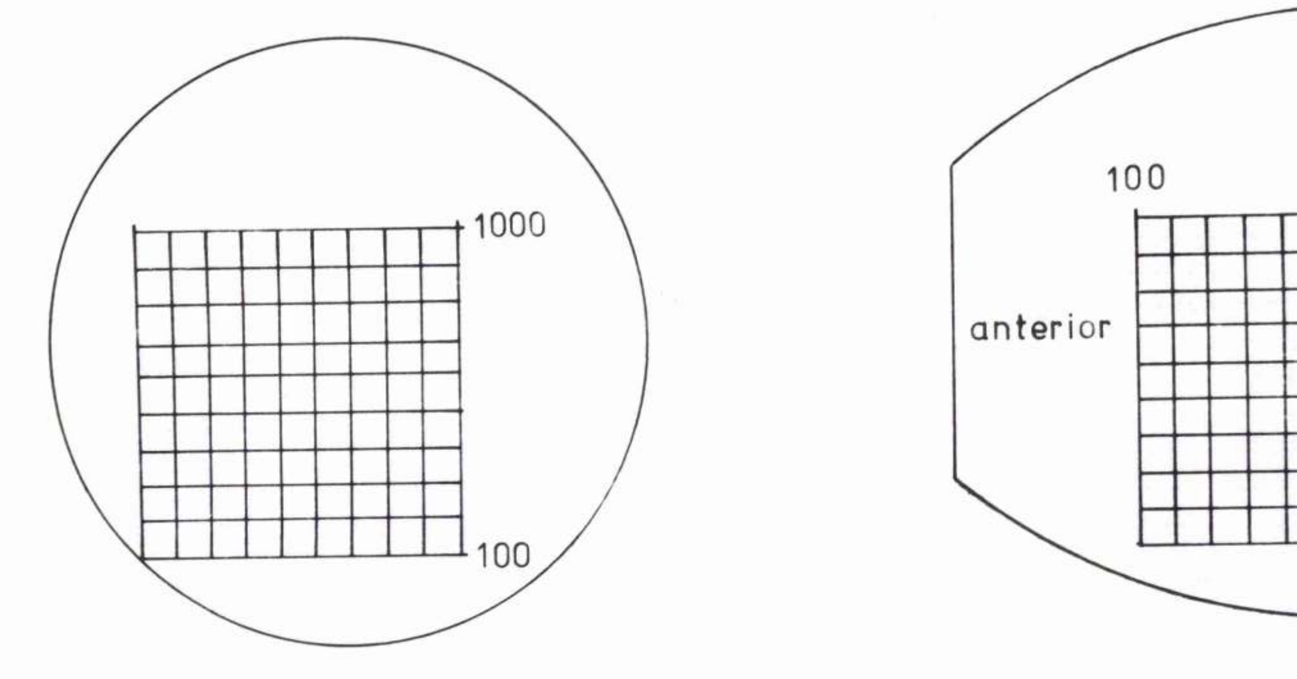
The contour maps labelled A42/10 - 100 correspond to the arrays of recordings A42/10 - 100 appearing before each contour map. They consequently represent isopotential

contours across frontal sections of the olfactory bulb. The contour maps labelled A42/S10 - S100 were drawn using the same data but reorganized so that each set of maps represents a sagittal section through the bulb. These two views of the array are shown in Figure A.1.

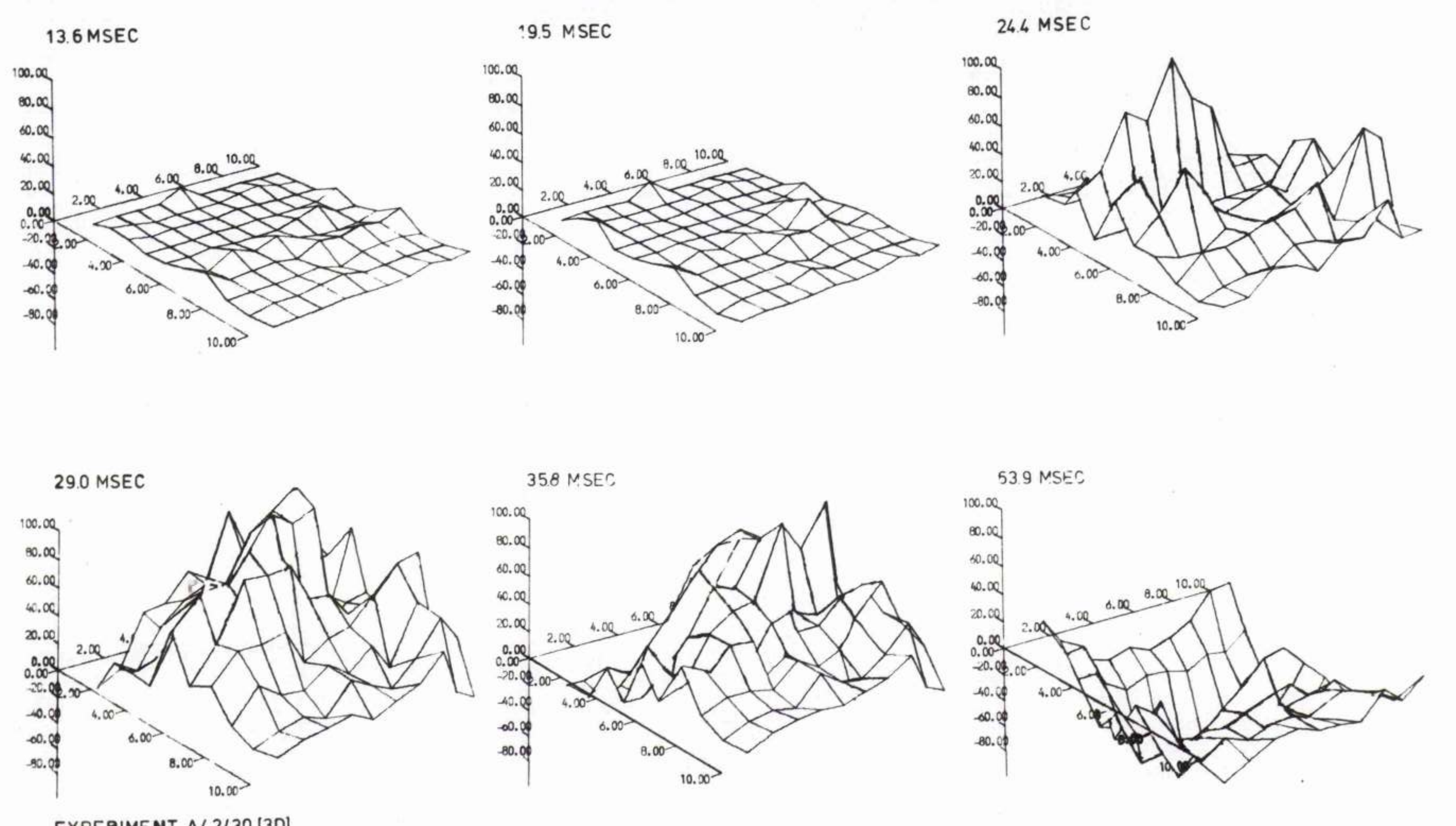
The one set of three-dimensional graphs shown is equivalent to the contour maps labelled A42/S30. The vertical axis represents voltage, the other two axes representing the edges of the recording array. This set of graphs has been included to emphasize the different distributions of potential at various time intervals. Particularly dramatic is the difference between 29msec. and 53.9msec. These two times represent the peaks of the $N_2$ positive reflection and the P wave negative reflection recorded in the granule cell layer.

On all figures in this Appendix, voltage is given in arbitrary units.

Figure A. 1: Diagram to illustrate the approximate position of the recording array



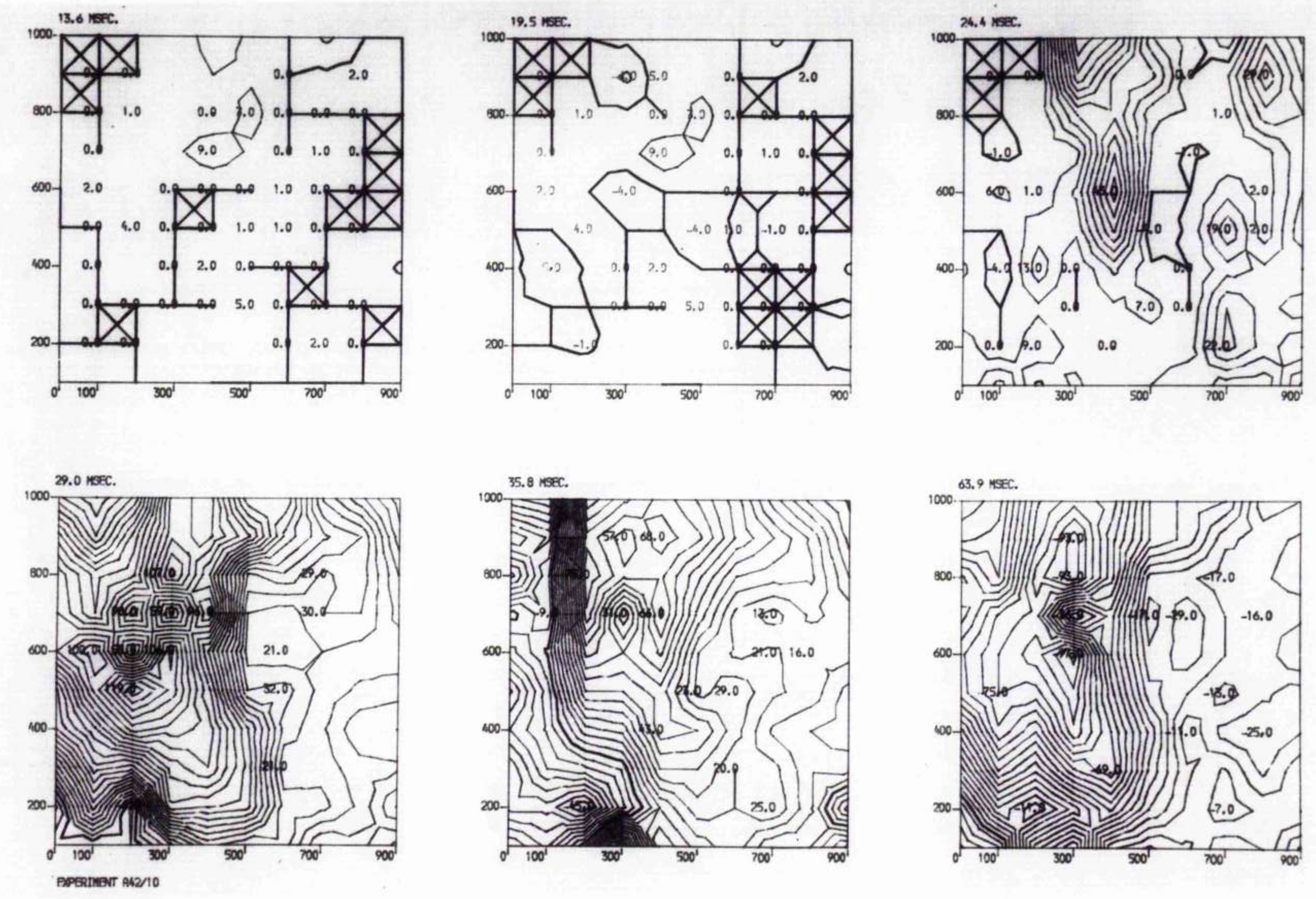
Three-dimensional representation of the contour map A42/S30.



A42/10

1000
900
800
700
600
500
400
300
200
100

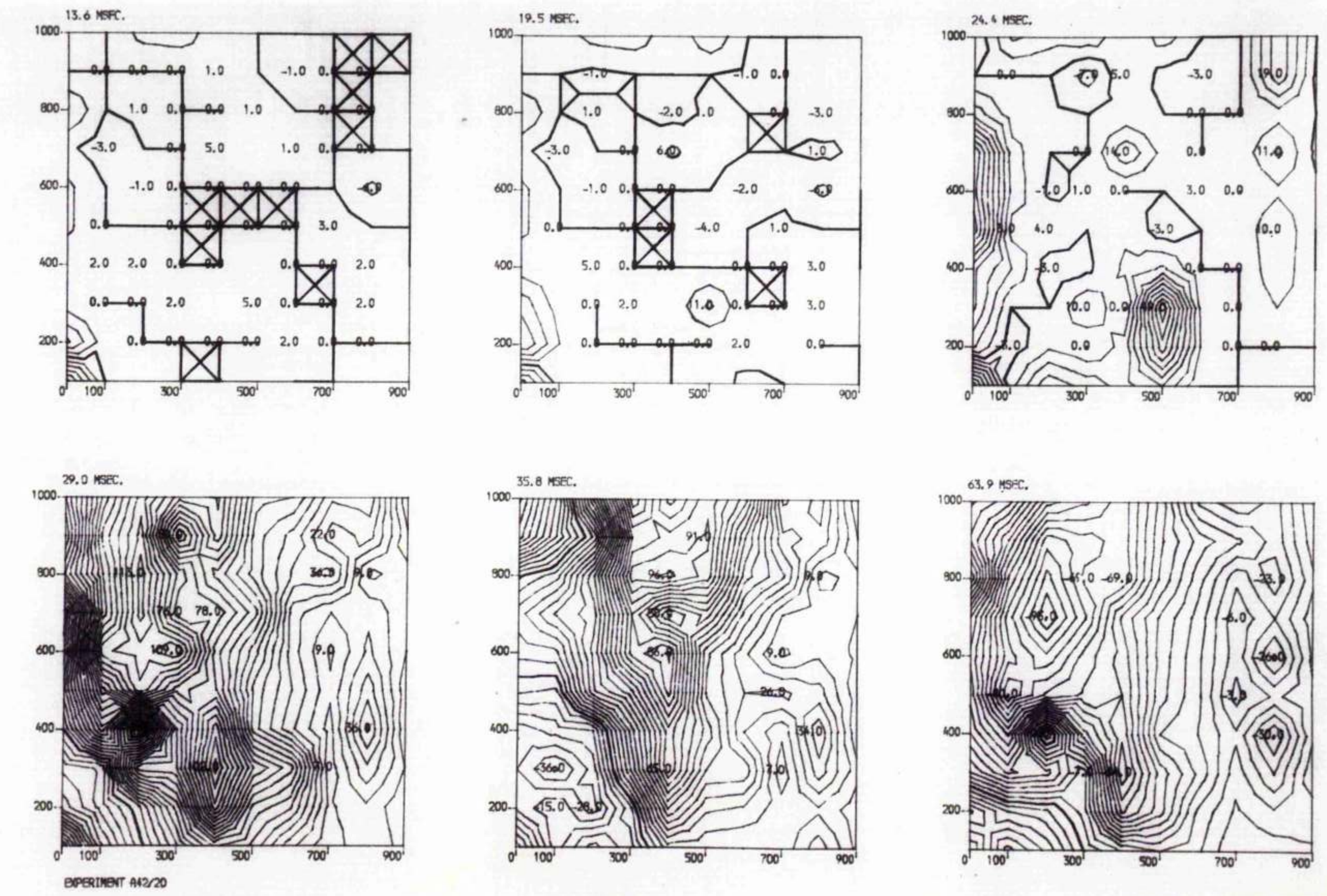
0 100 200 300 400 500 600 700 800 900


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT A42/10

A42 / 20

1000
900
800
700
600
500
400
300
200
100

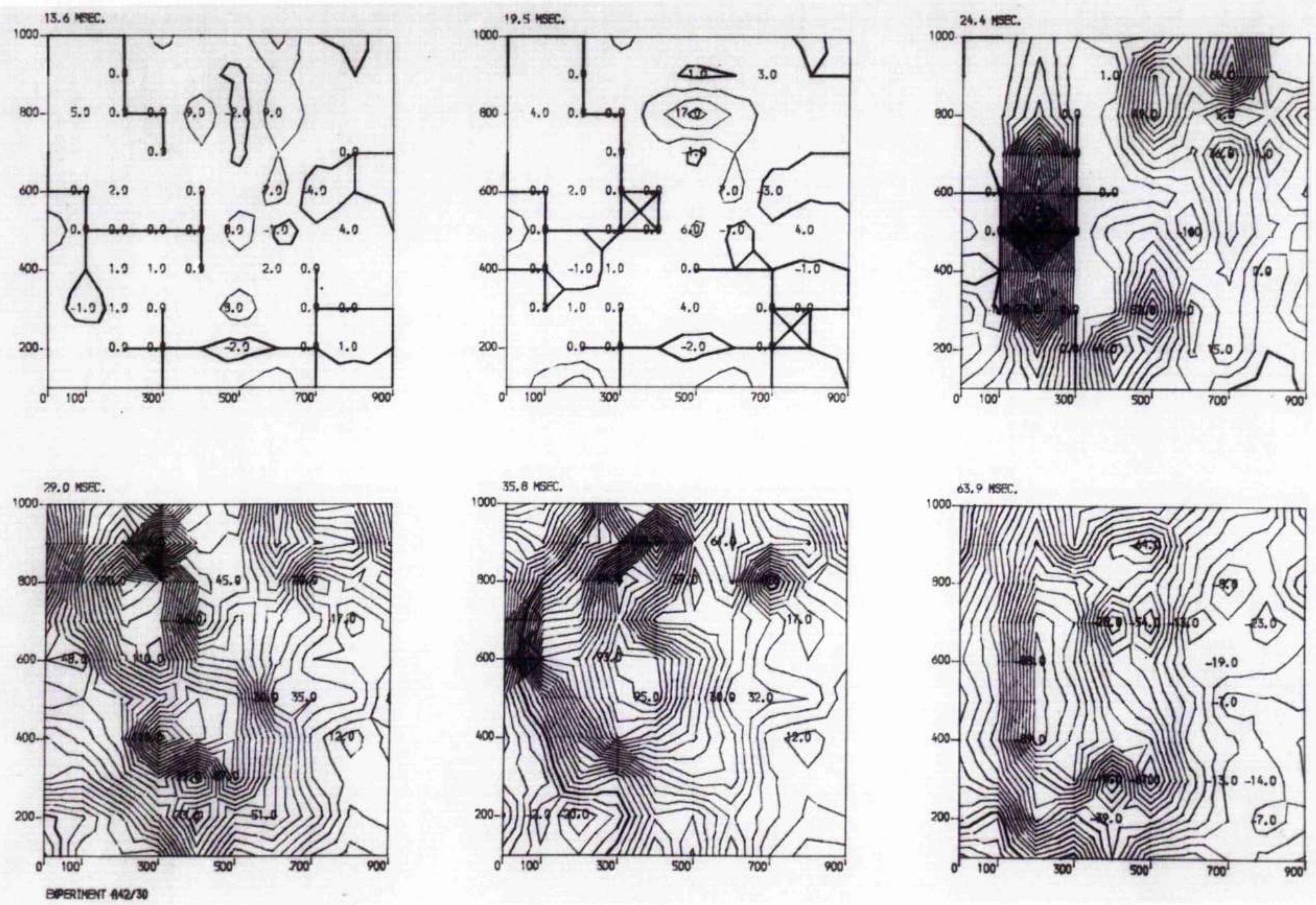
0 100 200 300 400 500 600 700 800 00


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT A42/20

## A42 / 30

1000
900
800
700
600
500
400
300
200
100

0 100 200 300 400 500 600 700 800 900

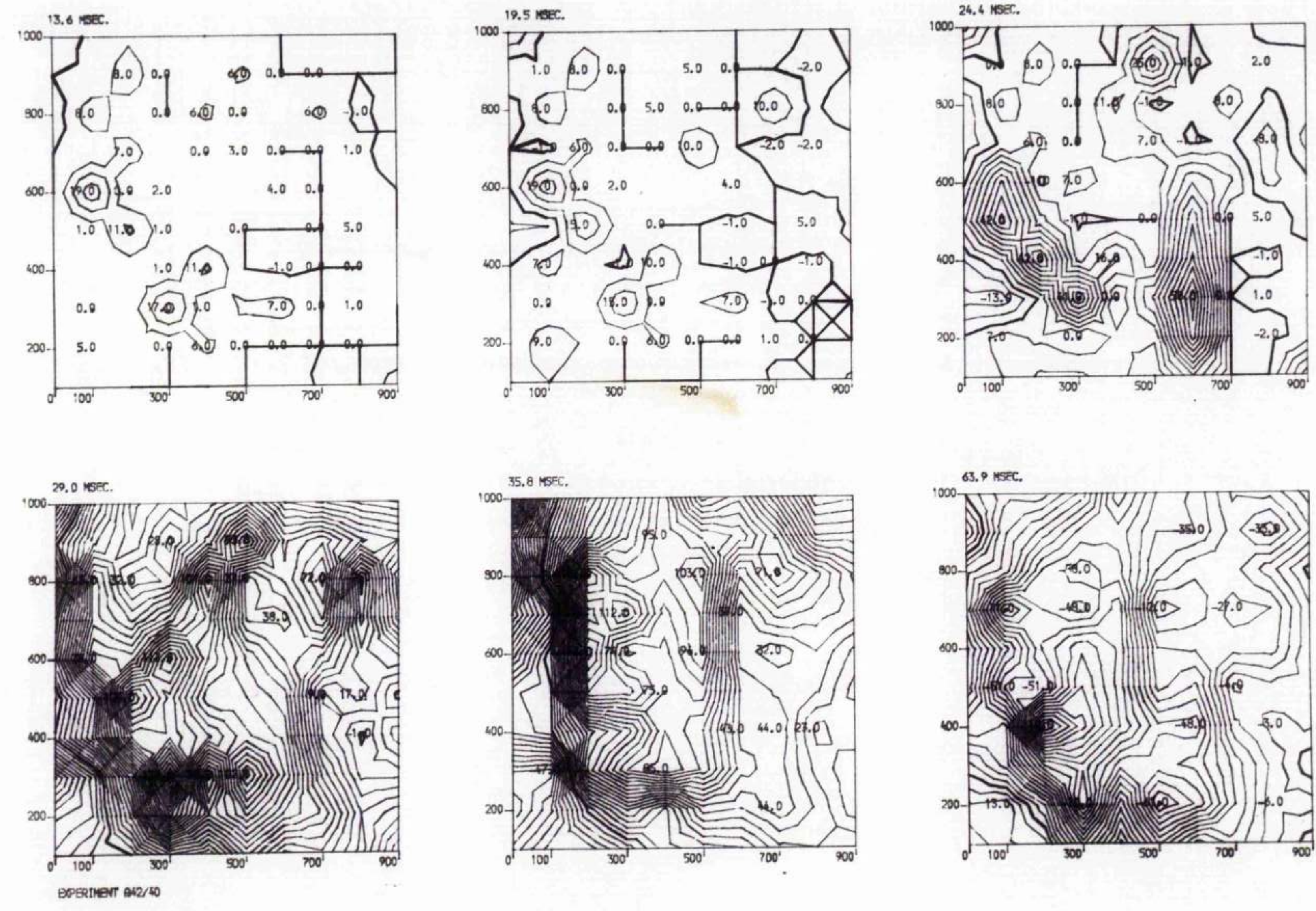


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT 642/30

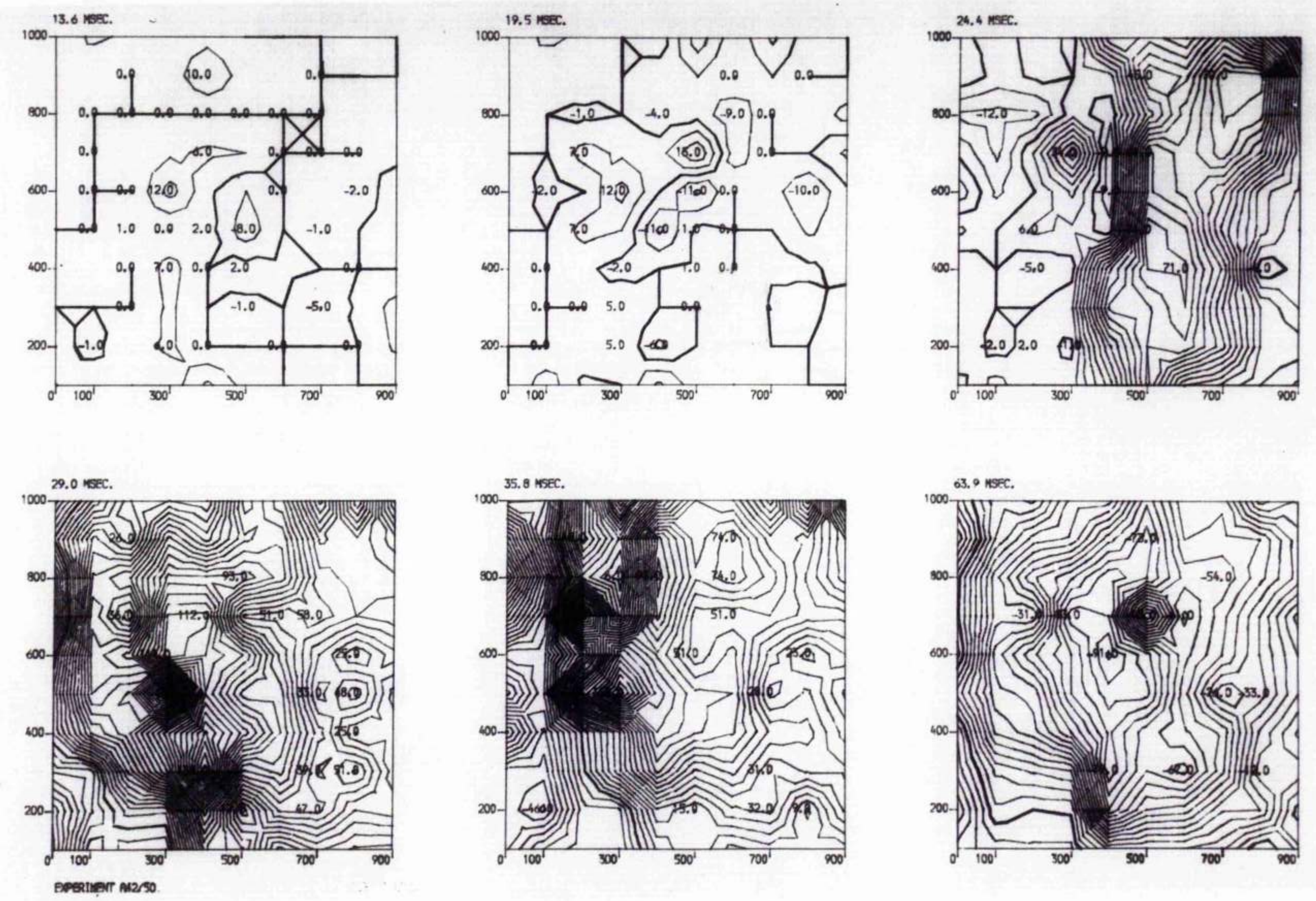
A42/40

1000
900
800
700
600
500
400
300
200
100

0 100 200 300 400 500 600 700 800 900


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT 842/40

## A42/50

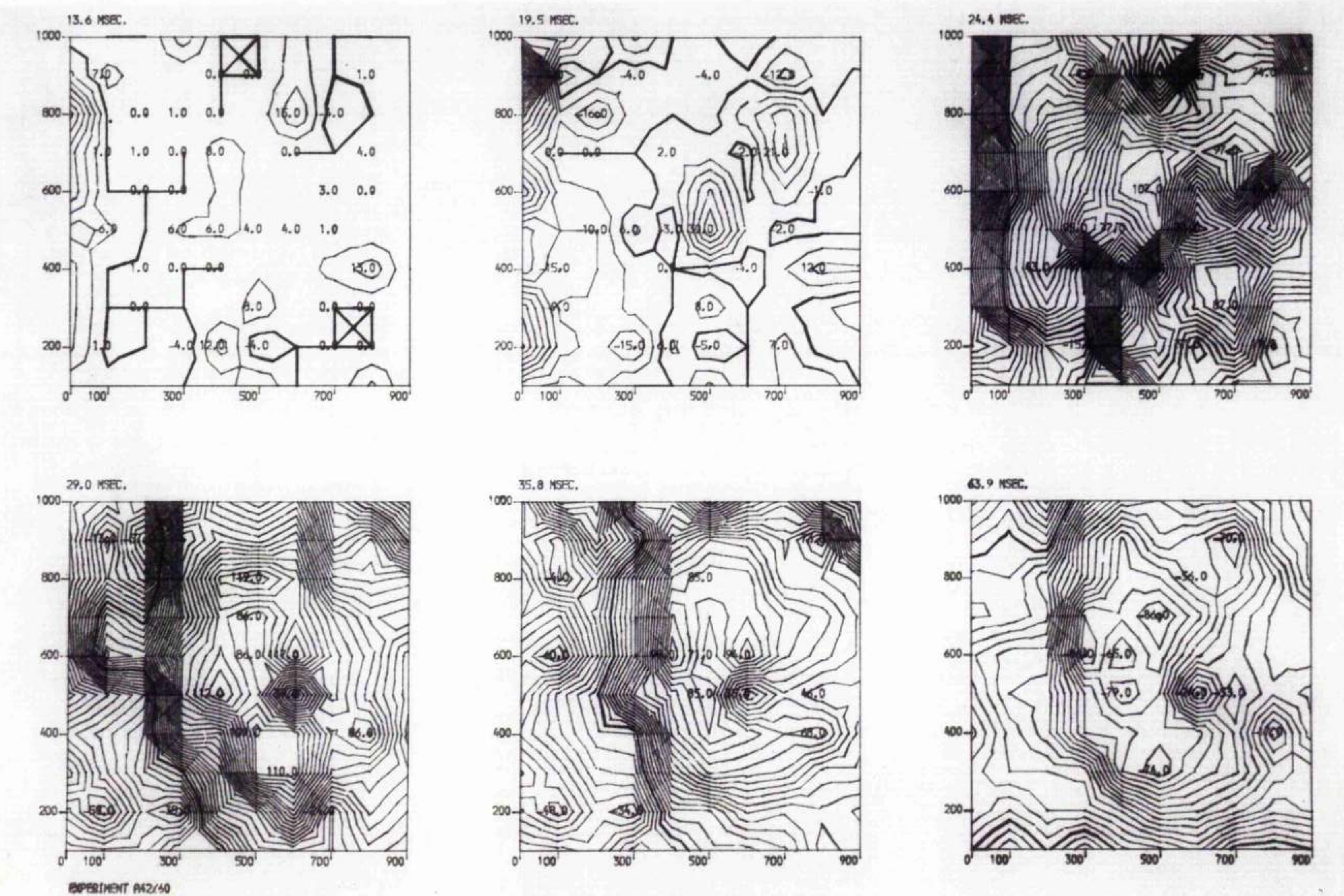


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT AN2/50

## A42/60

1000
900
800
700
600
500
400
300
200
100

0 100 200 300 400 500 600 700 800 900

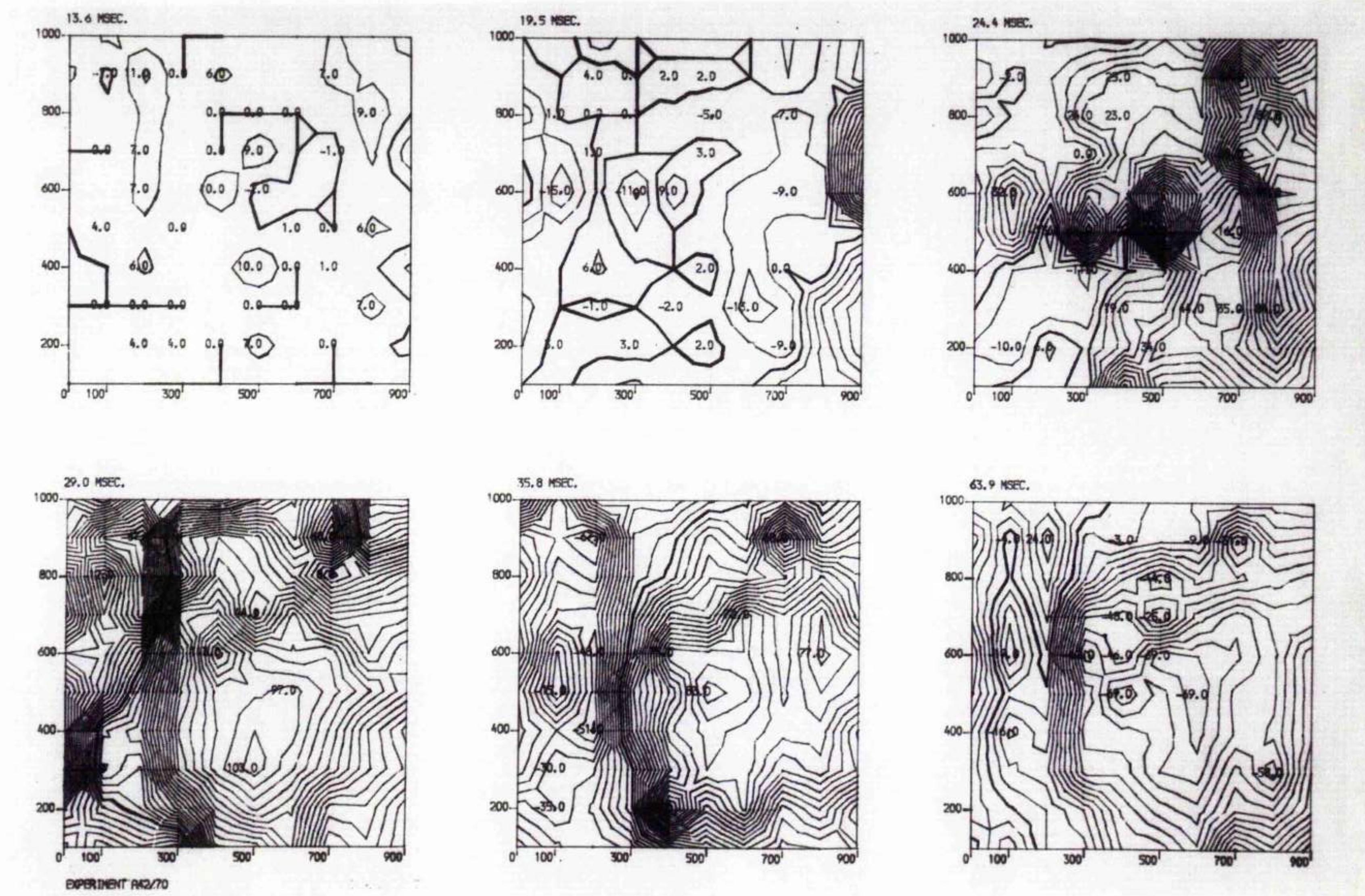


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.

## A42/70

1000
900
800
700
600
500
400
300
200
100

0 100 200 300 400 500 600 700 800 900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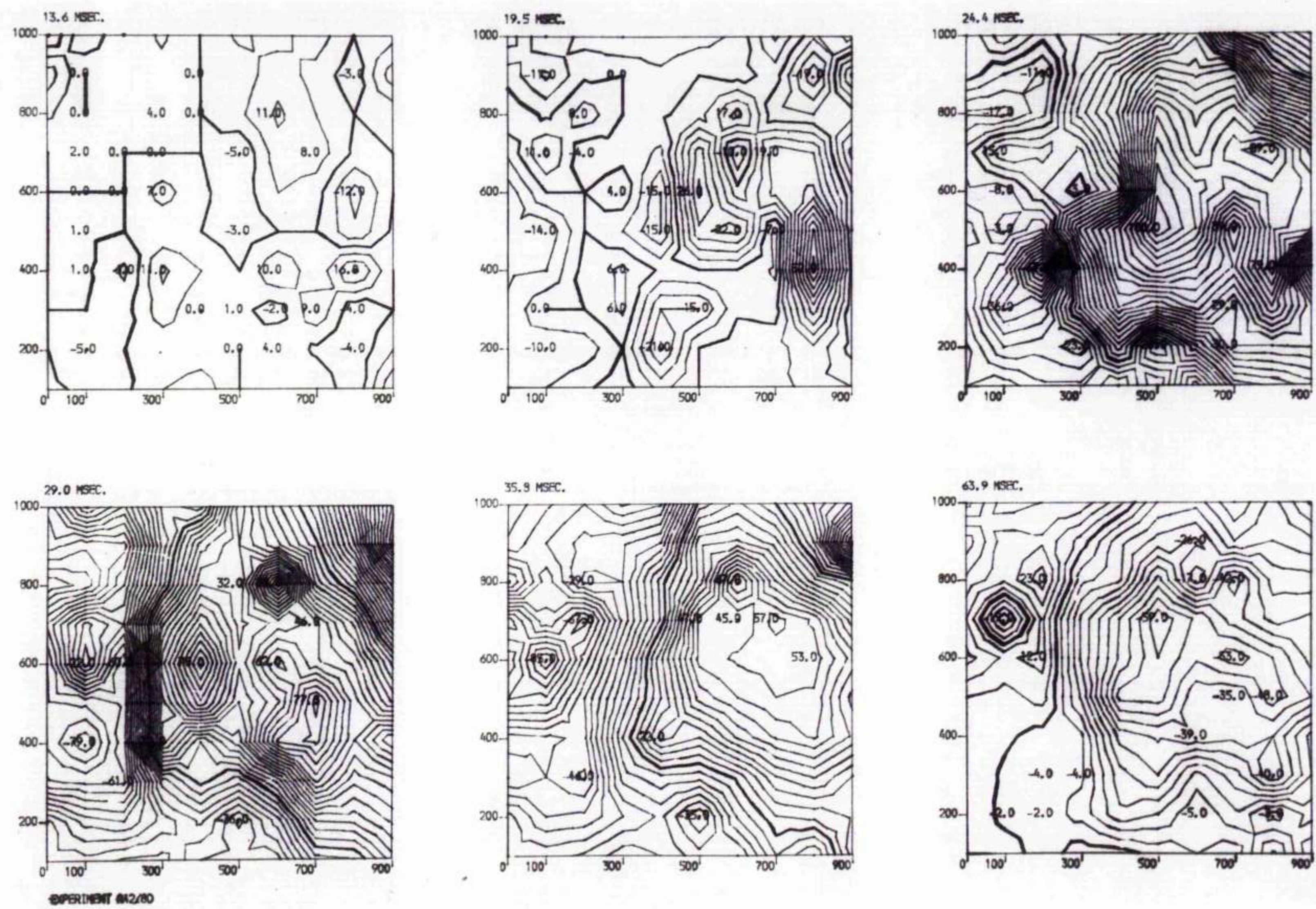
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT A42/70

A42/80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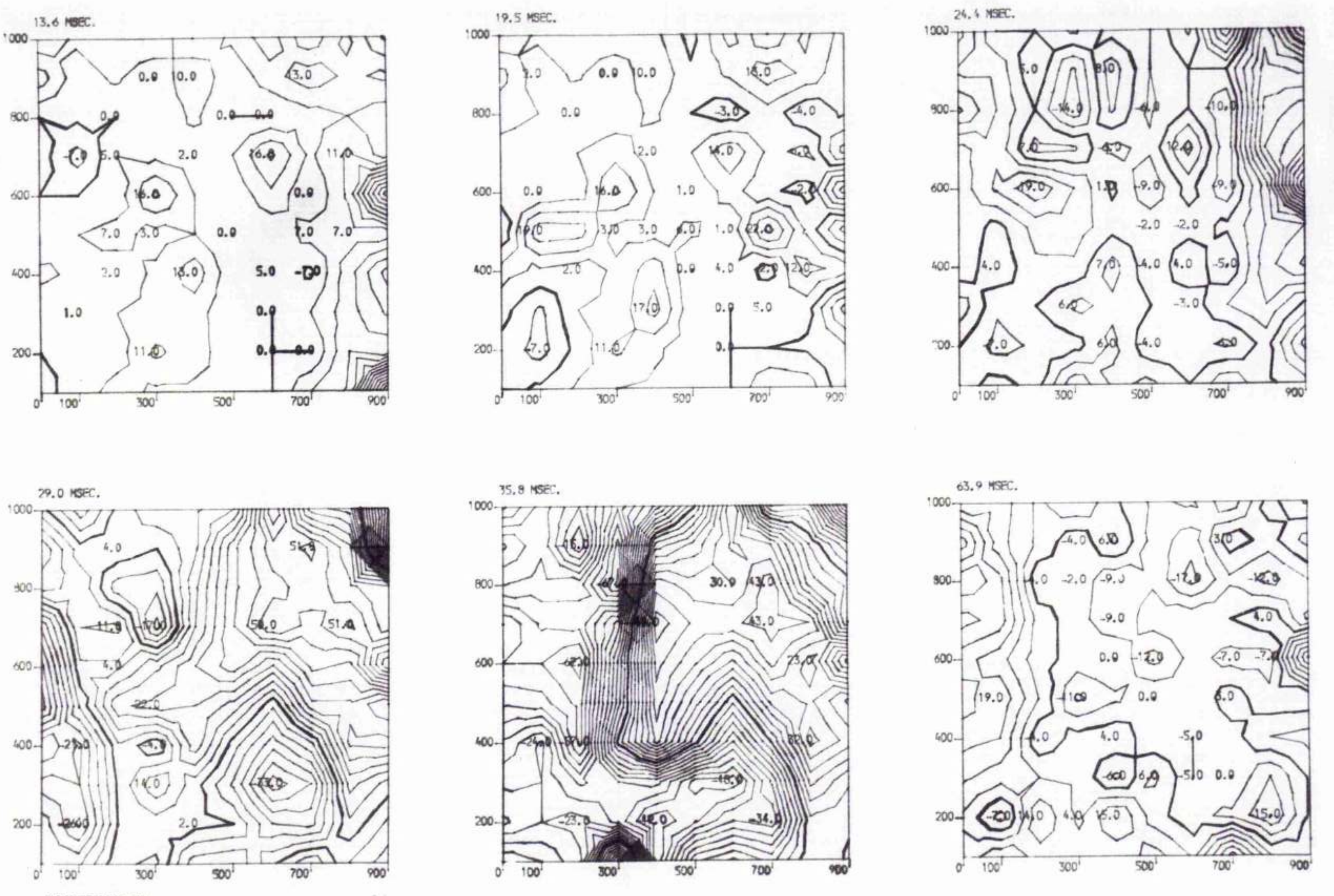
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.9 MSEC.
63.9 MSEC.
EXPERIMENT 8A2/80

442/90

1000
900
800
700
600
500
400
300
200
100

0 100 200 300 400 500 600 700 800 900

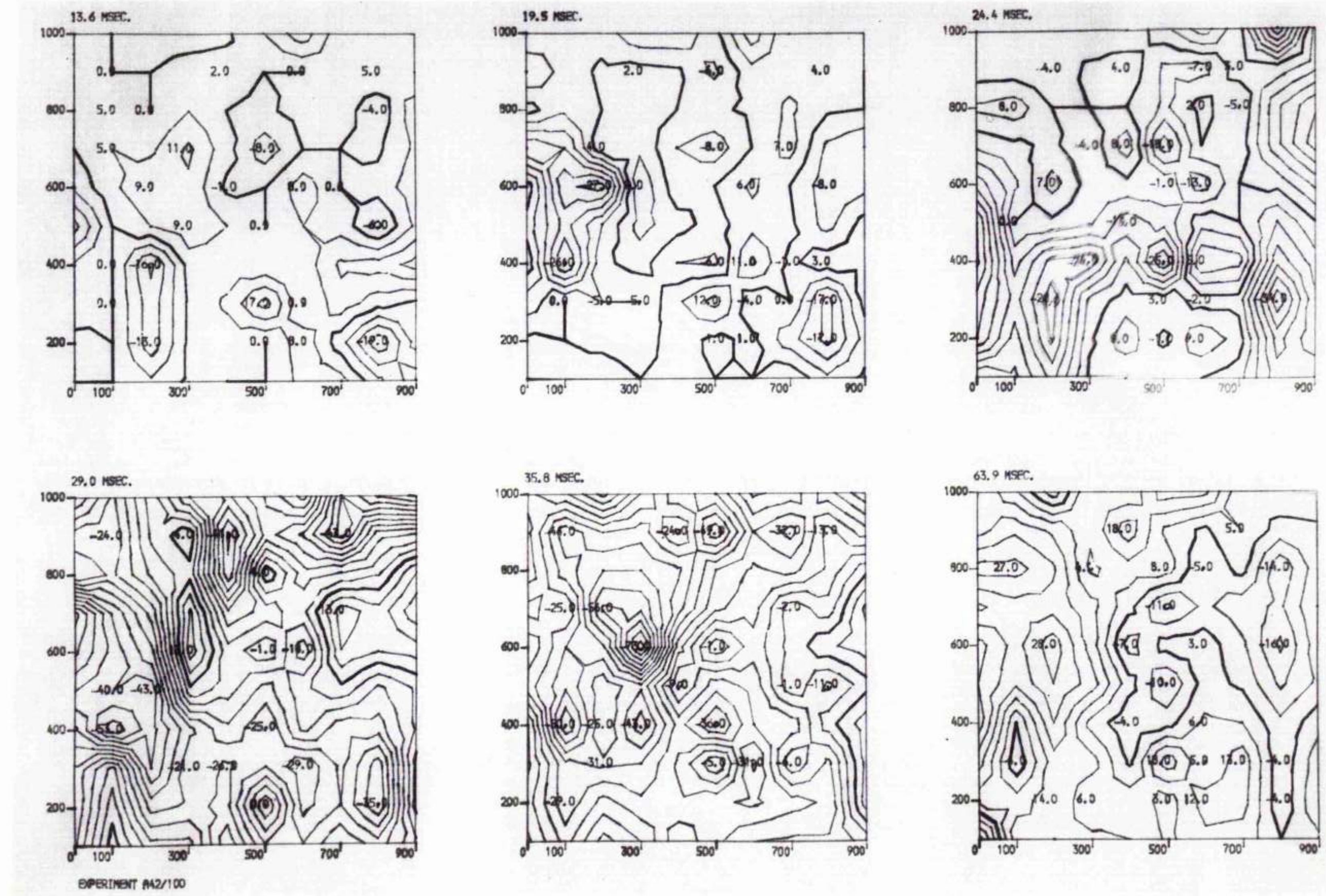


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT AA2/90

## A42/100

1000
900
800
700
600
500
400
300
200
100

0 100 200 300 400 500 600 700 800 900



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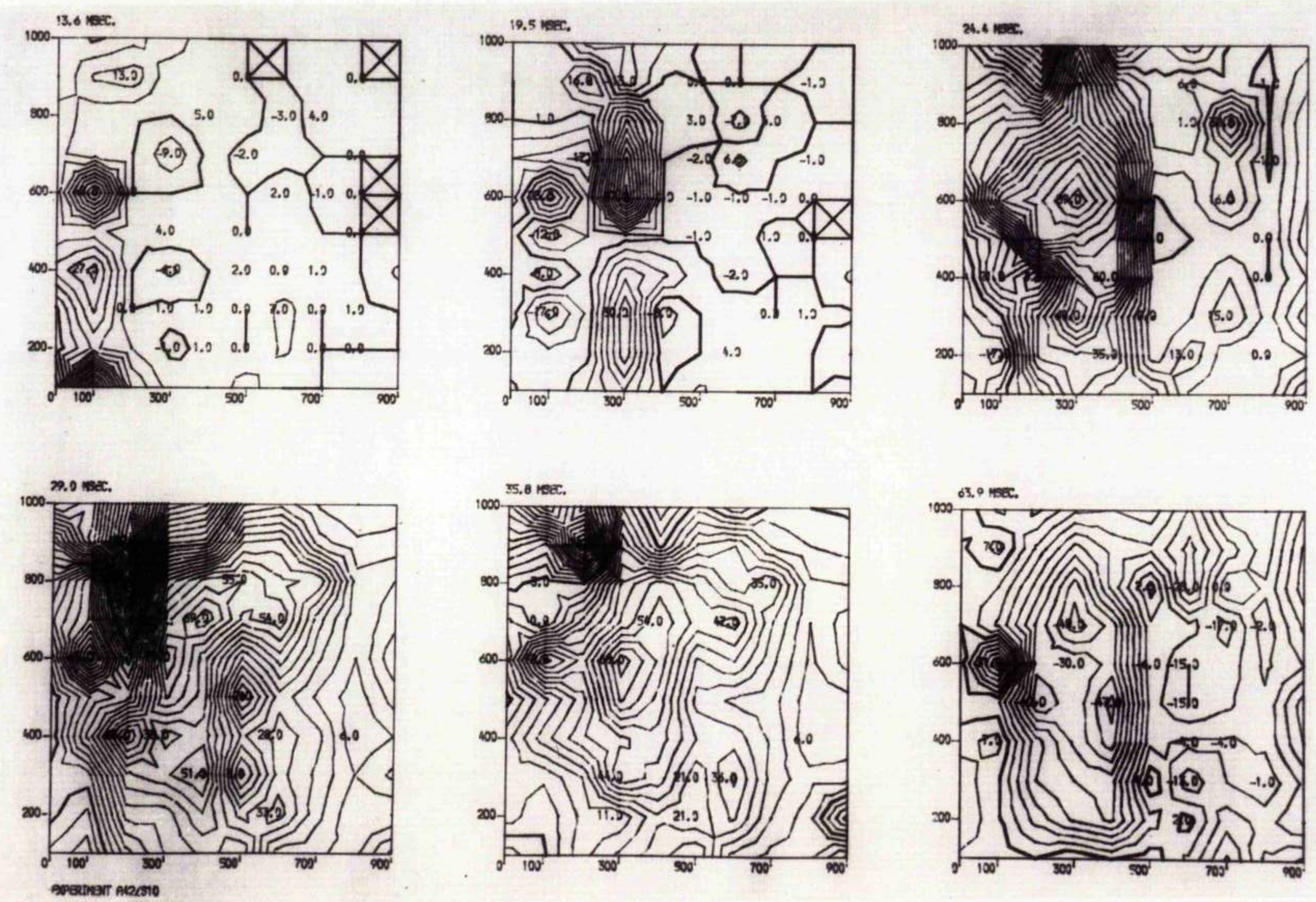
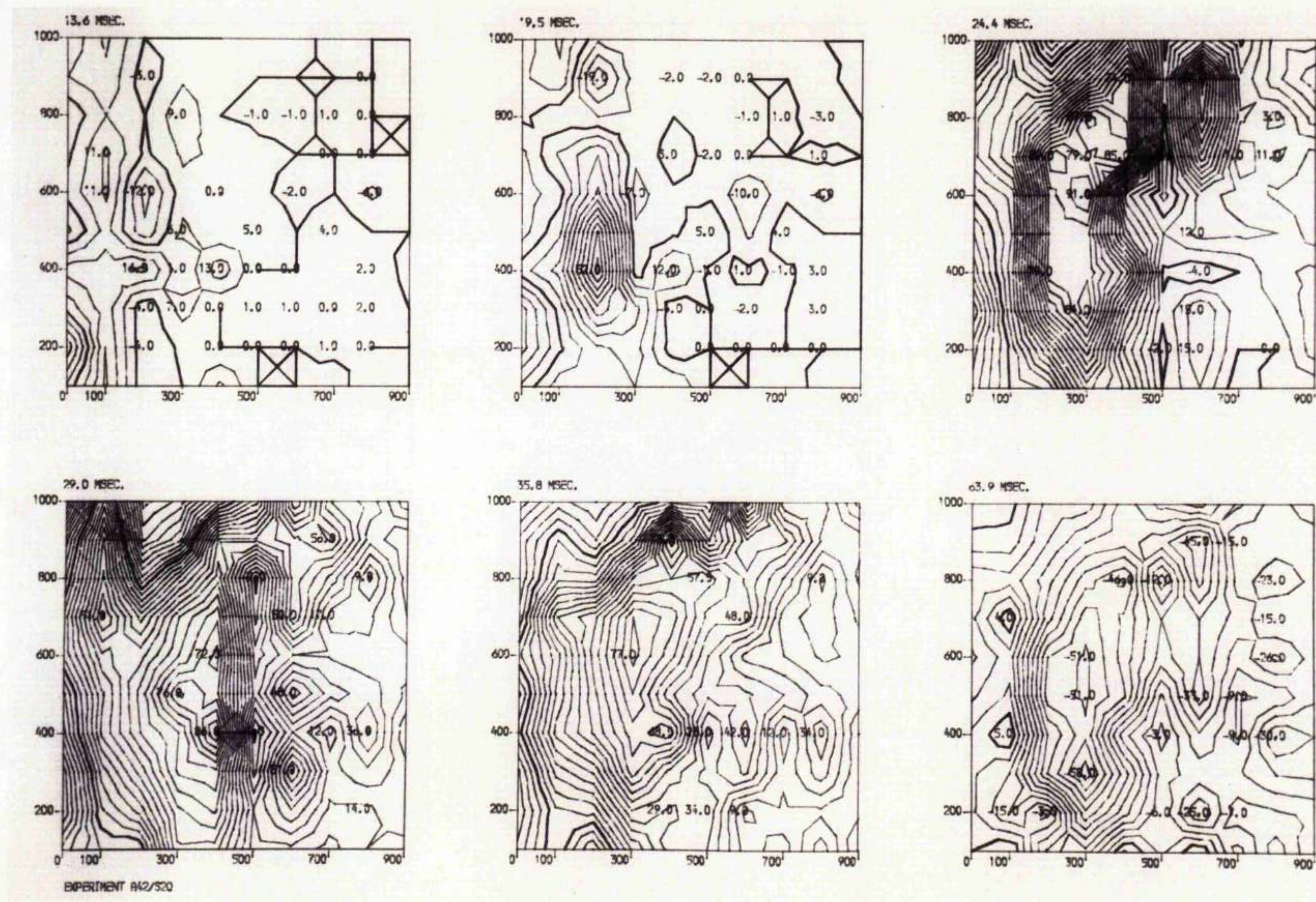
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT #A2/100



13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
1000
800
600
400
200
0
100'
300'
500'
700'
900'
EXPERIMENT A42/310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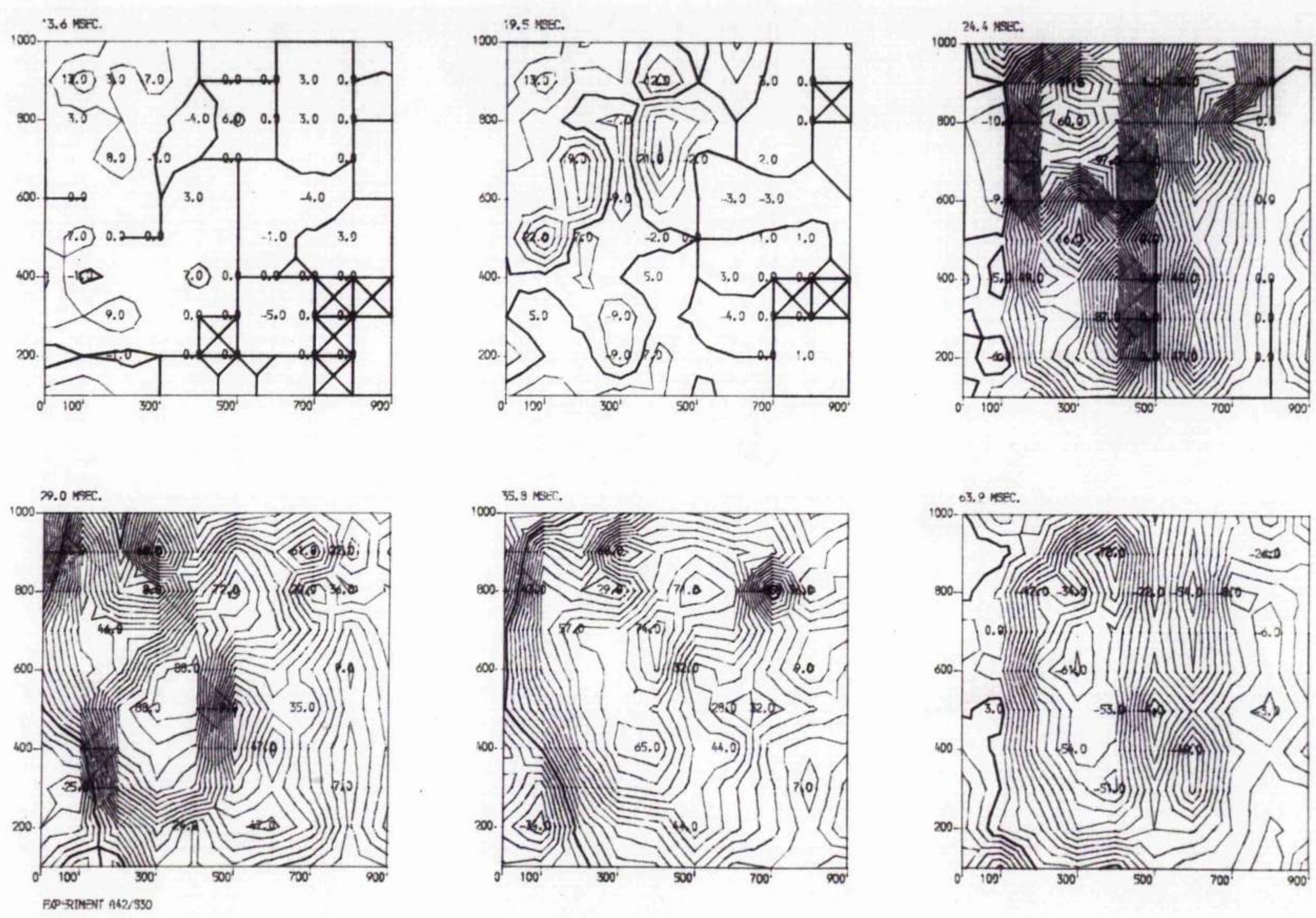
EXPERIMENT A42/S20



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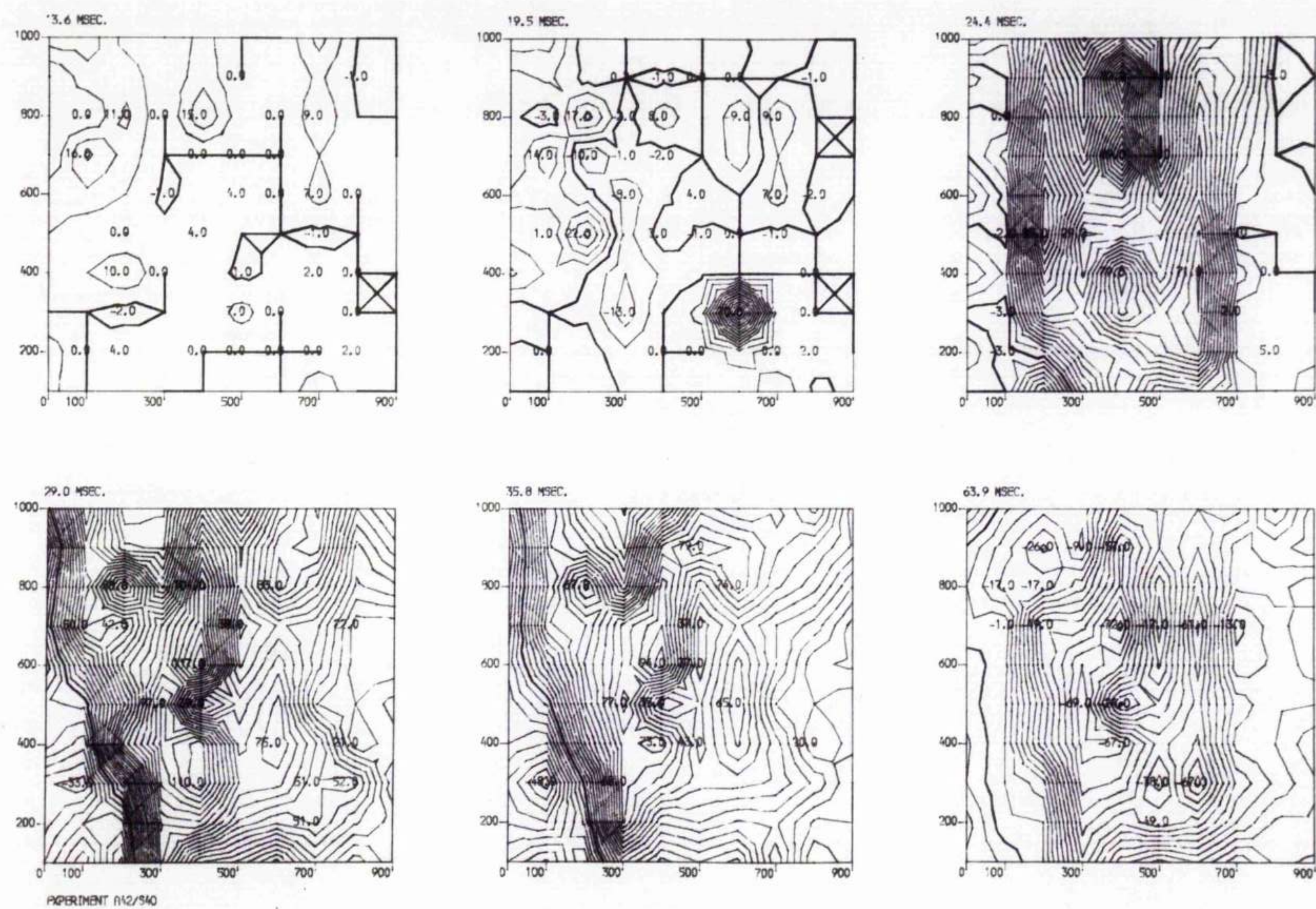
35.9 MSEC.
63.9 MSEC.
EXPERIMENT 842/330



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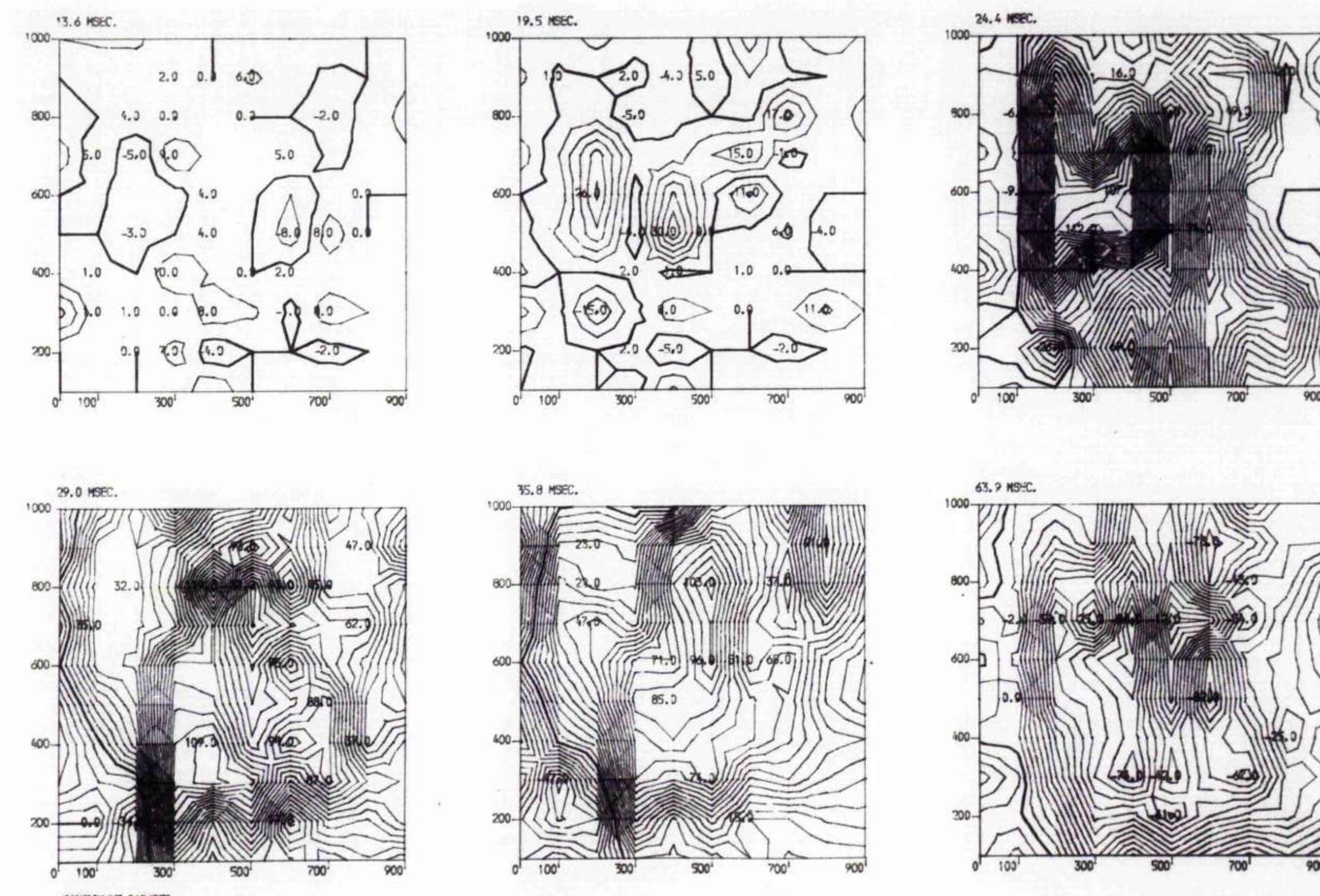
63.9 MSEC.
EXPERIMENT R12/940



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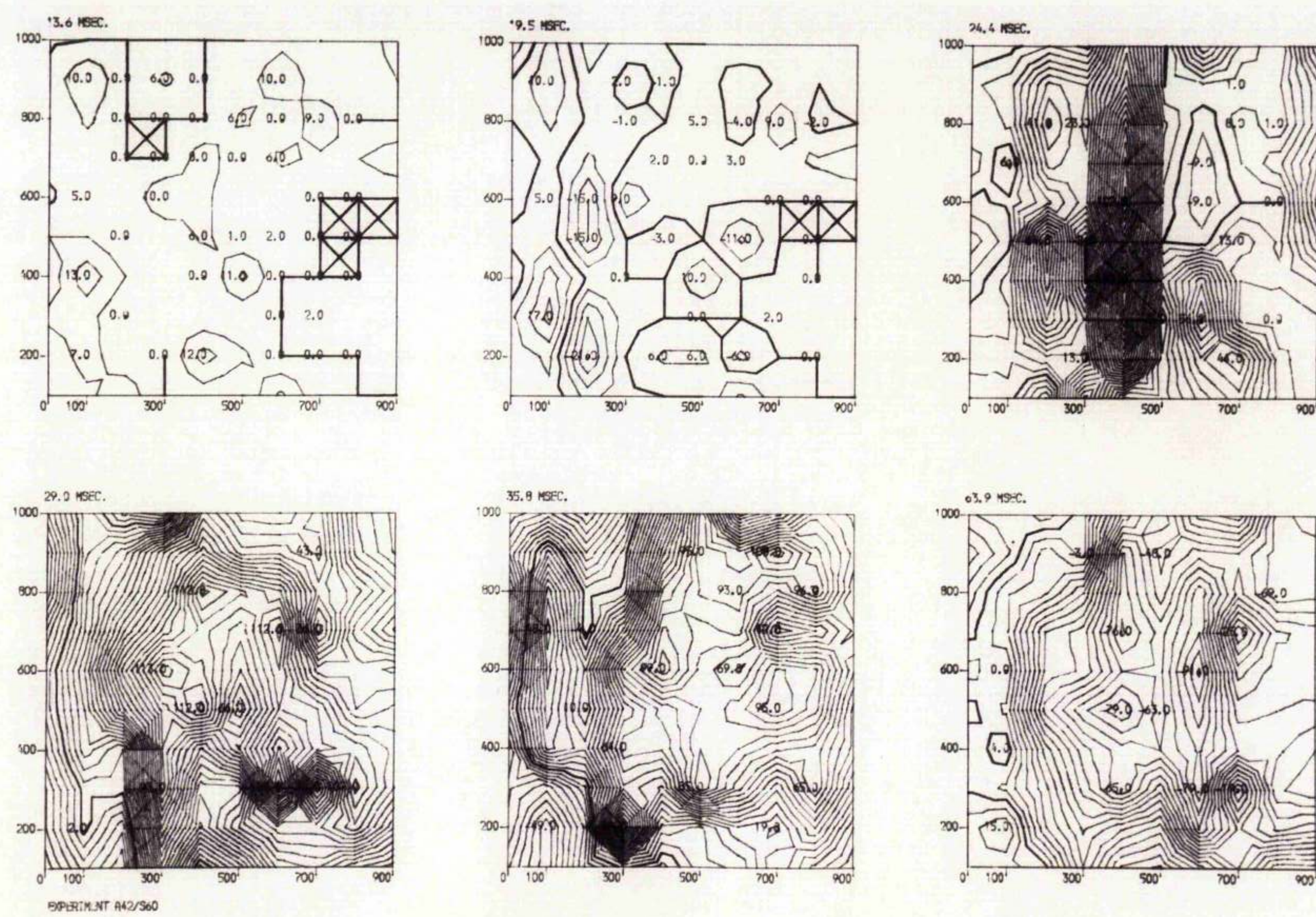
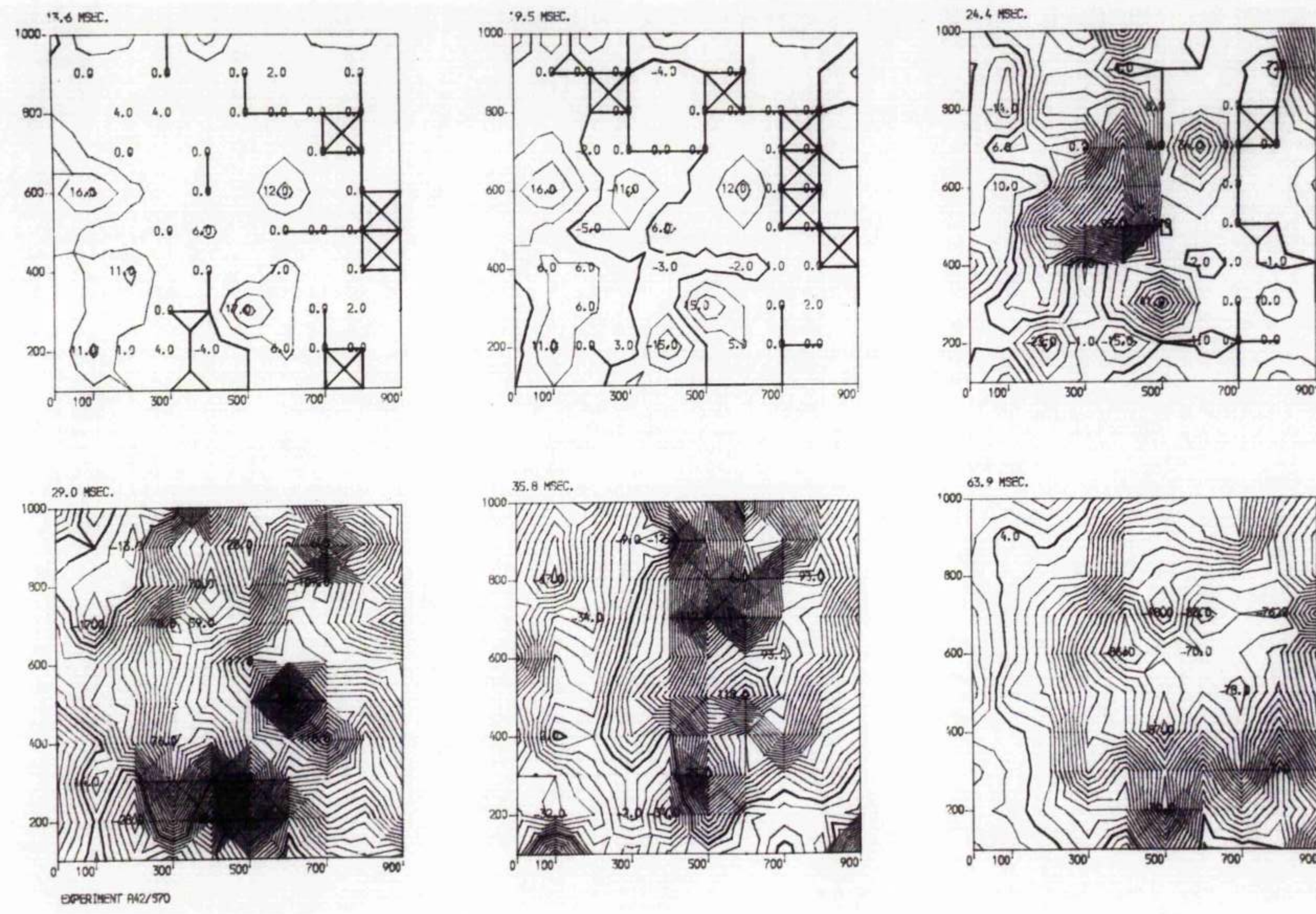
35.8 MSEC.
63.9 MSEC.
EXPERIMENT A42/S5D


24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
1000
800
600
400
200
0'
100'
300'
500'
700'
900'
EXPERIMENT A42/S60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29.0 MSEC.
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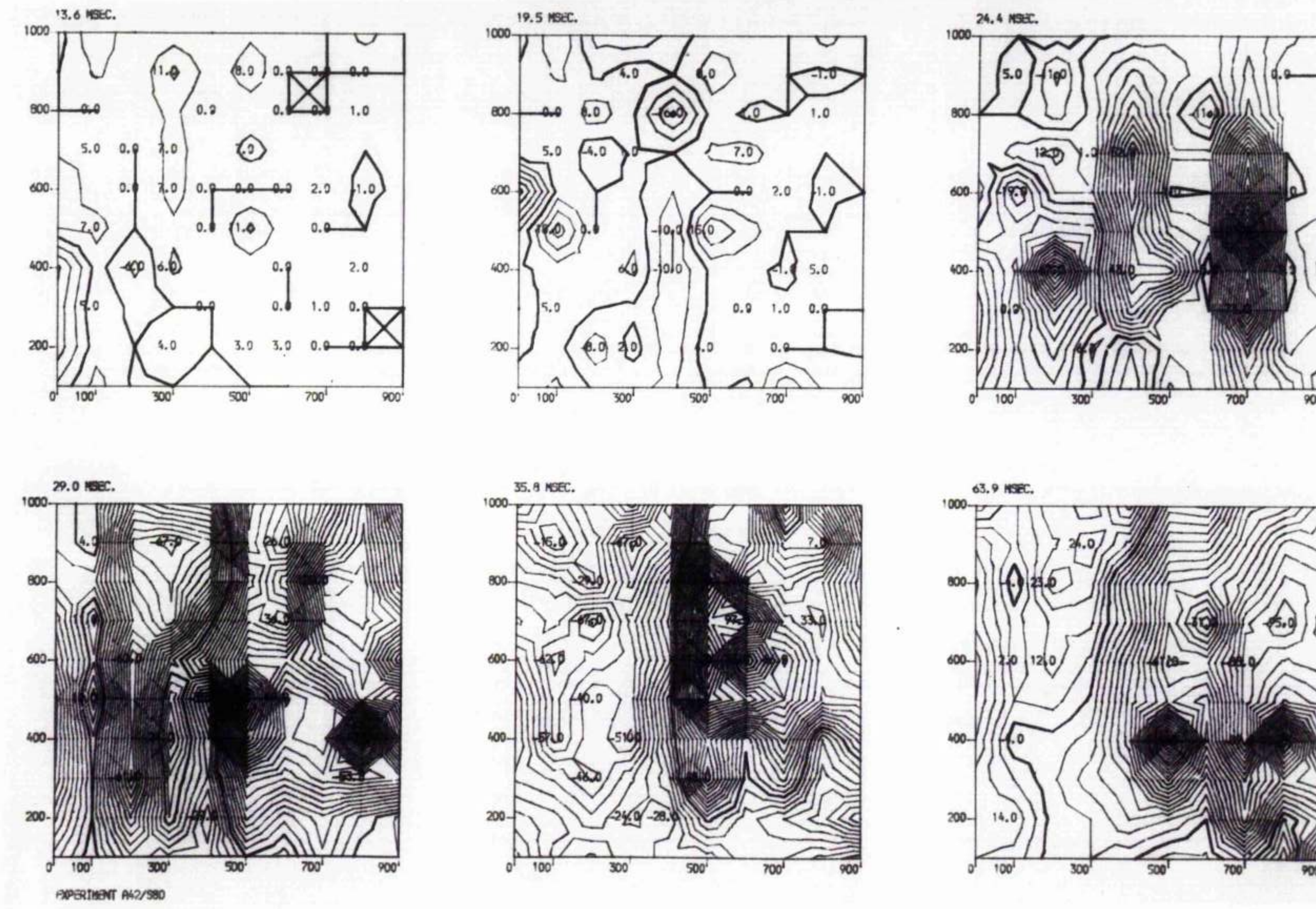
63.9 MSEC.
EXPERIMENT A42/970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35.8 MSEC.
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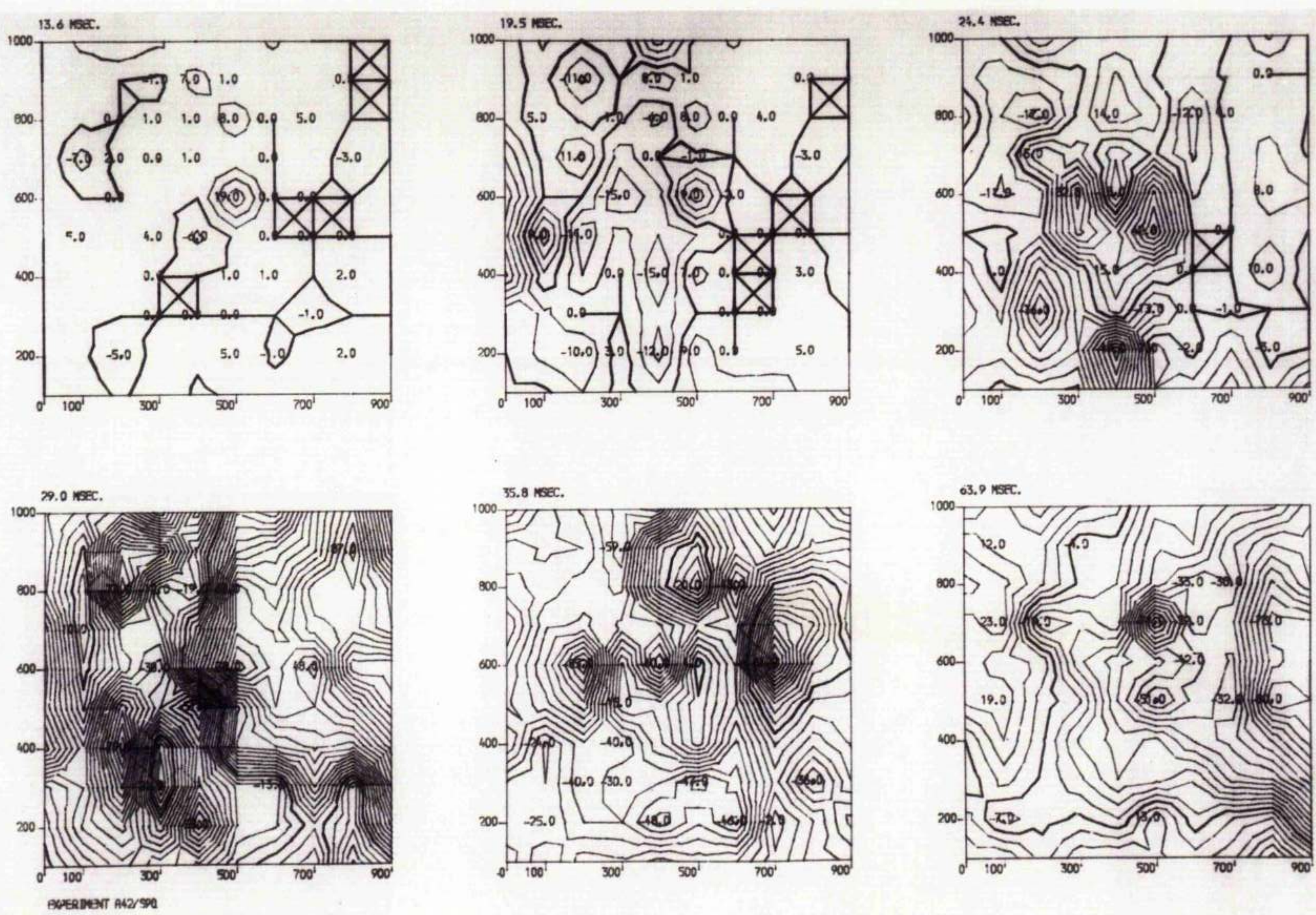
EXPERIMENT A42/S80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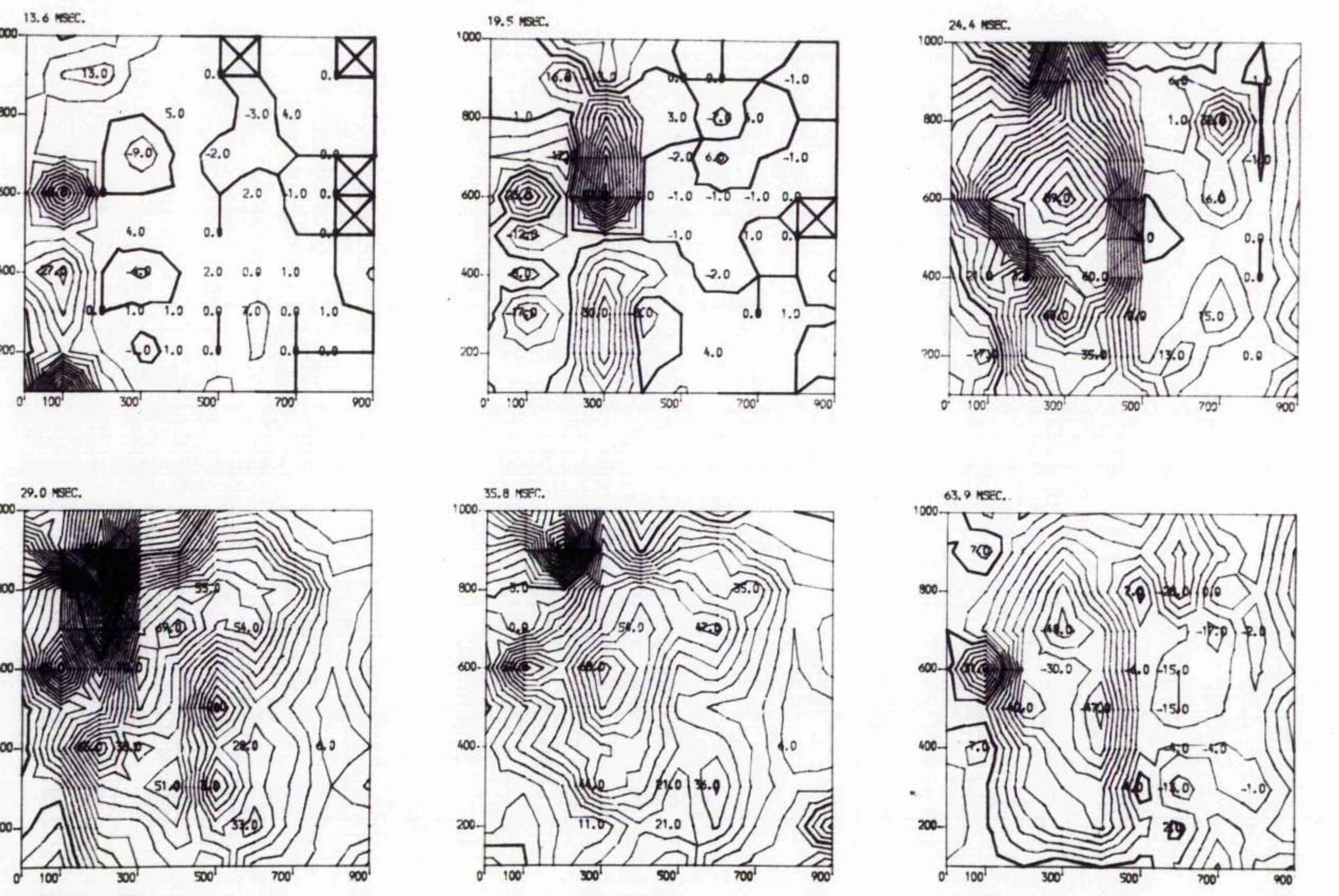
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT A42/SPQ


13.6 MSEC.
19.5 MSEC.
24.4 MSEC.
29.0 MSEC.
35.8 MSEC.
63.9 MSEC.
EXPERIMENT R42/S100

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