MOLECULAR STUDIES ON THE GABA-BENZODIAZEPINE RECEPTOR

by

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Abstract

The gamma-aminobutyric acid/benzodiazepine receptor (GABA/BZ) from mammalian brain has been investigated using biochemical and molecular biological techniques.

The receptor complex was solubilised in sodium deoxycholate and purified by benzodiazepine-sepharose affinity chromatography. Binding parameters of the soluble receptor complex were determined by radioligand binding assays ; identification of subunits involved in benzodiazepine binding by photoaffinity labelling and subunit composition by SDS polyacrylamide gel electrophoresis.

RNA was extracted from rat cerebral cortex and mRNA isolated by oligodT-cellulose chromatography. The suitability of the mRNA for translation was tested by formamide- and glyoxal-gel electrophoresis and translation activity assessed by rabbit reticulocyte lysate. The mRNA was then microinjected into Xenopus laevis oocytes. Functional receptor in the oocyte membrane was assayed electrophysiologically in a collaborative study. Biochemically, the presence of the receptor complex was detected by benzodiazepine-sepharose affinity chromatography, photoaffinity labelling and direct binding studies.

The results and their implications are discussed with reference to current work at the molecular level on this and other neurotransmitter receptors.

To Dorothy and George Bilbe

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ABBREVIATIONS

The abbreviations appear below and are also defined on their first entry in the text.

AMPS	ammonium persulphate
Bmax	maximal binding capacity
BSA	bovine serum albumin
BZ	benzodiazepine
CHAPS	3-[(3cholamidopropyl)-dimethylammonio]propane
CNS	central nervous system
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPC	diethyl pyrocarbonate
DHP	dihydropicrotoxinin
E.coli	Escherichia coli
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycoltetraacetic acid
FLU	flunitrazepam
GABA	gamma-aminobutyric acid
HEPES	hydroxyethylpiperazineethane sulphonic acid
Kd	dissociation constant
KOH	potassium hydroxide
KCl	potassium chloride
M.W.	molecular weight
MUS	muscimol
mRNA	messenger ribonucleic acid
NaOH	sodium hydroxide
NaH_2PO_4	sodium dihydrogen phosphate
Na_2HPO_4	disodium hydrogen phosphate
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PMSF	phenylmethylsulphonyl flouride
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
TBPS	t-butylbicyclophosphorothionate
TCA	trichloroacetic acid
TEMED	N'N'N'N'tetramethylethylenediamine
THIP	4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

CHAPTER 1

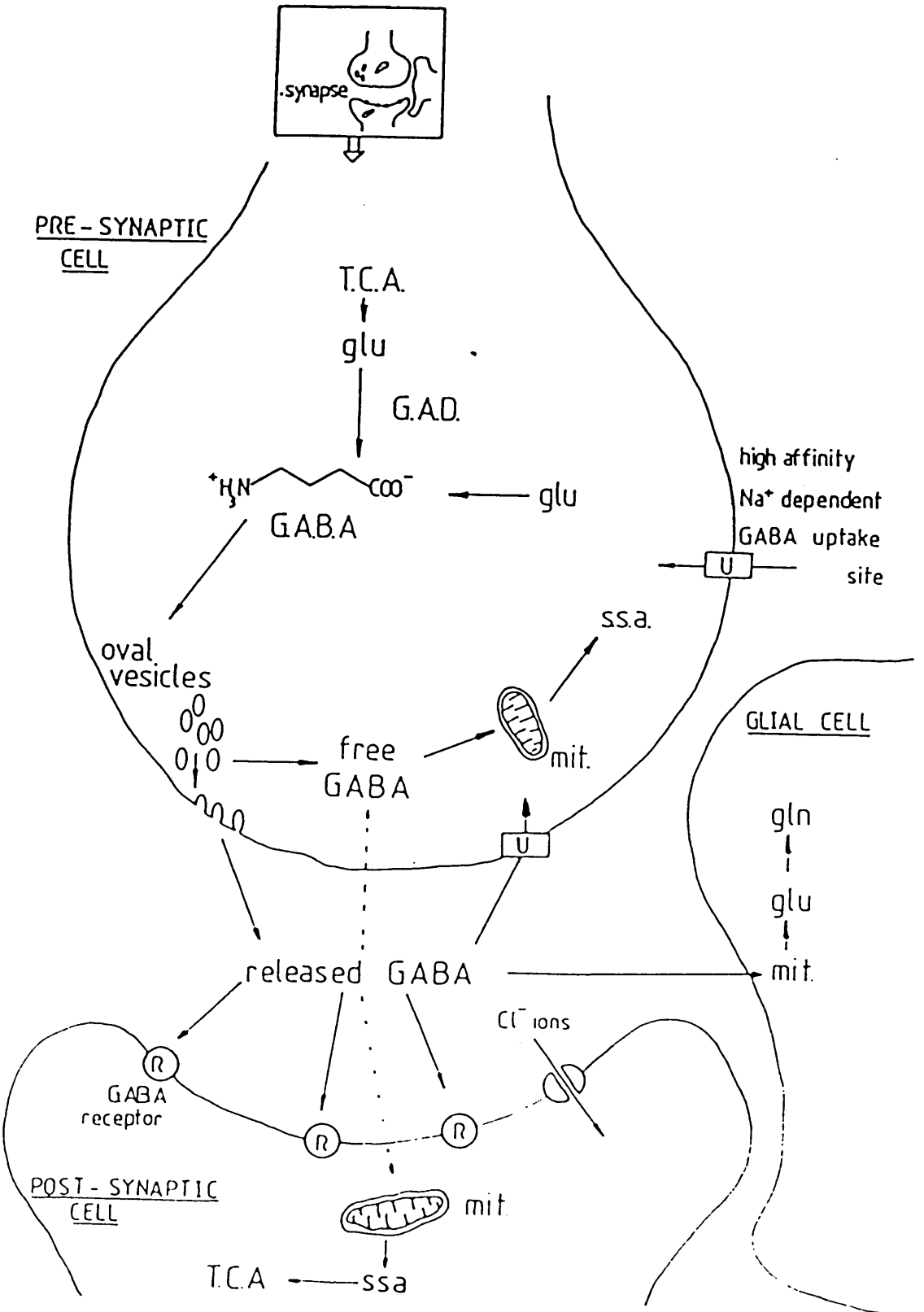
1.1.1. General Introduction

Within the mammalian central nervous system (CNS), neurones communicate with each other via specialised junctions (synapses) where the action potential carried along the pre-synaptic cell is transduced into a chemical transmitter (neurotransmitter). The neurotransmitter diffuses across the synaptic cleft and interacts specifically with a protein receptor in the post-synaptic cell membrane. This interaction results in an increase in permeability to ions which may either depolarise or hyperpolarise the membrane. Sufficient depolarisation will generate an action potential in the post-synaptic cell.

Excitatory and inhibitory synapses exist in the CNS. Catecholamines and amino acids such as glutamate and aspartate act as excitatory neurotransmitters propagating action potentials in the post-synaptic cell whereas gamma-aminobutyric acid (GABA) and glycine function at inhibitory synapses suppressing the production of action potentials (see Figure 1).

FIGURE 1

A GABA SYNAPSE



abbreviations

mit. - mitochondria, TCA - tricarboxylic acid cycle, ssa - succinic semialdehyde, GAD - glutamic acid decarboxylase; gln - glutamine, glu - glutamate

GABA was isolated from mouse brain extracts (Roberts and Frankel, 1950) and shown to have a neurotransmitter action by inhibition of crayfish stretch receptor neurone discharge (Florey, 1954). GABA is now known to be the major inhibitory neurotransmitter in mammalian CNS functioning at 30-40% of synaptic connections (Fonnum and Storm-Mathisen, 1978;).

1.1.2. GABA binding sites

A major breakthrough in understanding the mechanism of neurotransmitter action was the development of receptor-ligand binding assays. Radioactively labelled ligands have been used to study binding interactions between a receptor and its agonists and antagonists and the molecular events involved in receptor-drug interaction. Thus [^{3}H]-labelled GABA has been used to characterise the GABA receptor binding site and the properties of this receptor within the neuronal membrane.

Early attempts to identify GABA binding components in mammalian brain tissue used [^{14}C]-labelled GABA to characterise a sodium dependent site in membranes (Sano and Roberts, 1961). The introduction of a tritium labelled GABA ligand with a greater specific activity facilitated an investigation by Peck and coworkers (1973) who reported a binding site for [^{3}H]-GABA in rat cerebellar membranes. The binding of [^{3}H]-GABA was present in the absence of sodium ions and could be competitively inhibited by bicuculline, an alkaloid with an antagonistic action at this site. Sodium-free buffers are now used routinely in GABA-binding assays in order to minimise GABA association with uptake sites which are sodium dependent.

A single population of [^{3}H]-GABA binding sites was described in early reports (Zukin *et al.*, 1974; Enna and Snyder, 1975; see Table 1) with the dissociation constant *Kd*, ranging from 100-400nM and a maximum number of binding sites, Bmax, up to 5 pmol/mg protein. Further studies have improved binding by using brain membrane preparations which have been either frozen-thawed and

TABLE 1

[^{3}H]-GABA Binding to brain preparations

Effects of variations in treatment

Source	Treatment	Kd (nM)	Bmax (pmol/mg Protein)	Reference
Rat Cerebellar Cortex	None	21000	9	Peck et al., (1973)
Rat Whole Brain	None	100	ND	Zukin et al., (1974)
Rat Whole Brain	None	370	0.7	Enna and Snyder (1975)
Rat Whole Brain	Triton X-100 washed	16 130	0.6 5	Enna and Snyder (1977)
Rat and Bovine Brain	None	170	5	Lester and Peck (1977)
Rat Brain	Buffer washed	100	ND	Greenlee et al., (1978)
Rat Cerebral Cortex	Frozen and thawed Triton X-100 washed	20 111	1.8 5.3	Toffano et al., (1978)
Rat Cerebellum	Triton X-100 washed	4.5 30	1 4	Horn and Wong (1978)
Human brain Cortex	Buffer washed	18 180	0.3 1.5	Olsen et al., (1979)
Bovine Brain Cortex	Buffer washed frozen and thawed	13 300	0.33 1.8	Olsen et al., (1980)
Rat Brain Cortex	Buffer washed frozen and thawed Triton X-100 washed	10 170		Napias et al., (1980)
Rat Synaptosomal Membranes	Frozen and thawed	90 1600	2.7 7.7	Willow et al., (1981)
Rat Brain Cortex	Buffer washed	8.3 154	0.44 4.9	Burch et al., (1982)

ND = not determined

washed extensively in assay buffer to minimise vesicular structures and remove endogenous GABA (Greenlee et al., 1978; Napias et al., 1980) or treated with low levels of detergent (Enna and Snyder, 1977; Horng and Wong, 1978; See Table 1). These treatments have revealed two apparent populations of binding site; a high affinity site (Kd 4-90nM, Bmax 0.6-2.7 pmol/mg protein) and a low affinity site (Kd 130-1600nM) with a high binding capacity (Bmax up to 5 pmol/mg protein); these sites have been consistently observed in all brain regions, subcellular fractions and ages of mammalian species examined (Olsen et al., 1981). The functional significance of each site is unclear; the proportion of the two sites varying with brain region (Van Ness and Olsen, 1979) and in response to pH and temperature (Olsen, 1980).

The manipulations described previously have led to speculation that endogenous inhibitors may mask the high affinity site (Toffano et al., 1978/1980; Costa et al., 1978). One group has shown that Triton X-100 treatment removes a 15,000 molecular weight protein, GABA-modulin, concom itant with revealing the high-affinity GABA binding site,

although other groups have failed to repeat these results (Guidotti et al., 1978).

The GABA binding site can also be characterised using ligands such as muscimol, isoguvacine, piperidine-4-sulphonic acid and 4,5,6,7-tetrahydroisoxazolo [5,4-c] pyridin-3-ol (THIP - see Table 2). All these agonists show binding comparable to GABA (Kd values of 10-1000nM) and labelling of two sites in suitably prepared membranes.

[^{3}H]-bicuculline methiodide has been used to study GABA binding (Table 2, Mohler and Okada, 1977). These results demonstrated that [^{3}H]-bicuculline binding was unaffected by freeze-thaw and wash procedures and had a different regional distribution which mimicked in part [^{3}H]-GABA binding regions. These non-[^{3}H]-bicuculline labelled [^{3}H]-GABA sites have now been shown to be part of the $GABA_B$ receptor subtype which can be specifically labelled by [^{3}H]-baclofen (a hydrophobic GABA analogue) in a calcium ion dependent, bicuculline insensitive manner (see Table 2 and Figure 2; Hill and Bowery, 1981; Bowery et al., 1983).

TABLE 2

Binding of [^{3}H] labelled GABA agonists and antagonists to brain membrane preparations

Source	Ligand	Treatment	Kd (nM)	Bmax (pmol/mg Protein)	Reference
Rat Cerebellum	[^{3}H] Bicu-culline	None	380	4.5	Mohler and Okada (1977)
Rat Whole Brain	[^{3}H] Muscimol	Triton X-100 washed	2.7 2.2	3.3	Snodgrass (1978)
Rat Synaptic Membranes	[^{3}H] Muscimol	Frozen and thawed buffer washed	2.2 60	0.7 2.8	Beaumont et al., (1978)
Rat Whole Brain	[^{3}H] Muscimol	Buffer washed frozen and thawed	3 21	1.1	Greenlee and Olsen (1979)
Mouse Fore-brain Synaptic Membranes	[^{3}H]-IGV	Frozen and thawed buffer	10 90	0.33 0.7	Morin and Wasterlain (1980)
Cerebellar Membranes	[^{3}H]-IGV	Frozen and thawed	5.6 62.5	0.44 1	
Bovine Brain	[^{3}H]-P4S	Frozen and thawed washed	17 237	0.15 0.8	Krogsgaard-Larsen et al., (1981)
Whole Rat Brain	[^{3}H]-THIP	Sonicated frozen - washed	3 65 5000	0.029 0.44 6.4	Falch and Krogsgaard-Larsen (1982)
	[^{3}H]-GABA		4 193 5600	0.22 1.79 8.4	
	[^{3}H]-P4S		7 67 1800	0.56 0.97 3.9	
Rat Synaptic Membranes	[^{3}H]-GABA [^{3}H] Baclofen	Buffer Ca^{2+} addition	77 81.7 5300	1.22 0.68 6.9	Bowery et al., (1983)

Abbreviations: IGV = isoguvacine
P4S = piperidine-4-suphonate
THIP = 4,5,6,7-tetrahydroisoxazolo[5,4-c]pyridin-3-ol

It is now known that the GABA receptor exists as a membrane protein complex containing in addition to the GABA recognition site, a chloride ion channel and modulatory sites for two categories of drugs, the benzodiazepines (BZ) and the picrotoxinin/barbiturate drugs (Tallman et al.,1981; Olsen 1981). These two drug binding sites and their interaction with the GABA recognition site will now be discussed in relation to the receptor-ion channel complex.

1.1.3. Benzodiazepine binding sites

The benzodiazepines are a range of drugs based on diazepam (valium) and have been widely used in clinical practice as muscle relaxants, anti-convulsants, anxiolytics and tranquillizers. [^{3}H]-diazepam binds in a stereospecific, saturable manner to high affinity sites (Kd 3.6nM, Bmax Ø.3pmol/mg protein) in mammalian brain membranes (Squires and Braestrup, 1977; Mohler and Okada, 1977; see table 3) with the greatest binding found in the rat frontal and occipital cortex and intermediate levels in the hippocampus. A high affinity [^{3}H]-diazepam binding site has

TABLE 3

Benzodiazepine binding to mammalian brain preparations

Effects of GABA and barbiturates on binding properties

Source	Ligand	Kd (nM)	Bmax (pmol/mg Protein)	Compound and effect	Reference
Rat Cerebral Cortex	[^{3}H]-diazepam	3.6	0.3	ND	Mohler and Okada (1977)
Rat Fore-brain	[^{3}H]-diazepam	2.6	0.018	ND	Squires and Braestrup (1977)
Rat Brain	[^{3}H]-diazepam	12.2	0.88	10uM GABA increased Kd to 2.4nM	Martin and Candy (1978)
Rat Brain Synapto-somes	[^{3}H]-diazepam [^{3}H]-flunitraz-epam	14.8	0.36 ?	ND 0.37	Bosmann et al., (1978)
Rat Brain Cortex	[^{3}H]-diazepam	3.3	1.22	10uM musci-mol increased Kd to 2.6nM	Briley and Langer (1978)
Rat Cortex	[^{3}H]-diazepam	4.1	0.05	10uM GABA increased Kd to 2.3nM	Tallman et al., (1978)
Rat Brain	[^{3}H]-diazepam [^{3}H]-flunitraz-epam	4	0.05 1	ND 0.07	Squires et al., (1979)
Rat Cerebral Cortex	[^{3}H]-flunitraz-epam	1	1.8	10uM GABA increased Kd	Ehlert et al., (1981)
Rat Cerebral Cortex	[^{3}H]-diazepam	2.23	0.99	500uM pento barbital -> increased Kd to 0.86nM blocked by 10uM picro-toxinin	Leeb-Lundberg et al., (1980) (1981)

TABLE 3 CONTINUED

Source	Ligand	Kd (nM)	Bmax (pmol/mg Protein)	Compound and effect	Reference
Rat Cerebral Cortex	[^{3}H]-diazepam	2.33	0.99	a. 10uM GABA increased Kd to 0.94nM b. 500uM pento barbital increased Kd to 0.86nM c. 10uM etazolate increased Kd to 1.2nM	Leeb-Lundberg et al., (1981)
Rat Brain	[^{3}H]-diazepam	9	1.2	a. 10uM muscimol increased Kd b. 100uM pento barbital increased Kd to 4nM	Ticku (1981)
Rat Cortex	[^{3}H]-diazepam	9.22	1.8	a. 10uM muscimol increased Kd to 2.78nM b. 500uM pento barbital increased Kd to 5.2nM	Burch et al., (1982)
Rat Hippocampus	[^{3}H]-flunitrazepam	1.73	1.2	100uM GABA increased Kd to 0.72nM	Stapleton et al.,(1982)

ND = not determined

been described in kidney, liver, lung and several tissue culture cell lines, although the pharmacological profile of these sites differs considerably from the pharmacological profile in neuronal tissue (Braestrup and Squires, 1977)

Most active benzodiazepines bind to the brain high affinity site with an apparent single affinity, i.e. linear Scatchard plots and Hill coefficients (n_H) close to unity. However, the anxiolytic triazolopyridazine agents e.g. CL218,872 (3-methyl-6-[3-(triflouromethyl)-phenyl]-1,2,4, triazolo -4,3, pyridazine) competitively inhibited [^{3}H]-flunitrazepam binding in a manner characterised by shallow competition curves and n_H in the range Ø.5-Ø.7 (Squires et al., 1979; see Table 4). Similarly, alkyl-B-carbolines e.g. [^{3}H]-propyl-B-carboline-3-carboxylate, labelled a high affinity subpopulation of BZ receptor sites in cerebellum with a Bmax similar to the Bmax for [^{3}H]-flunitrazepam, but had a significantly lower Bmax than [^{3}H]-flunitrazepam in other areas such as forebrain (see Table 4, Braestrup et al., 1980; Nielsen and Braestrup, 1980; Ehlert et

Figure 2. THE CENTRAL ROLE OF $GABA_A$ RECEPTORS

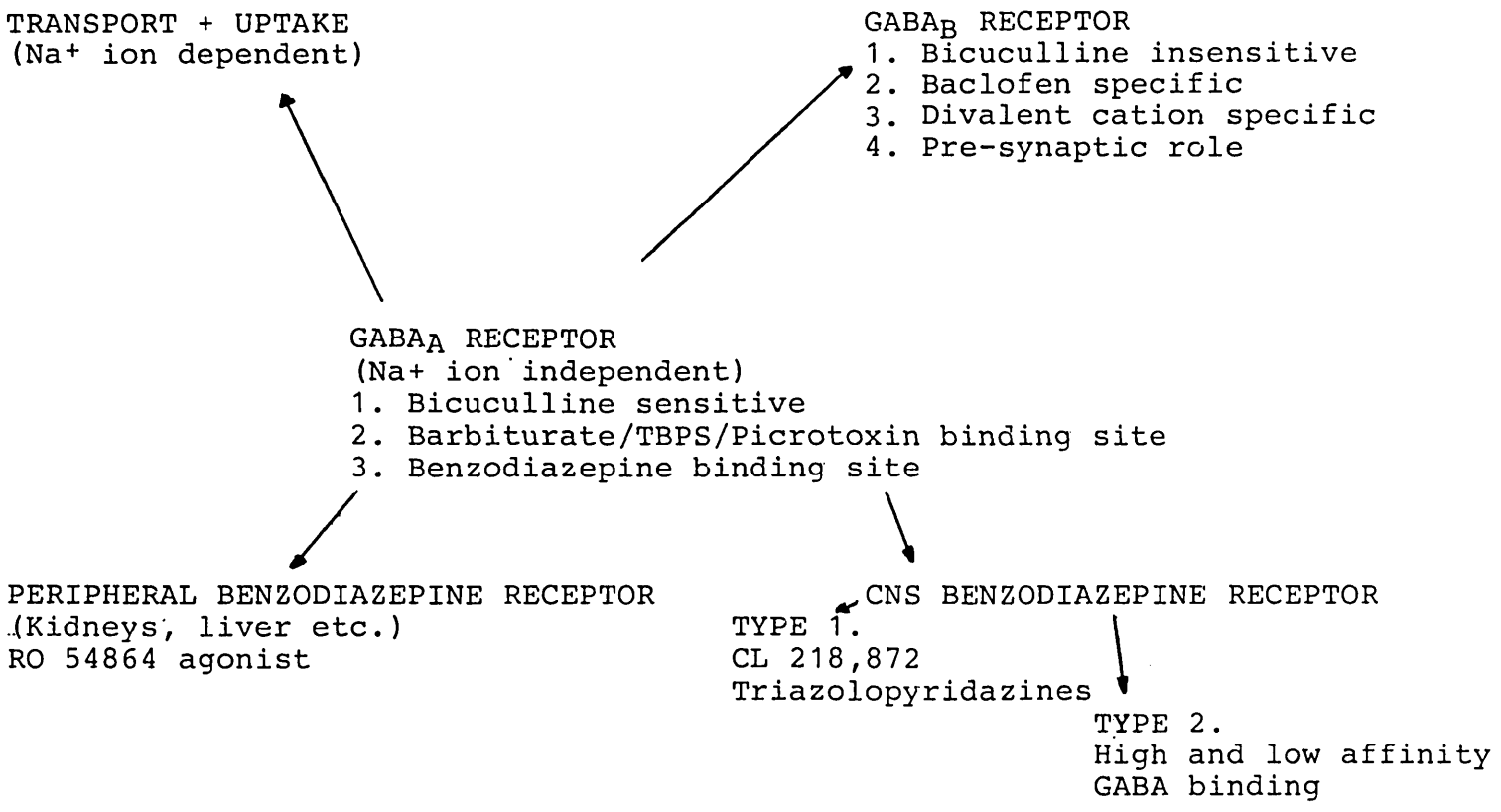


TABLE 4

Binding of B-Carbolines, triazolopyridazine and CL218,872 to rat brain

Effect of GABA, muscimol and pentobarbital

Source	Ligand	Kd (nM)	Bmax (pmol/mg Protein)	Effect	Reference
Forebrain	[^{3}H]-phenyl-B carboline-3-carboxylate	0.15-0.5	0.08	a. 100uM GABA 10uM muscimol	Nielsen et al., (1981)
Hippocampus	[^{3}H]-propyl-B-carboline-3-carboxylate	0.8	0.045	30% increase in binding	
Cerebellum	[^{3}H]-propyl-B-carboline-3-carboxylate	1.5 3	0.065	b. inhibited by 100uM bicuculline	
Cortex	[^{3}H]-CL218,872 [^{3}H]-TZP	26.5 611	ND ND		Yamamura et al., (1982)
Hippocampus	[^{3}H]-propyl-B carboline-3-carboxylate	0.8	0.7	100uM GABA increased Kd to 0.78nM	Stapleton et al., (1982)

al., 1983). Further evidence for heterogeneity has emerged from heat inactivation studies of BZ binding activity, in which two distinct subpopulations of the BZ receptor can be seen (Squires et al., 1979).

Flunitrazepam and clonazepam can be used as photoaffinity labels to identify the BZ receptor site (Mohler et al., 1980). Sieghart and Karobath (1980) demonstrated that [^{3}H]-flunitrazepam photoaffinity labelled a single 57,000 molecular weight polypeptide from cerebellar membranes whereas in hippocampus and striatum up to four additional polypeptides (53000-59000) were also labelled. From the kinetic, thermal inactivation and photoaffinity labelling data, benzodiazepine type I(BZ1) and type II(BZ2) receptor subtypes can be distinguished. Type I sites display a 10 fold higher affinity for B-carbolines and CL218,872 than benzodiazepines such as diazepam which have similar affinities for both sites.

1.1.4. Interactions between GABA and BZ binding sites

Evidence for a physical coupling between GABA and BZ binding sites was initially provided by experiments in which GABA protected BZ receptor binding against thermal inactivation (Squires et al., 1979). Subsequently many groups have reported that GABA and GABA receptor agonists enhance BZ binding by a reversible increase in affinity of the BZ binding site (see Table 3), an effect antagonised by bicuculline thus demonstrating this action was mediated by the $GABA_A$ receptor. In all brain regions GABA and GABA agonists enhanced [^{3}H]-flunitrazepam photoaffinity labelling of polypeptides, an action blocked by cold diazepam and consistent with a GABA/BZ receptor complex (Wastek et al., Karobath and Sperk 1979; Sieghart and Karobath 1980). Radiation inactivation is a technique used to detect coupling between membrane proteins (Kempner and Schlegel, 1979). Chang et al., (1981) used this technique to demonstrate that BZ and GABA binding sites (estimated with [^{3}H]-flunitrazepam and [^{3}H]-muscimol respectively), reside on the same membrane complex of apparent molecular weight 217000.

Guidotti et al., (1978) observed a potentiation of GABA binding affinity by pharmacologically active benzodiazepines, an effect not reported by other groups (Enna and Maggi, 1979; Olsen, 1981). However, Gavish and Snyder (1980) demonstrated diazepam was able to protect the GABA binding site from thermal inactivation or modification by iodoacetamide indicating benzodiazepines may interact with the GABA binding site in a manner which has yet to be elucidated.

The halides, thiocyanate and nitrides enhanced BZ base line binding in the presence or absence of GABA and GABA agonists, an observation which represents the coupling of the BZ receptor to the chloride ion channel (Martin and Candy, 1978; Mackerer and Kochman, 1978; Costa et al., 1979). This evidence thus proposed that the GABA and BZ binding sites reside together on a receptor- chloride ion channel complex.

1.1.5. Picrotoxinin/Barbiturate binding sites

Picrotoxinin, a low molecular weight convulsant of plant origin, has been used in electrophysiological experiments to non-competitively block GABA mediated synaptic events (Takeuchi and Takeuchi, 1969). Tritium - labelled dihydropicrotoxinin ([^{3}H]-DHP;Ticku et al., 1978) binds specifically to a site interrelated with the chloride ion channel (Ticku and Olsen, 1978; see Table 5), the binding being unaffected by GABA or GABA agonists. However this ligand is not widely used because of a relatively low affinity (Kd 1-2 uM) and high non-specific binding to fresh rat brain membranes. A new ligand, [^{35}S]t-butylbicyclophosphorothionate ([^{35}S]-TBPS;Squires et al., 1983) has been shown to bind competitively, with high affinity (Kd 17nM, Table 5) and exhibit similar but not identical properties to [^{3}H]-DHP for a chloride ion channel related binding site. The barbiturate and pyrazolopyridine anxiolytic drugs such as etazolate actively displace [^{35}S]-TBPS binding (Squires et al., 1983), an observation consistent with one report of low affinity [^{3}H]-phenobarbitone binding to synaptosomal

TABLE 5

Binding [^{3}H]-phenobarbitone, [^{3}H]-DHP and [^{35}S]-TBPS to rat brain membranes

Source	Treatment	Ligand	Kd (nM)	Bmax (pmol/mg Protein)	Reference
Brain Membranes	None	[^{3}H]-DHP	2000	5	Ticku and Olsen (1978)
Cerebral Cortex	None	[^{3}H]-DHP	2000	5	Ticku et al., (1978)
Cerebral Cortex	None	[^{3}H]-DHP	1000 - 2000	5-10	Leeb-Lundberg and Oben (1980)
Brain Synaptosomal Membranes	Frozen and thawed	[^{3}H]-phenobarbitone	100	0.8	Willow et al., (1981)
Brain Membranes	EDTA/water dialyzed	[35S]-TBPS	17	0.051	Squires et al., (1982)
Brain Membranes	EDTA/water dialyzed	[35S]-TBPS	25	1.4	Ramanjaneyvilu and Ticku (1984)

Abbreviations: DHP = dihydropicrotoxinin
TBPS = t-butylbicyclophosphorothionate

membranes (Kd 100nM; see Table 5; Willow et al., 1981). Both [^{3}H]-DHP and [^{35}S]-TBPS binding are inhibited by biologically active picrotoxin analogues, barbiturates and pyrazolopyridine drugs (Olsen et al., 1984), thus providing evidence for the existence of a specific picrotoxinin/barbiturate recognition site associated with the chloride ion channel.

1.1.6. Barbiturate/Picrotoxinin-GABA-Benzodiazepine Receptor Interactions

Benzodiazepine-barbiturate/picrotoxinin binding site interactions are well documented (see Table 3). Pentobarbital and etazolate significantly increase the affinity of [^{3}H]-diazepam, an effect dependent on certain anions (e.g. Cl^-,I^-,Br^-,ClO_4^-,SCN^-) and inhibited competitively by 1uM picrotoxinin and indirectly by bicuculline (see table 3; Leeb-Lundberg et al., 1980, 1981; Whittle and Turner, 1982). Picrotoxinin also allosterically inhibits BZ binding at 37^{o}C (Supavilai et al., 1982) and modifies heat inactivation rates (Squires and Saederup, 1982).

and barbiturate/picrotoxinin interaction are to maintain the integrity of the receptor complex, but also remove endogenous GABA and maintain activity of high and low affinity sites (see table 6). Bovine and rat brain membranes treated in this way display enhanced GABA receptor binding (an increase in the maximal number of binding sites) in the presence of barbiturates, pyrazolopyridines and picrotoxinin. This effect shows the same chloride dependence, barbiturate specificity and picrotoxinin and bicuculline sensitivity as the interactions with BZ binding. The observation that reciprocal interactions between receptors for barbiturates, benzodiazepines and GABA are maintained following detergent solubilization provides clear evidence that the three binding site complex exists (Stephenson and Olsen, 1982; Davis and Ticku, 1981a, 1981b).

TABLE 6

The effect of barbiturates on ^{3}H-GABA binding to brain membranes

Source	Treatment	Ligand	Kd (nM)	Bmax (pmol/mg Protein)	Effect of Barbiturate	Reference
Bovine Cerebral Cortex	Assay buffer washed	[^{3}H]-GABA	98	0.91	200uM pentobarbitone and 100mM KCl increased Bmax to 2.61	Asano and Ogasawara, (1981)
Rat and Bovine Brain	Frozed and thawed assay washed and dialysed	[^{3}H]-GABA	200	0.3	1. 200uM pentobarbitone 50mM KCl increased Bmax rat --> 0.5 bovine --> 2.2 2. Blocked by 10uM picrotoxinin	Olsen et al., (1981)
Rat Cerebral Cortex	Assay buffer washed and dialysed	[^{3}H]-GABA	15 150	- 0.79	1. 100uM pentobarbitone and 100uM KCl increased Bmax to 0.9 2. Abolished by 10uM picrotoxinin	Whittle and Turner, (1982)
Rat Brain Synaptosomal Membranes	Assay buffer washed	[^{3}H]-GABA	90 1600	2.7 7.7	100uM pentobarbitone increased Kd to 55nM	Willow and Johnstone, (1980)

1.1.7. Distribution and Ontogeny of the GABA/BZ Receptor Complex

The anatomical location of GABA binding sites is consistent with known GABA function. Thus, the highest [^{3}H]-GABA binding (up to 5 pmol/mg protein) is found in cerebellum, intermediate levels in frontal cortex, olfactory bulb, striatum and hippocampus and lowest (less than Ø.5 pmol/mg protein) in medulla and spinal cord (Enna et al., 1977; Placheta and Karobath, 1979). The distribution of picrotoxinin and BZ binding sites is similar to that for GABA receptors with intermediate levels in cerebellum and striatum and lowest levels in medulla and spinal cord (Figure 3).

The developmental time course for [^{3}H]-GABA binding in the rat brain parallels that for benzodiazepines, 1Ø-3Ø% being present at birth progressing to adult levels during post-natal weeks 1-4 (Coyle and Enna, 1976; Palacois et al., 1979; Mallorga et al., 198Ø). At all ages GABA receptor ligands enhanced BZ binding, an effect inhibited by bicuculline and manifested as a change in Kd (Braestrup and Nielsen, 1978; Candy and Martin, 1979;

FIGURE 3

RAT BRAIN

1) LATERAL VIEW

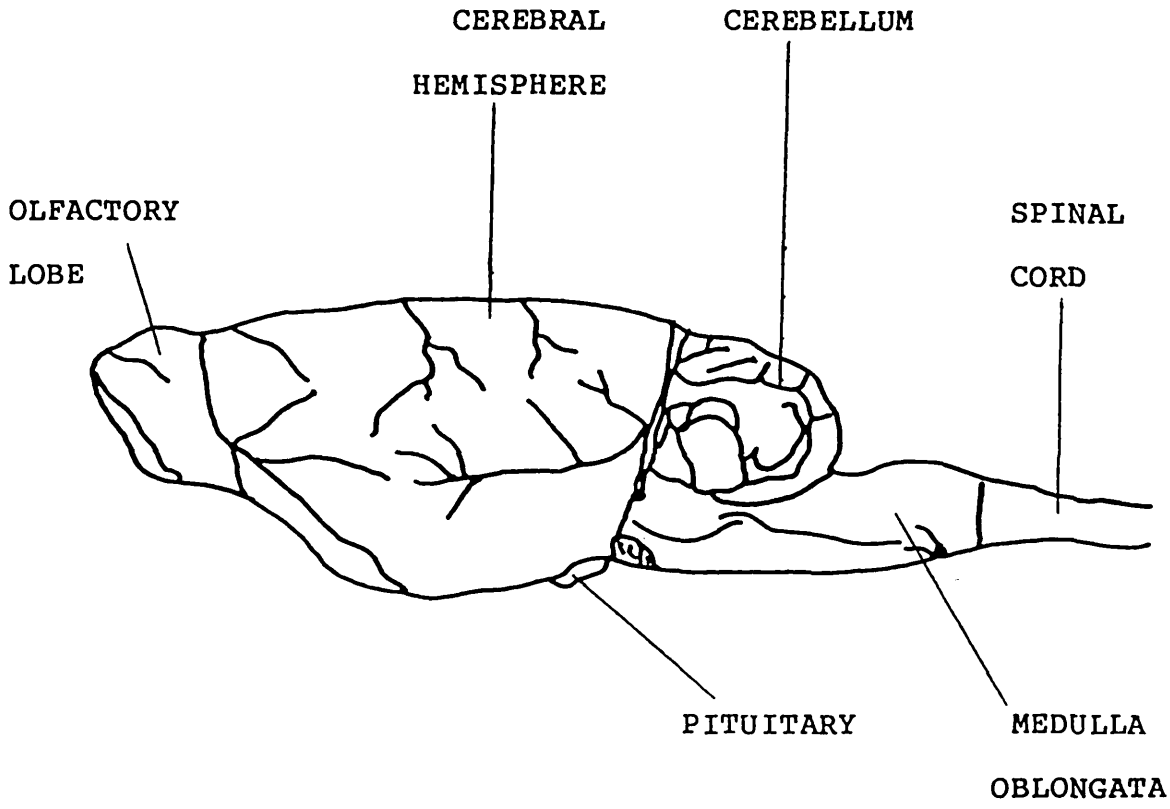
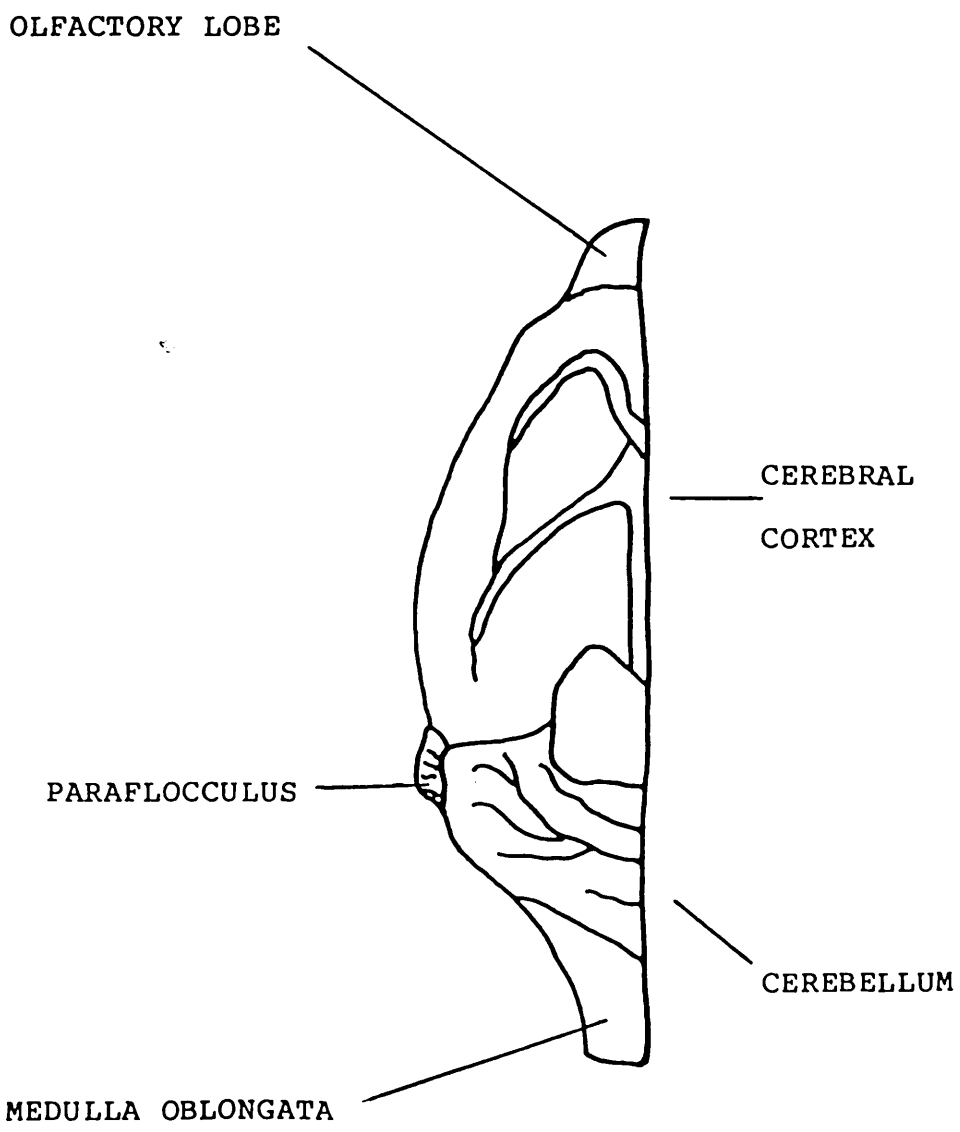


FIGURE 3 CONTINUED

2) DORSAL VIEW



Regan et al., 1979) with a greater degree of GABA enhancement in neonates than in the adult (Candy and Martin, 1979; Palacois et al., 1979). Thus, the GABA/BZ receptor complex develops early; uncoupled GABA receptors accounting for relatively more of the increased GABA binding in later stages of development.

1.1.8. Electrophysiological Studies on the GABA/BZ Receptor Complex

An electrophysiological characterisation of the GABA/BZ receptor complex differs from a biochemical characterisation for the following reasons: binding assays describe, in a rigorous quantitative manner, a recognition site that may or may not be associated with a particular biological response. An electrophysiological description of the GABA/BZ receptor complex assesses a system which when activated results in a specific biological response such as a change in conductance or membrane potential resulting from receptor-ligand interaction. To measure such a response the GABA/BZ receptor complex must remain intact.

The following section describes some of the electrophysiological properties of the GABA/BZ receptor. For a more detailed description see the following reviews; Johnston, 1978; Constanti and Nistri, 1979; Simmonds, 1983; Gallagher and Shinnick-Gallagher, 1983.

The response of a neurone to released GABA involves a rapid and transient increase in membrane permeability to chloride ions with a resultant hyperpolarisation (if the extracellular chloride concentration is above the intracellular concentration) (Johnston, 1978), a phenomenon which is non-competitively blocked by picrotoxinin (Takeuchi and Takeuchi, 1969). This effect correlates with binding data suggesting separate recognition sites for GABA and the toxin. Similarly, bicuculline is a competitive inhibitor presumably acting at the GABA binding site (Curtis et al., 1970).

Electrophysiologically, benzodiazepines have a dual role. Using cultured mammalian neurones MacDonald and Barker (1978) demonstrated that low doses of diazepam and chlordiazepoxide enhanced GABA

mediated inhibition, but at high doses depressed the action of GABA (in binding studies GABA is found to increase the amount of BZ binding). Similar studies also confirmed that the GABA binding site is distinct from the BZ recognition site (Simmonds, 1980; Study and Barker, 1981). Furthermore, Study and Barker (1981) proposed that benzodiazepines facilitate GABA-induced responses by increasing the frequency of ion channel opening events, whilst having a minimal effect on the average channel lifetime of the GABA receptor-chloride ionophore.

Barbiturates exert an electrophysiological effect by potentiating GABA-mediated inhibition; in binding studies barbiturates increase the affinity of the GABA receptor (Simmonds, 1980; Higashi and Nishi, 1982; Alger and Nicholl, 1982; for review see Willow and Johnston, 1983). Barbiturate action is competitive with picrotoxinin (suggesting action through the same site), and is proposed to decrease the frequency of channel opening events and increase the open-channel lifetime (Study and Barker, 1981).

Electrophysiologically therefore the GABA/BZ receptor-ionphore complex has been shown to consist of distinct GABA and BZ binding sites and a chloride channel which is associated with the barbiturate/picrotoxinin recognition site.

1.1.9. Solubilisation and Purification of the GABA/BZ receptor Complex

Solubilisation of the GABA/BZ receptor complex is necessary to allow a detailed study of those polypeptides involved in receptor function. Once purified to homogeneity, a determination of molecular weight, amino acid and carbohydrate composition and location of the binding site for GABA, benzodiazepines and barbiturates can be made in addition to the use of purifed protein in antibody production and reconstitution experiments.

Integral membrane proteins such as the nicotinic acetylcholine and GABA/BZ receptors reside in a lipid bilayer in which a substantial part of the protein is hydrophobically bonded to fatty acids/phospholipids or to non-polar portions

of neighbouring proteins; an interaction necessary for tertiary structure and activity. The hydrophobic interactions can be disrupted by detergents which substitute for the phospholipids in producing an artificial detergent-protein complex containing active and inactive receptor.

1.1.10. Solubilisation of GABA, BZ and Barbiturate Binding Sites

Detergents differ in the extent to which they solubilise a given protein-lipid complex, with stronger examples such as sodium dodecyl sulphate solubilising all membrane proteins, as well as partially denaturing the receptor complex. Early reports of solubilisation of GABA binding activity (Gavish et al., 1979; Chude, 1979; Greenlee and Olsen, 1979; see Table 7) from rat, mouse and bovine brain reported good yields (30-60%) using lysolecithin and deoxycholate, but did not report co-solubilisation of BZ binding activity. In the same year, solubilisation of a BZ binding site was reported (Yousufi et al., 1979; Lang et al., 1979; Gavish et al., 1979; see Table 7), but did not describe GABA enhancement. Yousufi et al., (1979) estimated

TABLE 7

Properties of the solubilised GABA/BZ receptor complex

Solubilisation conditions	Ligand	Kd (nM)	Bmax (pmol/mg protein)	Molecular Weight	Interactions	Reference
1. DIGITONIN 1%, rat brain	[^{3}H]-Flu	1.5	0.18	-	-	Gavish et al., (1979)
2. NONIDET P40 1%, rat brain		-	-	270,000 (gel filtration)	GABA/BZ (1982)	Ito and Kuriyama,
3. LYSOLECITHIN 0.25%, rat brain	[3H]-Muscimol	(i) 7 (ii)24	1 0.77	-	-	Gavish et al., (1979)
0.4%, mouse brain	[^{3}H]-Muscimol	(i) 3.5 (ii)52	2.8 14	-	-	Chude, (1979)
4. LUBROL PX 0.5%, rat brain	[^{3}H]-Diazepam	11	-	200-250,000 (gel filtration)	_ (1979)	Yousufi et al.,
1%, rat brain	[^{3}H]-DHP	(i) 38 (ii)1850	1.06 14.6	-	GABA/BZ/ Barbiturates	Davis and Ticku, (1981)
5. TRITON X-100 0.7-2%, rat cortex	[^{3}H]-Flu	12	1.8	12.85 (sucrose gradient)	-	Lang et al., (1979)
2%, bovine or rat cortex		-	-	9.45 (sucrose gradients) 220,000 (refined from (gel filtration) 220,000 (radiation inactivation)	GABA/BZ	Chang and Barnard, (1982) Lang et al., (1979)

TABLE 7 CONTINUED

Solubilisation conditions	Ligand	Kd (nM)	Bmax (pmol/mg protein)	Molecular Weight	Interactions	Reference
2%, calf brain	[^{3}H]-Muscimol	5	0.3	-	-	Lo et al., (1982)
	[^{3}H]-Flu	2	0.1			
6. CHAPS 20mM, bovine cortex	[3H]-Flu	8	1.4	550,000 (gel filtration in Triton X-100)	GABA/BZ - barbiturates	Stephenson and Olsen, (1982)
20mM, rat brain	[^{35}S]-TBPS	26	0.4	200,000 (gel filtra-	GABA/BZ - barbiturates	King and Olsen, (1984)
7. SODIUM DEOXYCHOLATE 2%, bovine brain	[^{3}H]-Muscimol	41	0.55	900,000 (gel filtration)	-	Greenlee and Olsen, (1979)
0.5%, calf brain	[^{3}H]-Flu	1.5	-	11S (sucrose gradient corresponding to 200-230,000)	GABA/BZ	Sherman-Gold and Dudai, (1980)
				51,000 subunit (photoaffinity labelled)		
0.2%, 1MKcl, rat brain	[^{3}H]-Muscimol	9.3	7.58	11.3S (sucrose gradient)	GABA/BZ	Asano and Ogasawara, (1981)
	[^{3}H]-Flu	3.1.	3.44	~200,000		
0.5%, bovine cortex	[^{3}H]-Muscimol	12	1.56	12.5S (sucrose gradient in both H_2O and D_2O)	GABA/BZ	Stephenson et al., (1982)

TABLE 7 CONTINUED

Solubilisation conditions	Ligand	Kd (nM)	Bmax (pmol/mg protein)	Molecular Weight	Interactions	Reference
	[^{3}H]-Flu	8	0.8	350,000 (gel filtration)		

A. Interactions refers to allosteric modulation by GABA or barbiturates of BZ binding.

Abbreviations:

[^{3}H]-Flu = [^{3}H]-Flunitrazepam
[^{3}H]-DHP = [^{3}H]-Dihydropicrotoxinin
[^{3}H]-TBPS = [^{3}H]-t-butylbicyclophosphorothionate

an apparent molecular weight for the BZ binding site of 200-500,000 by gel filtration in Lubrol PX. A similar figure was reported by Lang et al., (1979) when using sucrose density gradient centrifugation in Triton X-100. Similar Kd values were obtained by these two groups, Lang et al., (1979) noting that the Bmax figure could be increased if protease inhibitors were used, indicating the receptor complex was prone to proteolytic digestion and/or inactivation.

As with GABA binding to membrane preparations (mentioned previously), BZ binding activity could be enhanced if any endogenous GABA was removed before solubilisation, (Gavish and Snyder, 1980, Sherman-Gold and Dudai, 1980; Stephenson et al., 1982; Chang and Barnard, 1982), and a Triton X-100 solubilised BZ binding activity was enhanced up to 75% in the presence of 10uM GABA (Gavish and Snyder, 1980). Furthermore, the two binding activities were shown to comigrate on sucrose density gradients and gel filtration, indicating the GABA and BZ binding sites resided on the same protein complex (See Table 7, Asano and Ogasawara, 1981; Chang and Barnard, 1982, Stephenson et al., 1982).

Stephenson et al., (1982) reported differential solubilisation of BZ receptor sites in Triton X-100, but noted that 0.5% deoxycholate solubilised GABA and BZ binding sites. However, the deoxycholate extract was unstable and had to be assayed in Triton X-100 in which both activities were retained. The complex also showed chloride-dependent, picrotoxinin-sensitive, stereospecific barbiturate enhancement of GABA and BZ binding when first solubilised in the zwitterionic detergent, CHAPS, (3-[(3cholamidopropyl)-dimethylammonio]propane sulphonate), a characteristic of the membrane bound receptor (Stephenson and Olsen, 1982).

Two groups solubilised the picrotoxinin/barbiturate binding moiety; David and Ticku (1980) used Lubrol PX; Kind and Olsen (1984) used CHAPS detergent. In Lubrol PX, gel filtration separated the DHP binding from BZ binding, the former showing an apparent molecular weight of 185,000 (with enriched GABA binding) and the latter 61,000 (Davis and Ticku, 1981). However in CHAPS, flunitrazepam and TBPS binding co-migrated on gel filtration with an apparent molecular weight of 200,000 (King and Olsen, 1984; See Table 7).

There is now general agreement that these solubilisations are extracting the sodium-independent GABA binding site.Recent reports suggest CHAPS can solubilise all three binding activities, but the maximum recovery is only 74% of the activity solubilised by sodium deoxycholate (Stephenson and Barnard, in press). Crude detergent extracts have demonstrated that the GABA/BZ receptor complex is an acidic glycoprotein (Gavish and Snyder, 1981; Tallman et al., 1981; Chang and Barnard, 1982) and has a molecular weight in the range of 200-250,000 (Yousufi et al., 1979; Chang and Barnard, 1982; Stephenson et al., 1982) and in the presence of CHAPS or deoxycholate, GABA, BZ and barbiturate binding activities can be solubilised.

1.1.11. Purification of the GABA/BZ Receptor Complex

The GABA/BZ receptor complex has now been purified several hundred fold (Gavish and Snyder, 1981; Olsen et al., 1983), or thousand fold (Sigel et al., 1983) by benzadiazepine-sepharose affinity chromatography.

Early studies (Gavish and Snyder, 1981; Martini et al., 1982) on solubilisation of the receptor with Triton X-100 obtained [^{3}H]-muscimol and [^{3}H]-flunitrazepam binding activities which on subsequent photoaffinity labelling and SDS gel electrophoresis consisted of two 55,000 and 62,000 M.W. peptides (Gavish and Snyder, 1981). Following electrophoresis, Martini et al., (1982), obtained a 62,000 M.W. peptide by coomassie blue staining which corresponded to a similar M.W. peptide labelled in brain membrane preparations (Mohler et al., 1980).

Later work on purification has used the mild ionic detergent, sodium deoxycholate (Sigel et al., 1983; Schoch and Mohler, 1983; Olsen et al., 1983) for solubilisation, before elution from the affinity column and all subsequent operations in Triton X-100.

The purified receptor complex displays a single high affinity, bicuculline-sensitive, binding site for [^{3}H]-muscimol (Sigel et al., 1983) and a high affinity [^{3}H]-flunitrazepam binding site (Sigel et al., 1983; Olsen et al, 1984) exhibiting a binding ratio for GABA to BZ sites of 3.5 - 3.8 : 1; a value similar to

bovine brain membranes (Sigel et al., 1983). B-carbolines also bind to the BZ high affinity site (Schoch and Mohler, 1983). GABA enhanced BZ binding by 20% with respect to control values, showing that the binding properties of this deoxycholate solubilised complex were similar to the membrane bound complex (Sigel et al., 1983; Schoch and Mohler, 1983).

However, the picrotoxinin-barbiturate binding site was not extracted with deoxycholate. Using CHAPS to solubilise brain membrane, two groups reported a purified complex displaying the allosteric interactions of the membrane bound receptor (Olsen et al., 1984; Sigel and Barnard, 1984). A high affinity binding site was described for the cage convulsant, [^{35}S]-TBPS. This site was chloride dependent and binding could be displaced by picrotoxinin (Sigel and Barnard, 1984), GABA, barbiturates and cage convulsants (Olesn et al., 1984). GABA and barbiturates stimulated BZ binding (Sigel and Barnard, 1984) although muscimol binding had a lowered Bmax reducing the ratio of GABA to BZ sites close to 1, a phenomenon observed in crude CHAPS extracts (Stephenson et al., 1984). GABA binding was enhanced by luM pentobarbital

to 50% over control values (Olsen et al., 1984) demonstrating all three types of binding sites were present and interacted in the manner seen in membrane preparations.

A distinctive property of the GABA/BZ receptor complex in brain membrane is the production, by prior [^{3}H]-flunitrazepam photoaffinity labelling, of a polypeptide which migrates at a molecular weight of 53,000 on SDS-polyacrylamide electrophoresis thus providing an easy means of identification of the BZ binding subunit (Mohler et al., 1980; Sieghart and Karobath, 1980). In CHAPS and deoxycholate extracts of solubilised receptor a polypeptide of 53,000 molecular weight (Sigel et al., 1983; Sigel and Barnard, 1984) has been photoaffinity labelled. Following gel electrophoresis the purified receptor is revealed as consisting of two peptides, 53,000 and 57,000 apparent molecular weight (Sigel et al., 1981; Sigel and Barnard, 1984) by Coomassie blue staining. Using the same procedure, Olsen's group (1984) stained 4 bands of 56,000 (photolabelled with [^{3}H]-flunitrazepam), 60,000, 61,000 and 66,000 from a CHAPS extract of rat brain.

The native molecular weight of the GABA/BZ receptor complex is unclear but work from several sources indicates that it is approximately 230,000 daltons (Barnard et al., 1984; Olsen et al., 1984). This correlates with other studies for radiation inactivation of frozen purified receptor in Triton X-100 which gives a value of 197,000 for both GABA and BZ binding sites (Neilsen et al., 1984) and radiation inactivation of the native receptor of 220,000 in the membrane (Chang and Barnard, 1982).

Thus work on solubilised and purified receptor complexes show that biochemical and pharmacological properties reside within one macromolecular complex.

1.1.12. A Theoretical Model for the GABA/BZ Receptor Ionophore Complex

The complex binding properties of the GABA/BZ receptor can be summarised in a theoretical model after Olsen (1981 - Figure 4).

The receptor complex is described as containing 3 drug binding sites consisting of

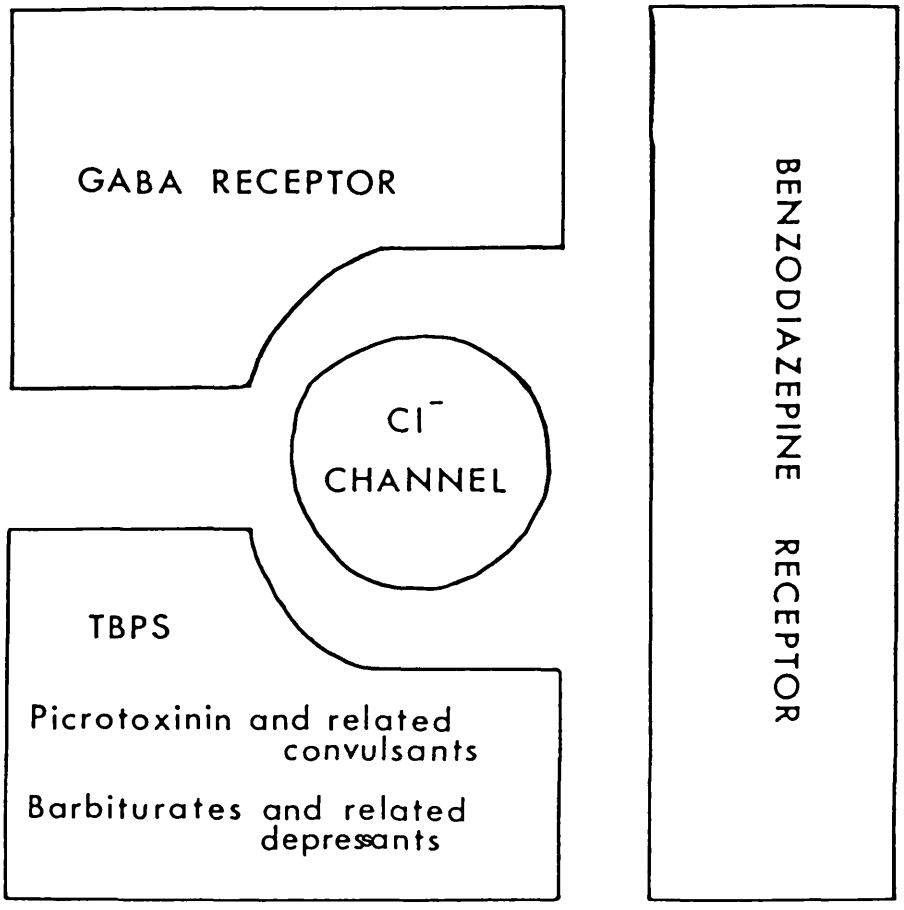


FIGURE 4

Theoretical model of the GABA/BZ receptor.

the GABA, benzodiazepine and picrotoxinin/ barbiturate receptor sites associated with an integral chloride ion channel. The opening of this channel is regulated by GABA agonists binding to the GABA receptor site. The activity of the chloride ion channel can be regulated either in a positive manner (depressant drugs e.g. benzodiazepines) to potentiate chloride ion permeability (and hence inhibition in the CNS), or in a negative manner (excitatory agents e.g. picrotoxinin) to block the chloride channel (and hence block CNS inhibition).

1.2.1. Molecular Biological Approach to Receptor Studies

Neurotransmitter receptor proteins are present in neuronal cell membranes at low concentrations (up to 1pmol/mg of protein) and are surrounded by the lipid-phospholipid bilayer which is required for receptor function. Detergents used to remove receptor components from the membrane exchange with the lipid envelope to produce an artificial detergent-protein complex containing active and inactive receptor. This decreases apparent receptor concentration making activity assessment difficult during purification. Receptors which contain an integral ion channel, such as the nicotinic acetylcholine receptor, represent one class of receptor. More complex types of receptor involve a second messenger system which is required to transduce the chemical signal. Purification of the proteins for both classes of receptor and their reconstitution into lipid bilayers is necessary for the complete evaluation of receptor activity. Purified protein subunits allow the comparison of receptor primary amino acid sequences, predicted secondary structures and patterns of

covalent modification; this permits investigations of the residues and structures involved in ligand binding and in the mechanism of ion channel opening and closing.

The nicotinic acetylcholine receptor is an example of an integral ion channel type and is abundant in the electric organ of Torpedo rays. It is easily solubilised by non-denaturing detergents such as Triton X-100, and can be purified by alpha-bungarotoxin affinity chromatography (Raftery et al., 1976). The receptor is composed of four subunits, $alpha_2$, beta, gamma and delta (Lindstrom et al., 1979). Microsequence analysis has proved difficult to obtain; only 50 N-terminal amino acid residues of the alpha subunit have so far been identified (Raftery et al., 1980; Conti-Tronconi et al., 1982).

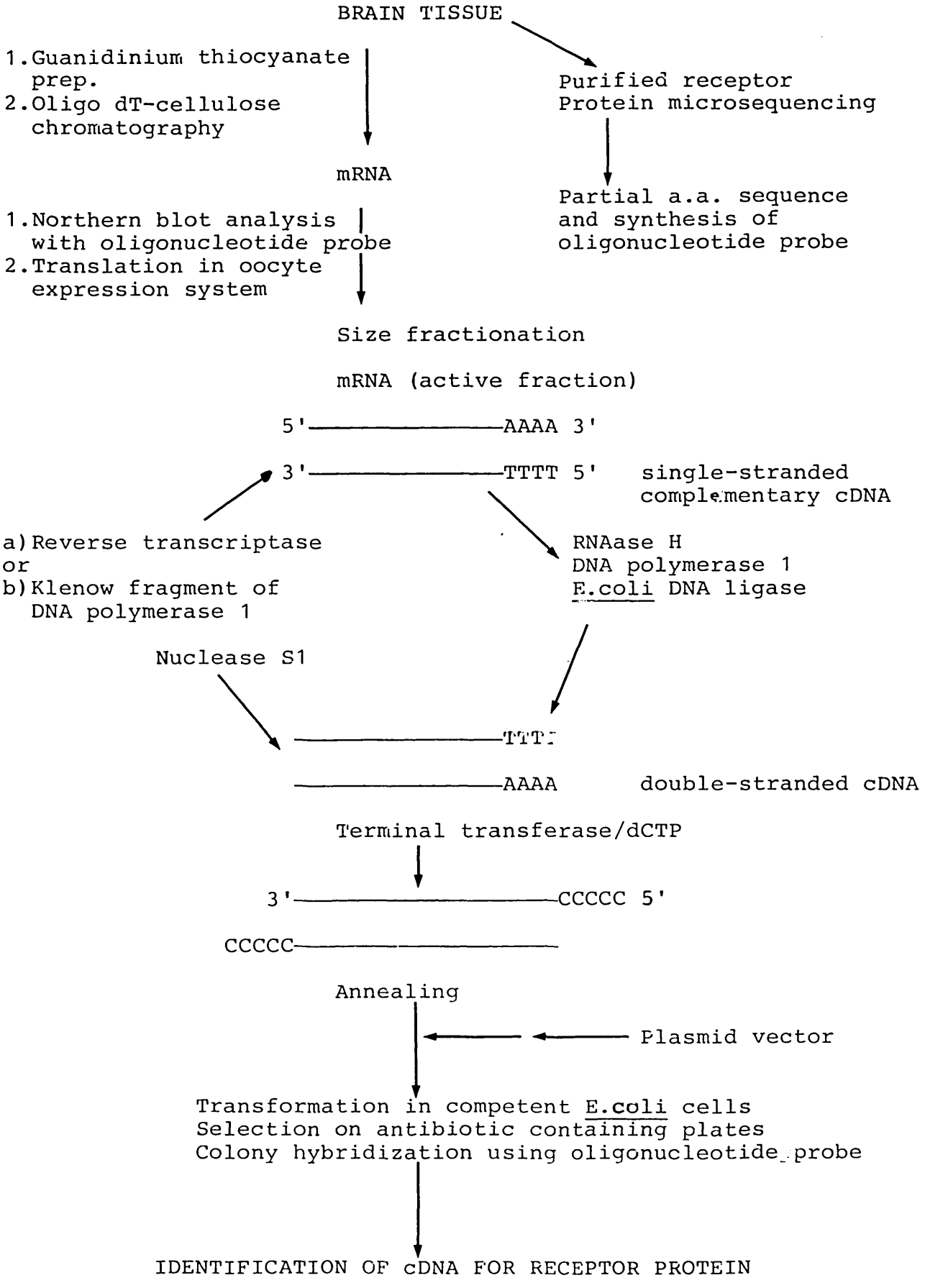
It is clear that, using current amino acid sequencing techniques, progress in the elucidation of receptor polypeptide primary sequence is difficult. An alternative strategy is to deduce the amino acid sequence from the nucleic acid sequence coding for the receptor components.

Many methods of complementary DNA (cDNA) cloning, which screen a cDNA library (made from mRNA) by functional assays, are difficult to apply to receptor cloning. The major reason for this is that often complex collections of single gene products are required for ligand binding, ion-channel opening/closing and other functions.
One successful approach to receptor cloning uses a short stretch of known amino acid sequence to predict a series of oligo-deoxynucleotides which allow for degeneracy in codon usage. Specific radiolabelled oligonucleotides are then used to probe cDNA libraries generated from brain mRNA to identify clones containing low abundance receptor sequences (Figure 5). For example, Sumikawa *et al.*, (1982) synthesised six oligonucleotides representing the coding sequences for four residues of the nicotinic acetylcholine receptor alpha subunit; one oligonucleotide was found to be complementary to mRNA extracted from *Torpedo* electroplax.

This approach relies on the isolation and partial sequencing of receptor-subunit protein. A translation system is also needed to identify potential sources of RNA and

FIGURE 5

A CLONING STRATEGY FOR LOW ABUNDANCE mRNAs



provide a means of measuring RNA enrichment by size selection using either sucrose density gradient centrifugation or gel electrophoresis. Expression of clones for the receptor subunits in this system would demonstrate that the receptor is fully functional 'in vitro'.

1.2.2. Translation of mRNA

Cell free synthesising systems have been commonly used for translating eukaryotic mRNA. Rabbit reticulocyte lysate (Pelham and Jackson, 1976) and wheat-germ lysate (Marcus et al., 1974) can be treated to give a low background activity of endogenous mRNA, by either a preincubation step which relies on endogenous nucleases to degrade RNA or by treatment with micrococcal nuclease. Micrococcal nuclease is a calcium dependent enzyme which is inactivated by the addition of the calcium specific chelating agent, ethyleneglycoltetraacetic acid, (EGTA), after the digestion of RNA. Translation is followed by radiolabelled amino acid incorporation. Production of protein reaches a maximum level in 10 to 15 minutes, but decreases rapidly after 60 minutes incubation at 37°C. The drop

in synthesis results from the exhaustion of components such as ribosomes and amino-acyl transfer RNAs. It is thought that the cell free system is incapable of multiple translations of a particular RNA molecule. Cell free systems are also contaminated with low levels of proteases and nucleases which degrade both mRNA and synthesised protein. For example, translation of acetylcholine receptor mRNA in cell free systems fails to produce acetylcholine receptor binding activity and, after immunoprecipitation by anti-receptor or anti-subunit antibodies, does not produce polypeptides corresponding to the native subunits (Sumikawa et al., 1981; Mendez et al., 1980; Anderson and Blobel, 1981).

Gurdon et al., (1971) demonstrated that mature oocytes of the South African clawed toad, Xenopus laevis, provide a highly efficient translation system for microinjected exogenous mRNA; a technique used for translating many mRNA species (for reviews see Gurdon, 1974; Asselburgs, 1979; Lane, 1983).

The Xenopus oocyte is specialised for production and storage of proteins for use in embryogenesis, including those systems required

for import and export of these proteins (Davidson, 1976; Dumont, 1978).

Oocytes have a high endogenous RNA activity, that is, injected mRNA has to compete with endogen ous mRNA in translation and processing events (Laskey et al., 1979). Following microinjection it is assumed that mRNA freely diffuses throughout the cell. Although, within minutes of injection some mRNA species are translated, most species reach maximum translation of protein products by seven hours (Gurdon et al., 1971). Assuming the mRNA species are stable, a steady translational and processing state is reached by 24 hours, (Asselbergs, 1979) and can last up to seven days (Gurdon et al., 1973). This suggests that even a low abundance mRNA species will be translated in the oocyte (Marbaix and Huez, 1980; Nudel et al., 1976; Richfer and Smith, 1981). For example, acetylcholinesterase mRNA directs the translation of sufficient protein for it to be detected within 48 hours (Soreq et al., 1982).

Oocytes can translate a wide variety of eukaryotic mRNAs including those from plants and animals as shown in Table 8 (Van Der Donk, 1975; Schroder et al., 1977). However bacterial and mitochondrial RNAs translate extremely poorly and fail to produce detectable amounts of protein product (Gurdon et al., 1971; Woodland and Ayres, 1974; Moorman et al., 1977).

The oocyte system is capable of post-translational modification of many proteins (Lane et al., 1971; Marbaix and Lane, 1982). When RNA codes for a large precursor protein, specific proteolytic cleavage in the oocyte will process such products into mature proteins (Knowland, 1974). Enzymes in the oocyte are also capable of phosphorylation, acetylation and glycosylation of synthesised peptides to produce biologically active proteins (Berns et al., 1972; Mous et al., 1982; Reynolds et al., 1975, see Table 8).

The synthesis of functional acetylcholine receptor is an example of the versatility of the oocyte system (Sumikawa et al., 1981). Biosynthesis of this receptor involves translation of mRNA for each subunit,

modification of subunit polypeptides and glycosylation. These subunits then interact in the endoplasmic reticulum membrane to produce functional receptor molecules which are transported to the cell membrane (Merlie, 1984). Synthesis of this receptor in Xenopus oocytes has permitted studies which avoid the interference in mixed-cell systems used in normal electrophysiological and biochemical assays.

Sumikawa et al., (1981) demonstrated that oocytes microinjected with Torpedo electroplax mRNA synthesised a protein that bound [^{125}I]-bungarotoxin. Subsequent incubation of injected oocytes with [^{35}S]-methionine permitted the isolation, by bungarotoxin-affinity chromatography, of the receptor molecule which had apparent subunit sizes of 40, 49, 57 and 63 kilodaltons. Similar sizes have been reported for receptors isolated from native tissue (Lindstrom et al., 1979; Raftery et al., 1980). The oocyte receptor was sized at 9S and 13S (monomer and dimer) by sucrose density gradient centrifugation. This corresponded to the sizes described for native receptor (Heidmann and Changeux, 1978; Vandlen et al., 1979).

TABLE 8

Production of Functional Proteins by Foreign mRNA in Xenopus Oocytes

Source of Messenger RNA injected into oocytes	Functional Protein	Assay	References
Human Fibroblast	Interferon	Inhibition of viral infection	Reynolds *et al.*, (1975)
Rabbit reticulocytes	Globin	Column chromatography binding of metal porphyrin	Gurdon *et al.*, (1971)
Xenopus Liver	Vitellogenin	Immunoprecipitation and co-electrophoresis with purified vitellogenin	Berridge and Lane (1975)
Honey Bee gland	Promelittin	Electrophoretic mobility, partialamino acid sequence	Kindas-Mugge *et al.*, (1974)
Rat Ventral Prostate	Prostatic binding protein	Electrophoretic mobility	Mous *et al.*, (1977)
Herpes Virus	Thymidine kinase	Enzyme assay	McKnight *et al.*, (1980)
Human Placenta	Human placental lactogen	Immunoprecipitation and co-electrophoresis	Mous *et al.*, (1979)
Torpedo electric organ	Acetylcholinesterase	Enzyme assay	Soreq *et al.*, (1982)
Torpedo electric organ	Nicotinic acetylcholine receptor	Electrical stimulation	Barnard *et al.*, (1982)

Electrophysiological assays were undertaken on the oocyte membrane to determine if the acetylcholine receptor had been inserted in the correct orientation and conformation for ion channel function.

The non-injected oocyte membrane contains adrenergic, dopaminergic and cholinergic (muscarinic) receptors which exert their action through transient opening of the chloride and potassium channels in the membrane (Kusano *et al.*, 1977; Miledi, 1982). Voltage dependent channels also exist for sodium and chloride ions (Band *et al.*, 1982). Therefore, rigorous controls need to be employed to ensure the observed response is due to assay conditions and not a function of the membrane itself.

Using a voltage clamp method with ionotophoresed acetylcholine, Barnard *et al.*, (1982) demonstrated a fast depolarising response to acetylcholine which was d-tubocurarine sensitive, in an oocyte previously injected with *Torpedo* electric organ mRNA. No response was observed in non-injected or sham-injected oocytes. Subsequent experiments (Miledi *et al.*, 1982)

using cat denervated muscle mRNA, showed that fully functional nicotinic acetylcholine receptors are expressed in the oocyte membrane.

In this thesis similar techniques have been used to examine the GABA/BZ receptor complex. As described, a comprehensive picture of the structural and functional properties of the nicotinic acetylcholine receptor is emerging. The results of these studies have been extended in this thesis to give further insights into a receptor with an integral ion channel. The allosteric interactions between the clinically important benzodiazepine and barbiturate drugs, the GABA binding site and the chloride ion channel provide a means with which to characterise and purify the solubilised receptor complex. Thus, this work on the GABA/BZ receptor complex has involved the purification of rat brain receptor protein and isolation of the receptor mRNA species. Rat brain mRNA has been micro-injected into Xenopus oocytes to produce 'in vitro' a receptor complex which is both biochemically and electrophysiologically similar to native receptor.

CHAPTER 2

2.1 Protein Chemistry

Introduction

The GABA/BZ receptor complex has been purified by benzodiazepine-sepharose affinity chromatography (Gavish and Snyder, 1981; Martinin et al., 1982, 1983; Sigel et al., 1983). Gavish and Snyder and Martini et al., solubilised the receptor protein in Triton X-100 whereas Sigel et al., used deoxycholate. In each report the purified protein was shown to contain [^{3}H]-muscimol and [^{3}H]-flunitrazepam binding sites which resided on one or more subunit polypeptides. [^{3}H]-flunitrazepam photoaffinity labelled peptides of approximately 53,000 (Sigel et al., 1983), 55,000 and 62,000 molecular weight (Gavish and Snyder, 1981), although only the former group described allosteric interactions in the deoxycholate solubilised receptor protein.

The purification of rat brain GABA/BZ receptor was based on the procedure developed by Sigel et al., (1983) who used the benzodiazepine, R07-1986/1, coupled to adipic dihydrazide agarose (Figures 6 and 7) for affinity purification.

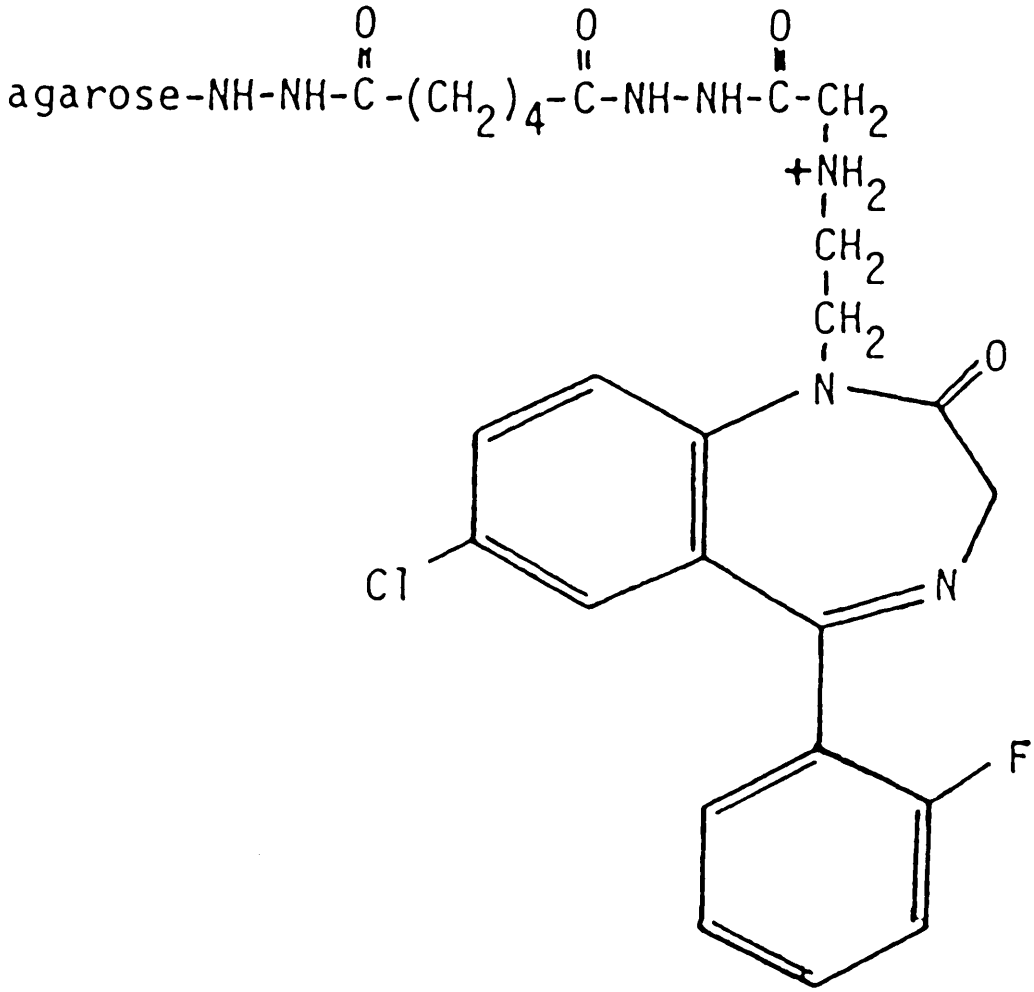


FIGURE 6

Structure of BZ, RØ7-1986/1 affinity gel used for purification of the GABA/BZ receptor protein (Sigel *et al.*, 1983).

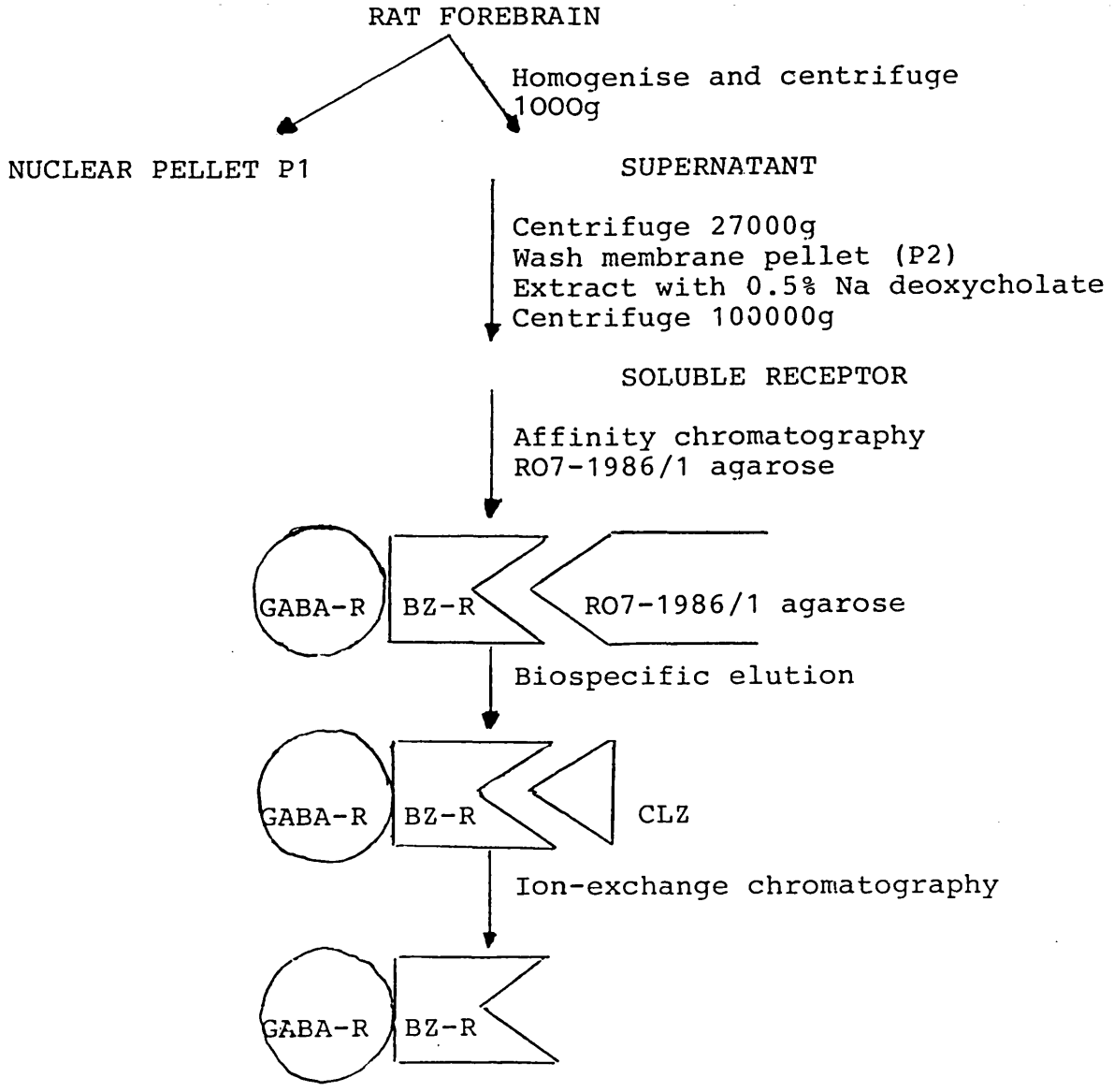


FIGURE 7

Schematic representation for the purification of GABA/BZ receptor protein.

Abbreviations: GABA-R = GABA Receptor

BZ-R = Benzodiazepine Receptor

CLZ = Chlorazepate

Briefly, the GABA/BZ receptor complex was solubilised from rat forebrain using sodium deoxycholate in the presence of protease inhibitors. The solubilised receptor extract was passed through the benzodiazepine affinity column which retains both GABA and benzodiazepine binding sites. The affinity column was then extensively washed in a high salt medium to remove proteins which may be non-specifically bound by either hydrophobic or electrostatic interactions. The GABA/BZ receptor was specifically eluted from the affinity column using a water soluble benzodiazepine, chlorazepate. The chlorazepate-GABA/BZ receptor complex was dissociated by ion exchange chromatography then bound to a DEAE Sephacel column which was washed until all of the chlorazepate was removed. The purified GABA/BZ receptor was recovered by high salt elution.

2.2. MATERIALS AND METHODS

2.2.1. Preparation of Soluble Receptor

(Sigel et al., 1983)

Adult Wistar rats were killed by decapitation, the cortex was rapidly removed, frozen in liquid nitrogen and stored at -80°C until used. Cortex (in 100g lots) was thawed and homogenized in a Waring blender in Buffer A (containing protease inhibitors to protect against inactivation/degradation and maximise recovery-500ml/50g) for 15 seconds at low speed, 15 seconds at high speed.

Buffer A:

10mM Hydroxyethylpiperazine ethane sulphuric acid (HEPES) (pH7.4 with KOH)
320mM sucrose
0.1mM Ethylenediamine tetraacetic acid (EDTA)
1mM benzamidine/HCl
0.5mM dithiothreitol
0.1mM phenylmethylsulphonyl flouride (PMSF)
0.02% (w/v) sodium azide
made up to 1.4 litres

All operations were carried out at 0-4°C. The homogenate was centrifuged in a GSA 600 rotor, Sorvall centrifuge, at 1000_{gmax} for 12 mins. The supernatant was decanted off and centrifuged at 27000_{gmax} for 30 mins. The pellet was resuspended in Buffer B using a

Teflon glass homogeniser and centrifuged at 27000gmax for 30 mins to give a membrane enriched pellet.

Buffer B:

10mM HEPES (pH 7.4 with KOH)
0.1mM EDTA
1mM benzamidine/HCl
0.5mM dithiothreitol
18mg bacitracin
0.02% (w/v) sodium azide
made up to 1.4 litres

The pellet was resuspended in Buffer B to a final volume of 170ml. The following were added with stirring

(a) 7.7ml of 3.5M KCl (final concentration 150mM)

(b) 18mg bacitracin

(c) 4.5ml of 20% (w/v) Sodium deoxycholate

(d) PMSF to 0.1mM

Stirring was continued for 10 mins, (timed after the last addition). This solution was then centrifuged in 30ml polycarbonate screw cap bottles, 60Ti rotor, in a Beckman ultracentrifuge for 70 mins at $100,000_{gav}$. The supernatant was used as the solubilised receptor preparation.

2.2.2 Affinity Chromatography

The GABA-BZ receptor was purified on a Ro 7-1986/1 adipic dihydrazide-agarose affinity column (E.Sigel *et al.*, 1983). After the supernatant was degassed, it was passed through the affinity column at 60ml/h and then washed overnight at 40ml/h with Buffer C, a high salt buffer containing Triton X-100 to exchange for deoxycholate in which the receptor complex was found to be unstable (Stephenson *et al.*, 1982), and sucrose and magnesium acetate to remove high molecular weight contaminants. The column was then washed with 25ml Buffer D at 40ml/h.

Buffer C:

10mM potassium phosphate (pH7.4 with KOH)
50mM potassium chloride
0.1mM ethyleneglycol tetra acetic acid (EGTA)
2mM magnesium acetate
10% (w/v) sucrose
0.2% (w/v) Triton X-100
0.02% (w/v) sodium azide
made up to 1 litre

Buffer D:

20mM potassium phosphate (pH7.4 with KOH)
2mM magnesium acetate
10% (w/v) sucrose
0.2% (w/v) Triton X-100
0.02% (w/v) sodium azide
made up to 250 ml.

The receptor was specifically eluted with an Elution Buffer containing 10mM chlorazepate (a water soluble benzodiazepine) at 20ml/h in 5ml fractions. Ten fractions were collected.

Elution Buffer:

The following were dissolved in 15ml distilled water:
80mg chlorazepate (10mM)
2g sucrose (10% w/v)
200ul 20% Triton X-100 (0.2 w/v)
200ul 1 M potassium phosphate (10mM)
adjusted to pH7.4 with 50mM phosphoric acid.
The following were then added:
20ul 2M magnesium acetate (0.2mM),
volume adjusted to 20ml and degassed.

2.2.3. Ion Exchange Chromatography

Fractions 3-9 from the affinity chromatography step were pooled (30ml), the pH adjusted to 6.5 with 50mM phosphoric acid and applied at 40ml/h to a DEAE-Sephacel column (10 x 1.9 cm Pharmacia) pre-equilibrated with Buffer E, to remove chlorazepate and concentrate the receptor. The column was washed for 2 hours with Buffer E (80ml).

Buffer E:

20mM potassium phosphate (pH6.5 with KOH)
2mM magnesium acetate
10% (w/v) sucrose
0.2% (w/v) Triton X-100
0.02% sodium azide
made up to 250 ml

Six, 1.5ml fractions were collected and frozen at -20°C until used.

2.2.4. Receptor Binding Assay

Soluble receptor activity was measured by a Polyethylene glycol (PEG) filtration assay (Sigel et al., 1983). The soluble receptor was diluted in assay buffer (180ul) and incubated on ice for 30 mins with either 20ul [^{3}H]-muscimol or for 60 mins with 20ul

Assay Buffer:

20mM potassium phosphate
0.1mM EDTA
0.1% Triton X-100
0.02% sodium azide
pH7.4 with KOH

[^{3}H]-flunitrazepam. The final concentration of these ligands was 30nM. After incubation, 15ul of 3.3% gamma - globulin followed by 85ul of 36% PEG were added to give a final volume of 300ul. The solutions were allowed to equilibrate on ice for 12 minutes and then duplicate 100ul aliquots were filtered under vacuum through Whatman GF/C glass fibre filters. The filters were washed three times with 3ml of wash buffer.

Wash Buffer:

20mM potassium phosphate
0.1mM EDTA
7.5% (PEG)
0.1% Triton X-100

The filters were dried and counted in a liquid scintillation cocktail containing 9% (v/v) soluene 350 (Packard) in toluene.

2.2.5. Binding Assay in Solution

The receptor complex (10ul) was diluted in assay buffer (160ul) with either 20ul of medium or 20ul of unlabelled muscimol or flunitrazepam (40 or 30uM final concentration respectively). [^{3}H]-muscimol or [^{3}H]-flunitrazepam was added (20ul) and incubated on ice for 30 mins and 60 mins respectively. The final concentration of the labelled ligands was 30nM. The receptor complexes were treated as in Section (2.1.4).

2.2.6. Embryonic Chick Brain Membrane Binding Assay

Embryonic chick brains (5g) were removed and Polytron homogenised in HEPES buffer (1g/20 volumes) with two, 10 second burst at three quarters maximum speed and a final 15 second burst at maximum speed. The homogenate was spun at 2000 gav. for 10 minutes, 4°C, in a Sorvall HB4 rotor. The supernatant was transferred to 30ml Oakridge centrifuge tubes and spun at 45000 gav., 30 minutes, 4°C in a Ti60 rotor, Beckman ultracentrifuge.

HEPES buffer:

50mM Trizma base
50mM potassium chloride
pH7.4 with KOH.

The supernatant was discarded and the pellets resuspended (10ml/tube) with ice cold wash buffer. The suspension was frozen to -20°C for 60 minutes, then thawed and spun again at 45000 gav., 4°C, 30 minutes. This was repeated three times. Following the final spin the pellet could be frozen until used.

For use in binding assays, the pellet was thawed and resuspended in wash buffer by 20 strokes of a teflon-glass Potter homogeniser at three quarters maximum speed, then centrifuged at 45000 gav., 4°C, 30 minutes. The supernatant was discarded and the pellet re-homogenised (5 strokes). The membrane pellet was resuspended in phosphate buffer. Assays in triplicate were carried out as follows.

Phosphate Buffer:

20uM potassium phosphate (pH 7.4 with K0H)
0.1mM EDTA

Membranes (400ul) received 50ul of phosphate buffer or 50ul of unlabelled flunitrazepam or muscimol (30 or 40uM final concentration). [^{3}H]muscimol or [^{3}H]-flunitrazepam was added (50ul) and the reaction mix was vortexed and incubated for 60 minutes, 4°C. Assays for GABA enhancement of [^{3}H]-flunitrazepam binding received 50ul of a 1mM GABA solution. The reaction mix was then spun at 15000 gav., 4°C, 15 minutes and the pellets were given a superficial wash of 0.2M NaCl. Each pellet was solubilised overnight in 400ul soluene 350 (Packard), scintillant (4 litres toluene/28g 2,5-Diphenyloxazole/2.4g of

1,4-Di-methyl-5-phenyl-oxazoyl-benzene/ 400ml soluene 350) was added and then counted.

2.2.7. Protein determination

A micro-Lowry method was used as modified by Markwell *et al.*, (1978). Samples and the standard solution (1mg/ml bovine serum albumin (BSA)) were diluted in solution A to

Solution A:

20g sodium carbonate
4g sodium hydroxide
10g sodium dodecyl sulphate (SDS)
in 1 litre distilled water

a protein concentration in the range 0-60ug/ml. 200ul aliquots of each solution were added in duplicate to Eppendorf tubes. Solutions B and C were mixed in the ratio 100:1 and 800ul of this mix was added to each tube.

Solution B:

As solution A plus 1.6g sodium tartrate

Solution C:

2g copper sulphate pentahydrate
50ml distilled water
Filtered

The tubes were vortex mixed and incubated at room temperature for 20 mins. Folin and

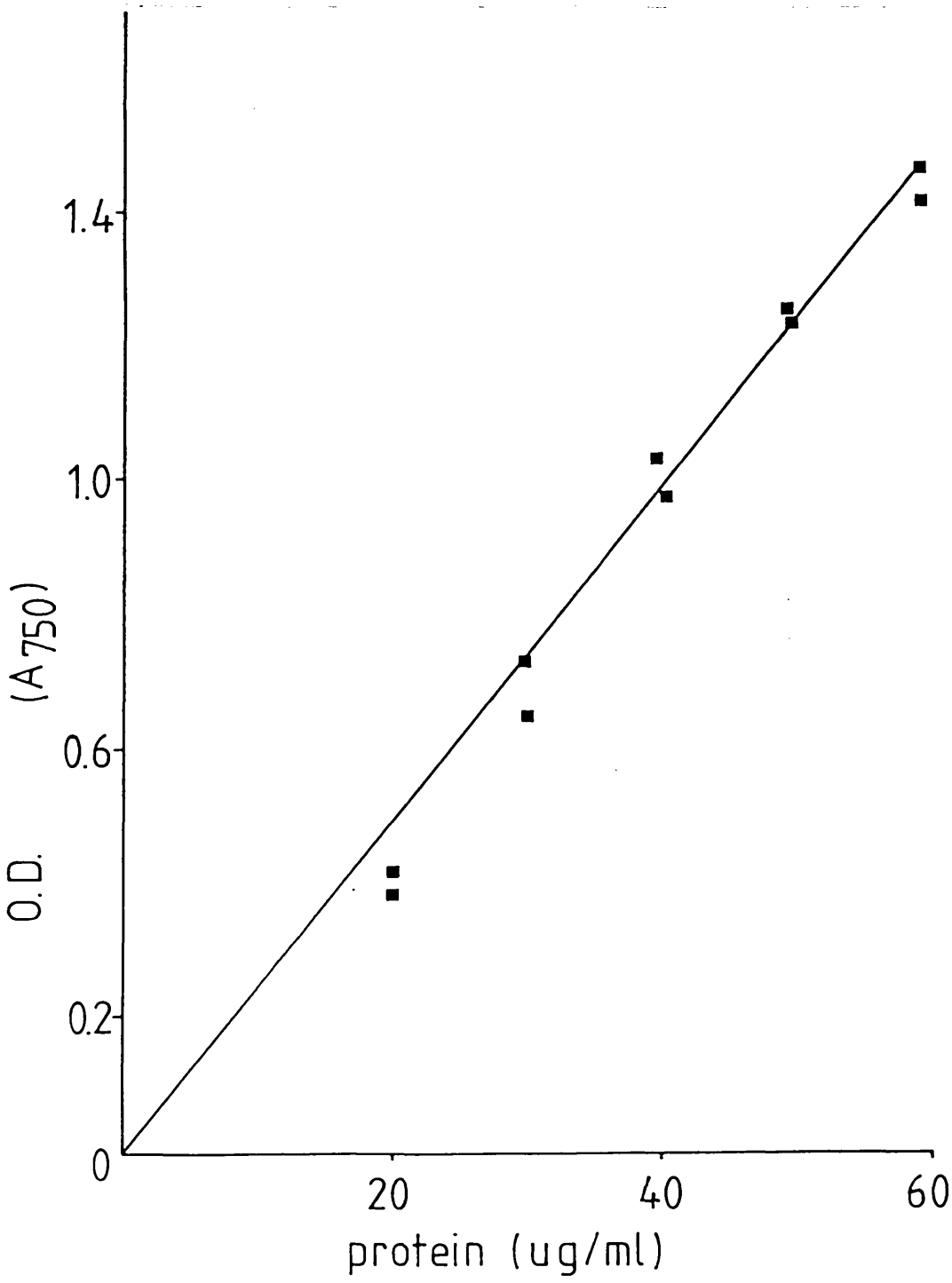


FIGURE 8

Folin-Lowry protein assay calibration curve. The curve represents duplicate determinations for each point.

Ciocalteau's phenol reagent was diluted 1:1 with water. Each tube received 60ul of this solution, was immediately vortexed and the reaction mix incubated for 40 mins at room temperature. Absorbance at 750nm was measured.

2.2.8. Polyacrylamide Gel Electrophoresis

2.2.8.1. Preparation of solubilised receptor protein for electrophoresis.

A 400ul aliquot of the receptor protein was taken and added to an Eppendorf tube containing 600ul distilled water and 360ul 45% Trichloroacetic acid (TCA), then left for 15 mins on ice. Following this interval, the tube was centrifuged for 15 mins in an Anderman 5154 bench top microfuge. The supernatant was decanted off, the pellet was washed in 500ul 12% TCA, and then recentrifuged for 15 mins. The supernatant was removed and the pellet washed and centrifuged with 500ul acetone. After removal of the supernatant, the pellet was desiccated under vacuum for 15 mins. The precipitated protein was dissolved in a 2 times concentration of Gel sample buffer

(50ul). Following heating at 100°C for 2 mins, the sample buffer was diluted to 100ul with distilled water.

Gel Sample Buffer:

2% (w/v) SDS
20mM Trizma base
10% (w/v) glycerol
0.1M dithiothreitol or 0.1M 2-mercaptoethanol
0.007% bromophenol blue

2.2.8.2. Preparation of markers and protein for electrophoresis

[^{14}C]-labelled radioactive molecular weight markers (Amersham), unlabelled markers (Pharmacia), or protein in solution were diluted to 100ul with sample buffer and heated at 100°C for 2 mins before use.

2.2.8.3 Preparation of electrophoresis gel

Preparation and electrophoresis of slab gels was performed using a modified Laemmli procedure (Laemmli 1970). Before pouring the gel the electrophoresis plates were scrubbed in a dilute solution of Decon 75 (Decon Laboratories), rinsed with tap water, then

distilled water and finally dried and wiped with an acetone-saturated paper cloth. Unless stated otherwise, 12.5% (w/v) polyacrylamide gels were used. The gels were made and poured into the plates as follows:

Resolving gel (12.5%)

30% (w/v) acrylamide	12.5ml
1% (w/v) methylene bis acrylamide	3.1ml
1.5M Trizma base pH8.7	7.5ml
10% (w/v) SDS	0.3ml
deionised distilled water	6.4ml
10% (w/v) Ammonium persulphate (AMPS)	0.1ml
N'N'N'N'Tetramethylethylenediamine (TEMED)	10.0ul

After thorough mixing the solution was poured and allowed to polymerise under a top layer of water saturated butanol. Once set the butanol layer was poured off and the top of the gel was washed with distilled water. The stacking gel was made up and poured on to the resolving gel. The samples were loaded and unless otherwise stated, the gel was run overnight at 50V.

Stacking gel (5%):

30% (w/v) acrylamide	1.6ul
1% (w/v) bis-acrylamide	1.3ul
1M Trizma base pH6.8	1.25ul
10% (w/v) SDS	100ul
10% (w/v) AMPS	50ul
TEMED	5ul
deionised distilled water	5.6ul

Running Buffer:
(5 times concentrate)

Glycine	144g
Trizma base	30g
SDS	5g

made up to 1 litre with distilled water

At the end of the run (signified by the dye-marker nearing the end of the gel) the gel was removed to a plastic container and fixed in 10% acetic acid in 50% aqueous methanol (v/v) for 30 mins before staining.

2.2.8.4. Gel Staining with Coomassie Blue:

After fixing, the gel was transferred to a staining solution containing 1.25% (w/v) coomassie blue in 10% acetic acid/50% aqueous methanol (v/v). The gel was destained in 7% acetic acid/10% aqueous methanol (w/v). Destaining could be accelerated by heating to 60°C or more commonly by the use of muslin bags filled with charcoal to act as an absorbent. Destained gels were placed on 2 sheets of Whatman 3MM filter paper and dried down by heating for 2 hours on a Bio-Rad slab gel dryer under vacuum at 60°C.

2.2.8.5. Rapid coomassie blue stain for radioactive gels

This method provided a quick check for gels which are to be used in autoradiography. The gel was fixed in 12.5% TCA for 15 mins. A

Ø.25% (w/v) coomassie blue solution (5ml) was added to 1ØØml distilled water and the gel stained for 3Ø mins. If necessary the gel was destained with a 5% (v/v) solution of acetic acid.

2.2.8.6. Ammoniacal silver stain

(modified from Marshall 1984).

This is a more sensitive staining method than coomassie blue, used for 1-2ug quantities of protein. Gloves should be worn at all times to avoid contamination of the gel. The gel was rinsed in distilled water, then fixed for 3Ø mins, in 1Ø% acetic acid/5Ø% aqueous methanol (v/v). The gel received 3 washes of 15% aqueous methanol for 3Ø mins each wash. After incubation (with shaking) in 1Ø% glutaraldehyde (v/v) for 3Ø mins the gel was given three, 4Ø mins washes of 15% aqueous methanol followed by three 1Ø mins washes of distilled water. The gel was developed in 1ØØul of 37% (v/v) formaldehyde with 2ØØul Ø.ØØ5% citric acid (w/v) for 15-2Ø mins or until light brown bands could be seen.

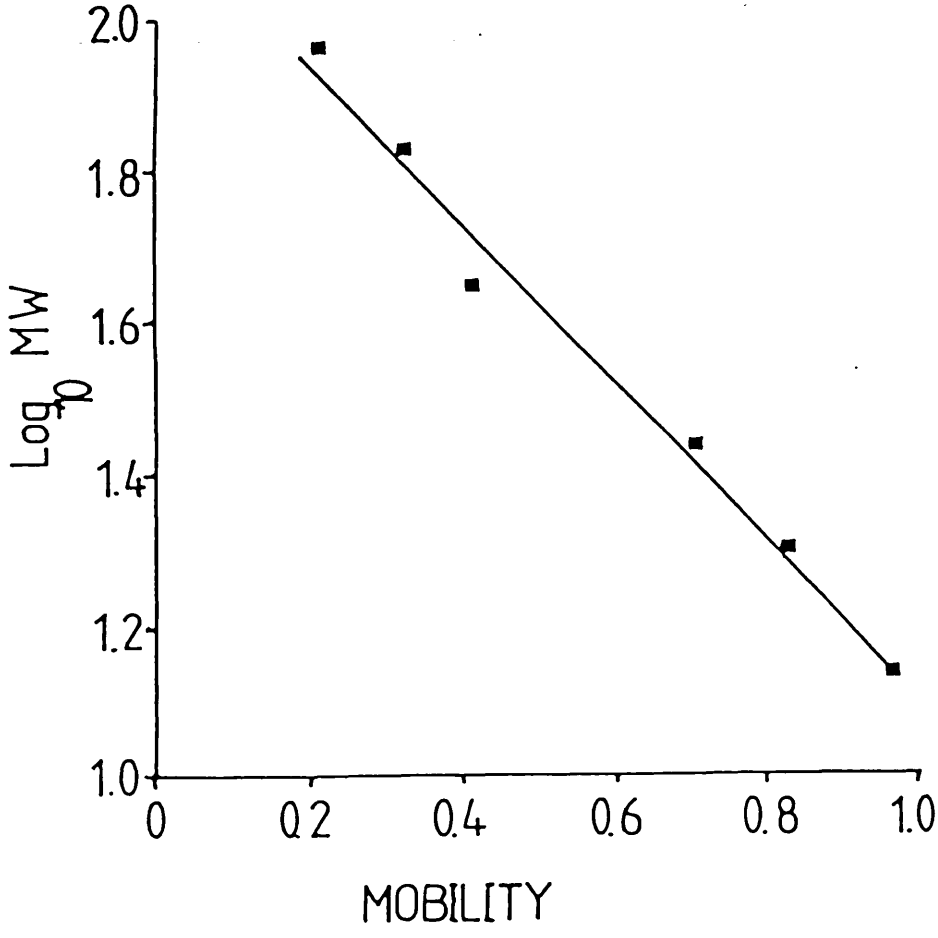


FIGURE 9

Molecular weight - mobility calibration curve for 12% polyacrylamide gel electrophoresis. The standard proteins are from left to right, phosphorylase b (92k), bovine serum albumin (67k), ovalbumin (43k), carbonic anhydrase (67k), soybean trypsin inhibitor (20.5k), alpha-lactalbumin (14.4k).

The reaction was stopped by washing 10 times in 200ml distilled water to neutralise the developer. Silver stained gels could be bleached in photoreducer and restained with coomassie blue (2.1.7.4).

2.2.9. Iodination of solubilised Receptor

2.2.9.1. Iodination using Chloramine T

This was the mildest of the 3 iodination methods undertaken being a modification of the method used by Froehner et al., 1978 for protein in detergent solution. An aliquot containing 2.5ug of purified receptor protein was placed in a siliconised Eppendorf tube and the following were added in a lead screened fume hood:

1. 10ul 0.5M potassium phosphate pH7.5
2. 500uCi $[^{125}]I$.
3. 10ul Chloramine T, prepared fresh by dissolving 5mg in 10ul 0.5M potassium phosphate.

The reaction mix was gently agitated and incubated for 2-3 mins at room temperature then quenched with 100ul saturated tyrosine in

phosphate buffered saline (PBS) and 100ul of 1% BSA (w/v) in PBS. The quenched iodination mix was added to a gel filtration column made from a spherical polyacrylamide gel, P6DG (Bio-Gel), swollen overnight in PBS and then packed into a 5ml pipette and washed prior to use with 3 column volumes of 1% BSA (w/v) in PBS, to reduce non-specific binding. The iodinated protein mix was added to the top of the column, desalted with 1% BSA (w/v) in PBS and collected in 0.5ml fractions. The fractions containing the iodinated protein peak, (in the void volume) were pooled and used in assays after determination of the amount of TCA precipitable protein.

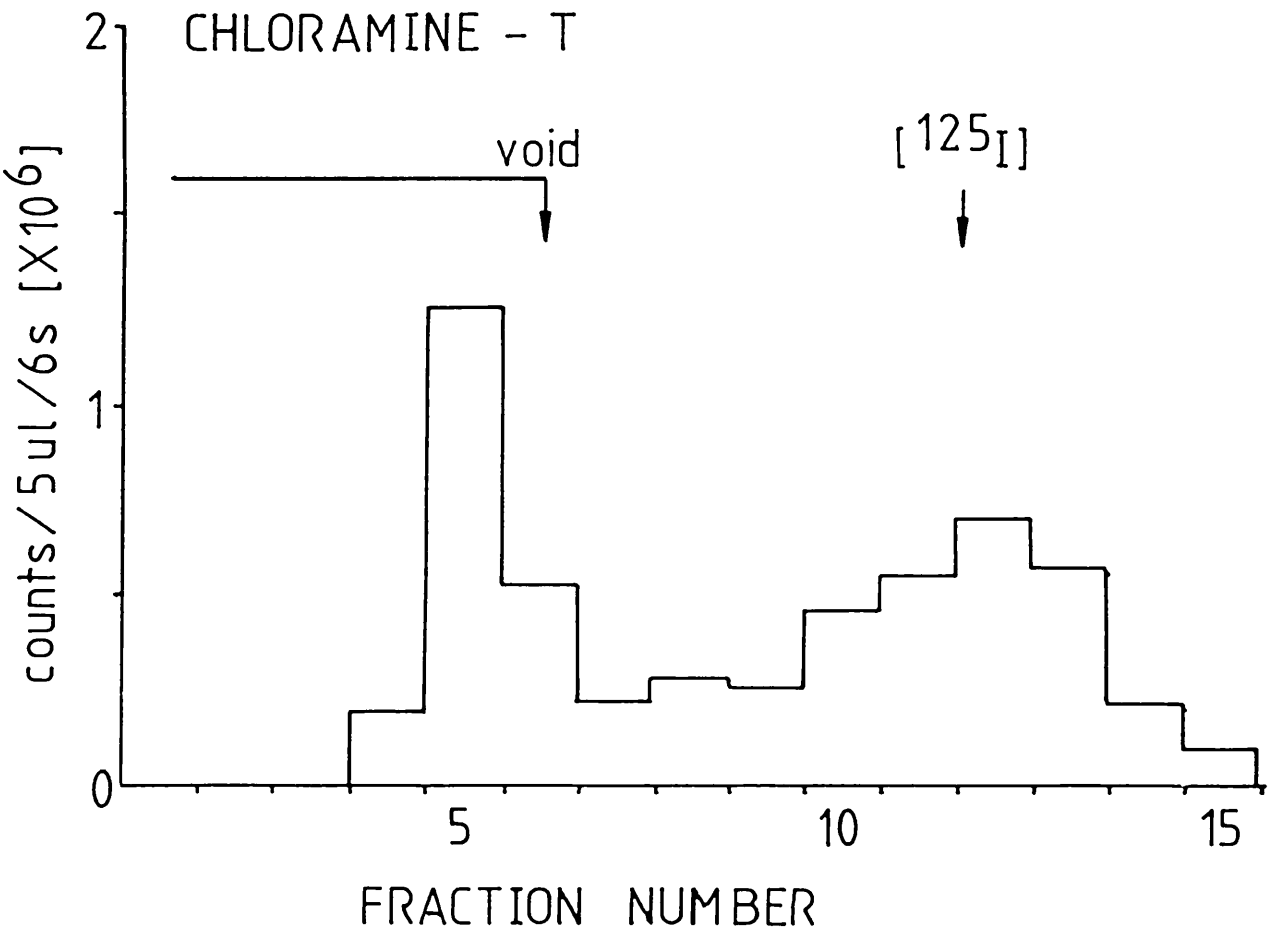


FIGURE 10

Chloramine T desalting column profile of radioiodinated GABA/BZ receptor. lug of iodinated receptor protein was added to the column and washed through with 1% BSA in PBS; 0.5ml fractions were collected. Iodinated protein in the void volume was confirmed by TCA precipitation.

2.2.9.2.[^{125}I] labelling using Iodogen

(Fraker P.J. and Speck J.C. 1978)

This method was very similar to the Chloramine T method except for the following alterations. Iodogen 400ng/lug protein was prepared by dissolving 1mg Iodogen in 100ul chloroform, aliquoted into 400ng lots, and dessicated with dried nitrogen gas and stored at -70°C. 30ul of potassium phosphate solution (0.5M pH8 prepared fresh) plus 250uCi [^{125}]I was added to an Eppendorf tube containing dried Iodogen. The reaction was started by addition of 25ul (1.25ug) soluble receptor and incubated at room temperature for 5 mins before desalting as for 2.1.8.1.

Both products of the Chloramine T and Iodogen methods were stored at 4°C or frozen at -20°C until used.

2.2.9.3 Iodination with Lactoperoxidase

(Powell-Jones et al., 1979)

Unlike the previous two methods, this iodination was effected by enzymic action requiring an alteration in the pH of the receptor solution to enable the enzyme to work efficiently.

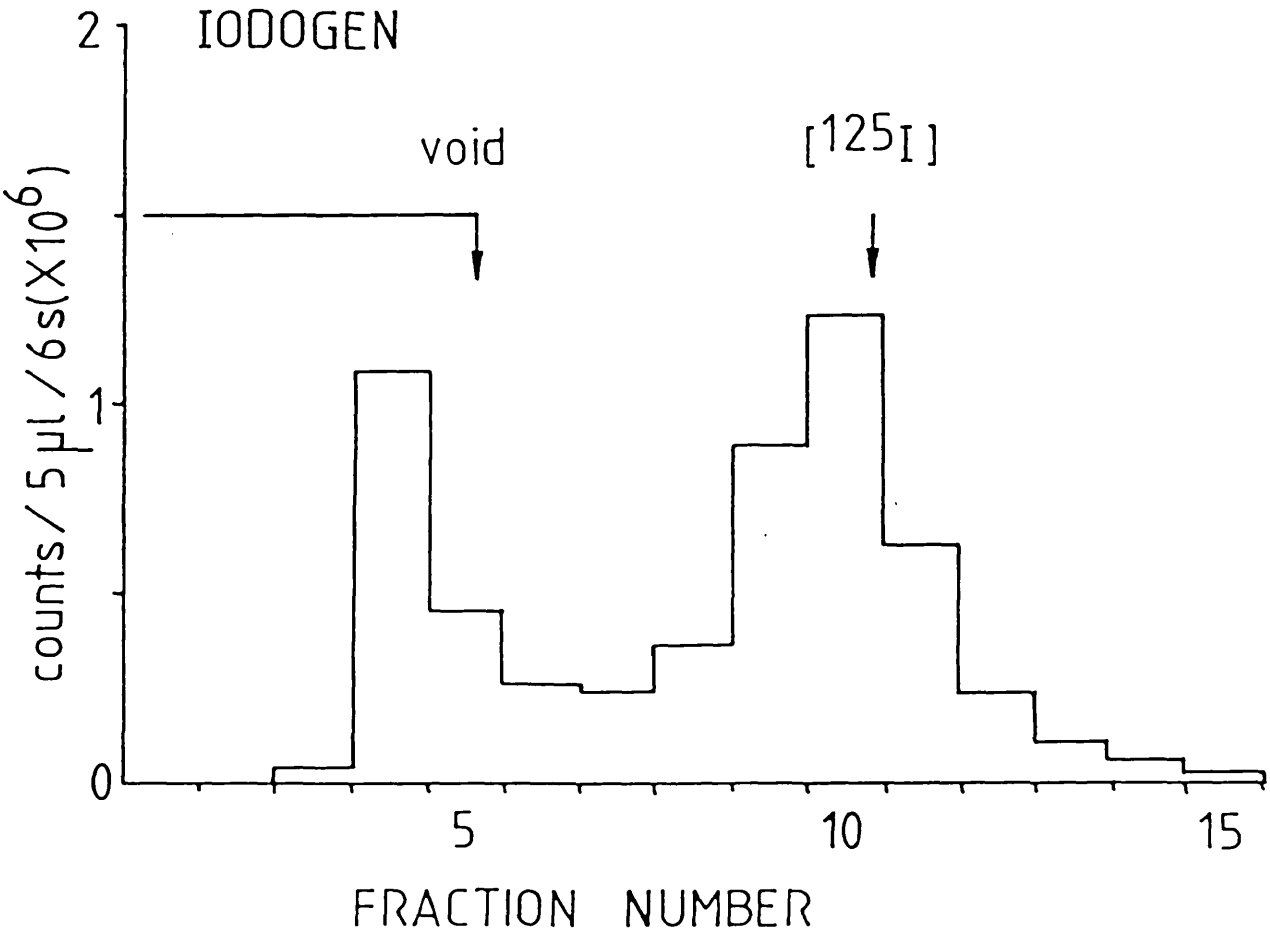


FIGURE 11

Desalting column profile of radioiodinated GABA/BZ receptor. lug of iodinated receptor protein was added to the column and washed through with 1% BSA in PBS; 0.5ml fractions were collected. Iodinated protein in the void volume was confirmed by TCA precipitation.

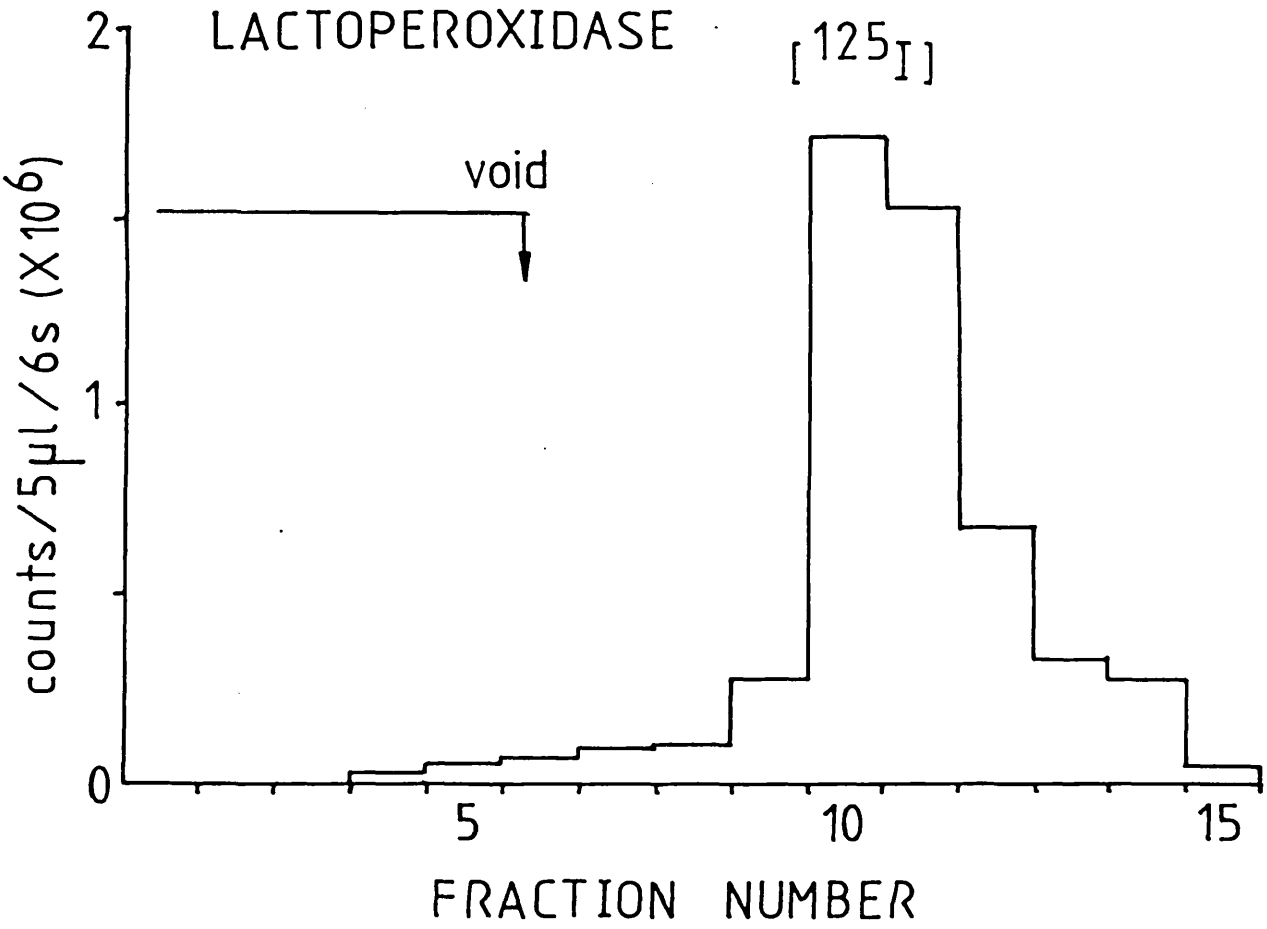


FIGURE 12

Lactoperoxidase desalting column profile for iodination of GABA/BZ receptor protein.

Following iodination of 2.5ug of receptor protein, the iodination mix was added to the column and washed through with 1% BSA in PBS. Fractions of 0.5ml were collected.

The pH of the soluble receptor protein was monitored by spotting 1ul onto pH paper. The pH was adjusted to pH6-6.5 by addition of 2ul of 1% (v/v) acetic acid solution and the pH rechecked after each addition. 500uCi of [^{125}I] was added plus 10ul of a 1 in 10 dilution of lactoperoxidase (1mg/ml) in 0.5M potassium phosphate pH6.5. The reaction mix was incubated at room temperature for 5 mins. The method was concluded as in 2.1.8.1.

2.2.9.4.TCA precipitation of Iodinated Protein

Triplicate aliquots of [^{125}I] labelled protein (5ul), were counted for 6 seconds in a gamma counter, (Nuclear Enterprises NE1600) and then received 100ul BSA (1mg/ml). This solution was made to 1ml with 15% TCA(w/v), mixed vigorously and left to stand at 4^{o}C for 30 mins before centrifugation in a benchtop microfuge for 15 mins. The supernatant was discarded and the pellet counted.

2.3 Results and Discussion

2.3.1 Purification of the GABA/BZ Receptor Complex

The GABA/BZ receptor complex was purified by benzodiazepine affinity column chromatography and biospecifically eluted using a water soluble benzodiazepine, chlorazepate. Chlorazepate was removed and the receptor was concentrated by ion exchange chromatography (Table 9).

Total loss of benzodiazepine binding sites during application of the soluble receptor preparation was less than 18% in all experiments; subsequent washing removed 3% of the applied activity. The yield of receptor in the affinity chromatography step was 18-22%. Following displacement from the ion exchange column with 0.8 M KCl, the recovery of [^{3}H]-muscimol binding activity was 60%. Table 1 represents the results of a typical isolation experiment.

The purification procedure permitted a final recovery of the receptor in a relatively concentrated form (400pmol of [^{3}H]-muscimol

TABLE 9

Purification of the GABA/BZ Receptor

Sample	Total Protein (mg)	Protein (mg/ml)	[^{3}H]-muscimol binding (pmol/mg protein)	[^{3}H]-flunitrazepam binding (pmol/mg protein)	Yield of [^{3}H]-Flunitrazepam binding sites (%)
1. Homogenate	13000	13	-	1.4	100
2. Crude synaptic	3116	16.4	0.06	2.45	42
3. Soluble receptor	2116	13.98	0.09	4.29	29
4. Column flow through	1225	8.75	0.057	1.25	8
5. Affinity column wash	2088	3.48	0.27	0.11	1.3
6. Affinity column eluate	<0.7	<0.02	>1050	-	-
7. Ion exchange eluate	0.2	0.1	2130	450	0.5

a. Chlorazepate interferes with the micro-Lowry protein assay (Sigel et al., 1983).
b. [^{3}H]-muscimol and [^{3}H]-flunitrazepam binding sites were determined at 30nM final concentration and were corrected to Bmax values using the Kd values (24 and 9nM respectively) determined for purified receptor.
c. Chlorazepate interferes with the binding assay.

binding sites/200ug protein in 3ml) within less than 30 hours. The yield of total [^{3}H]-flunitrazepam binding sites was 0.5%, a figure confirmed by other work on rat brain receptor purification (Olsen et al., 1984; Kuriyama and Taguchi, 1984; Ito and Kuriyama, 1982). However, of the solubilised applied activity, 29% was lost in column flow through and a further 3% by washing, suggesting that the capacity of the affinity column had been exceeded. The [^{3}H]-muscimol binding activity data in Table 9 was an underestimation of the GABA binding site concentration. This was due to high concentrations of endogenous GABA (Napias et al., 1980) or other endogenous ligands (Guidotti et al., 1978) in the crude extracts and the overestimation of protein concentration due to the micro-Lowry assay procedure.

Thus this procedure produced a BZ binding protein which also has a site for GABA ligands, but is this protein the GABA/BZ receptor? The following sections will discuss the experiments designed to answer this question.

2.3.2 Binding Properties of the Purified GABA/BZ Receptor Protein

The binding characteristics of this purified protein have been determined by direct binding studies. The protein had a single high affinity site for the GABA agonist [^{3}H]-muscimol, with a dissociation constant (Kd) of 26± 4nM (mean of 4 experiments, Figure 13). The concentration required for this binding site was up to three orders of magnitude lower (in the nanomolar range) than the concentrations of GABA needed to evoke GABA-induced conductance changes in neurones (uM). This lower concentration correlated with that for the membrane bound receptor complex (Olsen, 1982). Similar Kd's to the one reported here have been obtained by other groups for rat and bovine receptor preparations (Martini et al., 1982, 1983; Olsen et al., 1984; Sigel et al., 1983).

[^{3}H]-flunitrazepam also labelled a single high affinity site with a Kd of 9± 2nM (mean of 4 experiments, Figure 14). This is in the range reported by other groups (above). The ratio of [^{3}H]-muscimol to [^{3}H]-flunitrazepam binding sites was

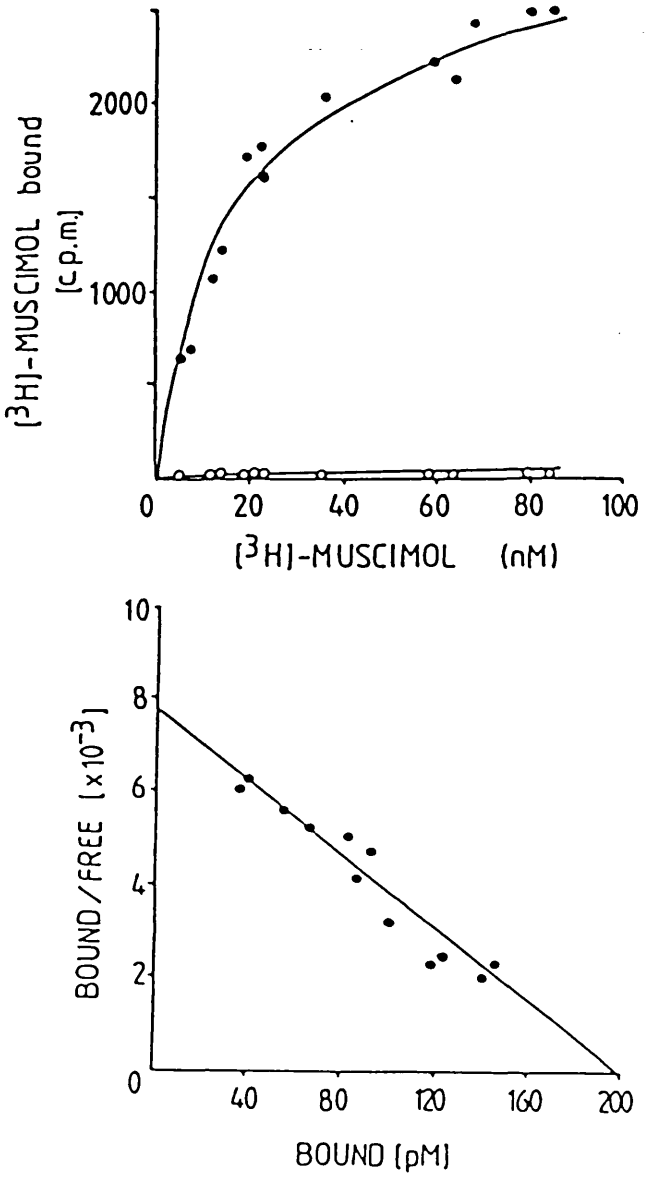


FIGURE 13

[^{3}H]-muscimol binding to the purified GABA/BZ receptor complex. This was assayed by the PEG precipitation method.

A. The receptor was incubated in the presence or absence of 40uM unlabelled muscimol; (•) specific binding; (o) non-specific binding.

B. Scatchard plot of [^{3}H]-muscimol binding to the purified receptor.

NOTE: The Figure represents duplicate determinations in which the error was less than 5% of the mean value. Protein concentration 50ug/ml.

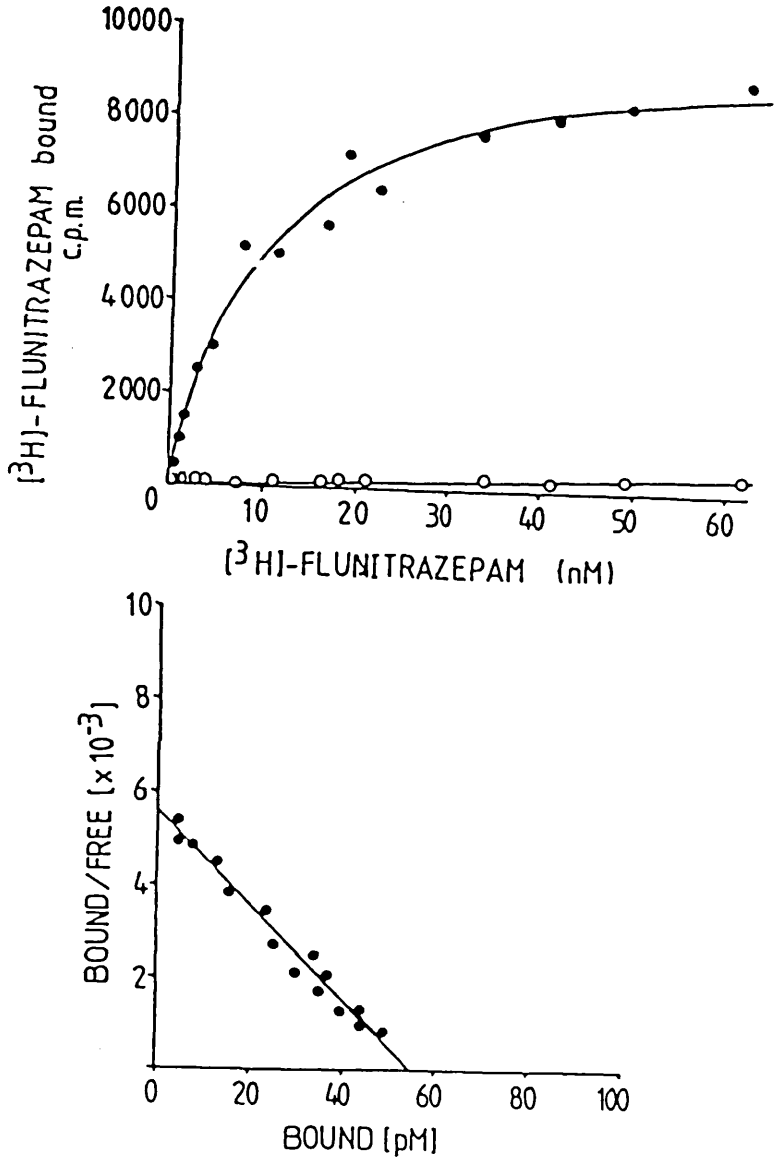


FIGURE 14

[^{3}H]-flunitrazepam binding to the purified GABA/BZ receptor complex. This binding was assayed by the PEG precipitation method.

A. The receptor was incubated in the presence or absence of 30uM unlabelled flunitrazepam; (•) specific binding; (o) non-specific binding.

B. Scatchard plot of [^{3}H]-flunitrazepam binding to the purified receptor.

NOTE: The figure represents duplicate determinations in which the error was less than 5% of the mean value. Protein concentration 50mg/ml.

determined from Bmax values of Scatchard plots and was in the range 3.5 - 3.7 to 1 in all experiments. This was similar to the value reported by Sigel et al., (1983), using a bovine receptor preparation, although lower values for this ratio have been determined using Triton X-100 (Martini et al., 1983) or CHAPS (Sigel and Barnard, 1984) detergents in the purification procedure. The maximum specific binding activity for [^{3}H]-muscimol was 4nmol/mg protein and for [^{3}H]-flunitrazepam, 1.1nmol/mg protein.

2.3.3 Subunit Composition and Photoaffinity Labelling of the Purified Receptor

SDS-PAGE of the purified receptor protein produced a simple polypeptide pattern which was similar after Coomassie blue or silver staining (Figure 15 and 16). The more intensely stained band (alpha) had an apparent molecular weight of 53,000 and the second more diffuse band (beta) 59,000 using Coomassie blue staining. The silver staining method was more sensitive and resolved the upper diffuse band slightly to show proteolytic breakdown which varied in extent from preparation to preparation. This demonstrates the need for

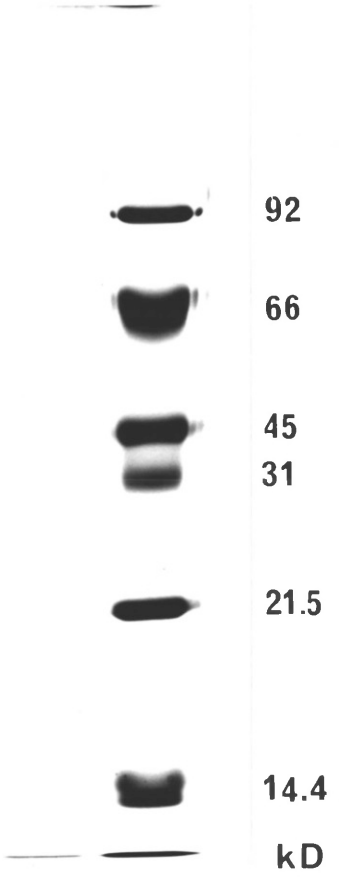


FIGURE 15

Coomassie blue stain of purified receptor complex. A 12% polyacrylamide slab gel received 5ug of receptor protein and was electrophoresed overnight at 50V. The molecular weight markers are phosphorylaseb (92000); bovine serum albumin (66,000); ovalbumin (45,000); carbonic anhydrase (31,000); soybean trypsin inhibitor (21,500); alpha lactalbumin (14.4000).

a

b

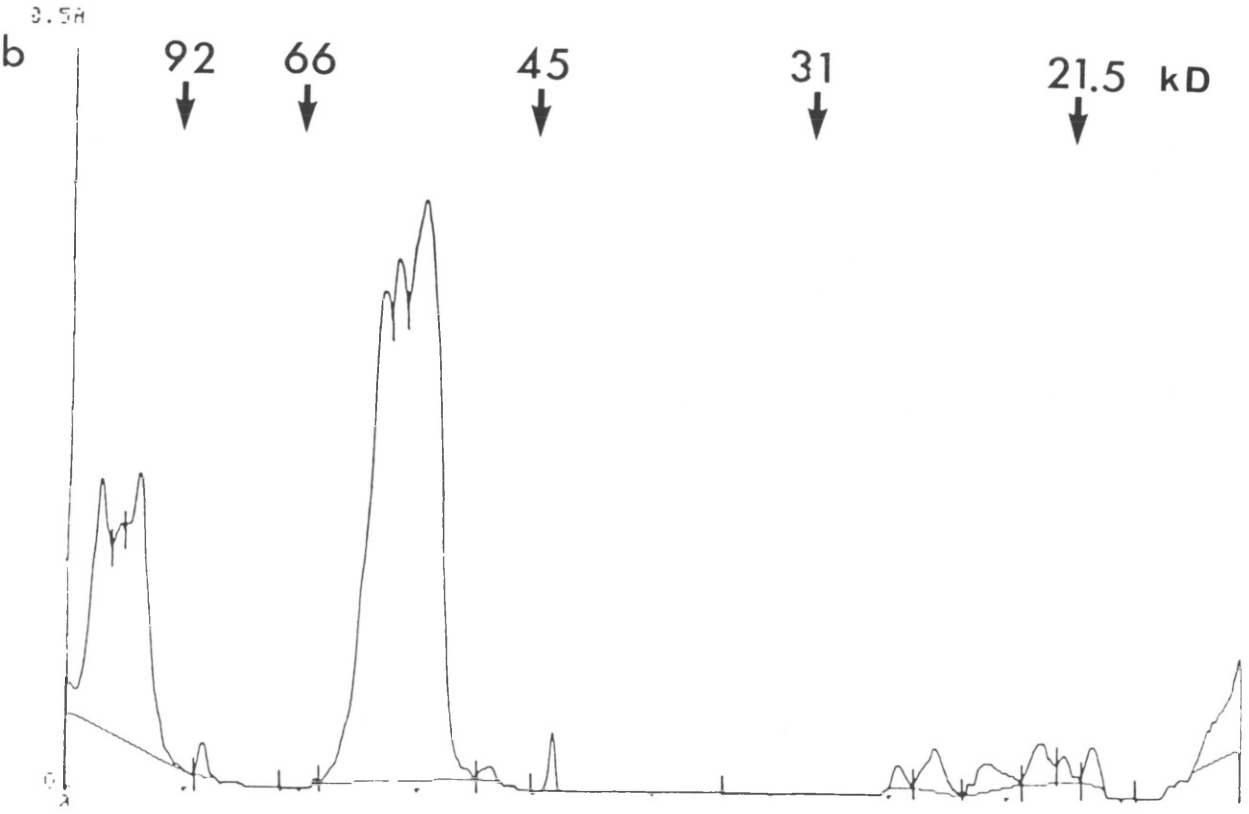


FIGURE 16

Silver stain of purified receptor complex.

a. A 12% polyacrylamide slab gel received lug of receptor protein and was electrophoresed overnight at 50V.

b. Scan of this gel.

protease inhibitors and careful handling of the purified receptor complex. Higher apparent molecular weight bands were observed intermittently at 62,000 and 65,000 daltons. These higher molecular weight subunits have also been reported by Olsen et al., (1984) who used flurazepam to elute the purified receptor complex from the affinity column. These authors offer no explanation for these bands, however, in my preparations they have been observed inconsistently from one preparation to the next. This was probably due to contamination of the purified protein resulting from exceeding the capacity and insufficient washing of the affinity gel.

A diagnostic feature of the GABA/BZ receptor complex is the ability to produce a [^{3}H]-flunitrazepam, photoaffinity labelled peptide in membrane preparation (Mohler et al., 1980). My purified receptor preparation exhibited a photoaffinity labelled polypeptide migrating at 53,000 apparent molecular weight. There was no incorporation of [^{3}H]-flunitrazepam in the presence of excess non-radioactive flunitrazepam or in the absence of U.V. light (Figures 17 and 18). The radioactivity on the top of the

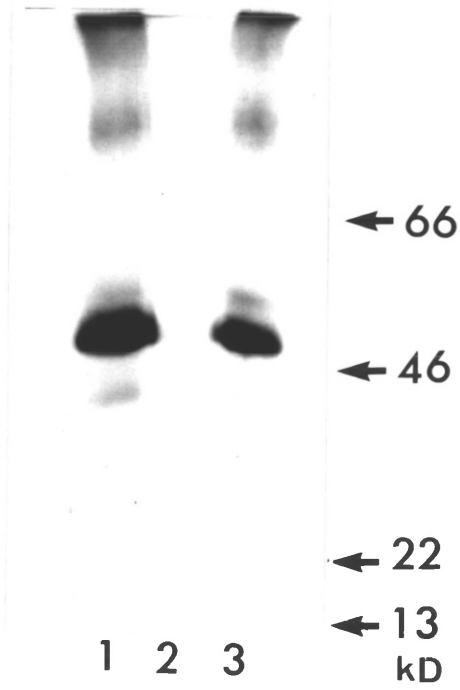


FIGURE 17

Autoradiograph of rat and bovine purified receptor following photoaffinity labelling. Purified receptor 10ug rat (Track 1), 5ug cow (Track 3) was photoaffinity labelled with 100nM [^{3}H]-flunitrazepam and then subjected to SDS-PAGE and processed for autoradiography. Track 2 contains rat receptor incubated with 100nM [^{3}H]-flunitrazepam and 10uM unlabelled flunitrazepam.

Bovine purified receptor kindly donated by Dr. F. A. Stephenson.

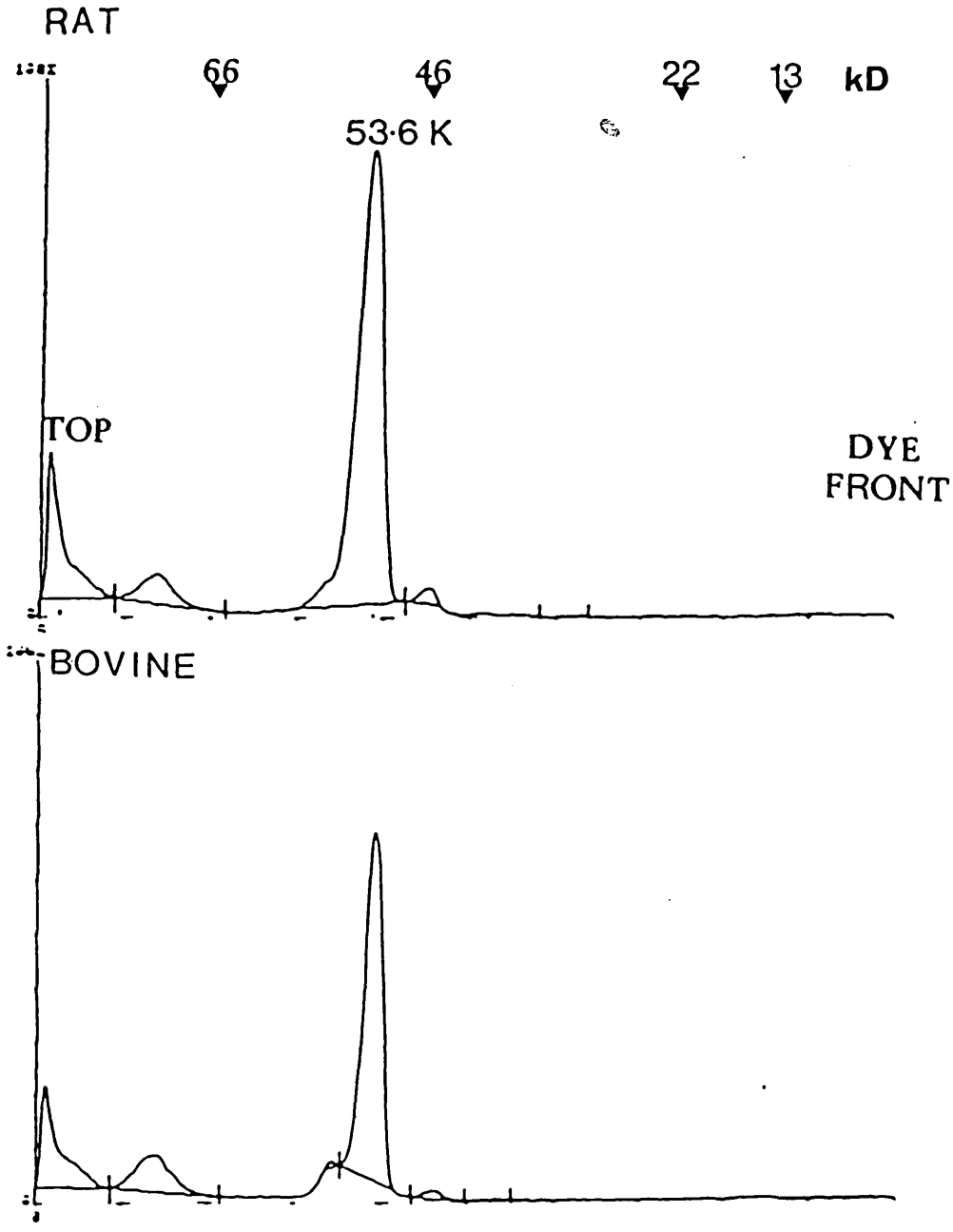


FIGURE 18

Scan of autoradiograph for photoaffinity labelled GABA/BZ receptor. Purified receptor (100ul) was incubated with 100nM [^{3}H]-flunitrazepam in the presence and absence of 10uM unlabelled flunitrazepam before being subjected to SDS-PAGE and exposure on photographic film. Markers as for Figure 3.

autoradiograph represents TCA precipitated protein which did not fully solubilise in the gel sample buffer. A similar molecular weight has been reported by other groups (Sigel et al., 1983; Olsen et al., 1984; Kuriyama and Taguchi, 1984). Of the two major bands, one can therefore tentatively assign the 53,000 species as containing the BZ binding sites.

2.3.4 Iodination of the Purified Receptor Complex

That 2 major subunits constitute the GABA/BZ receptor complex was confirmed by radioiodination of the purified protein. Of the 3 methods used Chloramine T and Iodogen effectively labelled the receptor complex with [^{125}I], producing peptides of approximate molecular weight 53,000 (alpha) and 59,000 (beta) (Figure 19). That [^{125}I] was bound to soluble receptor protein was confirmed by precipitation by 15% TCA of 99% of the radioactivity present in the iodinated mixture. Lactoperoxidase failed to iodinate the receptor protein. As this is an enzyme based reaction, many physical properties of the receptor could have hindered its action (Owen et al., 1982; Hissey, 1984), although

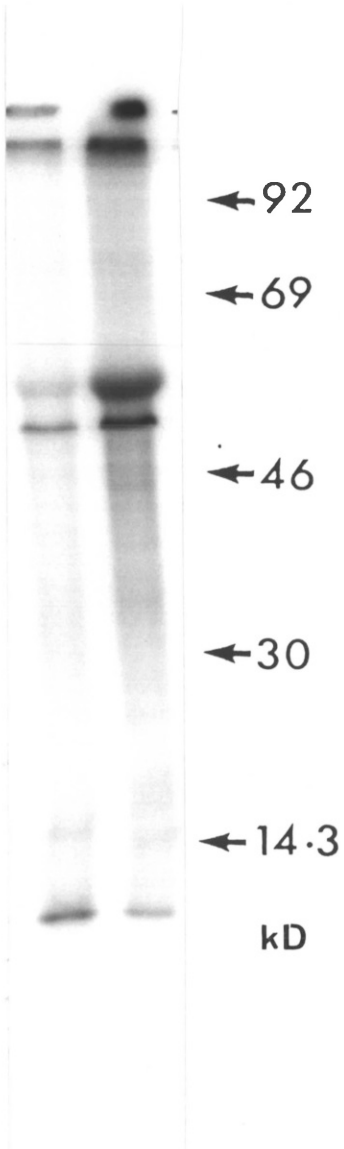


FIGURE 19

[^{125}I]-Iodine labelling of the purified GABA/BZ receptor. Each track received 5ul (~10^6 cpm) of radiolabelled receptor was subjected to electrophoresis and then treated for autoradiography.

1. Protein iodinated by the Chloramine T method.
2. Protein iodinated by the Iodogen method.

the likeliest explanation is that the high concentration of potassium chloride in the soluble receptor preparation was responsible.

2.4 Conclusion

The experiments described here permit the conclusion that the GABA and benzodiazepine binding sites coexist in one macromolecular complex. In all the purified preparations that have been described so far, it has not been shown that the purified protein when solubilised in deoxycholate has the binding site for picrotoxinin, TBPS or the barbiturates. Olsen *et al.*, (1984) are the only group to report binding of [^{35}S]-TBPS to purified rat receptor solubilised in deoxycholate. In this laboratory we have been unable to confirm this data in bovine or rat preparations (F. A. Stephenson, personal communication); however, using solubilisation in CHAPS detergent, this binding site has been purified with the GABA and BZ binding sites (Sigel and Barnard, 1984). The obvious next step from this work would be to solubilise the rat brain receptor in CHAPS detergent.

A purified rat brain GABA/BZ receptor protein is now available for microsequence analysis of one or more subunits for use in the construction of oligonucleotide probes.

CHAPTER 3

3.1 Molecular Biology

Introduction

Early studies on the purification and characterisation of receptor mRNA centred on those mRNA species coding for Torpedo nicotinic acetylcholine receptor, as described in Chapter 1. Subsequent attempts to express the nicotinic acetylcholine receptor of the central nervous system, using mRNA prepared from chick optic lobe, failed to obtain clear evidence that this receptor had been translated in Xenopus oocytes. However, a small response to GABA (at concentrations greater than 10^{-2} M) was observed in some oocytes, indicating that chick optic lobe mRNA contained a mRNA species coding for the GABA/BZ receptor complex (Miledi et al., 1982).

The GABA/BZ receptor mRNA species were identified by translation in Xenopus oocytes. This translation assay was used to study the characteristics of the oocyte produced protein and partially purify the receptor mRNA.

3.2. MATERIALS AND METHODS

3.2.1. Extraction of RNA

The two following methods involved the extraction of total cellular RNA by initially lysing the cells in a buffer designed to inhibit ribonuclease activity. In order to further minimise the latter, the lysis was carried out rapidly in dilute solution (grammes of tissue/ml of solution) (inhibitors present when necessary). All glassware was detergent and acid-washed and then baked in an oven for several hours and/or autoclaved. All buffers were made Ø.Ø5-Ø.1% (v/v) with diethyl-pyrocarbonate (DPC) and/or autoclaved. Large items of glassware were either autoclaved or rinsed well with lysis buffer and/or sterile distilled water. All operations were carried out wearing gloves to minimise contamination from ribonucleases.

3.2.1.1. The Phenol-Chloroform Method (modified from Palmiter, 1975)

A saturated phenol solution was made by dissolving 1kg of AnalaR grade phenol in 11Ømls pH9 buffer with 1g hydroxyquinolone (to act as a stability indicator).

PH9 Buffer:

Ø.2M Trizma base
1ØmM EDTA
5ØmM sodium chloride
Ø.5% (w/v) SDS

pH9 with HCl and autoclaved before use.

The source tissue was homogenised in pH9 buffer (1g/1Ø ml) with 1mg/ml heparin (added just before use), using a polytron homogeniser (15 seconds, 1/2 max speed). Following homogenisation, this mixture received a half volume of saturated phenol and was vigorously shaken for 5 mins. A half volume of chloroform was then added and the mix shaken again for 5 mins. The mixture was carefully loaded into 3Øml corex tubes (sterile, siliconised) and the aqueous phase separated by centrifugation in a Sorvall HB4 swing-bucket rotor, $11ØØØ_{gav}$, 5 mins, $4^{o}C$. For larger volumes 25Øml polypropylene bottles (autoclaved) and a Sorvall GSA rotor were used. The clear organic phase was carefully aspirated off and the remaining interface and aqueous phase are separated and treated as follows. The aqueous phase was extracted three more times with an equal volume of a

saturated phenol/chloroform mixture (1/1), the aqueous phase was separated each time by centrifugation for 5 mins. The aqueous phase was carefully removed (leaving behind any interface), and extracted twice more with an equal volume of chloroform. The interface was treated separately with a half volume of pH9 buffer and then extracted as above with phenol/chloroform and chloroform. The aqueous phases were removed, pooled and nucleic acids precipitated overnight at -20°C with 2.5 volumes of ethanol and 0.05 volumes of 4M NaCl. The precipitate was collected by centrifugation in a HB4 rotor at $11,000_{gav}$ for 30 mins at 4°C, washed 3 times with 70% (v/v) ethanol, and dessicated under vacuum for 15 mins. The pellet was then redissolved in 5ml 20mM Hepes pH7.5 and solid sodium chloride added to 3M final concentration. RNA was selectively precipitated overnight at -20°C. The precipitate was collected by centrifugation at $11,000_{gav}$ for 30 mins, 4°C in the HB4 rotor. The pellet was washed with 10ml of 3M sodium acetate (pH6), vigorously vortexed,

then centrifuged for 15 mins, 4°C, HB5 rotor. The wash was repeated 3 times. Finally the pellet was given 2 washes of 10ml 70% ethanol, dessicated under vacuum for 15 mins, redissolved in 2ml, 5mM Hepes pH7.5 and stored at -70°C. The amount of RNA obtained by this procedure was determined by measuring its Optical Density (a 1 in 50 dilution of the stock solution) at 260nm.

3.2.1.2. The Guanidinium Thiocyanate Method

(modified from Chirgwin et al., 1979)
This method gave a one step purification of RNA from protein and DNA by centrifugation of the homogenate through a caesium chloride cushion. Source tissue (1g/10-20ml) was homogenised in guanidinium thiocyanate solution with 2% (w/v) sodium lauroyl sarcosyl, with a polytron homogeniser for 15 secs at 1/2 maximum speed (at room temperature).

Guanidinium thiocyanate solution

4M guanidinium thiocyanate (Fluka Grade)
25mM sodium citrate
0.1M 2-mercaptoethanol
Spin and/or filter (millipore) to clear, adjust to pH7 with 1M NaOH

The homogenate was loaded into 30ml Corex tubes (sterile, siliconised) and centrifuged for 10 mins at 11000_{gav}, 4°C in the HB4 rotor. The supernatant was heated rapidly to 65°C for 2 mins then cooled rapidly on ice. Six 11.5ml polyallomer tubes, (rinsed with hydrogen peroxide and then given 3 rinses of sterile distilled water) received 2-5ml each of half saturated caesium chloride solution.

Half saturated caesium chloride solution:

5.7M caesium chloride (Hopkin Williams)
100mM EDTA
pH7.5 and autoclaved

The cooled guanidinium thiocyanate solution was carefully layered on top of the caesium chloride cushion and the tubes were centrifuged in a SW41 rotor for 18 hours, $151,000_{gav}$, 20°C (Beckman L8-70 ultracentrifuge). The tubes were removed, the supernatant aspirated off and the tubes inverted to drain. The lower 2 cm of each tube containing the pellet was severed using a sharp scalpel blade and the pellets resuspended in 5ml (total volume) solubilising buffer. This was then heated to 65°C for 5 mins and rapidly cooled on ice.

Solubilising Buffer:

10mM Trizma base
5mM EDTA
1% (w/v) SDS
pH7.5 with HCl

Ribonucleic acids were precipitated overnight with 2.5 volumes absolute ethanol and 0.05 volumes 4M NaCl solution, then pelleted by centrifugation in a Sorvall HB4 rotor, 11000_{gav}, 4_oC, 30 mins, dessicated under vacuum for 15 mins and redissolved in 5mM Hepes pH7.5. Optical density at 260nm of a 1 in 50 dilution of the stock gave a determination of the amount of RNA present.

3.2.2. Purification of messenger RNA

Messenger RNA (mRNA) was purified by binding of the poly(A)$^+$ RNA "tail" of the molecule to a matrix of cellulose and oligo-deoxythymidine (Oligo-dT) in the presence of a high salt concentration. Poly(A)$^+$ RNA (mRNA) was isolated by differential salt elution. Oligo-dT cellulose (P and L Biochemicals) was prepared for use by soaking 0.5g in a 5ml solution of 0.1% SDS, 5mM EDTA, 10mM Tris-HCl pH7.5. This was applied to a 10ml Biorad Econocolumn (internal diameter 0.9cm) and

washed with 20ml of 5mM Hepes pH7.5. Each time before use, the column was washed with 10ml 0.1% DPC(v/v) in 20mM Hepes pH7.5 (to inactivate ribonucleases) followed by 20ml 0.5M KCl in 5mM Hepes pH7.5, to remove traces of DPC. The column was then washed with 5-10 volumes of 0.1M NaOH to remove traces of non-specifically bound RNA and equilibr ated to pH7.5 with 0.5MKCl in 5mM Hepes pH7.5. The column was connected in series with a Gilson Minipuls 2 peristaltic pump and a Uvicord apparatus (LKB Uvicord II type 8300) to measure the absorption of light at 260 nm, thus monitoring the elution of RNA (see plate).

RNA was prepared for addition to the column by dilution with an equal volume of 1M KCl. The RNA/KCl solution was applied to the column at a rate of 2ml/min and followed by washing with 0.5M KCl in 5mM Hepes pH7.5 until the absorption at 260nm (A_{260}) had fallen to near background. The column was then washed with 0.1M KCl in 5mM Hepes, pH7.5 until the A_{260} had reached near background. The mRNA was eluted with 5mM Hepes, pH7.5 until the peak had reduced to near background. The collected mRNA was reapplied to the column and the procedure repeated.

After the second collection the mRNA was

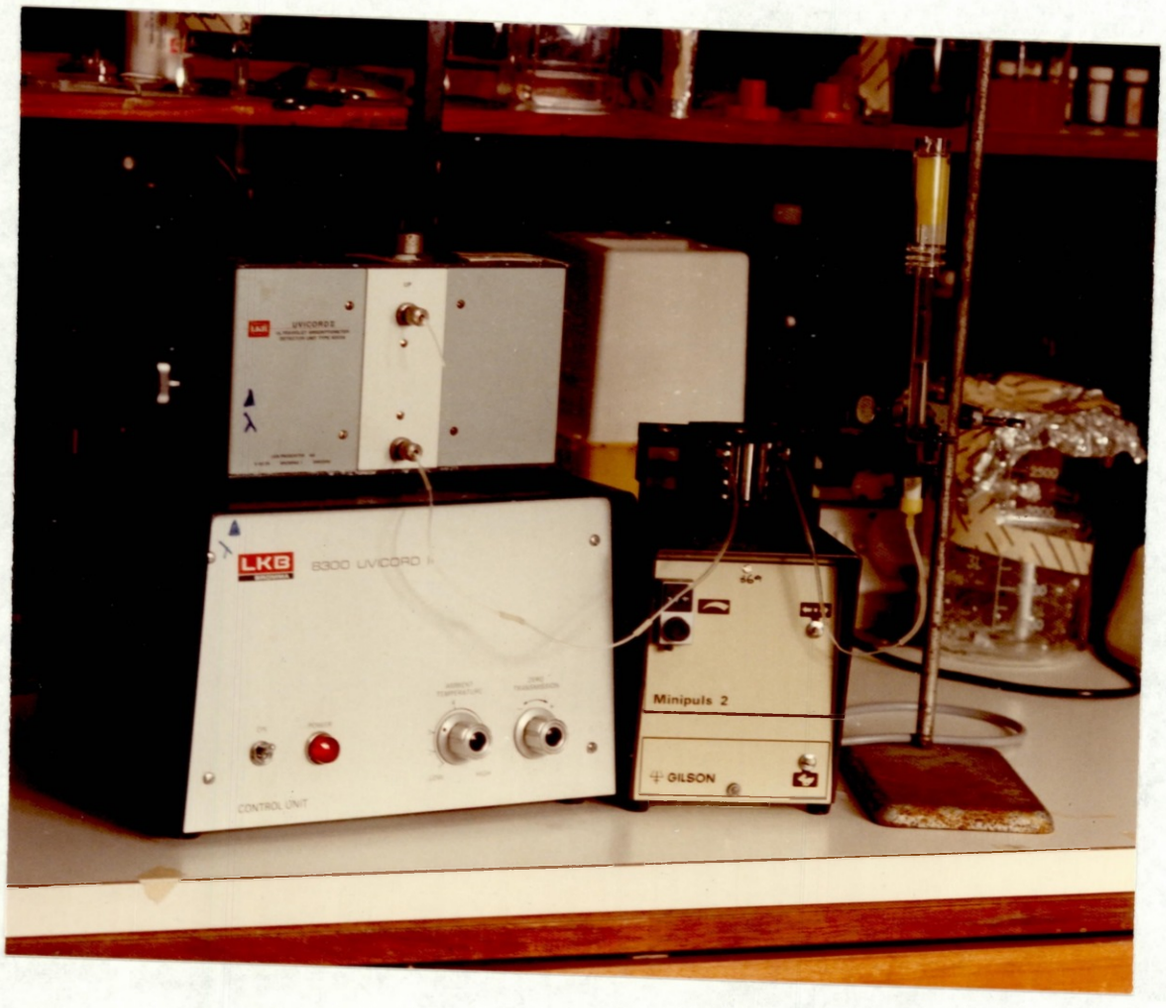


FIGURE 20

Equipment used in oligo dT-cellulose chromatography.

precipitated in 2.5 volumes absolute ethanol, 0.05 volumes 4M NaCl, precipitated overnight at -20°C, then centrifuged (HB4 rotor, 11000_{gav}, 10 mins, 4°C). The pellet was washed twice with 70% ethanol, dried under vacuum and resuspended in 100ul sterile distilled water. Optical density was measured at 260nM and mRNA stored at -70°C.

3.2.3. Detection of intact RNA

The following two methods were used as check assays to determine that the RNA obtained was suitable for use in translation. The formamide gel assay was used to determine that the heterologous RNA had not been degraded by nuclease action and was suitable for the preparation of messenger RNA. The glyoxal gel confirmed that the mRNA had not been degraded during the purification procedure.

3.2.3.1 Preparing and running formamide gels

Deionised Formamide was prepared by stirring 120ml formamide with 4g Amberlite monobed resin MB1 (BDH), gently, for 2 hours (covered with foil to protect from light degradation),

then filtered into sterile glassware. The following buffers were made up.

Concentrated Phosphate Buffer:

To 8ml distilled water were added:

340mg Na_2HPO_4 (Ø.24M)

900mg $NaH_2PO_4.2H_2Ø$ (Ø.58M)

the pH was adjusted to 7.5 with NaOH and the volume made up to 10ml.

Buffer Formamide:

5ml deionised formamide
70ul concentrated buffer

Running Buffer:

4.5g Na_2HPO_4

1.2g $NaH_2PO_4.2H_2Ø$

in 2 litres deionised water
pH adjusted with NaOH to pH7.5

Gels were cast in glass tubes of Ø.6cm internal diameter and 10cm length. To pour six, 4% running gels, the following mixture was prepared;

1.7g acrylamide

Ø.3g bis-acrylamide

50ml deionised formamide

1.35ml 10% (w/v) AMPS/concentrated buffer

200ul TEMED

The gels were poured, overlaid with a buffer running buffer (1/1) solution and allowed to polymerise overnight at 4°C. Total RNA (20ug) samples received 41.7ul of buffer mix and were then heated to 68°C for 5 mins.

Buffer Mix:

5ul buffer formamide
25ul bromophenol blue (50mg/ml)
25ul 1% (w/v) Xylene cyanol
Each sample was loaded and electrophoresis carried out at room temperature for about 4 hours at 5mA per tube. Gels were removed by water pressure after having been loosened with a syringe and needle. The gels were fixed in 30% (v/v) glycerol, 5% (v/v) acetic acid solution for three 20 mins cycles. The gels were scanned for absorption peaks at 260nm using a Joyce Loebl UV scanner.

3.2.3.2. mRNA Gel Electrophoresis on glyoxal gels

This agarose gel system was used to determine if any of the mRNA samples had been degraded by ribonuclease activity. Glyoxal (8M,BDH) was deionised by continuous passage down a mixed bed resin column, (BDH, MBI - ethanol/ether (1:1) washed and packed into a 10cm Biorad Econocolumn with dry resin). The pH of the glyoxal solution was checked on a pH meter after each pass down the column until it reached pH6, then used immediately or stored at -20°C.

The mRNA samples were treated as follows (at 4°c) then heated to 50°C for 1 hour.

mRNA (up to 5ug)	2ul
8M Glyoxal pH6	3ul
10mM sodium phosphate buffer pH7	5ul
Dimethylsulphoxide (DMSO) to 50% (v/v)	10ul
TOTAL	20ul

The samples were returned to ice and each received 5ul of loading buffer.

Loading Buffer:

10mM sodium phosphate pH7
0.05% (w/v) bromophenol blue
50% sucrose

If not used immediately the samples were kept at 4°C. A 0.8% (w/v) agarose gel (Sigma) was made up using 100ml 10mM sodium phosphate pH7 and left to cool for 45 mins. The mRNA samples were loaded with suitable markers (ribosomal RNA, 16S, 18S, 23S and 28S) and the gel was electrophoresed for 3 to 4 hours at 25mA constant current with continuous recirculation of buffer. At the end of this time the glyoxylate was removed from the gel by shaking in 50mM NaOH, 20°C, 40 mins. The gel was transferred to 10mM sodium phosphate buffer pH6.5 and shaken for 10 minutes with 100ml of buffer. This was repeated until the pH of the gel was pH6.5 (the pH of the gel was monitored using pH paper pressed to the gel surface). The gel was then stained using

2ug/ml (w/v) Ethidium bromide in 10mM sodium phosphate buffer pH7 and destained in the same buffer before scanning with UV light.

3.2.4. Rabbit Reticulocyte Lysate

The reticulocyte lysate assay was used as a routine screen to assess the activity of mRNA fractions. The lysate was intended as a general purpose assay of mRNA activity, judged by the incorporation of [^{35}S]-methionine into polypeptides (as shown by gel electrophoresis or TCA precipitation).

3.2.4.1 Time Course Study

The rabbit reticulocyte lysate used was a kit supplied by Amersham and the protocol used for the assay was as provided in the kit. This is summarised as follows. The lysate and mRNA to be tested were removed from storage (-70^{o}C) quickly thawed by hand heat and

gently but thoroughly mixed by vortexing. The following incubation mix was made up in a sterile, siliconised 1ml Eppendorf tube. These reaction volumes could be halved to be used in more mRNA assays.

Lysate	40ul
mRNA (0.5mg/ml)	2ul
L-[35S] methionine	50uCi
Total Volume	50ul

mRNA assays. The reaction mix was gently mixed by vortexing and placed in a water bath at 30°C. Samples (1ul duplicates) were withdrawn at the following time intervals - 0,5,10,20,30,40,60 mins, and added to 0.5ml of 1M NaOH, 5% (v/v) hydrogen peroxide (bought as a 30% solution from BDH), in a 3ml LP3 tube (Luckham). When all samples had been taken, the tubes were placed in a water bath at 37°C for 10 mins. to hydrolyse the amino-acyl-tRNA complexes. The tubes were removed to an ice bath and 2ml ice cold 25% (w/v) TCA solution was added with 100ul 1% (w/v) BSA. The tubes were mixed and left on ice for 30 mins.

The tube contents were then filtered through 2.5 cm Whatman GF/C glass fibre filters and washed 2 times with 3ml ice cold 8% (w/v) TCA solution. The discs were dried under an infra red lamp and counted using liquid scintillation in 4ml Biofluor (New England Nuclear).

3.2.4.2 Gel electrophoresis of lysate products

The lysate assay was performed as in 2.2.4.1 but the reaction was allowed to continue for 40 mins. and then stopped by the addition of gel sample buffer (see Section 2.1.7.1), gentle vortexing and heating to 100°C for 1 min. A 12.5% polyacrylamide gel was prepared and the samples loaded after a 2 minute spin in a benchtop microfuge (2.1.7.2). Following electrophoresis (3 to 4 hours at 50mV), the gel was fixed with 50% (v/v) aqueous methanol in 7% (v/v) acetic acid for 30 mins. This was followed by a rapid wash with 15% (v/v) aqueous methanol and distilled water. The gel was immersed prior to autoradiography in 100ml Amplify (Amersham) fluorographic reagent for 30 mins. Alternatively the gel was

stained with Coomassie blue (2.1.7.4) before autoradiography.

3.2.5. Fractionation of mRNA on sucrose gradients

(Brakke and Van Pelt, 1970)

This is a modified method using an isokinetic gradient of sucrose.

Acetate buffer, pH5.5 was made up and used for the following strength sucrose solutions; 31%, 27.5%, 23.2%, 18.2%, 11.8%.

Acetate Buffer:

10mM sodium acetate, pH5.5
1mM EDTA
0.1% (w/v) SDS

Prior to layering as follows into sterilised 11.5ml polyallomer tubes, the solutions were cooled at 4°C for 30 mins.

1ml	31 %
2.95ml	27.5 %
2.5ml	23.2 %
2.1ml	18.2 %
1.7ml	11.8 %
1.2ml	acetate buffer

The tubes were then left to stand for 30 mins before the addition of denatured mRNA. The mRNA was prepared for addition to the gradient

by dissolving 200ug mRNA in 200ul acetate buffer, heating to 70°C for 5 mins and cooled rapidly on ice before layering onto the top of the sucrose gradient.

Markers consisting of ribosomal RNA from E coli. MRE600 (23S AND 16S, 25ug-Pharmacia) and calf liver ribosomal RNA (28S and 18S, 25ug) were denatured as for mRNA in 200ul acetate buffer. The tubes were loaded into an SW41 rotor and centrifuged for 18hrs at $120,000_{gav}$, 20°C and allowed to decelerate with the brake off. 0.4ml fractions were collected at 0.5ml/min by displacement from the bottom of the tube with 60% sucrose using a Fison gradient collector. mRNA was precipitated with 2ul of transfer RNA (1mg/ml), 2.5 volumes ethanol, 0.05 volumes 4M NaCL, overnight at -20°C. The precipitate was collected by centrifuging the fractions for 15 minutes at maximum speed in a benchtop Eppendorf spinner (Anderman 5154) at 4°C. The supernatant was aspirated off, the pellet washed with 70% (v/v) ethanol, dessicated under vacuum for 15 minutes and finally redissolved in 10ul sterile distilled water and stored at -70°C.

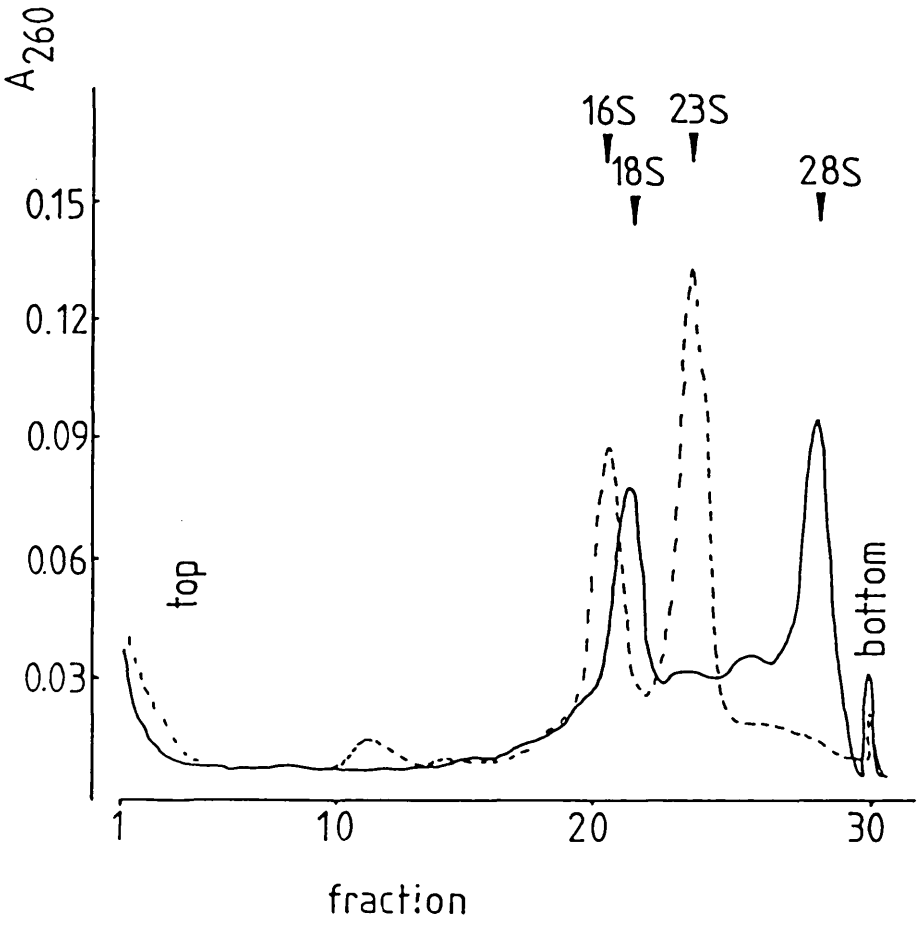


FIGURE 21

Sedimentation profiles of ribosomal RNA markers for calibration of sucrose gradients run in parallel. (---) represents a profile of 16S and 23S ribosomal RNA (25ug) derived from E.coli; (——) represents a profile of 18S and 28S ribosomal RNA (25ug) derived from calf liver.

3.2.6. PROCEDURES WITH OOCYTES

3.2.6.1. Obtaining Oocytes for Microinjection

Oocytes were obtained from females of the South African clawed toad, Xenopus laevis., (100-150g weight). The toads were kept in fish tanks (100cm x 30cm x 50cm), 10 to a tank, in 10cm of water and were fed on alternate days with a diet of fresh, chopped liver or trout food pellets (Xenopus Limited).
Oocytes were obtained from anaesthetised females (15 mins, in 10% (v/v) methanol) by a ventral abdominal incision and subsequent removal of ovarian tissue with forceps (see figures 22-26). The oocytes were placed in a 10cm petri dish containing 20ml Barth's medium (Barth and Barth, 1959).

Barth's Medium
(made and stored as a 5 times concentrate)

440mM NaCl
5mM KCl
12mM sodium hydrogen carbonate
4.1mM magnesium sulphate ($.7H_20$)

1.65mM calcium nitrate ($.4H_20$)
2.05mM calcium chloride ($.6H_20$)
37.5mM Trizma base buffered to pH7.6 with HC
Before use the concentrate was diluted 1:5(v/v) with distilled water and received 100ug/ml gentamycin solution.

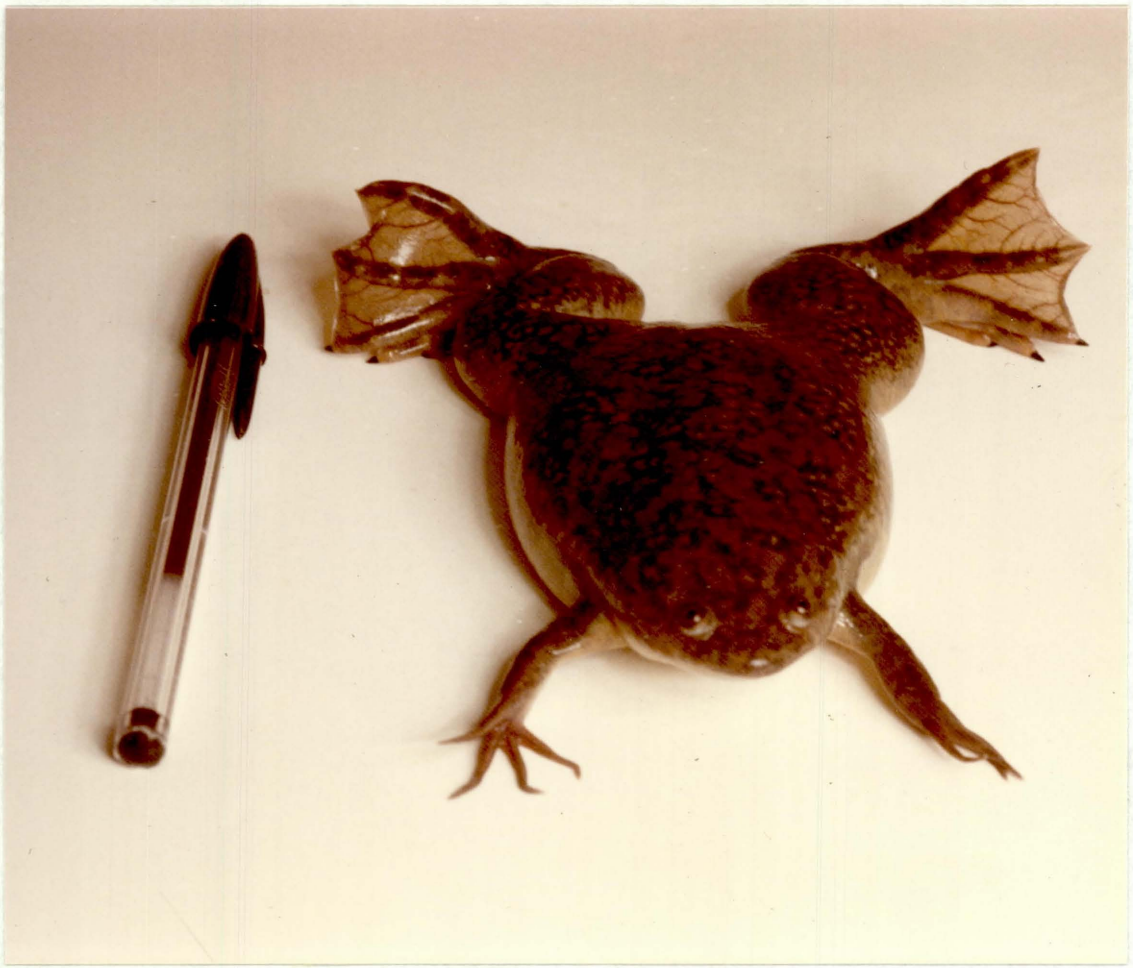


FIGURE 22

Adult female Xenopus laevis.

FIGURE 23

Xenopus placed ventral side uppermost before incision.

FIGURE 24

Ventral incision 1cm long through the body wall.

FIGURE 25

Removal of ovarian lobes with foreceps.

FIGURE 26

Repair of body wall incision.

The oocytes were washed several times in Barth's solution before being dissociated with forceps and sorted according to size and stage of development (Figures 27-29).

Finally oocytes of stage V and VI were pooled in a 10cm petri dish with Barths and kept in a 21°C incubator until used.

3.2.6.2. Microinjection needle preparation

Microinjection needles were prepared by heating hard glass capillary tubes (BDH) to melting point and then stretching to twice the original length. Tips of the correct diameter (20-50um) were made using a microforge. Finally, prior to use, the tips were trimmed using a pair of jewellers forceps, and calibrated by drawing up 1ul of sterile distilled water, marking the extent of uptake and dividing the resultant length of pipette into equal volumes. At those times when a more

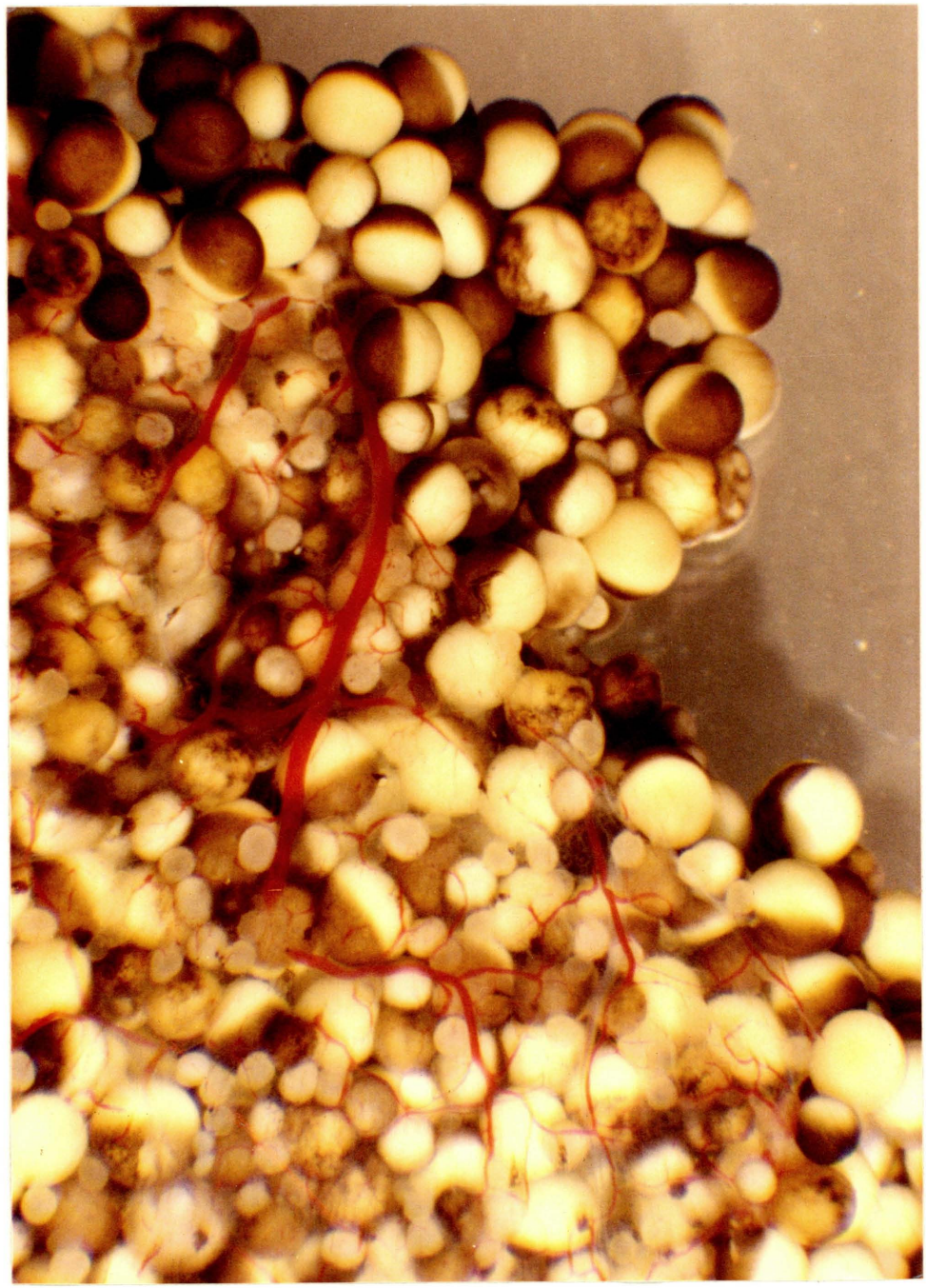


FIGURE 27

Xenopus ovarian tissue. This picture shows the blood supply and various stages of oocytes present in ovarian tissue (6 times magnification).

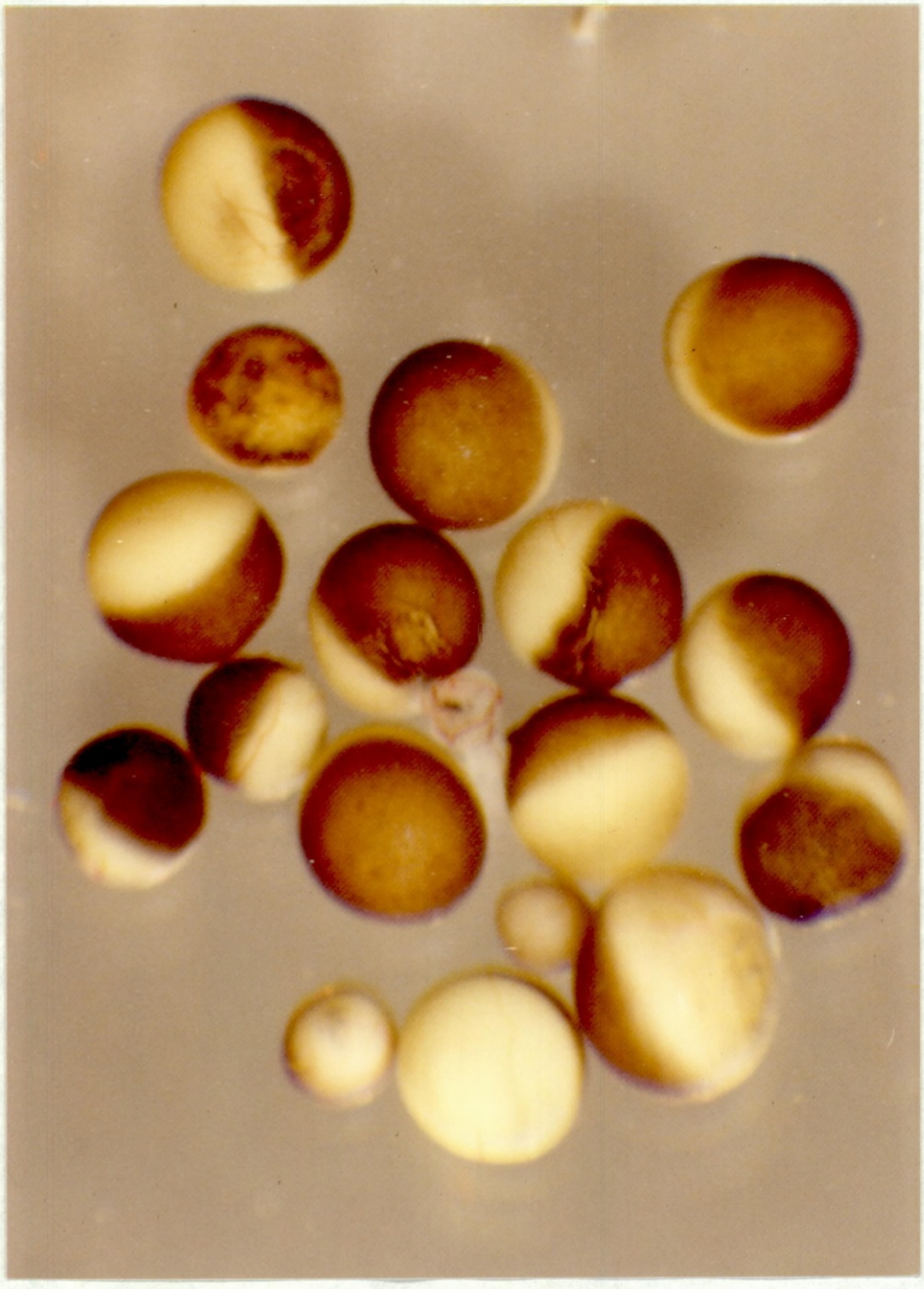


FIGURE 28

Stage V and VI oocytes. Mature dissociated oocytes (12 times magnification).

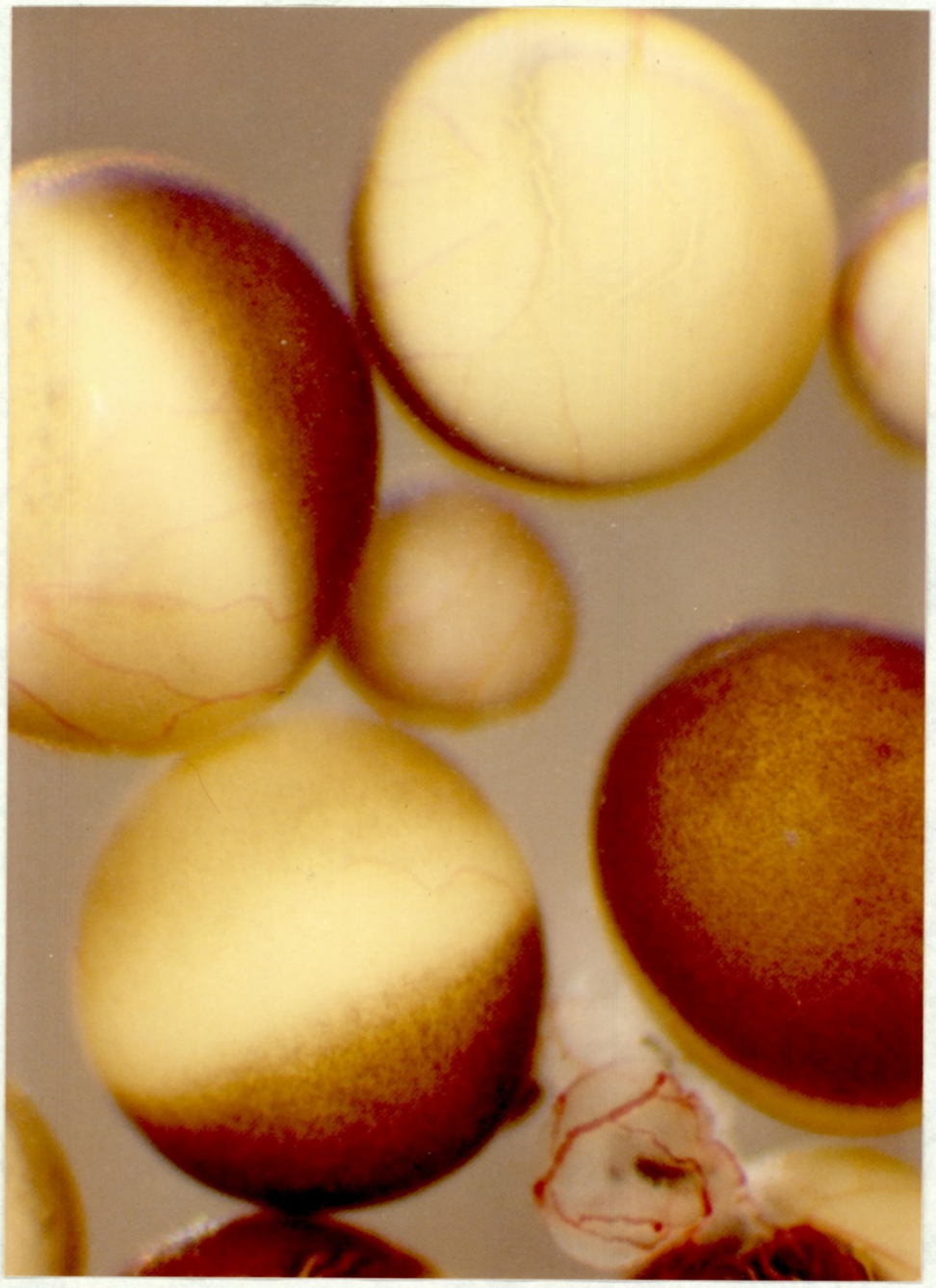


FIGURE 29

Close up of mature oocytes (31 times magnification). Note blood supply to each oocyte. Bright band around equatorial region is characteristic of mature stage V and VI oocytes.

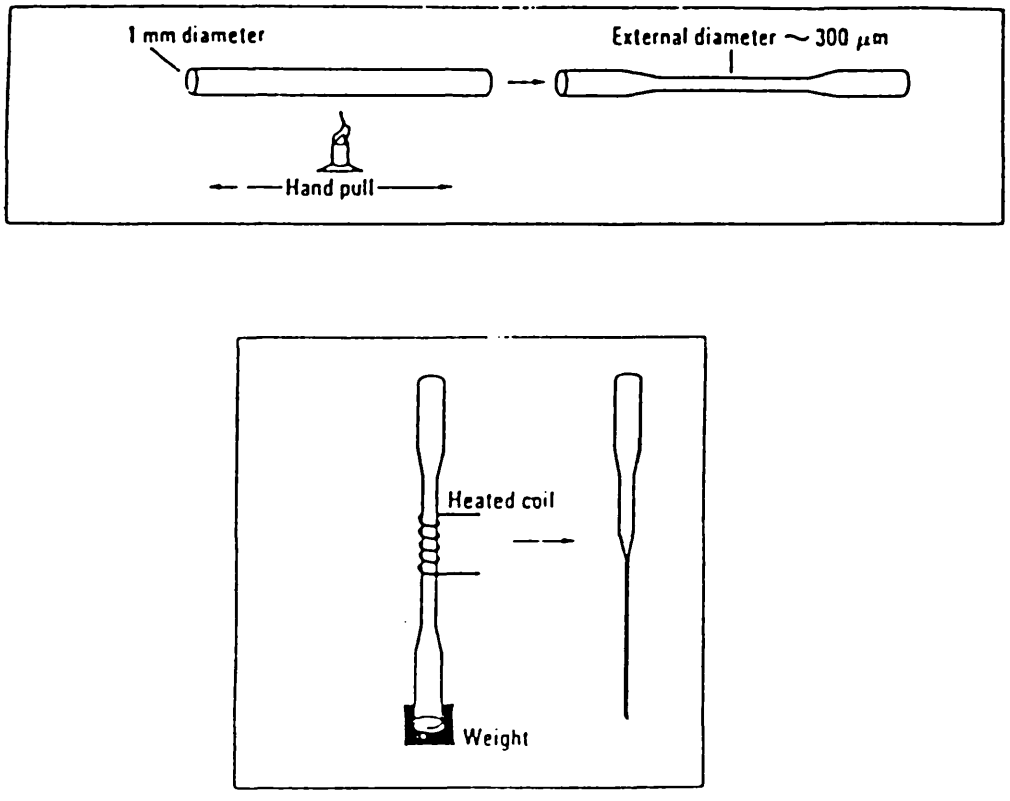


FIGURE 30

Manufacture of microinjection needles.

accurate calibration was needed, a small volume of radioactive solution was drawn up into a micropipette and then equal volumes (as above) were spotted onto filter discs and counted.

3.2.6.3. Microinjection of mRNA

Unless otherwise stated each oocyte received 40ng of mRNA (1mg/ml) in sterile distilled water. The oocytes were immobilised during the injection procedure using microforceps and were then transferred to a 5cm petri dish containing fresh Barth's medium and incubated at 21^{o}C.

3.2.7. Benzodiazepine-sepharose affinity column purification of oocyte produced receptor

300 oocytes each received 40ng whole rat brain mRNA and were incubated for 48 hours, 21^{o}C, with 0.3mCi[^{35}S]-methionine in Barth's medium. After excess medium was drained off, the oocytes were resuspended in HEPES buffer (10ml), and

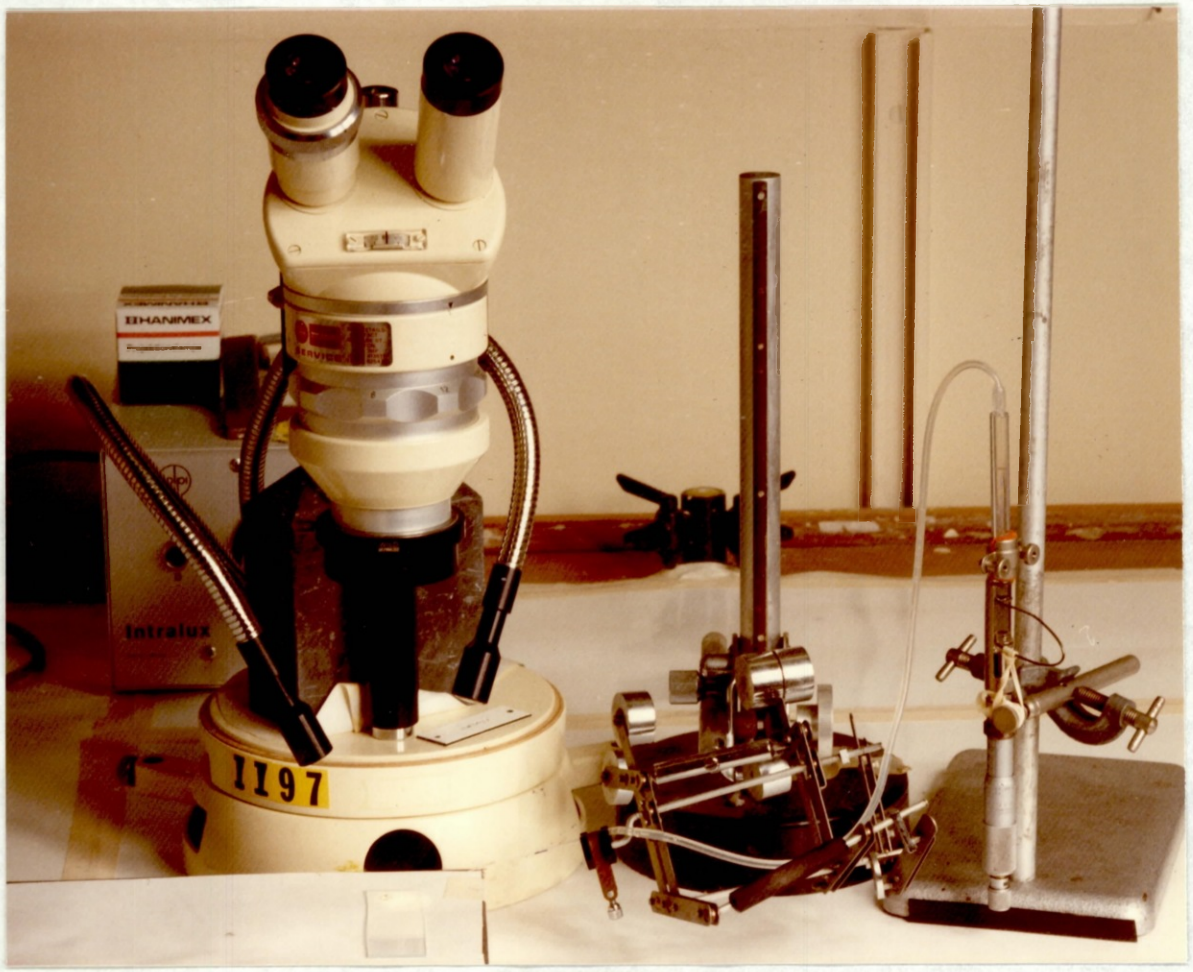


FIGURE 31

Microinjection equipment.

HEPES Buffer:

10mM HEPES pH7.5 with KOH
1mM EDTA
1mM EGTA
1mM benzamidine HCl
0.1mM phenylmethylsulfonylflouride (PMSF)

homogenised for 15 seconds at 3/4 maximum speed using a Polytron homogeniser. The volume was then adjusted to 20ml, stirred at $4^{o}C$ and the following were added dropwise; 20% sodium deoxycholate to 0.5% final concentration and 3.5M KCl to 150mM final concentration.

The HEPES buffer was left to stir for 30 mins after which the solution was loaded into 10ml polycarbonate screw cap bottles in a TI50 rotor and spun at $100,000_{gav}$ for 70 mins. (Beckman L2-65B ultracentrifuge). The supernatant was poured off and shaken for 1 hour with 0.5ml of Ro7-1986/1 adipic dihydrazide-agarose affinity gel (2.1.2) The gel solution was added to an 11ml Biorad disposo-column at $4^{o}C$ with the flow through repassed once (10ml/hour). The column was then washed three times with 5ml of Wash 1 buffer (10ml/hr). The column then received 5ml of wash 2 buffer (10 ml/hr).

Wash 1 Buffer:

10mM potassium phosphate pH7.4 with KOH
50mM potassium chloride
0.1mM EGTA
0.2% (w/v) sodium azide
2mM Magnesium acetate
10% (w/v) sucrose
0.2% (v/v) Triton X-100

Wash 2 Buffer:

20mM potassium phosphate
0.02% (w/v) sodium azide
2mM magnesium acetate
10% (w/v) sucrose
0.2% (v/v) Triton X-100

An Elution buffer was then made up in the following way.

Elution Buffer:

The following were dissolved in 15ml distilled water:
80mg chlorazepate (10mM)
2g sucrose (10% w/v)
200ul of 20% (w/v) Triton X-100 (0.2% w/v)
then add:
200ul 1M potassium phosphate (10mM)
and pH to 7.4 with 50mM phosphoric acid
finally add:
20ul 2M magnesium acetate (0.2mM)
adjusted volume to 20ml and degassed.

After addition of elution buffer, twenty 150ul fractions were collected and stored at -20°C.

3.2.8. Flunitrazepam photoaffinity labelling of oocyte homogenates

Batches of 50 oocytes were homogenised and rinsed twice in 1ml of ice cold phosphate buffer then homogenised in 1ml of the same buffer. The homogenate was transferred to a

siliconised eppendorf tube and centrifuged for 30 minutes, 4°C, in a bench top microfuge. The pellet was resuspended in 400ul phosphate

Phosphate Buffer

20uM potassium phosphate (pH7.5 with K0H)
0.1mM EDTA
1mM PMSF
0.02% (w/v) sodium azide

buffer. Injected and non-injected oocytes were used as follows. The oocyte homogenate received either 50ul phosphate buffer of 50ul flunitrazepam (10uM final concentration). [^{3}H]-flunitrazepam was added (50ul) to a final concentration of 100nM and the reaction mix incubated on ice in scintillation vial caps for 60 minutes. After the incubation, microstir bars were added, 1 per cap. The caps were transferred to a magnet stirrer cooled at 4°C and positioned six inches below a 360nm ultraviolet light source. The stirring reaction mix was given a six minutes exposure, then a 15 minutes cooling period on ice. This was repeated three times. Each vial cap then received 50ul of unlabelled flunitrazepam (final concentration 10uM) and incubated for 60 minutes in the dark. The reaction mix was transferred to a .liconised eppendorf tube and centrifuged for ten minutes at 4°C in a benchtop microfuge. The pellet was washed in 1ml phosphate buffer containing 25% (v/v) ethanol, then recentrifuged and resuspended in

1ml of this buffer. This was repeated five times. The final pellet was resuspended in gel sample buffer (see section 2.1.7.) before electrophoresis on tube gels.

Tube Gel Electrophoresis

Tube gels (13cm) were prepared as follows:

Resolving gel (10%)	
30% acrylamide, 0.8% bis-acrylamide	3.3ml
3M Trizma pH8.9	1.25ml
deionised distilled water	0.3ml
10% ammonium persulphate	35ul
TEMED	10ul

The gels were allowed to polymerise under a top layer of water saturated butanol. Samples were boiled for 5 minutes at 100°C with vigorous vortexing every two minutes. The samples were loaded and electrophoresed into the gel at 2 milliamps/tube. Once in the gel the samples were run at 5 milliamps/tube until the dye marker reached the bottom of the tube. The gels were removed from the tubes by positive pressure, sliced into 2mm sections and dissolved overnight in 400ul Soluene 350 (Packard) in capped scintillation vials. The samples then received 5.2mls of scintillant (1 litre Toluene; 5.5g permablend (Packard); 0.4ml glacial acetic acid.

3.2.9. Binding Assay to oocyte homogenates

Batched of 25 oocytes were microinjected with mRNA and incubated in Barth's medium for 48 hours, 21°C. Following homogenisation in phosphate buffer, assays in triplicate received the following;

Phosphate buffer

20mM potassium phosphate
0.1mM EDTA
0.02% (w/v) sodium azide
1mM PMSF
pH7.4 with KØH

Oocyte homogenate diluted to 450ul with phosphate buffer, 50ul phosphate buffer or 50ul unlabelled flunitrazepam (10uM concentration), and 50ul [^{3}H]-flunitrazepam (30nM final concentration). The reaction mix was incubated for 60 minutes, 4°C, then centrifuged in 15ml siliconised corex tubes, $14{,}000_{gav}$, 15 minutes, 4°C. The supernatant fluid was gently decanted off and the pellet superficially washed two times with ice cold phosphate buffer. The pellet was dispersed in 500ul Soluene 350 (Packard), then heated to 60°C for 60 minutes before receiving 4ml of scintillant (4 litres Toluene (AnalaR)/28g 2,5-Diphenyloxazole/2.4g, 1,4-Di-C4-methyl-5-phenyl-oxazoyl benzene (Fisons).

3.3. Results and Discussion

3.3.1. Preparation of Chick Brain mRNA by the Phenol/chloroform method

Fresh and frozen day 19 embryonic chick brain tissue was used to prepare RNA. To reduce carbohydrate and protein contamination of the nucleic acid extract, a high volume to tissue ratio (~1g tissue/10-20ml of phenol-pH9 buffer) was used. A critical step in this procedure was the first phenol extraction (at which most losses occurred). The interface between organic and aqueous layers required thorough re-extraction in phenol-pH9 buffer to partition all nucleic acids into the aqueous layer. The yield of total RNA following phenol/chloroform extraction was 0.1% of the starting tissue weight (mean of 5 experiments).

Before isolation of mRNA, the total RNA was routinely assayed by Formamide gel electrophoresis (Figure 32) to determine if there was any significant degradation of the RNA. Subsequent isolation of mRNA by oligo-dT cellulose chromatography gave a yield of 2.1%

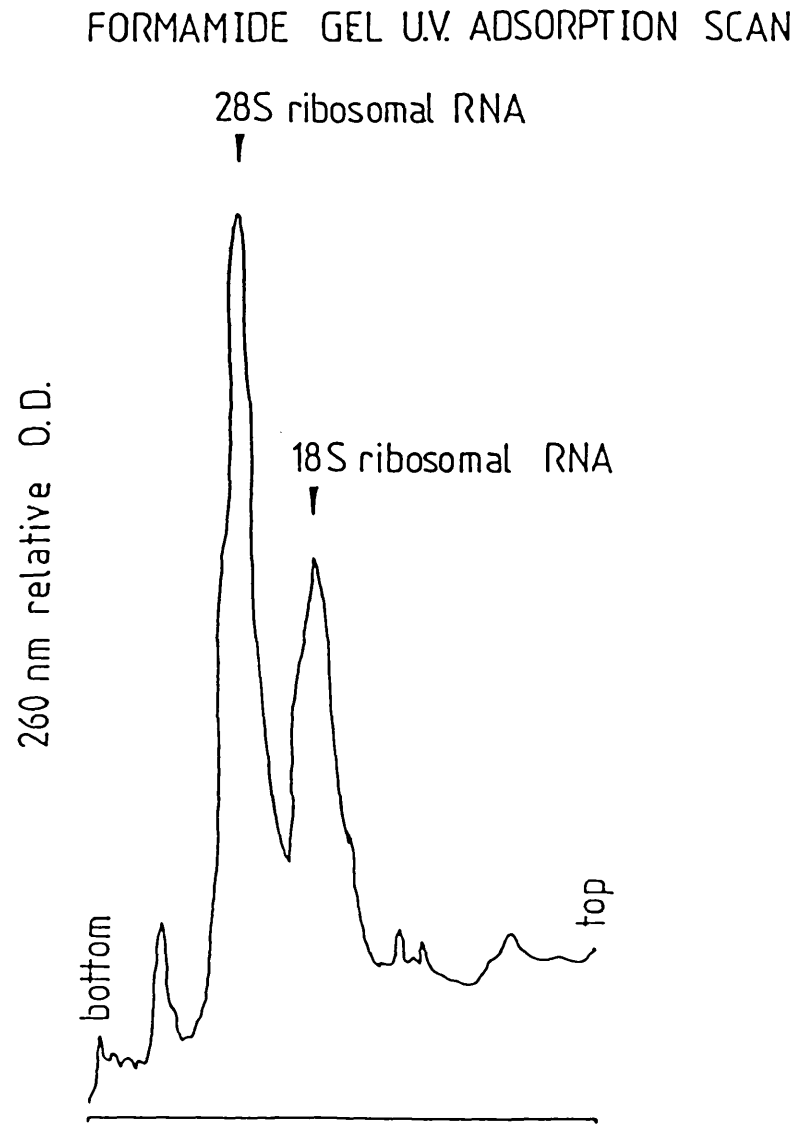


FIGURE 32

U.V. adsorption scan of a formamide tube gel. Each gel received 20ug total RNA or 20ug 18S/28S markers. The gels were run for 4 hours at 5mA. Absorption of U.V. light at 260nM was achieved using a gel scanner.

mRNA (mean of 5 experiments) (Figure 33).

Isolation of chick brain RNA by the phenol/chloroform method was long and required close attention to prevent a chance contamination by ribonculease. The drawbacks of this method were possible coprecipitation of carbohydrate (which was very difficult to remove) and a variable low yield. Formamide gel electrophoresis did confirm that most RNA prepared by this method was undegraded, but overall yield of mRNA was correspondingly low.

3.3.2 Assessment of Chick Brain mRNA Activity in Translation Assays

The suitability of mRNA for use in microinjection experiments was determined using a rabbit reticulocyte lysate assay to assess translation ability and gel electrophoresis to check for degradation of the mRNA extract.

The translation ability of mRNA was assayed in the cell free lysate by timed incorporation of [^{35}S]-methionine (Figure 34). Alternatively samples were taken at 20 and 40 min time points or more routinely after 60

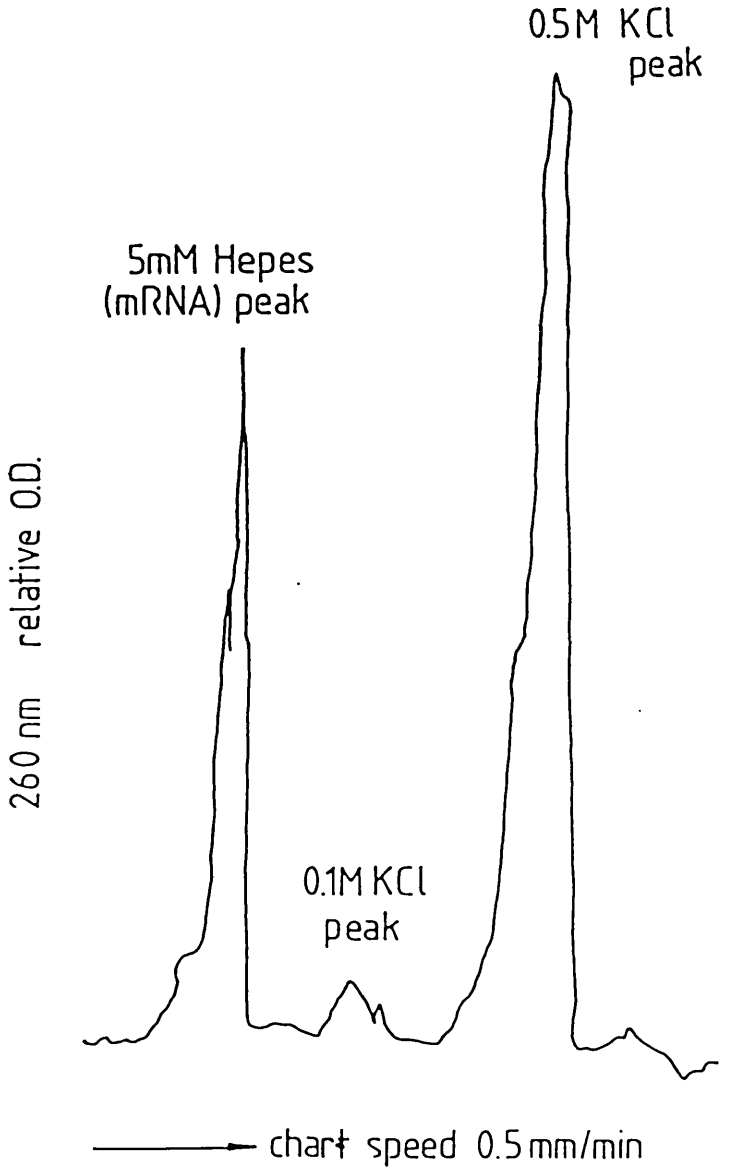


FIGURE 33

Typical elution profile for isolation of mRNA by oligo dT cellulose chromatography.

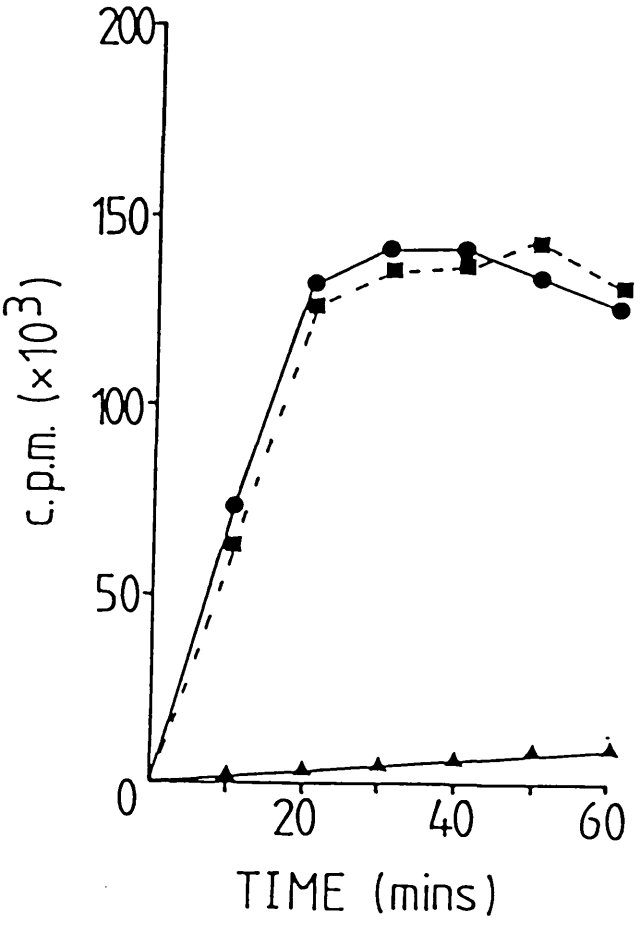


FIGURE 34

Time course incorporation of [^{35}S]-methionine by a rabbit reticulocyte lysate cell free assay. Each lysate mix (40ul) (run in duplicate) received lug of mRNA. (●) chick brain mRNA; (■) rat brain mRNA; (▲) no mRNA added.

minutes incubation, added to gel sample buffer and subjected to SDS polyacrylamide electrophoresis. The gel was then stained with Coomassie blue, dried and exposed for autoradiography (Figure 35).

Active intact mRNA displayed the following characteristics; a greater than 10 fold stimulation of incorporation of [^{35}S]-methionine label over endogenous (reticulocyte lysate mRNA) incorporation in parallel assays (Figure 34); production of labelled proteins over a long time period (40 - 60 mins), concurrent with production of higher molecular weight labelled protein as shown in Figure 2. mRNA prepared from fresh and frozen tissue displayed no measurable differences in activity in these experiments (Figure 35).

3.3.3. Microinjection of Chick Brain mRNA

Detection of mRNA coding for the GABA/BZ receptor was accomplished by microinjection of whole mRNA into oocytes of Xenopus laevis (40ng.oocyte) followed by electrophysiological assay of the expressed protein. These experiments were performed in

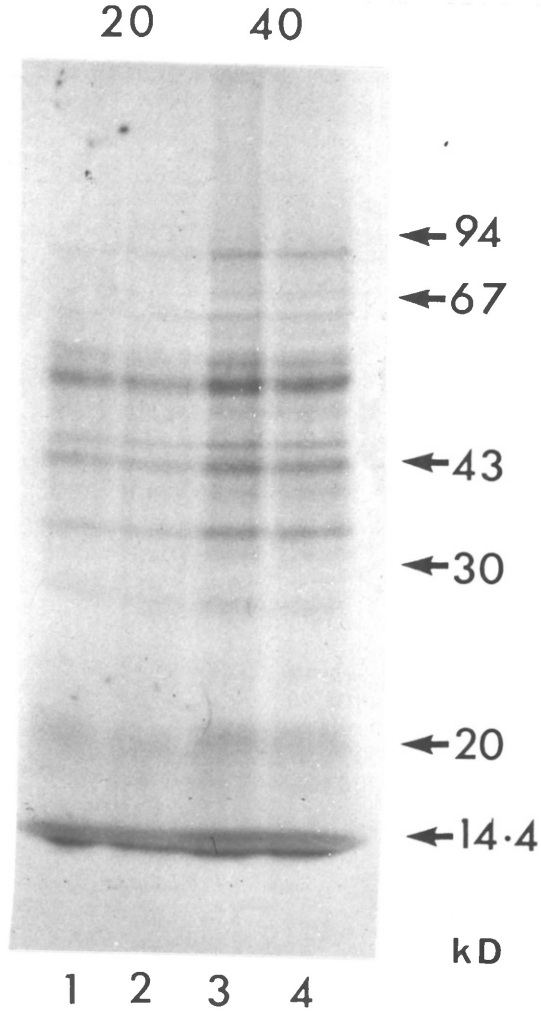


FIGURE 35

20 + 40 minute samples taken from a cell free lysate of mRNA (prepared from fresh and frozen chick brain), subjected to electrophoresis and autoradiography. Each lysate sample (40ul) received 1ug mRNA and was incubated at 37°C. 2ul samples were added to 50ul gel sample buffer heated to 100°C for 2 minutes and subjected to electrophoresis (12% polyacrylamide gel) at 50V for 16 hours then fixed and autoradiographed. Track 1 and 3 contains mRNA prepared from fresh chick brain. Track 2 and 4 contains translation products of mRNA prepared from frozen chick brain mRNA.

a collaborative study with Dr. A. Constanti, Dr. T. Smart and K. Houamed who performed the recordings shown.

Injected cells were kept in Tris buffered Barth's medium, plus 50 ug/ml gentamycin and incubated for 24 - 48 hours before use. Oocytes were secured to a sylgard base of the recording chamber by placing within a circle of pins. The cells were impaled with two microelectrodes (placed within 50 um of each other): one filled with 1.5 M potassium citrate for voltage recording, and the other with 0.6 M potassium sulphate for current injection. Potentials were monitored on a storage oscilloscope and recorded on a Brush-Gould 8000 chart recorder. Cells were continuously superfused with Frog Ringer solution (at room temperature, 20 - 23^{o}C) containing (uM); NaCl 118, KCl 1.9; $CaCl_2$ 2; Trizma base 2.5; buffered to pH 7.4 with HCl.

Non-injected and sham-injected oocytes displayed no response to bath applied GABA. Figure 36a shows typical responses to GABA in injected oocytes; the conductance response was directly proportional to GABA concentration (Figure 36b). Similarly GABA agonists,

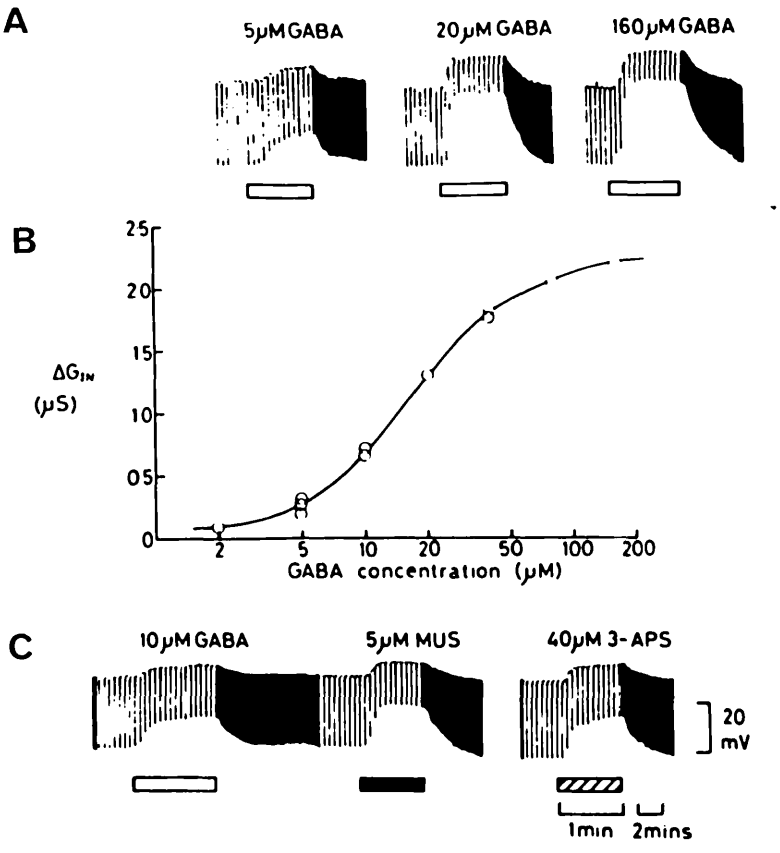


FIGURE 36

A. Membrane conductance changes evoked by bath applied GABA in a single Xenopus oocyte, recorded 1 day after injecting chick whole brain mRNA. Downward pen deflections are electrotonic potentials evoked by intracellularly applied hyperpolarising current pulses (40nA, 1s, 0.2Hz). Upward baseline shifts are membrane depolarisations produced by GABA (applied during periods indicated by bars). Note chart speed was slower during the offset of the responses. The resting potential was -35mV.

B. Corresponding GABA log dose/conductance relationship. Ordinate, change in input conductance (ΔG_{in};uS); abscissa, GABA concentration

C. (Different mRNA-injected oocyte; same batch) comparison of responses produced by GABA, muscimol (MUS) and 3-amino propanesulphonate (3-APS) on the same cell. The resting potential was -33mV; injected current pulses were 35nA, 1s, 0.2Hz.

muscimol and 3- amino propanesulphonate depolarised the oocyte membrane, an effect reversibly antagonised by 1Ø uM bicuculline (Figure 36c). Muscimol was 4 times more potent than GABA in producing a response. No responses to baclofen ($GABA_B$ ligand 1 uM) were found. All of these effects were reversible following a wash period in Ringer solution.

In accordance with other biochemical and eletrophysiological studies (Olsen, 1981; Simmonds, 1981) the induced GABA receptor response was enhanced in the presence of chlorazepate (1 - 1Ø uM) and pentobarbitone (25 - 1ØØ uM). Chlorazepate was more potent than pentobarbitone in enhancing the GABA response (Figure 37) which was reversibly blocked by bicuculline (1Ø uM). Both control and enchanced responses were blocked by picrotoxinin (1Ø - 1ØØ uM).

The pharmacological sensitivity of the GABA/BZ receptor population produced by chick brain mRNA-injected oocytes was representative of that for vertebrate central neurones, i.e. GABA, muscimol and 3-aminopropanesulphonate were all active. Benzodiazepine and barbiturate sites were present; bicuculline

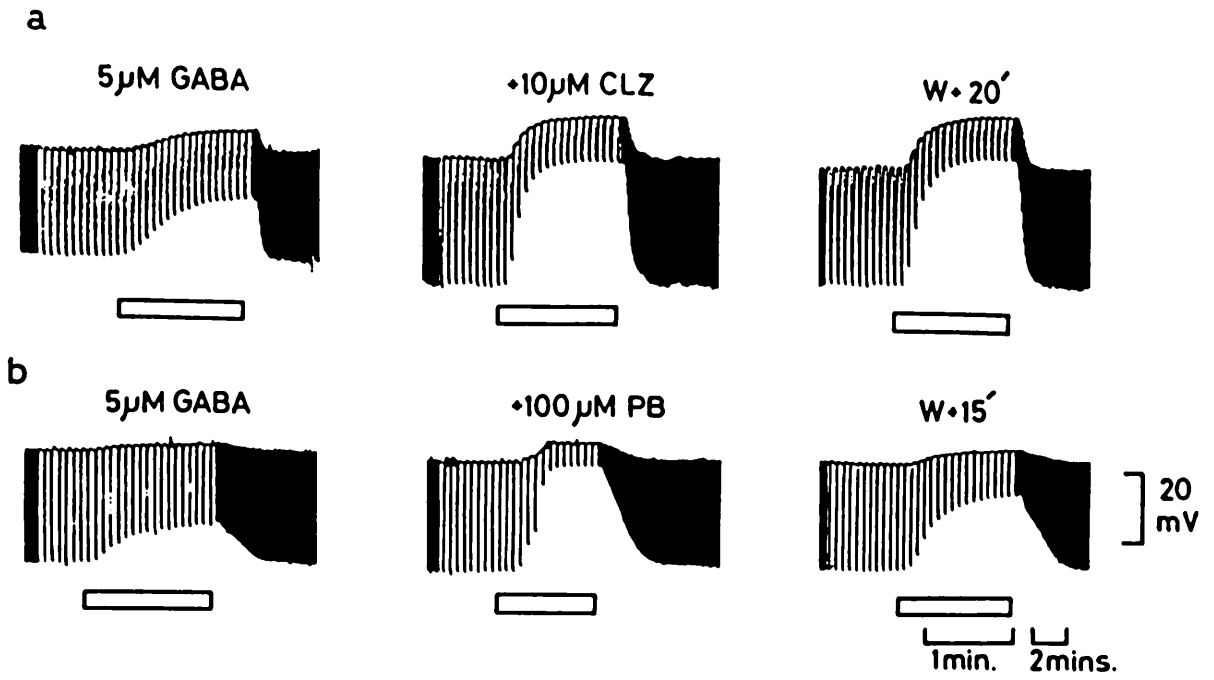


FIGURE 37

Enhancement of GABA-evoked conductance change in chick mRNA-injected oocytes by chlorazepate (CLZ) or pentobarbitone (PB).

A. Responses to GABA in control solution, after 12 minutes, 10um CLZ was added, then after a 20 minutes wash period with ringer solution (resting potential -40mV).

B. GABA response in control solution, after 10 minutes in PB, then after a 15 minute wash. (Different oocyte, same batch, resting potential -38mV). Note partial recovery of GABA response after PB but not after CLZ exposure.

and picrotoxinin had antagonistic action on the induced receptor. These observations were confirmed in part by Miledi et al., (1982), indicating that the partly purified chick brain mRNA can direct the synthesis and membrane insertion of the functional subunits comprising the GABA/BZ receptor.

Also the induced receptor responses in the Xenopus laevis oocyte correlated with radioligand binding experiments on 18 - 20 day embryonic chick brain membrane preparations. Frozen-thawed and washed membranes demonstrated binding of [^{3}H]-muscimol (0.5 - 0.7 pmol/mg protein) and [^{3}H]-flunitrazepam (0.6 - 1.6 pmol/mg protein) in a centrifugation assay (Stephenson et. al., 1982). That these binding sites resided on the same receptor complex was suggested by the allosteric enhancement of [^{3}H]-flunitrazepam binding by up to 20% in the presence of 0.1 mM GABA (Table 10).

3.3.4. Preparation of mRNA from Rat Brain

Rat brains varying in age from 24 hours pre- and post-parturition to 21 days were used to prepare mRNA by the

TABLE 10

Binding of [^{3}H]-muscimol and [^{3}H]-flunitrazepam to embryonic chick brain membranes

Age of embryonic chick brain	Binding (pmol/mg protein)		% enhancement in 0.1mM GABA	Activity in electrophysiology assay
	Muscimol (16nM)	Flunitrazepam (3nM)		
18	0.62	0.56	14	+
19	0.53	1.66	16	+
20	0.61	1.18	20	+

phenol-chloroform method. The yield of mRNA from total RNA in these experiments was 3.55% (mean of 12 experiments). The younger aged rat brain preparations (24 hours pre-parturition to 5 day old) gave slightly higher yields (4 - 4.2%) of mRNA.

Rat brain mRNA was assayed for activity in a similar way to that of chick brain mRNA (3.1.2). Parallel rabbit reticulocyte lysate assays of chick and rat brain mRNA produced high molecular weight, labelled proteins (Figure 38). There were no apparent differences between lysates of various age rat brain mRNA (Figures 39 and 40).

Rat brain mRNA produced a slightly different banding pattern following autoradiography of reticulocyte lysate products compared to chick brain mRNA, but there were no measurable differences in a time course study. Glyoxal gels also did not distinguish between rat and chick mRNA preparations (Figure 41), but did provide another method of determining that mRNA was not degraded.

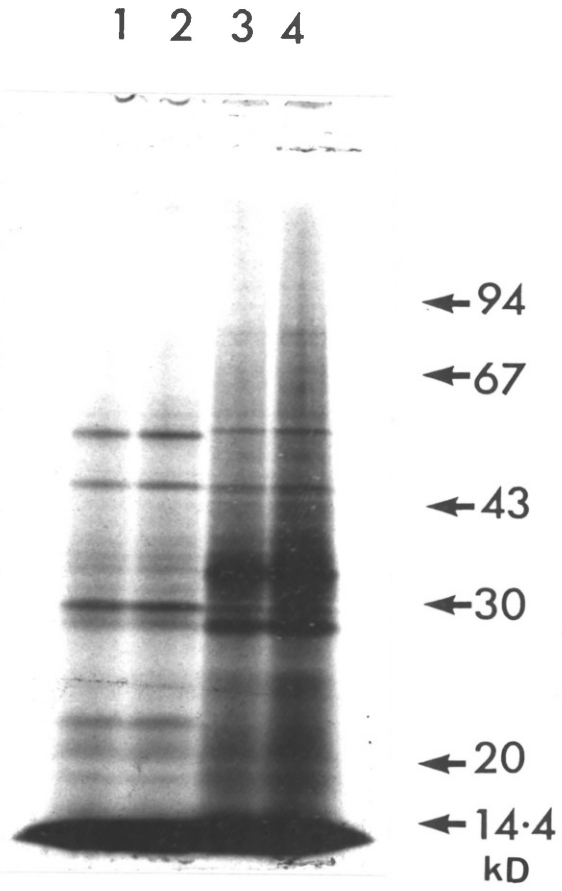


FIGURE 38

Autoradiograph of chick and rat brain mRNA cell free lysates following 12% polyacrylamide gel electrophoresis. Track 1 contains lysate proteins synthesised from frozen tissue day 19 embryonic chick brain mRNA (1ug mRNA/40ul lysate). Track 2 contains lysate proteins synthesised from fresh tissue day 19 embryonic chick brain mRNA (1ug mRNA/40ul lysate). Track 3 contains lysate proteins synthesised from 5 day old rat brain mRNA (2ug mRNA/40ul lysate). Track 4 contains lysate proteins synthesised from 8 day old rat brain mRNA (3ug mRNA/40ul lysate). The standard proteins were; phosphorylase b (94k); bovine serum albumin (67k), ovalbumin (43k), carbonic anhydrase (30k), soybean trypsin inhibitor (20.1k) and alpha-lactalbumin (14.4k).

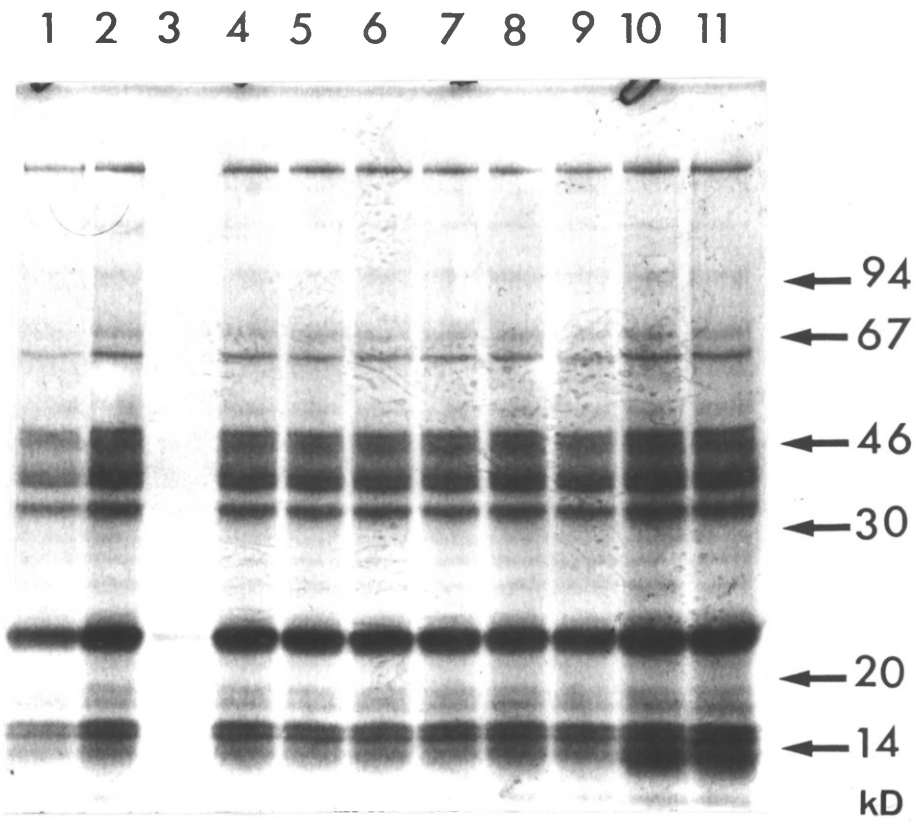


FIGURE 39

Coomassie blue stain of duplicate reticulocyte lysate assays after electrophoresis on a 12% polyacrylamide gel. Tracks 1 and 2 contained lysate proteins for day 19 embryonic chick brain mRNA. Track 3 no added mRNA. Tracks 4 and 5 day 21 rat brain mRNA. Tracks 6 and 7 day 14 rat brain mRNA. Tracks 8 and 9 frozen-thawed day 14 rat brain mRNA. Tracks 10 and 11 day 10 rat brain mRNA. Standards as for Figure 5. Each 40ul lysate mix received lug of mRNA.

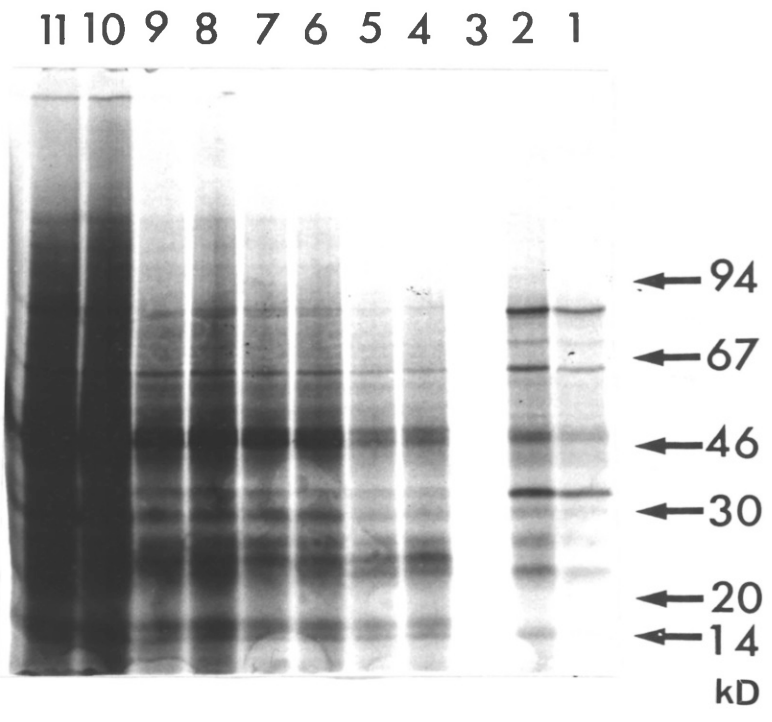


FIGURE 40

Autoradiograph of gel in Figure 39.

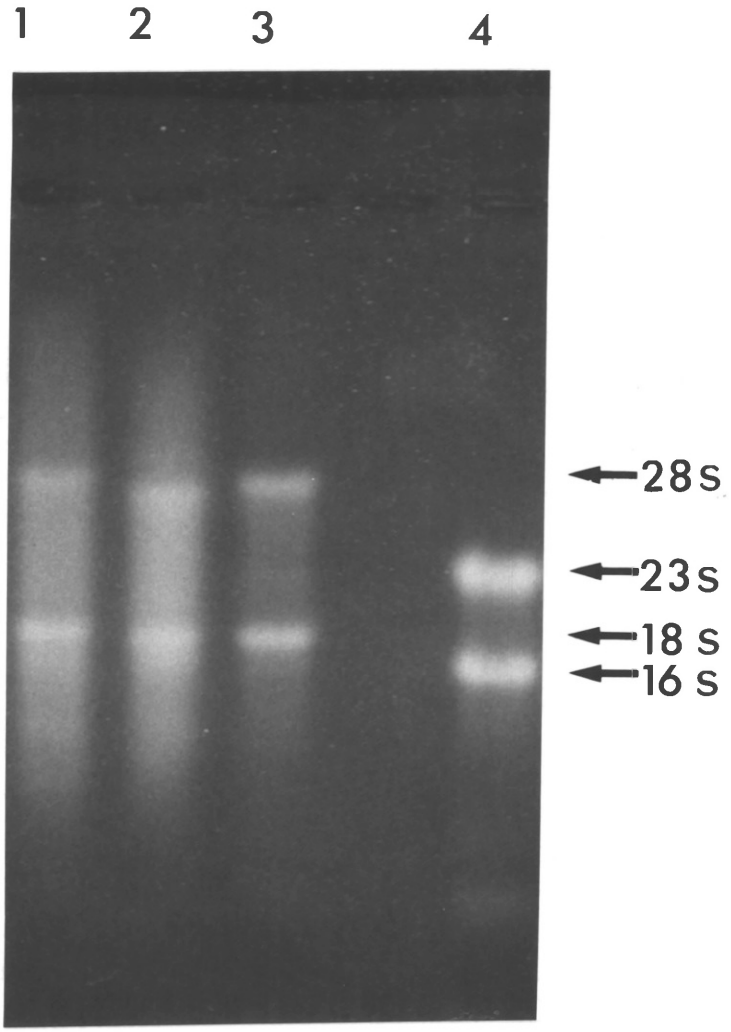


FIGURE 41

Gloxal-agarose gel of chick and rat brain mRNA.

Each track received approximately 5ug of mRNA and was electrophoresed fo 4 hours at 25mA.

1.rat brain mRNA

2.chick brain mRNA.

3.18-28 S markers.

4.16-23 S markers.

3.3.5. Microinjection of Various Age Rat Brain mRNA

Xenopus oocytes received 40 ng of these mRNA's per cell and were incubated for 48 hours, then subjected to the electrophysiological assay procedure. Day 14 rat brain mRNA-injected oocytes gave good responses to both applied GABA, muscimol being markedly more potent (about 4 times) than GABA in evoking a depolarising response, although these responses were less sensitive than those produced by chick brain mRNA-injected oocytes (Figure 42c). No activity was detectable in embryonic brain (Figure 42a) up to day 12 which exhibited a slight activity. The peak of activity was at day 14 then declined again to low levels at day 21.

The translation of day 14 rat brain mRNA in oocytes to produce GABA/BZ receptors was similarly displayed by 13-16 day rat brain preparations. Rats of this age were then routinely used as a convenient tissue source for mRNA preparation. This finding agrees with binding studies on developing rat brain, Palacois et al., (1979) demonstrated a 500% increase in [^{3}H]-muscimol binding to

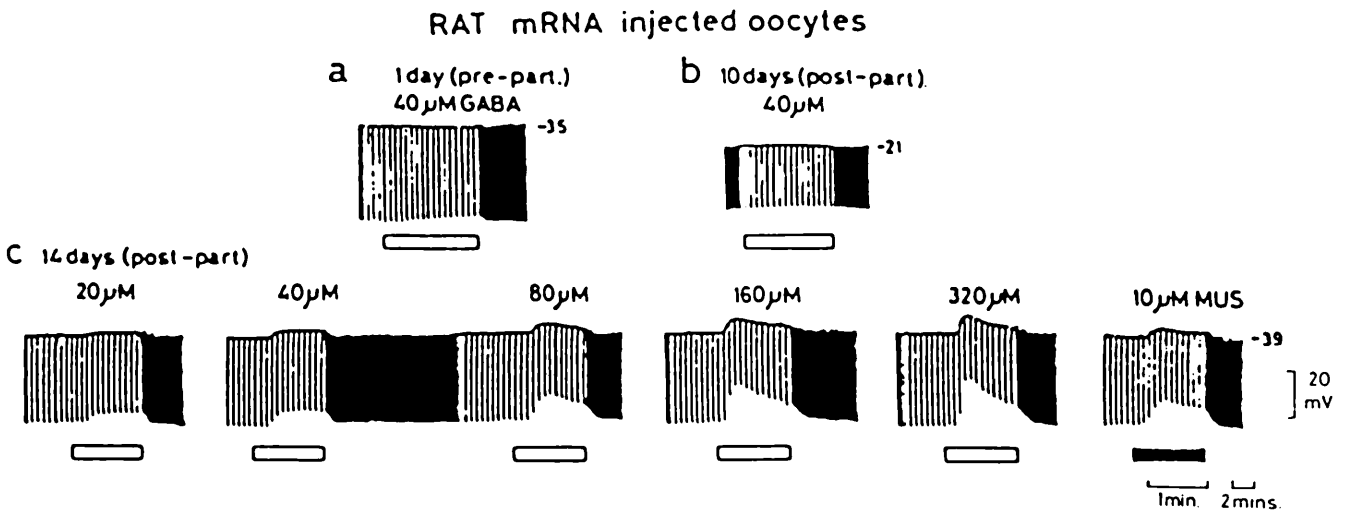


FIGURE 42

The GABA dependent conductance change in oocytes injected with mRNA extracted from whole rat brain during development.

A. From rats 1 day preparturition

B. 10 days post parturition

C. 14 days post parturition with dose-dependent responses to the GABA concentrations shown and to muscimol (MUS).

developing rat brain aged between two and three weeks post natal. To produce such an increase in receptor concentration requires a large pool of enriched receptor mRNA; subsequent isolation of mRNA at this age provided a mRNA preparation containing proportionally more mRNA for GABA/BZ receptor.

3.3.6. Preparation of mRNA by the Guanidinium Thiocyanate Method

This method was found to be much easier and quicker than the phenol/chloroform methods. It exploited the rapid solubilisation and denaturation of the source tissue by guanidinium thiocyanate and fractionation of RNA from protein, DNA, carbohydrate, etc., was achieved by centrifugation through a 5.7 M caesium chloride cushion. The buoyant density of the RNA was such that it passed freely through the cushion to form a transluscent pellet whilst DNA, protein, etc. remained in a cloudy layer above it.

When this method was combined with 2 runs down an oligodT-cellulose column the yield of mRNA (4% of total RNA - mean of 15 experiments) was greater than the phenol/chloroform method. The greater yield, ease of preparation and reproducibility of this method made it the method of choice for all further mRNA extractions. Following microinjection of *Xenopus laevis* oocytes, this mRNA not only produced the GABA/BZ receptor but also glycine, glutamate and kainate receptor types (Appendix 1). The results of these experiments will be discussed in the following sections.

3.3.7. Microinjection of day 14 Rat Brain mRNA prepared by the Guanidinium Thiocyanate Method

All injected oocytes responded to bath applied GABA by changing their resting potential and conductance. Control, i.e. non-injected or sham-injected oocytes (using distilled water or fibroblast mRNA) showed no sensitivity to GABA. Bath applied GABA produced a smooth membrane depolarisation

coupled with an increase in conductance which was reproducible and reversible in the same oocyte and from oocytes of several donors (Figure 42 and 43). The GABA antagonist, bicuculline (20uM), reversibly antagonised the GABA response, while picrotoxinin (1 - 5 um) reversibly depressed the GABA response (Figure 44). As for chick brain mRNA-injected oocytes, chlorazepate (10uM) and pentobarbitone (60uM) enhanced the GABA response. The 10uM GABA response was enhanced to the level of a 40uM GABA response in control solution as shown by the end response of Figure 45. Unlike chick brain mRNA-injected oocytes, rat brain mRNA was not stable to repeated freeze-thaw. The response of oocytes injected after a second freezing of the mRNA was remarkably low.

Rat brain mRNA injected oocytes displayed responses to GABA, benzodiazepines and barbiturates which are characteristic of eletrophysiological assays on neuronal membranes (Gallagher and Shinnick-Gallager, 1983) and confirmatory to a brief study by Gundersen et al., (1984). The induced oocytes response also suggested that ligand-binding sites were present as for membrane bound

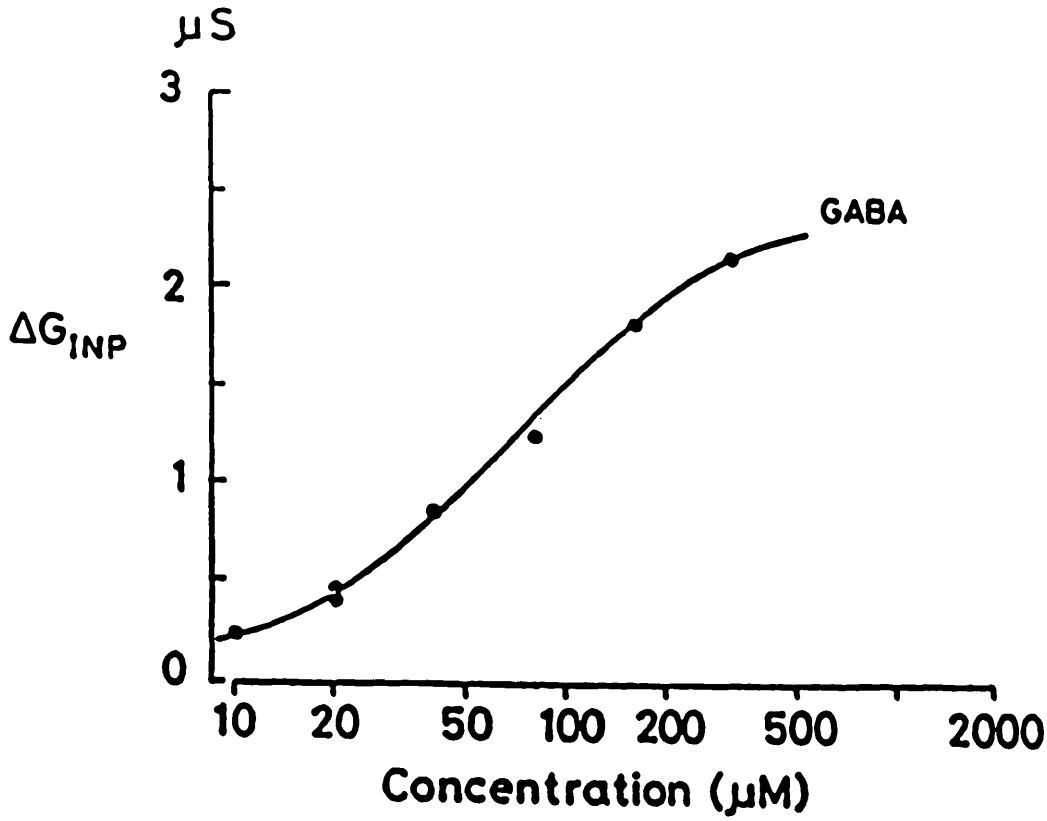


FIGURE 43

GABA dose-conductance curve. The membrane conductance changes were evoked by bath applied GABA in a single oocyte.

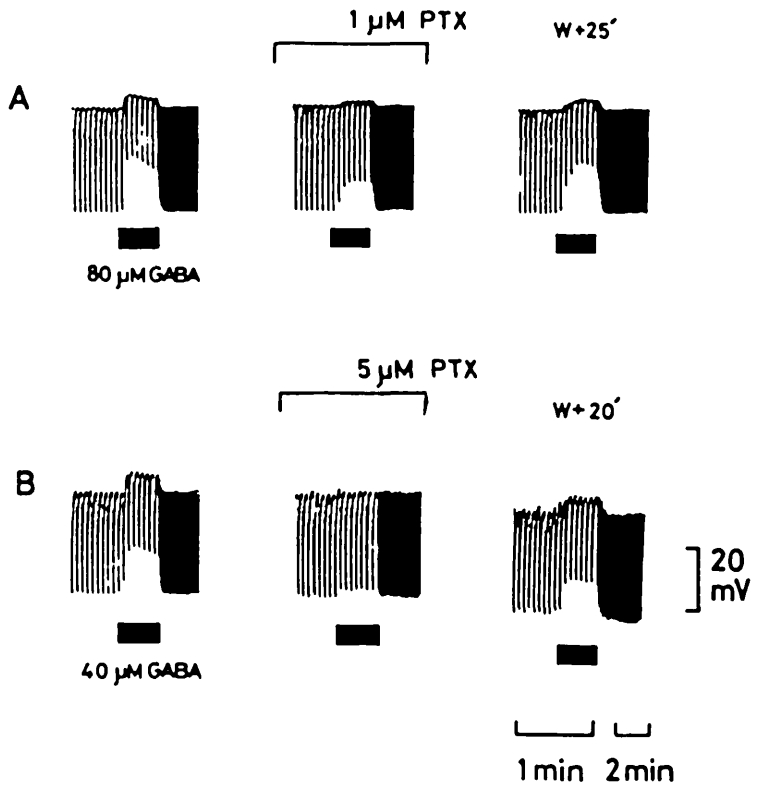


FIGURE 44

The effect of the antagonist picrotoxinin on the responses to bath applied GABA (responses obtained from two oocytes). Upward deflections of the trace denote membrane depolarisations and downward deflections are hyperpolarising electrotonic potentials.

A. Low-dose picrotoxinin (1uM), recovery after 25 minutes washing).

B. 5uM picrotoxinin, recovery after 20 minutes washing.

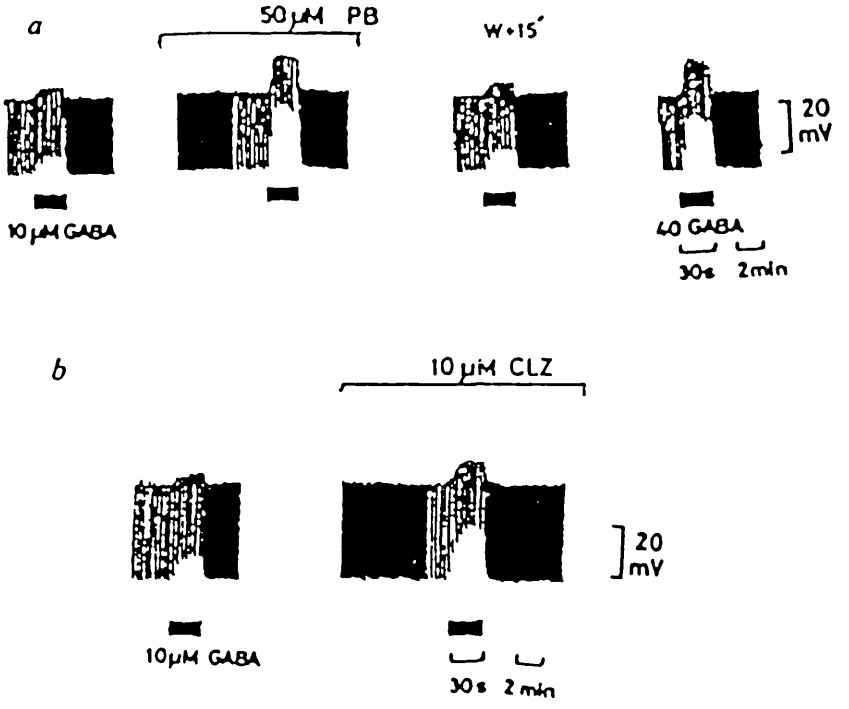


FIGURE 45

Enhancement of GABA evoked conductance changed by 50uM pentobarbitone (PB) and chlorazepate (CLZ) in two oocytes.

A. The response to 10uM GABA was recorded in control solution then after 5 mintues in PB and finally after a 15 minute wash in control Ringer. Resting potential -69mV. The 10uM GABA response was enhanced to the level of a 40uM GABA response in control solution as shown by the end trace.

B. The response to 10uM GABA in control solution then after 7 minutes in CLZ. Resting potential -62mV.

(Turner and Whittle, 1983) and purified receptors (Stephenson and Barnard, 1985). The stability of rat brain mRNA in these preparations is probably due to an artifact in the procedure.

To determine if glycosylation was necessary for the expressed receptor activity, injected oocytes (batches of 20) were incubated for 24 hours following injection in 2, 4, 6 and 8 ug/ml tunicamycin. Normal responses were obtained from all oocytes incubated in 2 ug/ml tunicamycin. Oocytes incubated in 4 ug/ml tunicamycin gave responses to 40 uM GABA which would normally be seen in a 10 uM GABA solution. Oocytes incubated in 6 and 8 ug/ml tunicamycin did not respond to bath applied GABA. Other expressed neurotransmitter receptors e.g. glycine and serotonin (see Appendix 1) were also absent at these concentrations, a property shared by chick and rat brain mRNA-injected oocytes.

The observed effect, that incubation of injected oocytes with tunicamycin stops expression of the receptor (as tested by this system) suggests, firstly, the receptor is

glycosylated; tunicamycin inhibits dolicholpyrophosphate-linked oligosaccharide synthesis, a primary step in the glycosylation process (Schwarz and Datema, 1980). Secondly, this glycosylation is required for receptor function in the oocyte membrane, i.e. under normal conditions (tunicamycin absent) the oocyte glycosylates the GABA/BZ receptor in such a way that a fully functional receptor is produced. This finding also agrees with studies on crude detergent extracts; that the GABA/BZ receptor is a glycoprotein (Gavish and Snyder, 1981).

3.3.8. Fractionation of mRNA by Sucrose Density Gradient Centrifugation

The sedimentation profiles of mRNA derived from day 19 embryonic chick brain and 14 day old rat brain are shown in Figures 46 and 47 respectively. To study the functional expression of the gradient fractions, oocytes were injected with 35 nl each of alternative fractions. The oocytes received between 5 and 25 ng of chick brain mRNA and between 8 and 23 ng of rat brain mRNA and were incubated for 24 - 48 hours, then tested for response to bath applied GABA.

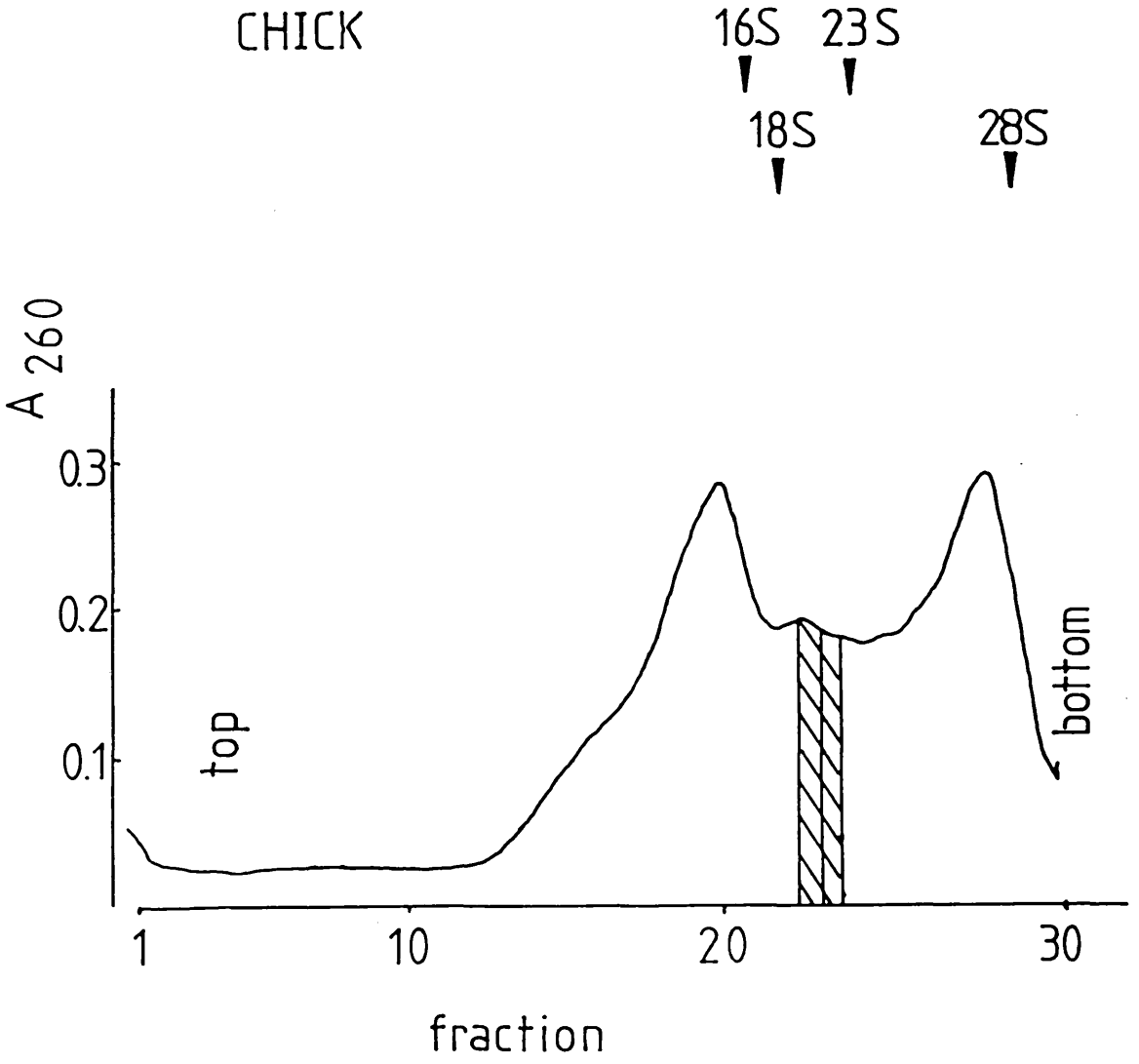


FIGURE 46

Sedimentation profile of poly(A)$^+$ mRNA derived from day 19 embryonic chick brain. After centrifugation, 0.4ml fractions were collected. The hatched bars represent those fractions which following injection into oocytes, responded to bath applied GABA.

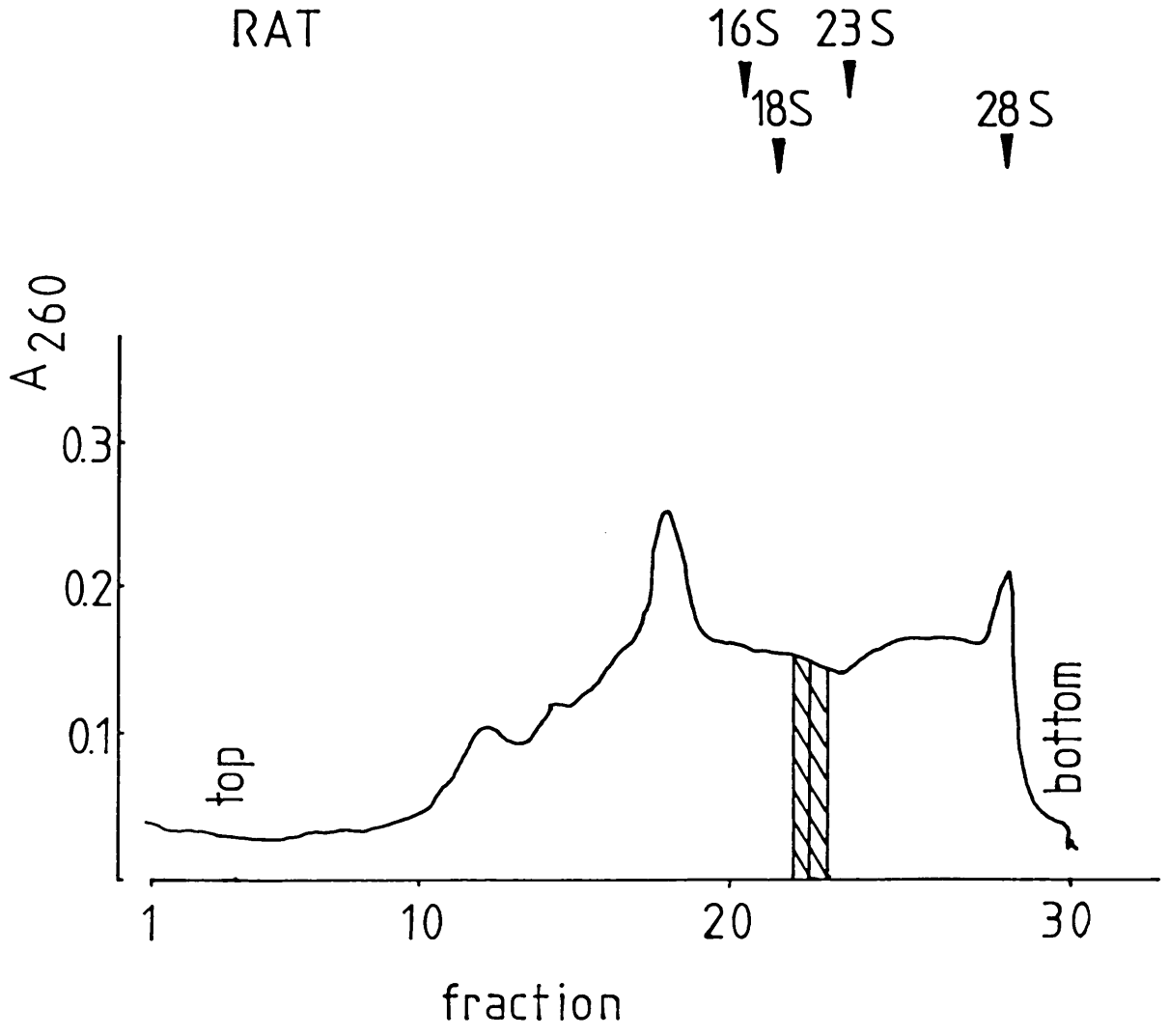


FIGURE 47

Sedimentation profile of 14 day old rat brain poly(A)$^+$ mRNA. After centrifugation 0.4ml fractions were collected. The hatched bars represent those fractions most responsive to bath applied GABA on injected oocytes.

In both chick and rat mRNA-injected oocytes the 21-22 S fractions responded to 1Ø uM GABA, although only the chick fractions exhibited the response seen with whole mRNA. The fractionated rat brain mRNA-injected oocytes were less sensitive to GABA (the response to a 4Ø uM dose was as for a 1Ø uM dose on the chick fractions), probably a result of the further 2 freeze-thaw cycles during the fractionation process.

If the GABA/BZ receptor was coded by a single mRNA species or by several mRNA species of similar size, then sharp peaks could be expected. But, as the GABA/BZ receptor is made of several subunits, all required to form a functional complex, then the mRNA species for these subunits would be of different sizes. if these were of disparate size, then they would sediment in different fractions. However, this is not what was found, because fractionated mRNA was effective in producing receptors. The profiles showed widespread distributions (over 5 fractions) which was probably due to mRNA aggregation. The size of the GABA/BZ receptor mRNA species, 21-22S, is for the fraction exhibiting the most marked sensitivity to bath applied 1ØuM GABA. This

finding was confirmed by Sumikawa et al., (1984), although these preparations required a higher concentration of GABA (1uM) and a longer oocyte incubation time (5 - 10 days).

3.3.9 Biochemical Assays on Oocyte produced Receptor

3.3.9.1. Affinity Column Purification of Rat Brain mRNA Injected Oocytes Incubated in [^{35}S]-methionine.

Batches of 300 oocytes were injected with rat brain mRNA and incubated for 48 hours in Barth's medium containing [^{35}S]-methionine. Table 11 represents the level of radioactivity at each stage in the purification procedure. The number of counts representing the purified material (chlorazepate eluate) was 0.0003% of the total counts present in oocyte homogenates. This compares favourably with the rat brain receptor purified protein which comprised 0.0008% of the total protein present in crude homogenates. Over 98% of the radioactivity applied to the column was washed off. Homogenates of non-injected oocytes incorporated only 20% of the total counts for injected oocytes.

TABLE 11

Yield of [^{35}S]-methionine labelled protein from affinity purification of oocyte produced GABA/BZ receptor protein.

Sample	Total cpm ($x10^8$)	% yield of total cpm
1. Homogenate	2.13	100
2. Soluble protein	1.82	85
3. Insoluble protein	0.314	15
4. Affinity column flow through	1.59	74.5
5. Wash no.1	0.145	6.8
6. Wash no.2	0.056	2.6
7. Wash no.3	0.0033	0.001
8. Chlorazepate eluate	0.00071	0.0003

Following elution with chlorazepate from the affinity column, an asymmetric elution peak was obtained (Figure 48). This may have been due to a slightly high flow rate; a similar effect described for bovine GABA/BZ receptor purification by Sigel et al., (1983). However, three different preparations displayed a similar profile and 66% of the eluted radioactivity was present in the first 10 fractions.

Samples from each stage of the purification procedure were electrophoresed and autoradiographed (Figure 49). Several bands in the predicted (from rat brain receptor) molecular weight range are apparent, but these bands are also present in the wash fractions. This suggests that the wash procedure was not extensive enough to remove radiolabelled protein contamination. The purification procedure was based on that for the rat receptor. In purifications of rat and chick brain GABA/BZ receptor, contaminating protein bands could be seen following electrophoresis when the protocol for the preparation of bovine receptor was followed (F. A. Stephenson, personal communication), suggesting the same wash procedure as

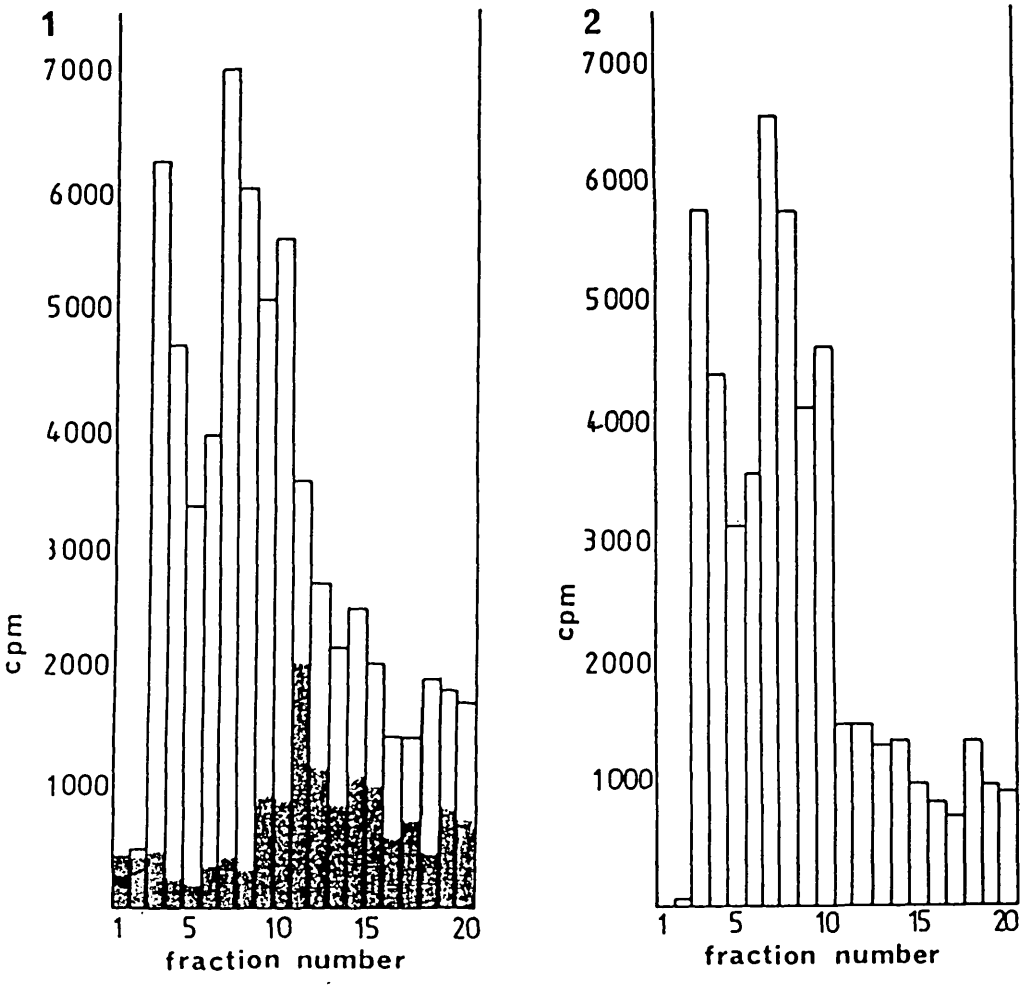


FIGURE 48

Histogram of chlorazepate eluate.

1. This graph represents total cpm in 150µl fractions of the chlorazepate eluate. The hatched area represents total counts from non-injected oocytes (same batch).

2. Specific counts remaining after subtraction of oocyte 'background' counts.

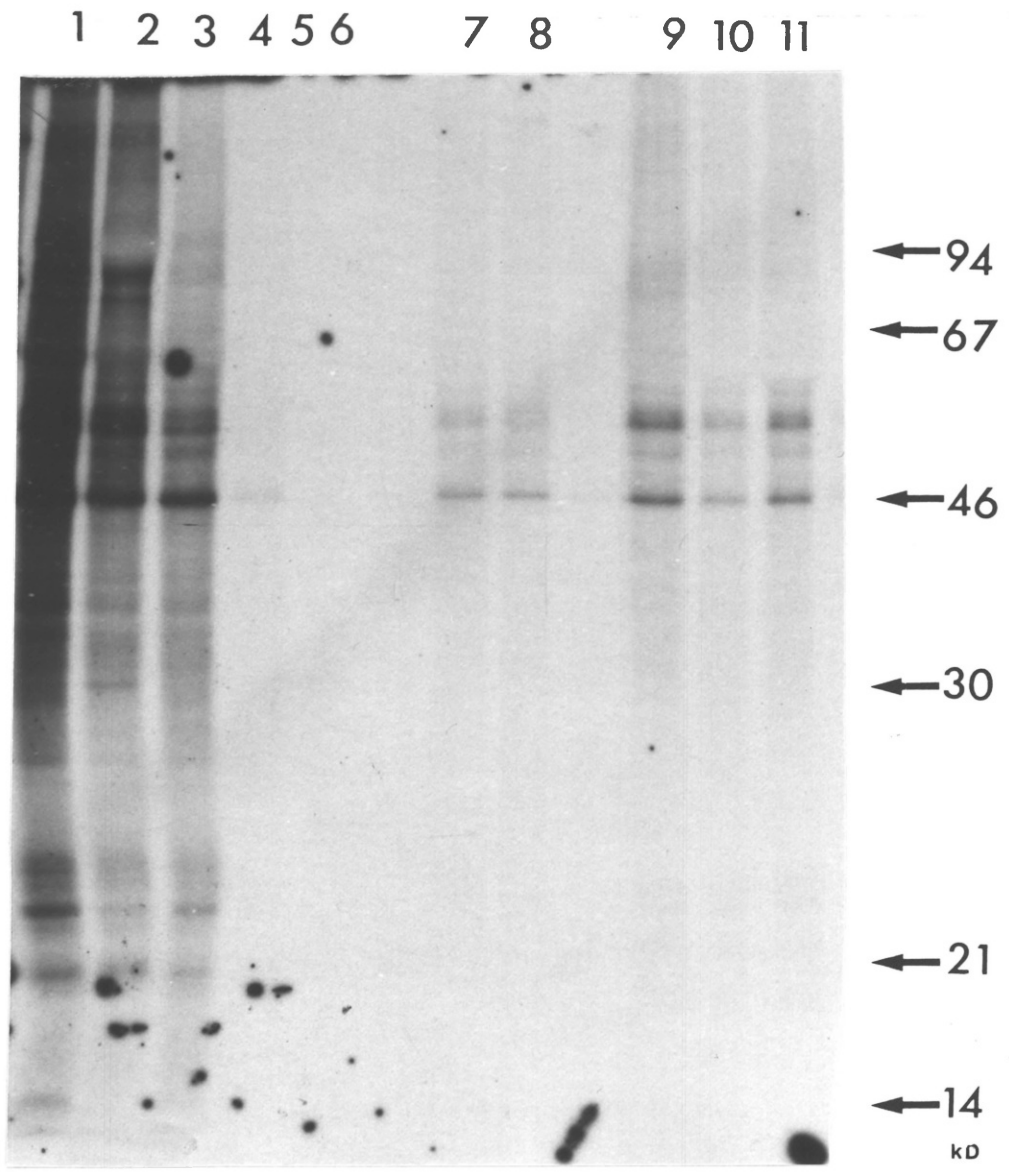


FIGURE 49

Autoradiograph of oocyte derived purification samples. 150ul aliquots of each sample were TCA precipitated and prepared for electrophoresis, then autoradiography. 1. soluble protein; 2.membranes; 3.Column flow through; 4.wash no.1; 5.wash no.2; 6.wash no.3; 7. elution fraction (ef) 3; 8.ef 5; 9.ef 10; 10.ef 14; 11.ef 20.

developed for rat brain requires modification to be more suitable for removal of other brain protein contaminants. To thoroughly remove oocyte protein contaminants will require an alteration in wash time and/or wash buffer composition to obtain optimum results. Thus, high percentage contaminants in the final extract may have masked the purified receptor protein.

3.3.9.2. Photoaffinity Labelling of Oocyte Homogenates

Batches of 50 oocytes microinjected with rat brain mRNA were subjected to photoaffinity labelling with [^{3}H]-flunitrazepam. Following electrophoresis in tube gels, slicing and counting, a peak of radioactivity migrating at approximately 53,000 molecular weight could be seen (Figure 50). The number of specific [^{3}H]-flunitrazepam counts in this peak was low but repeatedly observed in five experiments. High background levels of counts were present in control experiments (when unlabelled flunitrazepam was present) reflecting the low specific activity of the labelling ligand, low abundance of receptor protein in the oocyte

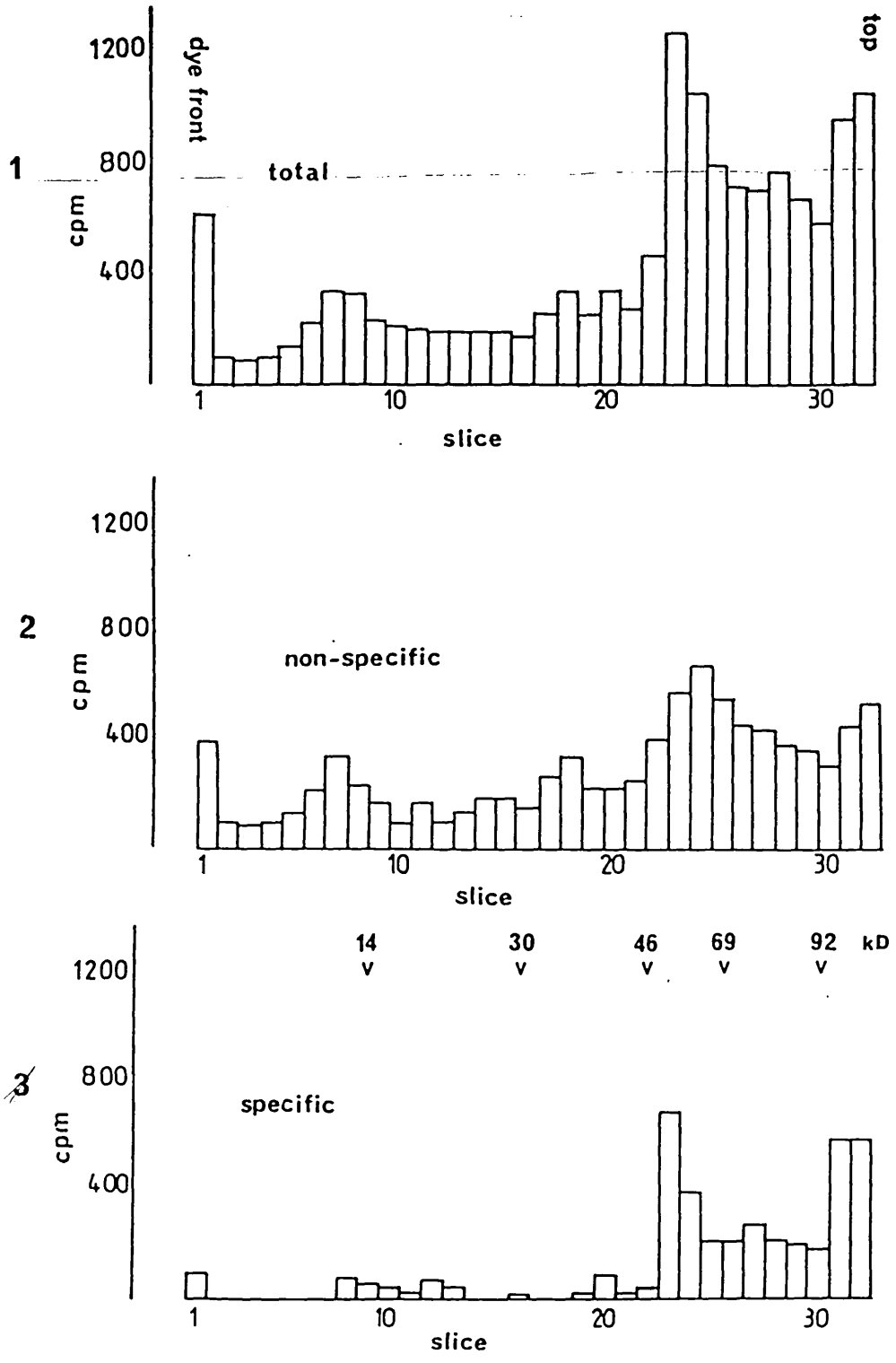


FIGURE 50

Photoaffinity labelling of oocyte homogenates.

Batches of 50 oocytes were incubated with [^{3}H]-flunitrazepam with or without unlabelled flunitrazepam before exposure to U.V. light and electrophoresis.

1. Total [^{3}H]-flunitrazepam label incorporated into protein.
2. Non-specific.
3. Specific incorporation.

homogenates and difficulty in removing the unbound and non-specifically bound tritiated ligand. Following exposure to U.V. light the homogenates were given repeated washes of ice cold phosphate buffer with 20% ethanol to partition out free [^{3}H]-flunitrazepam. Running the tube gels was particularly difficult due to high oocyte protein/low labelling efficiency. This resulted in the high level 'shoulder' towards the top of the gel. Photoaffinity labelling with [^{3}H]-flunitrazepam in parallel with binding experiments has been found to label only 30% of the sites seen by binding assay (Mohler and Okada, 1978),hence the number of sites labelled in oocyte homogenates would not reflect the number achieved in binding assays. Non-injected oocytes displayed a similar profile to that for the non-specific in Figure 50.

3.3.9.3. Binding Assays to Oocyte Homogenates

These experiments were less conclusive than the previous ones being technically difficult and difficult to reproduce consistently (Table 12). The binding assay was based on the centrifugation assay of

Stephenson et al., (1982). Following incubation, oocyte protein was pelleted by centrifugation. This procedure effectively trapped a lot of unbound [^{3}H]-flunitrazepam present. This pellet was superficially washed with ice cold assay buffer with or without 20% ethanol. At each stage inaccuracies are involved, hence the variability between the results in Table 12. Oocytes from various donors also produced varying levels of receptor expression making assessment of binding effectiveness difficult between batches. To try to overcome some of these problems, filters were used to separate the free and bound ligand. Unfortunately, the high protein concentration per assay did not lend itself to reproducible, fast, efficient washing of the filter discs, often causing clogging and slowing repeated washes of the filter.

Although not conclusive these three biochemical assays for GABA/BZ receptor expression in Xenopus oocytes will, when combined with studies using monoclonal antibodies to probe receptor expression, provide a quick and convenient system for the assessment of receptor mRNA and later those cDNA sequences required for full receptor function.

TABLE 12

Binding of [^{3}H]-flunitrazepam to oocyte homogenates.

a) Rat brain mRNA injected oocytes.

Donor	Injected oocytes (cpm)		Non-injected oocytes (cpm)	
Xenopus	Total	Non-specific	Total	Non-specific
1.	59700	48800	39000	39700
	Specific cpm : 10900			
2.	31500	19700	22400	25000
	Specific cpm : 8500			

b) Chick brain mRNA injected oocytes.

Donor	Injected oocytes (cpm)		Non-injected oocytes (cpm)	
Xenopus	Total	Non-specific	Total	Non-specific
2.	69000	61500	20000	19500
	Specific cpm : 7500			

Note The above represent means of triplicate determinations in which the standard deviation was 12-18%.

3.4 General Discussion and Future Perspectives

3.4.1 The Present Work

The protein purification procedure described in Chapter 2 has provided a method for the simple and reproducible preparation of purified GABA/BZ receptor from rat brain. The receptor protein had subunit composition, size and binding properties which were similar to purified preparations of other groups. Further analysis of subunit amino acid sequence, secondary structure and tertiary conformation is difficult to obtain by conventional methods and some of these questions can be answered by a study of subunit cDNA sequences. Here, the purified protein is an initial requirement. Partial microsequencing of one or both subunits for the GABA/BZ receptor provides a short segment of amino acid sequence for the construction of oligonucleotide probes and primers for cDNA synthesis. The amino acid sequence would also be helpful for identifying subsequent cDNA clones of receptor subunit genes.

The other essential requirement for this approach to gene cloning is an mRNA population containing GABA/BZ receptor mRNA.

This has been provided by translation studies in Xenopus oocytes, as described in Chapter 3. The mRNA species in question have been shown to direct the synthesis, processing and membrane insertion of oocyte synthesised receptor protein in the correct orientation for ligand binding and channel gating functions to be studied. These initial studies on the oocyte produced protein have confirmed that it displays properties of the receptor found 'in situ'. Further studies using monoclonal antibodies to purify oocyte-produced receptor will confirm that its subunit size and composition are similar to that for the purified protein. The apparent size of receptor mRNA on sucrose density gradients has been achieved by translation of fractions in the oocyte system. A partially pure preparation of receptor mRNA is now ready.

3.4.2. Applications of the Oocyte Translation System to Receptor Analysis

The oocyte translation system provides an expeditious means for biochemical and electrophysiological studies of

neurotransmitter receptor protein in one cell.

As shown in this thesis, the oocyte translation system permits investigations at the level of mRNA, to recognise mRNA populations coding for receptors and confirm that the mRNA fraction under study codes for all receptor functions. When cDNAs coding for receptor subunit genes are obtained and the corresponding mRNA synthesised, coinjection and cotranslation should produce a fully functional receptor molecule. The practicality of this approach has been demonstrated by Mishina *et al.*, (1984).

These workers expressed the cDNA sequences for *Torpedo* acetylcholine receptor by transfection into Cos monkey cells and isolation of the corresponding mRNAs. Coinjection of these mRNA species produced a functional receptor by the criteria previously described.

Isolation of the respective subunit cDNA clone allows studies which examine the contribution of individual subunits to receptor function by site directed mutagenesis. Site directed mutagenesis of *Torpedo* acetylcholine receptor alpha subunit

cDNA has permitted the location of functional regions for this subunit polypeptide (Mishina et al., 1985). Construction of specific probes from known cDNA sequences of receptor protein opens the way for a detailed study of pre-synaptic and extra-synaptic forms of GABA and other neurotransmitter receptors and is useful for identifying differences in mRNA species appearing in defined biological states. For example, the alteration of receptor function during development as for the ontogenetic change in benzodiazepine modulation by the $GABA_A$ receptor (Mallorga et al., 1980; Regan et al., 1980).

Finally, the accessibility of a single oocyte (which does not suffer the complications of multicellular complexes used in current electrophysiological investigations) permits a detailed analysis of the receptor ion channel which can then be studied by patch-clamp techniques (Sakmann et al., 1983). Studies of induced receptors are in the controlled oocyte environment and phospholipid, fatty acid and cholesterol content of the oocyte plasma membrane can be varied experimentally to explore the effect of membrane composition on synaptic and extra-synaptic receptor action.

APPENDIX 1

Receptors Induced in the Xenopus Oocyte after Injection of mRNA from the Brain of 14-day Rats.

Agonist	Threshold dose(μM)[a]
GABA	20
glycine	20
L-glutamate	50
L-aspartate	50
kainate	5
quisqualate	0.5
serotonin	0.001
β-alanine[b]	200

[a] The lowest dose giving reproducibly a significant response under the recording conditions of Fig.9. The membrane potential was in the range -60mV to -84mV in this series.

[b] D-aspartate at 100μM, L-cysteic acid at 100 μM and taurine at 500 μM, were inactive on the same cells.

Differential Drug Responses at GABA or Glycine Receptors Induced in Xenopus Oocytes Following Microinjection of their Rat Brain mRNAs.

	Drug[a]	$GABA_A$ receptor	Glycine receptor
(i)	Agonists	(μM)	
	GABA	10	-
	muscimol	2.5	-
	isoguvacine	10	-
	3-aminopropane sulphonate	40	-
	glycine	-	100μM (See Fig.5)
(ii)	Antagonists		
	bicuculline (10)	(~50%I)	-
	strychnine (10)	(very small effect)	(~100%I)
	picrotoxinin (10)	(~50%I)	(~50%I)
(iii)	Potentiators		
	chlorazepate (10)	(~200%)	-
	pentobarbitone (50)	(~300%)	-

[a] The concentrations of GABA-mimetics giving of the same order of response are shown, in (i). In (ii), the approximate magnitude of the inhibition (I) obtained at the stated concentration (in μM) is shown, at 10 μM GABA (40 μM with bicuculline) or 800 μM glycine. In (iii) the approximate magnitude of the potentiation of the response to 10 μM GABA is given.

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SYNTHESIS OF FUNCTIONAL CHICK BRAIN GABA–BENZODIAZEPINE–BARBITURATE/RECEPTOR COMPLEXES IN mRNA-INJECTED *XENOPUS* OOCYTES

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Membrane conductance changes evoked by bath-applied GABA (2–640 μM) and some related agonists were recorded intracellularly in *Xenopus* oocytes previously injected with chick brain mRNA. Muscimol and 3-aminopropanesulphonate were ~ 4 and 0.25 times as potent as GABA in producing a conductance increase. GABA responses were antagonized by bicuculline (10 μM) or picrotoxinin (10–100 μM) but were clearly enhanced by the benzodiazepine (BZ) receptor ligand chlorazepate or by pentobarbitone. We conclude that an appropriate fraction of brain mRNA was capable of directing the synthesis and correct insertion of functional GABA–BZ–barbiturate/receptor complexes in the oocyte membrane.

Recent reports from two of our laboratories [1, 7] have demonstrated the usefulness of *Xenopus* oocytes for studying functional neurotransmitter receptors translated from injected mRNA. Two such receptors have been successfully transposed in this manner. The microinjection of mRNA, extracted from *Torpedo* electric organ [1] or denervated cat muscle [8], has led to de novo synthesis and insertion of nicotinic acetylcholine (ACh) receptors in the oocyte membrane. Similar attempts to induce 'brain' nicotinic ACh receptors [10] using chick optic lobe mRNA resulted instead in the appearance of functional receptors for γ-aminobutyric acid (GABA), mediating an increase in membrane chloride conductance [7].

The aim of the present experiments was to investigate whether the mRNA fraction coding for the chick brain GABA receptor also directs the synthesis and membrane insertion of those modulatory subunits which respond to benzodiazepines (BZ) and barbiturates.

However, it was important to examine chick brain, firstly for GABA receptors and secondly to establish whether these receptors also possessed the appropriate modulatory binding sites (e.g. for benzodiazepines) in the native cell membrane.

This was demonstrated by the saturable binding of [^{3}H]muscimol (0.5–0.7 pmol/mg protein) and [^{3}H]flunitrazepam (0.9–1.2 pmol/mg) to frozen-thawed embryonic chick brain membranes, which was detectable after 18–20 days in ovo using a centrifugation assay [13]. Flunitrazepam binding was enhanced by 20% in the presence of 0.1 mM GABA, suggesting associated binding sites for GABA and BZ in this tissue.

Poly(A)-mRNA was extracted from embryonic chick whole brain and microinjected into *Xenopus* oocytes (~ 40 ng per cell) [7]. Injected cells were kept in Tris-buffered Barth's medium [2], plus 50 μg/ml gentamycin, for 1–2 days before use. Oocytes were secured to the Sylgard base of the recording chamber either by lightly sucking onto the tip of a polythene cannula or by placing within a circle of pins. The cells were impaled with two microelectrodes (placed within 50 μm of each other): one filled with 1.5 M potassium citrate for voltage recording, and the other filled with 0.6 M K_2SO_4 for current injection. Potentials were monitored on a storage oscilloscope and recorded on a Brush-Gould 8000 chart recorder. Cells were continuously superfused with frog Ringer solution (at room temperature, 20–23°C) containing (mM): NaCl 118; KCl 1.9; $CaCl_2$ 2; Tris base 2.5, buffered to pH 7.4 with HCl. All drugs (BDH or Sigma Ltd.) were dissolved and applied in this medium. This report is based on data obtained from 18 cells chosen from 6 injected batches of oocytes.

After inserting both microelectrodes, the recorded resting potential of the oocytes was usually between −20 and −30 mV [5]; however, after 20–30 min of Ringer superfusion (~ 30 ml/min), the resting potential gradually increased (−35 to −70 mV) and the input resistance attained a high stable level (~ 0.5 MΩ). Slow, spontaneous depolarizations with accompanying increases in conductance were also observed in some cells as previously reported for uninjected oocytes [5]. The current–voltage relation was generally linear for ~ 30 mV either side of the resting potential [5].

It was initially important to establish the reproducibility of the oocyte GABA response before testing for any drug-induced changes. Fig. 1a shows some typical responses to bath-applied GABA in an mRNA-injected oocyte. GABA (2–640 μM) increased the membrane input conductance and depolarized the cell in a reversible, dose-related manner (E_{Cl} is ~ −15 mV in these cells [5]). The effect usually equilibrated within 30 s of solution changeover, and the original conductance was regained within 1–4 min of washout. A slow 'fading' (desensitization?) of the response was observed in the continued presence of high doses of GABA (Fig. 1b) so a dose-interval of at least 10 min was necessary to obtain reproducible responses for a dose/conductance curve (Fig. 1c). The curve agrees well with that reported previously [7] for GABA-induced membrane currents. Fig. 1d shows, for comparison, the responses evoked by the analogues muscimol and 3-aminopropanesulphonate (3-APS). These compounds were ~ 4 and 0.25 times as potent as GABA respectively, and cross-desensitization between the three agonists could readily be

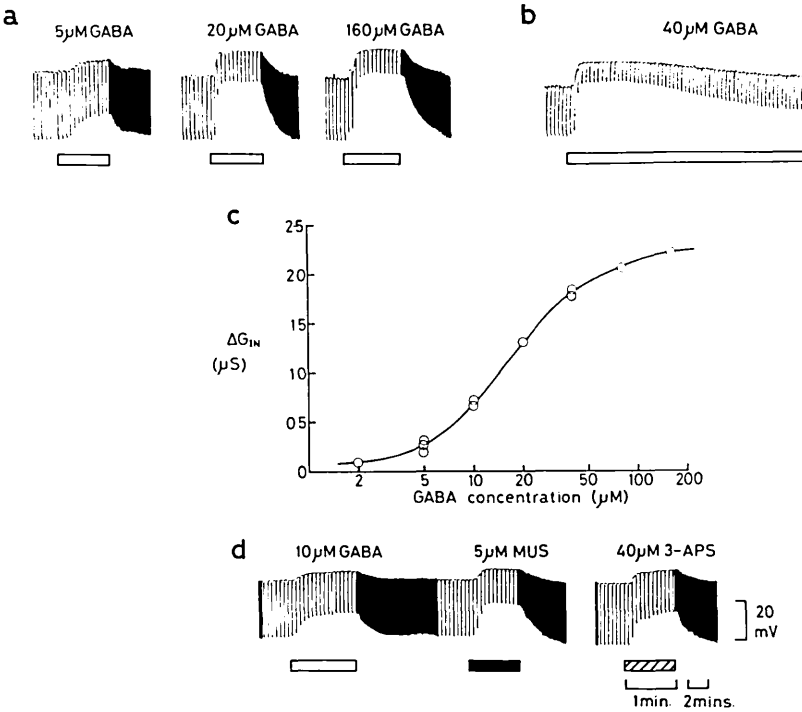

Fig. 1. a: membrane conductance changes evoked by bath-applied GABA in a single *Xenopus* oocyte, recorded 1 day after injecting chick whole brain mRNA. Downward pen deflections are electrotonic potentials evoked by intracellularly applied hyperpolarizing current pulses (40 nA, 1 s, 0.2 Hz). Upward baseline shifts are membrane depolarizations produced by GABA (applied during periods indicated by bars). Note chart speed was slower during the offset of the responses. The resting potential was −35 mV. b: fading response produced by a 5 min application of 40 μM GABA in the same cell. c: corresponding GABA log-dose/conductance relationship. Ordinate, change in input conductance (ΔG_{in}; μS); abscissa, GABA concentration (μM). d: (different mRNA-injected oocyte; same batch) comparison of responses produced by GABA, muscimol (MUS) and 3-aminopropanesulphonate (3-APS) on the same cell. The resting potential was −33 mV; injected current pulses were 35 nA, 1 s, 0.2 Hz.

demonstrated. No responses to baclofen (1 mM) or L-glutamate (1 mM) could be obtained on these cells. The pharmacological sensitivity of the oocyte GABA receptor population was thus more representative of that seen on vertebrate central neurones, than that in invertebrate systems, where 3-APS is only a very weak agonist [9].

In addition, the GABA receptor induced in the oocyte was, in accordance with other electrophysiological studies [3, 4, 6], clearly influenced by the presence of a benzodiazepine (BZ) or barbiturate. Fig. 2 shows that both chlorazepate, a potent water-soluble BZ receptor agonist [11] (1–10 μM), and pentobarbitone (25–100 μM) were capable of enhancing the conductance change and depolarization evoked by

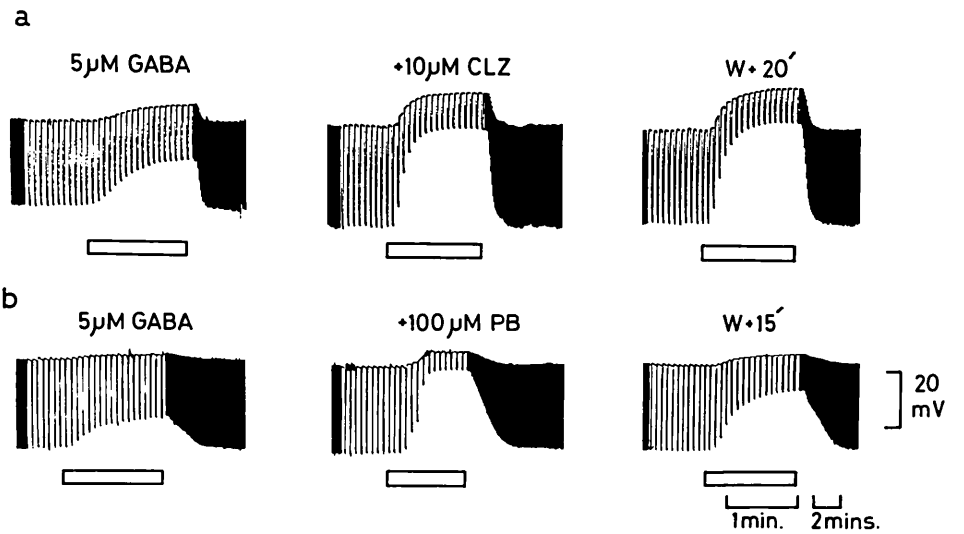

Fig. 2. Enhancement of GABA-evoked conductance change in chick mRNA-injected oocytes by a benzodiazepine (chlorazepate, CLZ) or barbiturate (pentobarbitone, PB). a: responses to GABA recorded in control solution, after 12 min in 10 μM CLZ, and then after a 20 min wash (W) in normal Ringer solution (resting potential −40 mV). b: (different cell; same batch as in a; resting potential −38 mV) GABA response in control solution, after 10 min in PB, then after a 15 min wash. Note partial recovery of GABA response after PB but not after 10 μM CLZ exposure.

GABA, chlorazepate being the more potent in this respect (Fig. 2a). Interestingly, the GABA response did not recover to the control level after exposure to this BZ, even after 1 h of washing. Both control and enhanced responses to GABA were reversibly antagonized by bicuculline (10 μM) and also picrotoxinin (10–100 μM; only slowly reversible). At the concentrations used, chlorazepate and pentobarbitone had no direct effect on the oocyte membrane conductance.

These results indicate that the partly-purified mRNA fraction extracted from chick brain can direct the synthesis and membrane insertion of not only the GABA receptor itself but also those functional subunits responsible for the enhancement of the GABA-evoked conductance change by benzodiazepines and barbiturates. Recent evidence [12, 14] supports the existence of a single protein carrying both the GABA and BZ binding sites. It is not yet known whether the barbiturate binding site and the chloride ionophore are synthesized as separate subunits, or are merely created by the topography of the oocyte-expressed GABA-binding protein in the membrane.

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Acetylcholine and GABA Receptors: Subunits of Central and Peripheral Receptors and Their Encoding Nucleic Acids

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Insights at the molecular level into the complex proteins that constitute the receptors for neurotransmitters and hormones—which should be securely based on an intimate knowledge of their architecture—have hitherto been severely limited, although they have been attained in the cases of many enzymes. An understanding in depth of receptor function has been elusive because of a number of special and major difficulties inherent in the nature and role of these systems:

1. These receptors in nearly every case are present in extremely minute amounts, which may reach down to the level of only a few molecules per active cell.
2. The receptors under consideration here are embedded in the cell membrane, and hence can only be extracted and studied in isolation by replacing the natural lipid environment with a detergent envelope; this frustrates many types of investigations.
3. In many cases, the last-named factor has impeded a purification of the receptor, if the detergent interferes with specific ligand binding or with affinity chromatography.
4. X-ray diffraction analysis, the method that has deciphered the secondary, tertiary, and quaternary structures of other proteins, is not applicable at sufficiently high resolution to membrane receptors, at least in the present state of the art, since (due to factor 2) they have resisted crystallization.
5. The components responsible for the transduction or other transmembrane signaling by the receptor are, in most cases, another set of proteins working in tandem. Thus, many receptors employ cyclic nucleotide second messengers. Present models indicate that these may open channels by interacting with at least four other proteins: adenyl cyclase and guanine nucleotide regulatory protein (Rodbell 1980; Limbird 1981) and further, it is presumed, a specific protein kinase and a protein substrate of phosphorylation (Greengard 1980; Schwartz et al., this volume). Hence, the link between ligand binding and membrane channel opening is not direct but rather complex and is dependent on the steric relationships and mobilities of these components in the membrane. There are other classes of receptors where not even an identification of the components involved has been possible by conventional techniques. Obviously, extra orders of difficulty are introduced into the analysis of a receptor in all of these cases.
6. After solubilization of a receptor, its overall activity (as distinct from ligand binding alone) can, by necessity, only be monitored in some fully reconstituted system in a lipid bilayer. This has been accomplished only in a very few cases, and in most attempts, with only limited fidelity.

For all of these reasons, most progress has been made with a limited class of neurotransmitter receptors that carry a gated ion channel in their own structure. The case par excellence here is, of course, the nicotinic acetylcholine receptor (AChR), which, from a great deal of painstaking evidence accumulated in a number of laboratories, is known to contain an integral small-cation-selective gated channel. The limitation of quantity (factor 1) is far less onerous when fish electric organ is used as a source. Furthermore, the functions of this receptor are exceptionally resistant to nondenaturing detergents (Conti-Tronconi and Raftery 1982). However, some degree of complexity is introduced by the presence of four different types of subunits (of stoichiometry $\alpha_2\beta\gamma\delta$) combined in its "monomer" structure, a multiplicity that is being encountered now with other receptors. For this and related reasons, chemical amino acid sequencing has proven difficult, and published results have not gone beyond 54 aminoterminal residues of any of the chains from *Torpedo* as source (Raftery et al. 1980) or 24–35 residues from other chains (Conti-Tronconi et al. 1982), despite the exploitation of current gas-phase microsequencing technology. Obviously, therefore, for all of the other enormously less abundant receptors noted above, determination of the entire structure by chemical sequencing is not a realistic goal.

It cannot be assumed that the principles deduced from the wealth of studies of the AChR of the electric organ, a highly unusual tissue, apply to all receptors of the direct channel-gating class. Nor can this be assumed even for other nicotinic AChRs, especially those in the brain. The latter, for example, are not blocked by α-bungarotoxin or, in certain cases, α-bungarotoxin binds and blocks but far more reversibly than it does at the electric organ receptor (Oswald and Freeman 1981; Norman et al. 1982). We shall review here, therefore, our recent studies on three other receptors that likewise

have the advantage of being in the direct channel-gating class but are, biologically, progressively more distant from the electric organ receptor. These are the nicotinic AChRs of vertebrate skeletal muscle, the equivalent receptor of chick brain, and the receptor for γ-aminobutyric acid (GABA) in mammalian brain (which carries a gated chloride channel). In particular, this paper focuses on initial applications of molecular genetics in analyzing those systems.

The immensely powerful methods of current molecular genetics can, indeed, provide an escape from many of the problems enumerated above. If the cDNAs encoding a receptor are sought and can be obtained, its entire protein sequence is rapidly provided. This can be expected, due to the considerations outlined above, to become the method of choice for structural analyses of receptor proteins. In general, it may be possible to operate at the nucleic acid level for many (though not all) types of study, and hence avoid the major difficulties for receptors in operating at the level of the purified protein. A variety of specific questions on structure-function relations can be framed and answered in terms of translations of the mRNAs for the receptor subunits, as will be illustrated below. Illuminating homologies can be sought, for example, to ask whether structural similarities occur between receptor types having a given type of ion channel in common, or an agonist in common (e.g., nicotinic vs. muscarinic receptors). Furthermore, studies on receptor DNA and mRNA can offer new types of information on receptors, at the level of their genetic control.

Torpedo Receptor mRNA and cDNA

Translation of receptor mRNA in a foreign environment. When we first attempted the cell-free translation of mRNA from *Torpedo marmorata* electric organ, using immunoprecipitation by polyclonal anti-receptor antibodies of the products, followed by SDS-polyacrylamide gel electrophoresis, no bands could ever be obtained at the positions of the four receptor subunits, and there was no α-bungarotoxin-binding activity. The evidence indicated that synthesis of the polypeptides in the reticulocyte lysate (or wheat germ) translation system is insufficient for their assembly into the active receptor oligomer; we pursued instead, therefore, synthesis in a cellular system, the *Xenopus* oocyte. Only inactive smaller subunits, immuno-cross-reactive with the native subunits, were produced in the cell-free system (Sumikawa et al. 1981), in agreement with the findings of Mendez et al. (1980) and Anderson and Blobel (1981) for cell-free translation of this mRNA.

In the *Xenopus* oocyte (with [^{35}S]methionine in its medium), microinjection of the *Torpedo* mRNA led to the synthesis of four mature subunits (^{35}S-labeled and visualized in autoradiographs of SDS gels) which now comigrated with the α-, β-, γ-, and δ-subunits from purified *Torpedo* receptor protein (Sumikawa et al. 1981). Binding of [^{125}I]α-bungarotoxin occurred normally to the product, nicotinic ligands competed specifically with this binding, and the product was in the form of the 9S receptor monomer (Fig. 1). The induced receptor is incorporated in the oocyte membranes and can be demonstrated on the external surface of that cell (Barnard et al. 1982). The synthesis of these subunits on the *Xenopus* polysomes is truly directed by the *Torpedo* mRNA, and not by activation of the *Xenopus* genome, as shown by the characteristic peptide map of the *Torpedo* α-subunit, which is, in fact, given by the corresponding subunit produced in the oocyte by the mRNA injection (Fig. 2A). The peptide map of a given subunit of this receptor is highly species-specific, since the amino acid sequence varies considerably with the

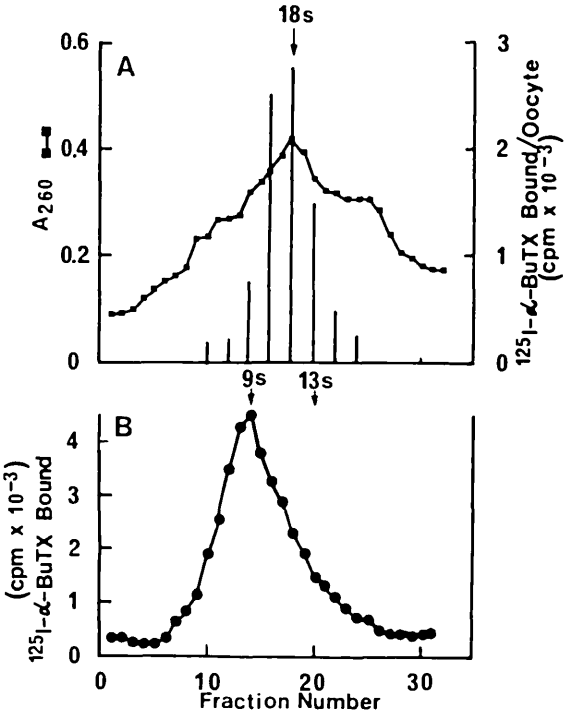

Figure 1. (*A*) RNA fractions containing the mRNAs coding for the AChR. Poly(A)$^+$ mRNA, prepared from *T. marmorata* electric organ, is shown after separation on a 10–31% isokinetic sucrose gradient containing also 10 mM HEPES (pH 7.5), 1 mM EDTA, and 0.1% Li dodecyl sulfate at 2°C. The nucleic acid content is shown by A_{260} nm (■), with human fibroblast rRNA used (in a parallel gradient) as the 18S marker. The vertical bars show the activity of the fractions in directing, in the oocyte translation assay, the synthesis of the product that binds [^{125}I]α-bungarotoxin. (*B*) The oligomeric (9S) form of the AChR is produced in the oocyte translation. After injection of the peak active fraction of mRNA from *A* into 30 oocytes, followed by receptor extraction, the extract was sedimented on a 5–20% sucrose density gradient. The [^{125}I]α-bungarotoxin-binding activity was assayed in every fraction. The arrows indicate the positions to which the 9S (monomer) and the 13S (dimer) forms of the native AChR purified from *Torpedo* sediment in a parallel sucrose gradient (from Sumikawa et al. 1982d). The same profile was obtained when the oocytes used had been injected, instead, with the unfractionated poly(A)$^+$ mRNA from the electric organ. Note that the 9S form assembled in the oocyte corresponds to the pentameric receptor ($\alpha_2\beta\gamma\delta$), and that the dimer of that pentamer (13S form) is not produced.

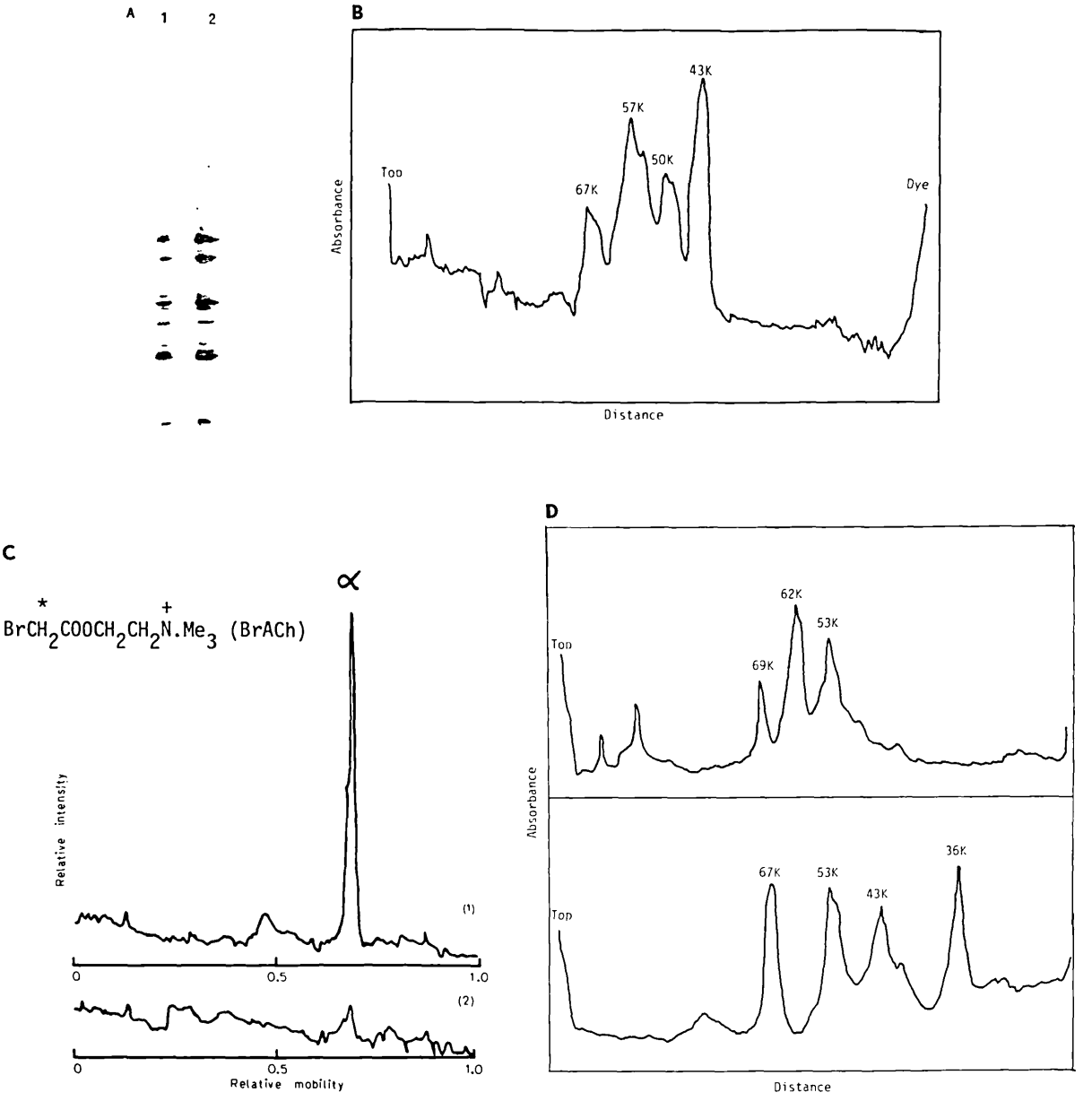

Figure 2. (*A*) Comparison by peptide mapping of the α-subunit produced in *Xenopus* oocytes after injection with *Torpedo* electric organ poly(A)$^+$ mRNA (track *2*) with the α-subunit of the purified AChR from *Torpedo* (track *1*) (Sumikawa et al. 1982c). The former preparation was labeled biosynthetically with [^{35}S]methionine and the latter by ^{125}I iodination on the purified receptor protein. In each case, the four subunits were separated by SDS gel electrophoresis and isolated; limited proteolysis by *S. aureus* V8 protease of the α-subunit produced the peptide maps, using the methods of Sumikawa et al. (1982a). Peptide pattern *1* is highly characteristic of the *Torpedo* α-subunit, and its identity with pattern *2* is evidence for the identity of the *Torpedo* subunits produced by translation in the foreign cell. (*B*) Subunits of the AChR from chicken muscle. The purified (Sumikawa et al. 1982b) receptor from chicken muscle was subjected to electrophoresis in an SDS-polyacrylamide (1-mm-thick) slab gel using the procedures of Sumikawa et al. (1982a), except that 8.75% acrylamide was used. Staining was with Coomassie Brilliant Blue: A scan at 550 nm is shown. M_r values were obtained from a linear plot given by four protein standards run in a parallel track (as shown in *D*, which also shows typical background). The four labeled bands represent (*right to left*) the α-, β-, γ-, and δ-subunits of this receptor. The 43,000 M_r (43K or α) polypeptide, when eluted and analyzed by microsequencing, gave the sequence shown in Table 2. The 50K and 57K polypeptides have been tested similarly: They have given aminoterminal sequences homologous with that of the 43K, confirming that all are receptor subunits. The sequencing has shown that there are four homologous polypeptides in the receptor, in all. (*C*) Affinity labeling of the muscle receptor α-subunit. The affinity reagent, bromoacetylcholine (BrACh), and its position of ^{3}H labeling (*) are shown. Trace *1* shows purified chicken muscle receptor, prelabeled with [^{3}H]BrACh after dithiothreitol reduction, analyzed on a gel as in *B*, but showing (by densitometry of a fluorogram) the radioactivity present. Trace *2* shows a control in which an equal quantity of the same receptor was labeled likewise but in the presence of an excess of α-cobra toxin. The result shows that of the four subunits, only α is labeled in the specific binding reaction. For details, see Sumikawa et al. (1982a). (*D*) Subunits of the chick optic lobe AChR. (*Upper trace*) The receptor, purified by affinity chromatography on α-bungarotoxin-Sepharose and elution (method modified from Norman et al. 1982), was analyzed as in *B*. (*Lower trace*) Four protein standards used for the calibration.

species. The maps obtained after the oocyte translation are fingerprints characteristic of the *Torpedo* subunits.

Further studies have been directed to the question of whether the specific ion channel of the peripheral nicotinic AChR is encoded by the same mRNA. An interesting finding emerged here. When Professor R. Miledi was asked to test the oocytes injected with *Torpedo* electric organ poly(A)$^+$ mRNA for a possible response to ACh, it was discovered that, indeed, the AChR channel had become inserted in the oocyte membrane. Microiontophoretic application of ACh to the voltage-clamped surface of the preinjected oocytes elicited a conductance change characteristic of nicotinic AChRs (Barnard et al. 1982). This response has since been obtained reproducibly. It is (1) specific for nicotinic agonists; (2) absent when the injection is of the other gradient fractions of the *Torpedo* mRNA that do not yield receptor subunits or of fibroblast mRNA; (3) absent when ACh or other agonists are applied, instead, to the inside of the oocyte membrane; and (4) yields an ACh dose-response curve similar to that obtained for receptors on a muscle surface. The response is blocked by *d*-tubocurarine and by α-bungarotoxin (4 min at 1 μg/ml) (Sumikawa et al. 1982c). The effects of modifying the bathing ions, and the reversal potential of -8 mV found for the response (Barnard et al. 1982), indicate that the channel has the original Na^+, K^+ selectivity. The uninjected oocyte has no nicotinic ACh channels of its own, and we must conclude that when this mRNA fraction produces the subunits and the 9S α-bungarotoxin-binding oligomer, the reception ion channel spontaneously forms in the membrane.

The binding of lectins (Sumikawa et al. 1981) showed that the receptor produced in the oocyte is glycosylated as is (Vandlen et al. 1979; Lindstrom et al. 1979) the native *Torpedo* receptor on all of its subunits. Moreover, when tunicamycin (2 μg/ml) was present in the medium during the normal incubation of injected oocytes, production of assembled receptor was totally blocked (K. Sumikawa and E.A. Barnard, unpubl.). The translational capacity to form the AChR resides in a restricted fraction of the poly(A)$^+$ RNA, centered on the 18S position when the latter is separated on a density gradient (Sumikawa et al. 1982d). From all of the above-mentioned results, we conclude that the four mRNAs (see below) coding for the α-, β-, γ-, and δ-subunits in the electric organ can be faithfully translated by the oocyte, processed, transported to the cell membrane, assembled to the full receptor structure with all of its functions present, and inserted with correct orientation in that foreign cell membrane. We presume that all of the information necessary for these steps is contained in the mRNA and subunit sequences. However, a final proof of that will come from experiments in which the pure mRNAs are obtained from their cloned cDNAs and are microinjected into oocytes with no contaminating *Torpedo* messengers present.

Cloning of receptor subunit cDNA. An approach to gene cloning that has facilitated the exploitation of other low-abundance mRNAs is the use of even a short stretch of known amino acid sequence of the gene product for construction of the corresponding DNA fragment as a probe for hybridization screening. We therefore used the aminoterminal sequence of the α-subunit, known from the work of Raftery et al. (1980) and Devillers-Thiery et al. (1979) and identical in *Torpedo californica* and *T. marmorata* (which we used) for the first 20 residues. ^{32}P-labeled oligodeoxyribonucleotides were synthesized corresponding to codons 22–25 of the mature α-subunit (Fig. 3A). Of the possible 12-nucleotide probes, 6 were tested for the ability to act as a primer with the fractionated receptor-specific poly(A)$^+$ mRNA. The products of reverse transcription primed with those components were analyzed on DNA gels, and a positive result was obtained with only one probe, α2, giving a single band corresponding to a segment of 275 nucleotides. This was specific, also, to the mRNA fractions that were active in receptor biosynthesis (Fig. 1B). The sequence of this DNA was obtained, and it showed an encoded amino acid sequence that agreed with the published aminoterminal sequence of the α-subunit, together with a further pre-aminoterminal sequence (Fig. 3B) corresponding to a signal peptide (Sumikawa et al. 1982d). The 12-nucleotide primer itself was insufficiently selective as a hybridization screening probe, but the 275-base fragment of genomic sequence thus recognized was used to construct an authentic 19-nucleotide probe (Fig. 3B). This acted as a very efficient screening probe. A *Torpedo* recombinant DNA library was cloned in the disabled *E. coli* strain DH1, using the plasmid pAT153 (Sumikawa et al. 1982d). Using the 19-base probe, a series of clones were identified that proved, on DNA sequencing, to contain partial and full-length inserts of the α-subunit cDNA. Using those to cover both DNA strands, the α-subunit cDNA sequence was determined. This revealed a single open reading frame, which starts with the codons for a typical signal peptide (of the type [Blobel 1980] that determines membrane insertion) of 24 mainly hydrophobic residues (Fig. 3B). This is followed immediately by codons for the known aminoterminal sequence of the α-subunit. This confirmed the identity of the cDNA cloned.

The entire cDNA sequence to the termination codon codes for a mature subunit of 437 amino acid residues. This sequence differs at five positions from the otherwise identical sequence of the α-subunit of *T. californica* published by Noda et al. (1982) shortly after our cloning was reported (Sumikawa et al. 1982d) (Table 1). These five substitutions are all due to 1-base changes and are regarded as true species differences. This is confirmed at one of them (our Asn-42) where Ser in *T. californica* is present both in the cDNA-cloning sequence and in the chemical sequencing of Raftery et al. (1980) for that species.

The full sequences for the three other subunits determined by cDNA cloning have since been reported, and there is considerable homology among all four, as discussed by others in this volume. (The sequences for the

A

Primers		
	α1	3′ CAT·CTT·GTG·GTG 5′
	α2	3′ CAT·CTC·GTG·GTG 5′
	α3	3′ CAG·CTT·GTG·GTG 5′
	α4	3′ CAG·CTC·GTG·GTG 5′
	α5	3′ CAA·CTT·GTG·GTG 5′
	α6	3′ CAA·CTC·GTG·GTG 5′

B

					−20											−10				
	Met	Ile	Leu	Cys	Ser	Tyr	Trp	His	Val	Gly	Leu	Val	Leu	Leu	Leu	Phe	Ser	Cys	Cys
GAAGA	AUG	AUU	CUG	UGC	AGU	UAU	UGG	CAU	GUC	GGG	UUG	GUG	CUA	CUG	UUA	UUU	UCG	UGU	UGU
				1			10				20				30				40
					−1	1								10					
	Gly	Leu	Val	Leu	Gly	Ser	Glu	His	Glu	Thr	Arg	Leu	Val	Ala	Asn	Leu	Leu	Glu	Asn	Tyr
	GGU	CUG	GUA	CUA	GGU	UCU	GAA	CAU	GAA	ACA	CGU	UUG	GUU	GCU	AAU	UUA	UUA	GAA	AAU	UAU
				60			70				80				90				100
						20										30			
	Asn	Lys	Val	Ile	Arg	Pro	Val	Glu	His	His	Thr	His	Phe	Val	Asp	Ile	Thr	Val	Gly	Leu
	AAC	AAG	GUG	AUU	CGU	CCA	GUG	GAG	CAU	CAC	ACC	CAC	UUU	GUA	GAU	AUU	ACA	GUG	GGG	CUA
				120			130				140				150				160
						40										50			
	Gln	Leu	Ile	Gln	Leu	Ile	Asn	Val	Asp	Glu	Val	Asn	Gln	Ile	Val	Glu	Thr	Asn	Val	Arg
	CAG	CUG	AUA	CAA	CUC	AUC	AAU	GUG	GAU	GAA	GUA	AAU	CAA	AUU	GUG	GAA	ACA	AAU	GUG	CGC
				180			190				200				210				220

Figure 3. Construction of a probe for screening the clones, and identification of the cloned cDNA for the α-subunit. (*A*) Residues 22–25 of the known aminoterminal sequence of the mature α-subunit of *Torpedo* receptor (shown in *B*), Val Glu His His, were chosen and the corresponding 12-base deoxyribonucleotide primers shown were synthesized in ^{32}P-labeled form. Primer α2 was the only one of these to give a positive result in the priming of the *Torpedo* 18S poly(A)$^+$ mRNA. (*B*) Pre-aminoterminal and aminoterminal regions of the α-subunit and the corresponding mRNA sequence. The numbered amino acid residues (1–54) are those known (Raftery et al. 1980) to be at the amino terminus of *Torpedo californica* receptor mature α-subunit. The bar shows the tetrapeptide sequence used in *A* for the priming. The cDNA obtained from the α2 priming as the sole electrophoretic product (275 nucleotides) was identified by sequence correspondence in the appropriate region of the α-subunit. This led to the choice of the 19-base sequence marked by arrows, for synthesis of a ^{32}P-labeled DNA fragment (CTTTGTGCAAACCAAC-GAT, 3′–5′) and its use as a hybridization screening probe. The cloned cDNA thus obtained included in its sequence the entire region shown (in mRNA form). The pre-aminoterminal peptide (codons −24 to −1) is a typical signal sequence, in that 11 of its 24 residues are Leu, Ile, or Val, it contains Cys, and it ends (at −1) in a very small residue. (The codon found at position 42 codes for Asn, not the Ser in the known *T. californica* α-subunit aminoterminal segment.) (Data from Sumikawa et al. 1982d.)

Table 1. *Torpedo* α-Subunit Protein Sequence Differences

	T. californica[a]	*T. marmorata*[b]
Number of residues	437	437
Molecular weight	50,116	50,186
Coding sequence differences	1. Ser-42 AGU	Asn-42 AAU
	2. Gly-230 GGA	Val-230 GUA
	3. Ile-291 AUU	Val-291 GUU
	4. Asp-318 GAU	Asn-318 AAU
	5. Ser-424 AGC	Cys-424 UGC
Untranslated sequence differences	three 1-base changes in first 85 bases at 5′ end of mRNA	

[a]Data from Noda et al. (1982).

[b]Data from Sumikawa et al. (1982d) and unpublished results, and confirmed by Devillers-Thiery et al. (1983). In the latter study, there is one coding difference from ours, at codon 323, given as Leu (CUU) in contrast to our Val (GUU); the latter, however, is found both by us and (in *T. californica*) by Noda et al. (1982).

α-subunit obtained in our laboratories [Sumikawa et al. 1982d] and by Noda et al. [1982] have recently been further confirmed by Devillers-Thiery et al. [1983].) Hence, the entire primary structure (other than the disulfide bridge locations) of this receptor has been determined with remarkable speed, once the cloning technology was found applicable to it.

Antisera raised against the four *Torpedo* subunits, when renatured from SDS, show considerable cross-reactivity among all four chains (Mehraban et al. 1982). This is to be expected from the degree of homology now seen between the four subunits.

Folded structure and membrane insertion. Where a polypeptide is destined to remain in the membrane and not to be secreted through it, it is assumed that there is additional information to direct this in the form of "stop-transfer" and "insertion" sequences in the mature polypeptide chain (Blobel 1980). These are likely to be one or more hydrophobic transmembrane helices flanked by polar residues. It is interesting to note that the amphibian oocyte recognizes whatever the insertion signal is in this *Torpedo* sequence, since we found that the AChR in the microinjected oocytes was not secreted but accumulated as a firmly bound membrane protein.

The aminoterminal half of the sequence contains the only site (Asn-141) in the subunit for N-glycosylation, a modification that is known (Vandlen et al. 1979) to occur on that subunit. The amino terminus formed by signal peptide cleavage is expected to be on the extracellular side of the membrane (Blobel 1980). An ACh-binding site is present on each of the two α-subunits, from the evidence on the BrACh affinity labeling (Wolosin et al. 1980). This binding site is adjacent to a disulfide (Karlin et al. 1976) and must be located in an extracellular projection on the aminoterminal half.

Attempts to use the amino acid sequence in the secondary structure prediction method of Chou and Fasman (1978) or in assigning transmembrane helices by inspection of hydrophobic regions (methods since used by others [Noda et al. 1982, 1983a; Devillers-Thiery et al. 1983]) gave ambiguous results. The initiation points of the α-helices are uncertain, as are their precise lengths, and, depending on exactly how they are chosen in the hydrophobicity or secondary structure plots, different helices can be equally well predicted. These would present different distributions of the polar residues present, over a given face. In fact, it has been shown (Green and Flanagan 1976; Geisow and Roberts 1980) that the Chou-Fasman procedure, which uses a globular protein data-base, considerably underpredicts the occurrence of α-helices in α-helix-rich membrane proteins. Also, different hydrophobicity indices in use give different profiles here. The aminoterminal half of the subunit sequence shows much greater polarity than the carboxyterminal half, as assessed by all those indices, and very probably is nonhelical. We conclude that it is not safe to predict the topology of such a membrane subunit, nor the alignment of the channel, by the above-mentioned methods.

Subunit sizes. It is interesting to note that the true molecular weight of the *T. marmorata* α-subunit is 50,186, as calculated from the sequence. The apparent molecular weight, deduced from mobilities in SDS-polyacrylamide gels, has long been found to be 39,000–40,000 (Karlin et al. 1976; Raftery et al. 1980). That value is for the fully glycosylated subunit, but cell-free synthesis without glycosylation (but with the signal peptide attached) gives an apparent size that is even smaller (38,000), not greater (Anderson and Blobel 1981). Hence, the anomaly seems to come from its particular sequence. Likewise, the δ-subunit of *T. californica* has a sequence molecular weight (Noda et al. 1983b) of 57,565, about 7000 below that usually found in the gels. These effects in opposite directions demonstrate that errors of up to at least 20% in M_r are liable to be made on either side of the true value, in gel analysis of receptor subunits.

Furthermore, the molecular weights from the four sequences of *T. californica* (Noda et al. 1982, 1983a,b; Claudio et al. 1983) give a sum of 267,751. This compares, for example, with a value of 250,000 ± 7,000 (± S.E.) obtained in solution by a rigorous sedimentation equilibrium method using D_2O balance (Reynolds and Karlin 1978). Values in the range 240,000–270,000 were obtained by several other techniques in which the interference of the detergent is eliminated (for review, see Barnard 1983).

These comparisons, and also model building based on the sequences, would be in error if the primary translation product of the mRNA is cleaved at sites other than at the signal peptide. The occurrence of paired basic residues present toward the carboxyl terminus has suggested (Noda et al. 1982) the possibility that the α-subunit might be further processed to a smaller size, closer to that estimated from gel analysis. This argument can be discarded, however, since some peptides that have been obtained and identified in chemical sequencing studies of the *T. californica* α-subunit (B.M. Conti-Tronconi and M.A. Raftery, pers. comm.) fit our amino acid sequence up to Glu-397.

Muscle AChRs

The receptor from innervated muscle is present at a very low level compared with that in *Torpedo* electric organ—often down to one-thousandth, as in chicken muscle (Sumikawa et al. 1982b). Obviously, cDNA technology can be of real advantage here. Chicken muscle is suitable for such use, since the chick embryo is a good source of muscle-specific mRNAs. Even so, the receptor mRNA is a very minor component of these. AChR from chick muscle (adult innervated, denervated, or embryonic) has been purified to homogeneity and several criteria of purity met (Fig. 4). The AChR species purified from those three types of chicken muscle could not be distinguished physically (Fig. 4B) nor by subunit analysis (Sumikawa et al. 1982a).

In SDS gel electrophoresis, three subunits were clearly seen (after taking full precautions against proteolysis during the isolation) (Sumikawa et al. 1982a);

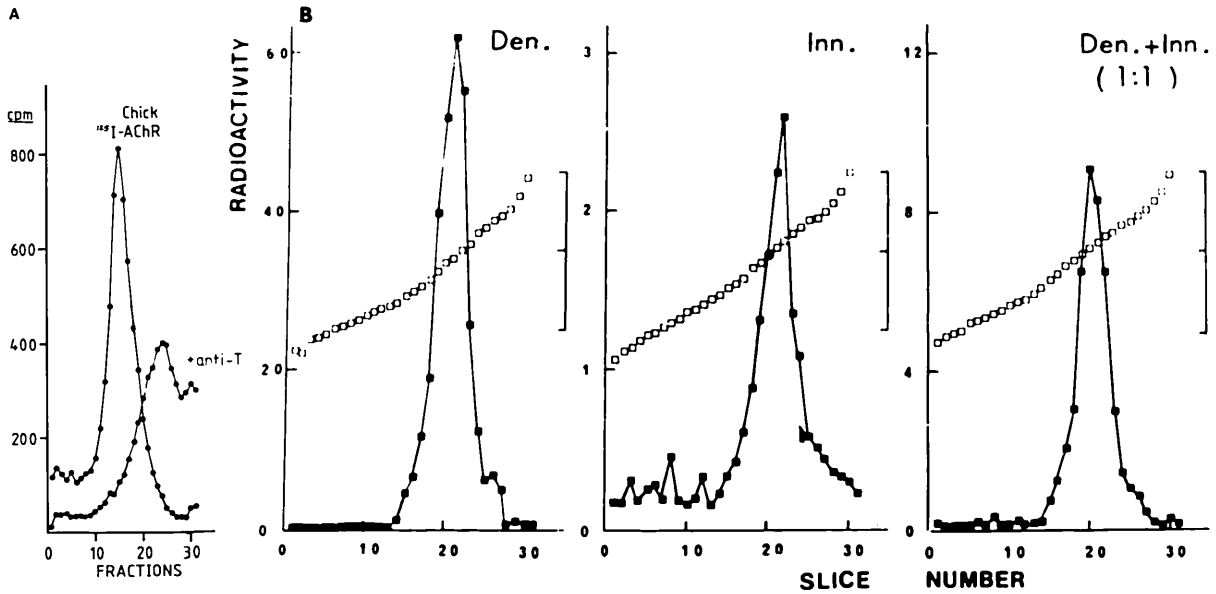

Figure 4. Some criteria of the purity of the isolated AChR from chicken muscle, synaptic and extrasynaptic. (*A*) The free receptor, ^{125}I-labeled ([^{125}I]AChR), sediments on a sucrose density gradient as a single peak of sedimentation constant 9S. Further, on addition of an equivalent amount of a cross-reacting antibody directed against the pure AChR from *T. marmorata* electric organ to the peak fractions from the gradient in a parallel sedimentation, all the ^{125}I present is seen to be moved to a zone lower in the gradient, corresponding to receptor-antibody complexes. Hence, all of the protein in the purified preparation is immunochemically characterized as AChR. (*B*) The complex of the pure, unlabeled receptor with [^{125}I]α-bungarotoxin was isolated and subjected to gel isoelectric focusing (narrow-range, pH 4-6 [□] on right-hand scales). The radioactivity in each slice (■) was measured (in cpm × 10^{-2}, on left-hand scales). (*Left panel*) Extrasynaptic receptor, from denervated (adult) chicken muscle; (*center panel*) synaptic receptor, from innervated (adult) chicken muscle; (*right panel*) a 1:1 mixture of these two forms. Note that a single species (by charge) is present, indistinguishable in the two forms. (Reprinted, with permission, from Sumikawa et al. 1982b.)

with recent improvements in the procedure, however, four are obtained (Fig. 2B), the heaviest band now becoming clear. Similar methods applied to a muscle from the guinea pig yield a receptor with protein purity and four subunits, similar to those shown here for the chicken. The band at 40,000–43,000 M_r is the α-subunit, since this is labeled specifically with [^{3}H]BrACh after disulfide reduction (Fig. 2C).

Amino acid sequence of muscle receptor subunits. The pure chicken muscle receptor was separated in preparative SDS gel electrophoresis, and the individual subunits electroeluted. Automated analysis by gas-phase sequencer carried out at the California Institute of Technology yielded the aminoterminal sequence shown in Table 2. It can be seen that the α-subunit is homologous to the *Torpedo* α-subunit. Other subunits of the chick muscle AChR, for which shorter stretches of sequence have recently been determined, also show homologies with the corresponding *Torpedo* subunit. Also marked in Table 2 are positions in this region that are completely conserved in all the subunits in the AChRs from all of the sources where sequencing has been performed. It can be seen that a degree of homology exists between all of the chains.

mRNA studies of the chick receptor α-subunit. mRNA from embryonic chick muscle was extracted (as for *Torpedo* mRNA) and microinjected into the *Xenopus* oocyte. AChR was again produced, this being shown by (1) the binding of [^{125}I]α-bungarotoxin to the product, and the blockade of that binding by cholinergic ligands; (2) total precipitation of the product complexed with [^{125}I]α-bungarotoxin by anti-AChR antibody; (3) a dependence of the receptor production on mRNA added, which was the same as that (Sumikawa et al. 1981) found for *Torpedo* mRNA. The production in the oocyte of AChR of muscle is illustrated in Figure 5A, where the activity is seen to be restricted to a fraction of the total poly(A)$^+$ mRNA. Similar results have been obtained with the mRNA from denervated cat muscle.

The aminoterminal sequences of the subunits of AChR from chick muscle have been used for an approach to cloning similar to that described above. For example, the α-subunit sequence in Table 2 was used to select a 14-base sequence as marked there, and 16 suitable probes were synthesized. One batch of four of these gave positive priming with gradient-purified mRNA from chick muscle (Fig. 5B). As before, a 275-nucleotide segment was recognized specifically, corresponding to a pre-aminoterminal and aminoterminal stretch of this subunit. The 14-base probe, which was positive for this, could itself act highly selectively in hybridization screening. By cloning as before, but using chick DNA, positive clones were obtained. Determination of the full sequence of subunits of the chick muscle receptor is thus in progress.

Table 2. Aminoterminal Sequences of Muscle and *Torpedo* AChR α-Subunits

1									10										20										30										40									
	*	*	*	*	*																																										
S	E	H	E	T	R	L	V	D	D	L	F	R	D	Y	S	K	V	V	R	P	V	E	N	H	R	D	A	V	K	V	(N)	V	G	L	A	(L)	I	?	L	I	N	V	D	E[a]
S	E	H	E	T	R	L	V	A	N	L	L	E	N	Y	N	K	V	I	R	P	V	E	H	H	T	H	F	V	D	I	T	V	G	L	Q	L	I	Q	L	I	N	V	D	E[b]

[a]Chick muscle AChR determined by chemical microsequencing on the purified α-subunit. ? = residue uncertain. ( ) = major residue detected, but other(s) significant. The asterisks mark the set of residues used for construction of oligonucleotide probes based on possible codons (Fig. 5B).

[b]*T. marmorata* (from Raftery et al. 1980). Single underline = residue identical with chick α-subunit residue (29 out of 45). Double underline = residue identical in all of the ACh receptor α-subunit sequences so far known (8 out of 45 positions).

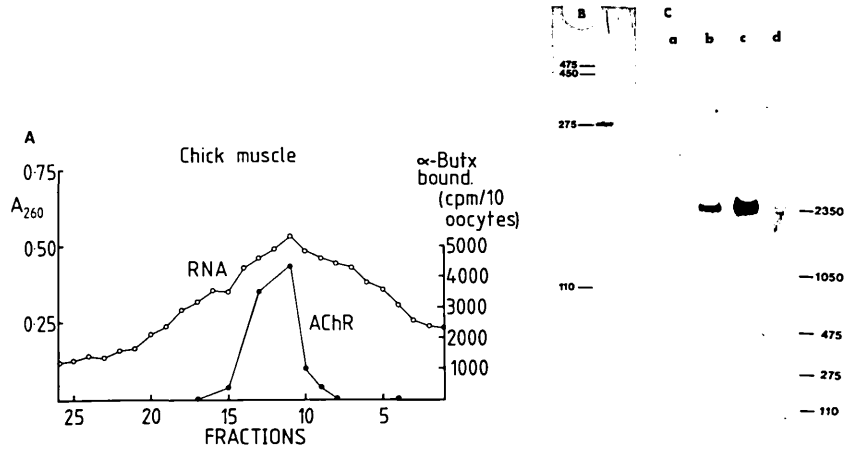

Figure 5. Chicken muscle receptor α-subunit nucleic acids. (*A*) Identification of the mRNA for this subunit. The poly(A)$^+$ mRNA isolated from the thigh muscles of 12-day chick embryos was fractionated (○) on a sucrose gradient (as in Fig. 1A). In the oocyte translation assay, the activity (●) of the fractions in directing the synthesis of the receptor was determined by the specific binding of [^{125}I]α-bungarotoxin that appeared, in batches of 10 oocytes (right-hand scale). The peak position corresponds to 18S. The fractions here are numbered from the bottom of the gradient. (*B*) cDNA segment coding for chicken muscle receptor α-subunit. The codons marked in the aminoterminal region of this subunit in Table 2 were used to construct a set of 14-base deoxyribonucleotides. One of these, (CTTCGTCTTTGGGC, 3′–5′) only, was positive in the priming of the reverse transcription of the poly(A)$^+$ mRNA from embryonic chick muscle (peak fraction 11 from *A*). This yielded a 275-base product, as shown in the DNA gel electrophoresis, with positions of markers indicated. In an adjacent track (not visible because totally negative), the products were run from similar priming of mRNA fraction 6 of the gradient in *A*, corresponding to much larger mRNA. Other procedures were as noted for Fig. 3. (*C*) RNA hybridization with 14-base DNA probes specific for chicken muscle α-subunit and for *Torpedo* α-subunit. The ^{32}P-labeled 14-base probe corresponding to codons 2–6 in each case was hybridized to glyoxal-denatured poly(A)$^+$ mRNA size-fractionated on a 1.4% agarose gel, by standard Northern blotting technique. The chicken α-subunit probe was that used in *B*, and the *Torpedo* probe contained the corresponding nucleotides as shown in the sequence in Fig. 3B, i.e., they differed in three bases. (Track *a*) *T. marmorata* electric organ poly(A)$^+$ mRNA was run (negative with the chick probe); (*b*) 12-day embryonic chicken muscle poly(A)$^+$ mRNA (positive with the chick probe); (*c*) the *Torpedo* sample again (positive with the *Torpedo* probe); (*d*) the chicken sample again (negative with the *Torpedo* probe). The positions marked were calibrated using denatured *Hin*dIII-restricted phage PM2 DNA; RNA sizes are slightly smaller than these. It is seen that these probes are specific for their corresponding receptor mRNAs and that these mRNAs are of equal size in the two sources.

Brain Nicotinic AChRs

As noted above, mammalian brain contains nicotinic AChRs that fail to show physiological blockade by α-bungarotoxin or related toxins; in the optic lobe of lower vertebrates, however, they do show such blockade (Oswald and Freeman 1981). We have therefore purified the receptor from chick optic lobe, by methods adapted (to the very low concentrations involved and the reversibility of α-bungarotoxin binding there) from those used with muscle (Norman et al. 1982). The same receptor from the rest of the chick brain (where there is no electrophysiological evidence that α-bungarotoxin blocks) was purified similarly. The products satisfy criteria of purity, behaving as a single component (after ^{125}I iodination) in narrow-range isoelectric focusing or density-gradient centrifugation (Norman et al. 1982). The pharmacological properties of the product, and the immunological cross-reactivity observed with the muscle AChR (discussed below), provided evidence that a brain nicotinic receptor has been isolated from the optic lobe.

In the AChR of chick optic lobe, three subunits can be demonstrated in SDS gels, of apparent M_r 53,000, 60,000, and about 70,000 (Fig. 2D). The 53,000 M_r subunit can be labeled specifically with [^{3}H]BrACh after disulfide reduction (Norman et al. 1982), indicating it is the equivalent of the muscle α-subunit. The higher subunit weights and sedimentation constant (in Triton X-100 medium) suggest a somewhat different structure for this receptor. It may be that a fourth subunit is present as in muscle and *Torpedo* AChRs, but is poorly resolved or poorly stained or labeled in the gels. Separation of the subunits and sequencing thereof is in progress. Clearly, one can proceed eventually to the structure determination via cloning as before. The chick embryonic optic lobe is a suitable source of mRNA for this task, despite the fact that levels of the brain receptor are lower than in muscle. Comparison of the coding sequences and flanking genomic sequences for the subunits of peripheral and brain AChRs should be of interest with respect to the neurobiology of brain receptors in general.

Relatedness of brain and muscle receptors and the use of monoclonal antibodies. The question of possible cross-hybridization of muscle and brain DNA sequences in the AChR genes, as a short-cut in coding the latter, also merits investigation. The choice of the same species, chick, for both is aimed to facilitate this. Relatedness of the two receptor proteins is evident from their antibody cross-reactivity. Polyclonal antibodies to muscle AChR generally show little cross-reactivity to brain receptors. However, one out of ten antibodies to

muscle receptor (in this case cat) showed definite cross-reactivity with chick optic lobe receptor (Norman et al. 1982).

Hybridomas secreting monoclonal antibodies provide more opportunity to find such a probe and to prepare it in adequate quantities. Apart from the information yielded directly, such an antibody could then be used also in translation assays. More recently, therefore, we raised monoclonal antibodies to the pure AChR of chick muscle (Mehraban et al. 1983) and have been able to apply them as probes of this kind. Thus: (1) Purified AChR from chick optic lobe was specifically precipitated by two of the monoclonal antibodies so far characterized (Table 3; Fig. 6A). The same extent of reactivity was seen with a given monoclonal antibody toward both the optic lobe receptor and the similar component from chick brain cortex. (2) In purified preparations from CNS, the total protein present (as shown by radiolabeling with ^{125}I) was immunoprecipitated in parallel with the immunoprecipitation of the [^{125}I]α-bungarotoxin-receptor complex (Fig. 6A). (3) Ultracentrifugation after complexing of these CNS proteins with a reactive monoclonal antibody showed that they could be shifted to a lower position on the gradient (Fig. 6B), in the same way that the muscle AChR (Fig. 4A) can be. The size of the immune complexes with antibody 7B2 corresponds to a 1:1 attachment of this monoclonal antibody to the AChR (see Fig. 6B), for both the brain and the muscle AChRs. (4) The subunits (M_r = 53,000, 60,000, and 70,000, discussed above) detectable in the purified AChR from chick optic lobe could be specifically and simultaneously precipitated in the reaction of monoclonal antibody 7B2 with the native AChR. In the immunoprecipitate, these subunits were visualized by means of SDS gel electrophoresis and autoradiography.

These observations confirm that these two types of brain "AChR" are related structurally to the peripheral AChR. Observations 2, 3, and 4 confirm that all of the protein isolated in the purification from optic lobe is AChR. Observation 4 shows that the subunits identified therein are all part of the intact receptor molecule.

Other properties of these monoclonal antibodies are themselves of interest here. Very varied cross-reactivities are displayed (Table 3) toward the AChRs from diverse sources by the different monoclonal antibodies. The one exception is for the optic lobe receptor, compared with the "receptor" from the rest of the chick brain. These two components always behaved identically in antibody reactions. Another feature of interest is that all of the mice inoculated with the cloned cells producing such an anti-muscle-receptor antibody developed experimental autoimmune myasthenia gravis (Mehraban et al. 1983). This indicates that the mouse AChR (which is the origin of this response) possesses an antigenic determinant in common with the AChR from chick muscle, which is exposed extracellularly.

We conclude that the optic lobe receptor and the component from the rest of the brain which is purified similarly on immobilized α-bungarotoxin are so far indistinguishable. There is no difference discernible in their protein properties, subunit structures, binding parameters for a series of ligands (Barnard and Dolly 1982; Norman et al. 1982), and immunological cross-reactivities with a series of antibodies. They are both labeled in the same way, and on the 54,000 M_r subunit, with BrACh (Norman et al. 1982). They may be truly identical; any apparent difference in blockade could then arise from different cellular locations with respect to electrophysiological testing. Alternatively, they may be very similar proteins but with a subtle difference in their toxin-binding sites.

Table 3. Cross-reactivities with Various AChRs' Proteins of Monoclonal Antibodies Raised against the AChR from Chicken Muscle

Receptor antigen[a]	Titer (%)[b] 2A3	5B2	7B2	7B3
Chicken muscle				
denervated	100	100	100	100
innervated	100	126	100	200
embryonic	92	100	85	100
Chicken				
optic lobe[c]	0	0	11	15
brain cortex[d]	0	0	11	12
Cat muscle (denervated)[c]	0	4	25	12
T. marmorata electric organ	0	5	5	34

[a]The AChR in a crude extract was used, except where noted, as the source, prepared in the presence of a set of protease inhibitors as in Sumikawa et al. (1982b). In all cases the [^{125}I]α-bungarotoxin receptor complex was prepared and used as the antigen.

[b]Expressed as a percentage of the apparent titer against the homologous antigen, for each of the four monoclonal antibodies listed, all raised against the AChR purified from chicken denervated muscle. The values quoted are the mean of three or more experiments with duplicate samples; % errors were approximately 5%. Apparent titers were measured as described by Mehraban et al. (1983).

[c]The pure receptor was used as the antigen here.

[d]α-Bungarotoxin-binding component (pure) was used as the antigen here.

Brain GABA Receptors

The $GABA_A$ receptor of vertebrate brain is known from electrophysiological analysis (for review, see Study and Barker 1981; Haefely and Polc 1983) to open a chloride channel at a major class of GABA-mediated inhibitory synapses of the brain. The $GABA_A$ receptor is distinct from the $GABA_B$ receptor: The latter class has been defined by Hill and Bowery (1981) as another form of receptor, binding GABA but (unlike $GABA_A$) requiring Ca^{++} for this, sensitive to baclofen, and insensitive to bicuculline. The $GABA_B$ receptors have a different mechanism of action and a different distribution in the CNS. The $GABA_A$ receptors themselves probably comprise more than one subtype (Olsen 1981; Haefely and Polc 1983); we shall be concerned here with the major subtype of the $GABA_A$ receptor.

At these $GABA_A$ receptors in the neuronal membrane, the response to stimulation is extremely fast, typical rapidly gated channels are opened under the in-

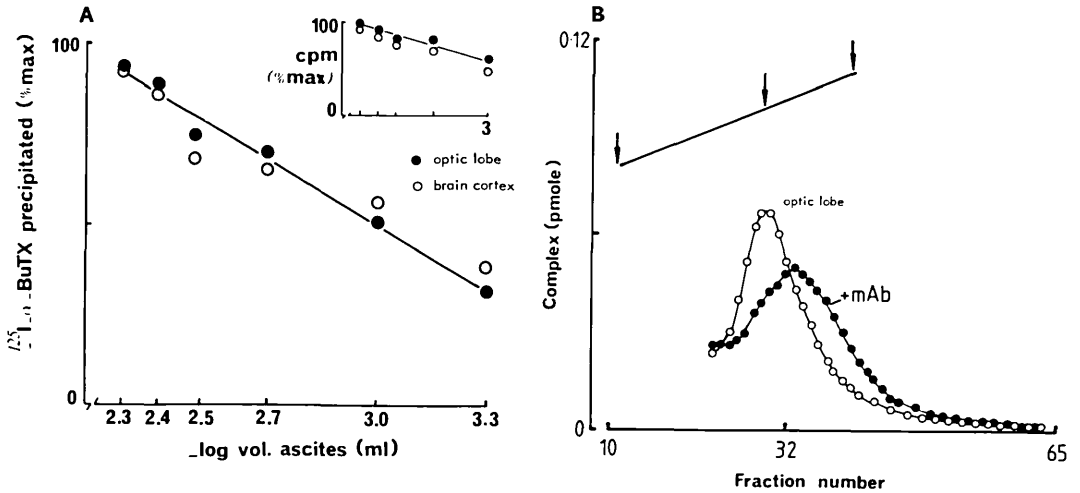

Figure 6. Reaction of chick brain receptors with monoclonal antibody directed against muscle AChR. (*A*) The receptor purified from 1-day chicks, in the form of its complex with [^{125}I]α-bungarotoxin, was incubated (8 nM) with dilutions of the ascites fluid containing monoclonal antibody 7B2 (see Table 3) overnight at 4°C. The product was immunoprecipitated in a procedure using Protein A. The percentage of the amount precipitable at the maximum, on the linear ordinate, is plotted against a logarithmic scale representing decreasing antibody additions. This titration was performed with the optic lobe AChR (○) or the α-bungarotoxin-binding "receptor" from the rest of the chick brain (●) as the antigen. (*Insert*) A similar plot was constructed, but using another preparation of each antigen, in the form of the free receptors, the protein being ^{125}I-labeled. In both cases, it is seen that the behaviors of the optic lobe receptor and of the brain "receptor" are identical. They give the same high affinity in the binding to this monoclonal antibody, which was raised against the authentic AChR from chick muscle. (*B*) AChR purified from the chick optic lobe (1 pmole) in Triton X-100 solution was complexed with [^{125}I]α-bungarotoxin. An aliquot was treated (4 hr at 23°C) with a 600-fold excess of monoclonal antibody 7B2 (see Table 3), raised against chick muscle AChR. Sedimentation on a 5–20% sucrose gradient is shown. It is seen that all of the complex is moved to a position toward the heavier (high fraction numbers) side which corresponds roughly to a 1:1 receptor-IgG complex. Unlike the other reactive antibodies, excess of this antibody did not lead to aggregates appearing near the bottom of the gradient (fraction 65); hence, it is concluded that this particular monoclonal antibody binds to the AChR in a 1:1 mode, i.e., its two binding sites recognize two copies of a determinant on one receptor molecule (i.e., on the two α-subunits or in two extremely homologous regions on two different subunits). This same behavior was shown by this monoclonal antibody, also, with the "receptor" from the rest of the chick brain, and likewise with its homologous muscle receptor antigen. Hence, the double determinant pattern is common to the chick muscle, chick optic lobe, and chick brain AChR oligomers. This reinforces the conclusion that the muscle and brain AChRs are related proteins. Arrows show the linear calibration with three marker proteins. (The fractions before 20 contained some free toxin only, which is not shown.)

fluence of GABA, and no evidence for involvement of any second messenger system is obtainable (Olsen 1981). All of the properties of this response (for review, see Krogsgaard-Laarsen et al. 1979; Haefely and Polc 1983) indicate that it involves the direct opening of a chloride channel by the binding of agonist to the receptor. Furthermore, the benzodiazepine (BZ) drugs (tranquilizers) potentiate the electrophysiological response to GABA (Brown and Constanti 1978; Study and Barker 1981; Haefely and Polc 1983) and are believed to exert therapeutic effects by means of their binding to the $GABA_A$ receptor (Olsen 1981). Binding of those drugs at nanomolar levels has been demonstrated to occur at a specific set of sites in brain membranes, and—highly significantly—this BZ binding interacts with GABA agonist binding, with the affinity for the BZ being increased severalfold when the GABA sites are all occupied (for review, see Olsen 1981). From these and a variety of other findings that testify to an interaction of the two sites, it is generally considered now that the $GABA_A$ receptor contains in its total structure a BZ-binding unit (for references, see Olsen 1981; Chang and Barnard 1982).

Other sites therein can also be defined pharmacologically. Barbiturates (Barb) bind to a distinct site associated with the $GABA_A$ receptor; this binding enhances both BZ binding and GABA agonist binding (Leeb-Lundberg et al. 1980; Olsen 1981). Barbiturates also enhance the electrophysiological response to GABA application (Brown and Constanti 1978; Study and Baker 1981; Haefely and Polc 1983). Specific blockers of the chloride channel are also known: Picrotoxinin binds selectively, does not affect any of the aforementioned ligand sites, but blocks channel opening (Olsen 1981; Haefely and Polc 1983). The $GABA_A$ receptor therefore contains at least three interacting sites for different ligand classes, plus a chloride channel structure that a picrotoxinin-binding site can regulate. We can represent it as a complex $(G\text{-}BZ\text{-}Barb)Ch_{pic}$, where G = GABA, indicating an unknown relationship of those three ligand sites in the structure, and the simultaneous presence within the complex of a chloride channel with its regulatory picrotoxinin site (Ch_{pic}).

Molecular size. When the $GABA_A$ receptor is in the membrane, the complex has a molecular weight of about 220,000, as determined by radiation inactivation target size analysis; moreover, the binding of BZ and the binding of a $GABA_A$ agonist were lost at precisely the same dosage rate (Chang and Barnard 1982). This

supports the concept of a single protein structure in the membrane containing both sites. When solubilized in Triton X-100, the properties were again consistent with those of a unit with an approximate molecular weight of 220,000. Furthermore, the binding capacity for [^{3}H] muscimol, a $GABA_A$ agonist, migrated either in sucrose gradient centrifugation or on a gel filtration column as a single peak coinciding exactly with the BZ-binding capacities (Chang and Barnard 1982). All of these observations support the existence of the single complex noted above.

Purification of the $GABA_A$ receptor complex. Complete purification of this receptor has recently been obtained for the first time by E. Sigel and A. Stephenson at our Imperial College laboratory (Sigel et al. 1982, 1983). Bovine brain cortex was the source, the receptor being extracted from it by sodium deoxycholate. An immobilized BZ ligand was used for affinity chromatography, with specific displacement by another BZ compound, followed by ion-exchange chromatography. The product, 1800-fold enriched in binding sites, is a protein carrying both the BZ site and the $GABA_A$ agonist site, both retaining their high affinity. In SDS gels, the protein yields two major bands of apparent M_r 53,000 (α) and 57,000 (β; Fig. 7). The α-subunit becomes strongly labeled by photoaffinity reaction with the photosensitive BZ ligand [^{3}H]flunitrazepam, both in the brain membranes and in the purified product, suggesting that the BZ site resides, at least in part, on this α-subunit (Sigel et al. 1983). This subunit has been isolated from the pure preparation run in SDS gels and its aminoterminal sequence determined by microsequencing (B.M. Conti-Tronconi et al., unpubl.). The

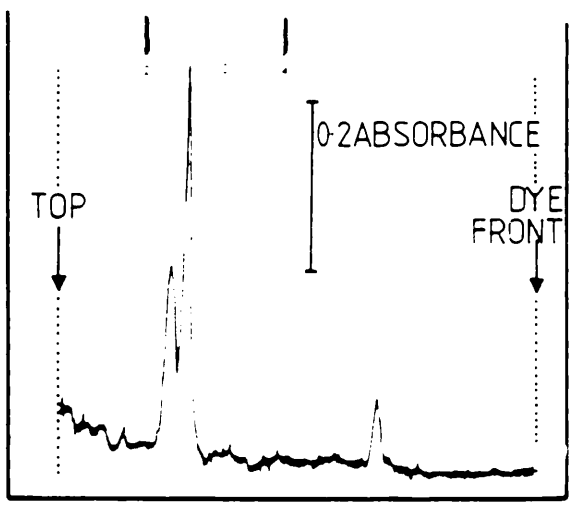

Figure 7. Detectable subunits of the GABA receptor. The purified GABA/BZ receptor was analyzed similarly. Two receptor subunits, at apparent M_r 53,000 and 57,000, are seen. (The small peak at 22,000 M_r is from a trace of soybean trypsin inhibitor, added earlier as an anti-protease.) The upper (parallel) track shows the positions of the five standards used, with two of them (serum albumin and carbonic anhydrase) seen. (Reprinted, with permission, from Sigel et al. 1983.)

application of sequence data for probe construction and gene cloning of the α-subunit of the $GABA_A$ receptor is in progress.

The purification of the GABA-binding protein on a BZ affinity column, along with all of the other observations reviewed here, confirms that the GABA-binding and BZ-binding components are together in a single complex. Specific ligand binding of the picrotoxinin-binding structure (Ch_{pic}) has been demonstrated to be retained in this receptor isolated in detergent solution (E. Sigel and E.A. Barnard, unpubl.), and reconstitution experiments in lipid bilayers have likewise resulted in a product in which the pure receptor retains all of the interacting sites described above.

To screen for a cDNA that encodes the α-subunit, it is not necessary that the receptor preparation used as the starting point have all of the true receptor subunits present and identified in it. It could be, for example, that additional subunits exist but are not seen in the gel analysis of the pure receptor protein, for one reason or another. However, so long as at least one subunit is isolated, this can be used either to design an oligonucleotide probe or to raise antibodies for applications, as discussed below, in the cDNA cloning procedures.

Channel formation induced by mRNA. Identification of mRNA coding for the proteins of the $GABA_A$ receptor complex was again achieved in the oocyte cellular translation system. Initially, the chick optic lobe has been used as the source, because this receptor complex accumulates in vivo faster in the developing chick than in the developing rat. This difference is associated with the known development in the rodent of many brain functions and components only over the first 2 weeks of postnatal life, whereas the newly hatched chick is more active and precocious. In fact, the linked GABA- and BZ-binding components were found to develop particularly rapidly in the optic lobe of the chick just before hatching (Smart et al. 1983).

Poly(A)$^+$ mRNA was prepared from chick optic lobe and was microinjected into *Xenopus* oocytes (40 ng per cell). After incubation for a day, the oocyte surface was shown to have developed functional GABA receptors (which do not exist in these oocytes prior to this treatment). Application of GABA produced an increase in Cl^- conductance (Fig. 8A): This response is dose-dependent (compare control responses to GABA at 5 μM and 40 μM in Fig. 8A). A smooth dose-conductance curve has been obtained with the half-maximal response at 15 μM GABA (Smart et al. 1983), in accord with the sensitivity to GABA of brain neurons in situ. The response can be blocked by physiologically active concentrations of the $GABA_A$ antagonist bicuculline or of the GABA receptor chloride channel blocker, picrotoxinin (Fig. 8A). Moreover, the response to GABA that is induced in the oocytes by the mRNA shows the specific phenomena of potentiation by BZ drugs and by barbiturates (Fig. 8B). These effects are all reversible, as the responses after wash-out (Fig. 8A,B) show. The pharmacology of the GABA receptor induced in the

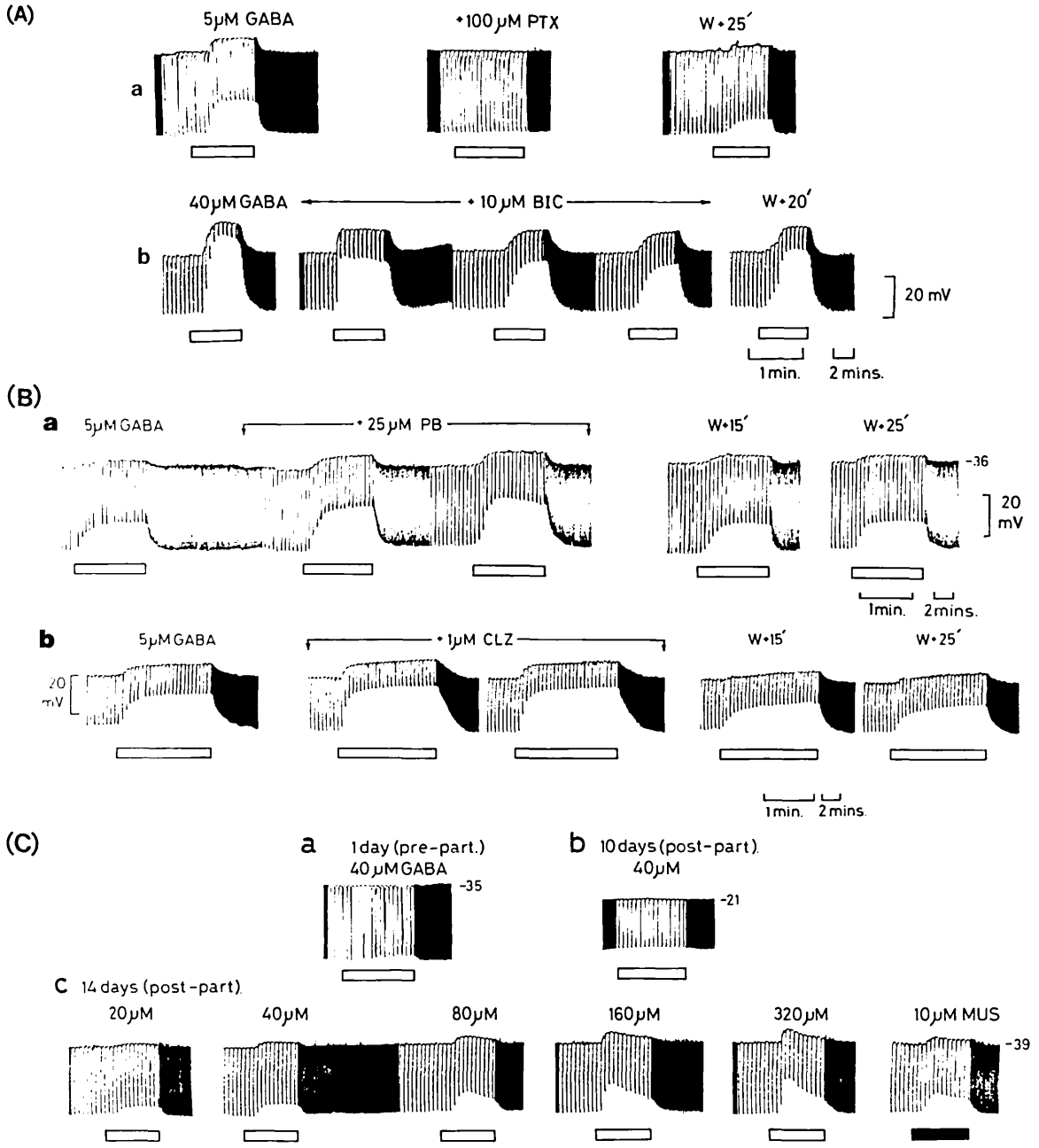

Figure 8. Responses of GABA receptors induced in the *Xenopus* oocyte cell membrane by the injection of brain mRNA. (*A*) Antagonism by picrotoxinin (PTX) or bicuculline (BIC) of the GABA-dependent membrane conductance change in oocytes 1 day after the injection of poly(A)$^+$ mRNA from the optic lobe of 19-day chick embryos. Downward deflections are hyperpolarizing electrotonic potentials evoked by injecting 35-nA current pulses (1 sec width; 0.2 Hz). Upward movements in the baseline represent membrane depolarization. Chart speed was reduced for drug offsets. (*a*) Control response to 5 μM GABA (applied in the superfused amphibian Ringer at room temperature) after 15 min in 100 μM PTX; further, after washing for 25 min in normal Ringer. (*b*) (Different cell) Control response to 40 μM GABA; three successive responses after 10 min in 10 μM BIC; after a 20-min wash. Open bars indicate periods of GABA application. Note the partial recovery obtained by washing, after either antagonist. (*B*) Enhancement of the GABA-induced conductance change by pentobarbitone (PB) or the benzodiazepine drug chlorazepate (CLZ) in oocytes injected (as in *A*) with chick optic lobe mRNA. (*a*) Response to 5 μM GABA in normal Ringer; two successive responses to 25 μM PB after 10 min (note the onset of desensitization in the later conductance change); two recovery responses after washing for 15 min and 25 min, respectively. (*b*) (Different cell) Response to GABA (5 μM) in control solution; two successive responses in 1 μM CLZ after 15 min (note a small enhancement); two recovery responses at 15 min and 25 min. The recovery from the enhancement due to CLZ or PB is always complete. (*C*) The GABA-dependent conductance change in oocytes injected with mRNA extracted from the whole rat brain during development. (*a*) From rats 1 day preparturition; (*b*) 10 days postparturition; (*C*) 14 days postparturition, with dose-dependent responses to the GABA concentrations shown and to muscimol (MUS). Note that only the oocytes injected with the 14-day brain mRNA exhibit a significant response to GABA. Note also that muscimol is more potent than GABA, as in the rat brain, and that the conductance change desensitizes at 80 μM GABA concentration and at 10 μM muscimol. (Scales as in *B*,*a*.)

oocyte has recently been found (Smart et al. 1983) to be as for the neuronal $GABA_A$ receptor. Baclofen (1 mM, in the presence of Ca^{++}) has no effect on it. All of the observations cited confirm that a functional receptor of the $GABA_A$ class is formed and becomes inserted in the oocyte membrane when the exogenous mRNA is translated. The entire complex (G-BZ-Barb)Ch_{pic}, is obviously assembled correctly in the membrane following the translation of the mRNA fraction used.

These observations have recently been extended to the $GABA_A$ receptor of mammalian brain (the source used for the subunit analyses). Rat whole brain was used as the source of poly(A)$^+$ mRNA, at different developmental stages. Only by 14 days after birth was the receptor mRNA activity fully present, and no activity was ever detectable in the embryonic brain (Fig. 8C). This is in accord with the ontogeny of the BZ-linked $GABA_A$ receptor complex in the rat brain (Olsen 1981). The formation of the $GABA_A$ receptor complex in the oocyte membrane showed the same properties when rat brain mRNA was used as when chick optic lobe mRNA was used. The increasing responses, up to saturation, to increasing doses of GABA are illustrated in Figure 8C. As also shown there, the $GABA_A$ agonist muscimol is about four times more potent than GABA, in agreement with the behavior seen at GABA-mediated neurons of the mammalian brain (Brown and Constanti 1978).

Hence, in two very different and complex receptor structures—the nicotinic AChR and the $GABA_A$ receptor (in each case from diverse sources)—the mRNAs are able to specify in the oocyte the synthesis of all of the component parts and the correct assembly in the membrane of the complex with its ion channel.

CONCLUSIONS

Requirements for Cloning of Receptor Genes

The major difficulties in the analysis of receptors, enumerated at the beginning of this paper, should to a considerable degree be overcome by exploiting the DNAs coding for receptor subunits and also by exploiting their mRNAs. The approaches at those two levels that have been adopted in our own initial studies are designed to be applicable to receptors generally, i.e., all of the other receptors of nervous systems that do not enjoy the unique tissue selectivity and concentration of the nicotinic AChR in the electroplax of *Torpedo*—a receptor source of which we can observe sadly, with Hamlet, "We shall not look upon his like again."

In this approach, there are three initial requirements:

1. Purification of a small quantity of the receptor protein and separation of at least some of its subunits.
2. The amino acid sequence of a short segment of one or more of those subunits, determined at present by aminoterminal microsequencing, applied to the subunit eluted after SDS-polyacrylamide electrophoretic separation.
3. An assay system for mRNA that monitors the receptor functions. Preferably this should detect binding functions *and* overall activity (e.g., channel opening). This is the value of the oocyte system.

Point 1 is needed for point 2. It is also needed for obtaining antibodies that would be used to aid the assays, perhaps also for immunoadsorptive purification of more receptor—especially employing anti-subunit antibodies—and perhaps for use in gene expression screening. The raising of monoclonal antibodies against the receptor in impure form will in many (but not all) cases be a rapid route to achieving requirement 1. Another good reason for pursuing requirement 1 as far as possible is that the information obtained, on the size of the oligomer and the number and types of subunits and their approximate sizes, is invaluable in searching for the correct gene products and in interpreting what is found.

Point 2, amino acid sequence information, is needed for identifying the clones unequivocally. It is also used, of course, for constructing primers or probes for screening. Examples of the power of the latter approach to receptor gene cloning are seen in the work reviewed above and in the remarkable progress of Dr. Numa and his colleagues (Noda et al. 1982, 1983a,b) on various subunits of nicotinic AChRs.

The above requirements need not apply where a given receptor gene, once obtained, is sought in other species. At least if the nicotinic AChR is a general precedent, a given subunit of a receptor is likely to be strongly conserved in structure. The muscle AChRs of different vertebrates generally strongly cross-react immunologically (see an example in Table 2; also Lennon and Lambert 1980; Souroujon et al. 1982). Likewise, their α-subunits are all in the molecular-weight range (apparent value, in SDS gels) of 40,000–44,000 and strong homology is expected. Cross-hybridization will be the method of choice here for obtaining the cDNAs for the corresponding subunits of the receptor from other species, once one muscle receptor cDNA is obtained. More generally, this possibility constitutes one of the important advantages of the cDNA approach over conventional protein isolation and analysis. The isolation of the receptor protein from the first source is a dead end so far as other species go; one must start the isolation anew, and the problems noted earlier are encountered with equal force for every new source. Not so at the DNA level. One of the laws of molecular biology that neurobiologists must constantly recall is that like nucleic acids hybridize with each other, but like proteins do not. Therefore, we should remain at the nucleic acid level as far as possible in comparing sequences from different species—one cDNA being used to isolate the next, by cross-hybridization. At least for each subunit type, say of the AChR, we should proceed by steps of cross-hybridization between species, and conceivably between ACh-binding proteins of all types.

The Oocyte Translation and Assembly System

If requirement 3 in the list above is met, this can greatly facilitate the molecular genetic analyses for re-

ceptors at two different levels. First, it can identify an mRNA population active in producing receptor subunits, for use in DNA cloning. We have seen that translation to form the subunits of a receptor protein in a *cell-free* assay system is unlikely to produce the active receptor. In the case of relatively unknown receptors, therefore, the production of the receptor chains in conventional cell-free translation may be difficult to recognize unequivocally.

Second, the oocyte system can verify in such a case that the mRNAs produced lead not only to the structure in the receptor that binds a ligand, but also to the full structure that maintains the channel gating or other effector function. We can test here for the coding for all of the components that contribute to an assembly of functions, as in the GABA receptor example. These binding activities for one ligand might arise by translation to give only a part of the receptor structure.

Related to this second level of mRNA analysis, the oocyte system is one in which a range of different types of expression experiments can readily be conducted. Amenable to analysis, there will be the product of an individual subunit mRNA, or of mRNAs for selected incomplete sets of subunits, or of specifically altered mRNAs derived through DNA mutagenesis experiments or by hybridization to synthetic probes. The evidence so far is encouraging for the prospect for such defined manipulations based on the sequence and on structure predictions. That evidence suggests that they may be pursued in the oocyte system, at the levels of binding functions, oligomer assembly, plasma membrane insertion of the receptor, channel function, and regulation of channel function.

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THE PURIFIED GABA/BENZODIAZEPINE COMPLEX: RETENTION OF MULTIPLE FUNCTIONS

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Summary The GABA/benzodiazepine (BZ) receptor has been purified to physical homogeneity from bovine, rat and chick brain cortex. It preserves the binding sites for GABA agonists and antagonists, BZ agonists and antagonists, β carbolines, pentobarbital and bicyclophosphate channel modulators and the characteristic interactions between these sites. The protein has mol. wt. ~200,000. Reconstitution into liposomes brings its functions closer to those of the native state. mRNAs from chick or rat brain can produce in the membrane of Xenopus oocyte the fully functional receptor/ion channel.

The GABA/BZ receptor has been purified in two different media with different results (Sigel et al. 1983). In (A), extraction was in deoxycholate (with protease inhibitors and 10% sucrose). In (B), 1.5% CHAPS/.15% Asolectin was used for solubilisation.

In both media, the receptor is obtained as a single protein species: in gel isoelectric focussing it gives a single sharp symmetric peak, pI 5.6 (Barnard et al., 1984). In sucrose density gradient centrifugation in 0.5% Triton X100/1M NaCl it gives a single peak, apparent sedimentation coefficient 7.4S. In gel filtration on Ultrogel ACA-22 in the same medium it gives a single peak of Stokes radius 7.3nm. The binding sites for [^{3}H] flunitrazepam and [^{3}H] muscimol co-migrate completely in these separations. The receptor has 3, perhaps 4, subunits, in SDS/polyacrylamide gel electrophoresis; M_r 53000 and two subunits close together near 57000. A faint band about 65000 is sometimes also seen. The 53000 band is labelled most strongly by 70nM [^{3}H] flunitrazepam in photoaffinity labelling experiments.

By sedimentation in H_2O and D_2O to give correction for the bound detergent, the true sedimentation constant of the receptor-detergent complex is found to be 8.5S in 0.5% Triton X100/1M NaCl and, in combination with the Stokes radius of 7.3nm, a value of about 230,000 is obtained for the protein molecular weight.

In preparation (A), the binding of a number of ligands for the various sites of this complex was retained (Table 1). In preparation (B), these were also observed and the binding of the channel-modulatory ligand [^{35}S] t-butylbicyclophosphorothionate (TBPS) (Table 1).

In preparation (B) above, the enhancement by GABA of BZ binding and the enhancement by pentobarbital of BZ binding were also retained (Table 1).

Antibodies have been raised to the pure receptor: these precipitate all of the GABA/BZ receptor protein present from the crude detergent solution (Barnard et al., 1984).

Messenger RNA was extracted from chick optic lobe or rat brain. In a collaboration with D.A. Brown., A. Constanti., K. Houamed and T.G. Smart at the London School of Pharmacy, it has been shown that a response to GABA is then introduced into the oocyte plasma membrane. All of the above-mentioned pharmacological properties of the $GABA_A$ receptor are produced, together with the GABA-opened chloride channel (Smart et al., 1983). The polypeptides of the GABA receptor are assembled into that complex in the membrane in the correct orientation. The electrophysiological evidence is covered by our collaborator Dr. A. Constanti in this symposium.

Table 1. The properties of purified bovine GABA/BZ receptor.

Property	Preparation A	Preparation B
[^{3}H] Flunitrazepam	K_D = 10nM	K_D = 11nM
[^{3}H] Muscimol	K_D = 12nM	K_D = 33nM*
[^{35}S] TBPS	N.D.	K_D = 90nM
GABA stimulation of BZ binding	Weak	EC_{50} = 2μM
Pentobarbital stimulation of BZ binding	N.D.	EC_{50} = 300μM
Subunit structure †	M_r 53000, 57000	M_r 53000, 57000
Molecular size Δ	~230,000	~230,000

Preparation A is bovine brain membranes solubilised with 0.5% sodium deoxycholate and subsequently purified in the presence of 0.5% Triton X100. Preparation B is bovine brain membranes solubilised with 1.5% CHAPS/0.15% Asolectin and subsequently purified in the presence of 1.5% CHAPS/0.15% Asolectin; * [^{3}H] muscimol binding activity measured in the presence of Triton X100; N.D. is not detectable; † is the minimum polypeptide composition and Δ molecular size is determined by H_2O/D_2O sucrose density gradient centrifugation and gel filtration measurements.

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Expression of functional GABA, glycine and glutamate receptors in *Xenopus* oocytes injected with rat brain mRNA

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The use of Gurdon's[1] *Xenopus* oocyte translation system has allowed the production of neurotransmitter receptors in a foreign cell membrane, following the translation of microinjected mRNA isolated from various sources[2–4]. This very accessible and relatively simple preparation permits the study of the requirements for receptor–ionophore function, assembly and membrane integration. This analysis is presently feasible for the peripheral nicotinic acetylcholine receptor[2,3], the chick brain γ-aminobutyrate (GABA) receptor[4] and the rat brain serotonin receptor[5]. We now report the novel and successful expression of the GABA, glycine, glutamate and related acidic amino acid receptors of mammalian brain, and show that they exhibit pharmacologically separate identities when their mRNAs are processed in the amphibian oocyte.

mRNA was extracted from day 14 Wistar (WA/KIR) rats using a guanidinium thiocyanate method[6]. Poly(A) mRNA was isolated by oligo(dT)–cellulose chromatography and injected into *Xenopus laevis* oocytes (~40 ng per cell) as previously reported[4]. After microinjection of the mRNA, cells were maintained in an incubator at 21 °C for 24 h before recording. They were superfused at room temperature (20–23 °C) with frog Ringer containing (mM): NaCl 118; KCl 1.9; $CaCl_2$ 2.0; Tris 2.5 buffered to *p*H 7.4 with 1 M HCl. All drugs (BDH or Sigma) were dissolved and applied in this medium. This study was based on data obtained from 47 cells chosen from 4 injected batches of oocytes. Recording conditions and the two-electrode current clamp were as previously described[4].

All injected oocytes examined responded to bath-applied GABA and glycine by changing their resting membrane potential and conductance; control, non-injected cells showed no sensitivity to these neutral amino acids. Both GABA (2–640 μM) and glycine (20 μM–1.6 mM) evoked a 'smooth' membrane potential depolarization (compare with the oscillatory responses to acetylcholine[7] obtained in uninjected oocytes, and also serotonin[5] and, in this work, glutamate in mRNA-injected oocytes) which was coupled with an increase in membrane conductance (Fig. 1*b*). These responses to GABA and glycine were quite reproducible, enabling dose–conductance curves to be constructed (Fig. 1*a*); GABA was invariably more potent than glycine (2–20-fold, dependent on individual oocytes). The low dose thresholds suggest a minimal influence of uptake mechanisms on the responses, although individual GABA responses always exhibited a rapid fade or desensitization, as frequently seen in rat central nervous system (CNS) neurones (a feature not observed with responses from chick brain GABA receptors expressed in oocytes[4]). Equivalent glycine responses exhibited less desensitization.

When the membrane potential was progressively depolarized by current injection, previously matched responses (depolarizations) to doses of GABA and glycine decreased in amplitude and eventually reversed at approximately −25 mV (Fig. 2). This

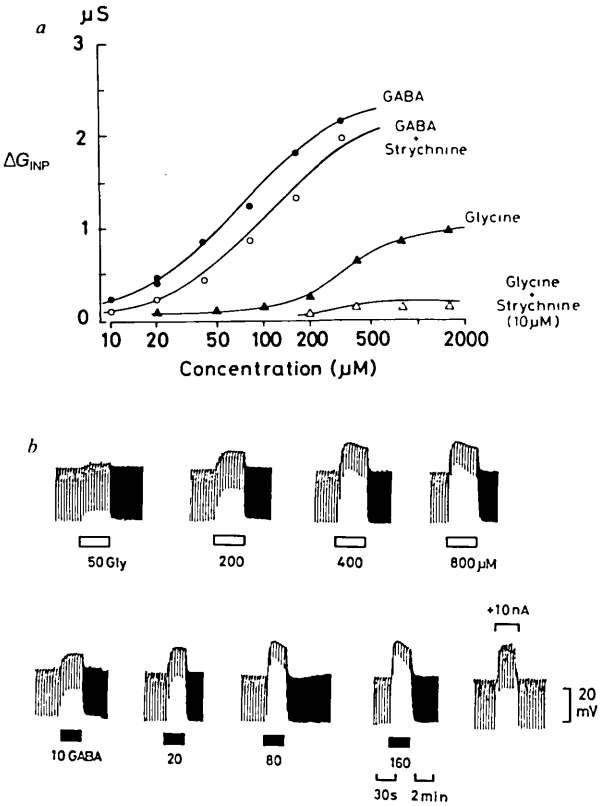

Fig. 1 *a*, GABA and glycine dose–conductance curves in the presence and absence of 10 μM strychnine. Data were obtained from a single oocyte by applying control doses of GABA (●) and glycine (▲) in the absence of strychnine and then repeating these in the presence of 10 μm strychnine (○, △, respectively). Note the small lateral shift in the GABA curve and the marked 'non-competitive' depression of the glycine curve in antagonist solution. The amino acid evoked input–conductance change (ΔG_{inp}) was uncorrected for membrane rectification. *b*, Membrane conductance changes evoked by bath-applied GABA (lower traces) and glycine (upper traces) in a single oocyte. Downward deflections are hyperpolarizing electrotonic potentials recorded in response to d.c. current injection (20 nA, 1 s duration, 0.25 Hz). Upward deflections of the baseline indicate membrane depolarizations induced by GABA or glycine applied during periods indicated by the bars. Resting potential, −70 mV. GABA produces a greater maximal conductance change than glycine. Desensitization is more apparent in the GABA traces (compare 80 μM GABA with 200 μM Gly). The evoked conductance changes were largely attributable to the amino acids and not solely due to membrane rectification as shown by the end response in the lower trace, where d.c. current depolarized the cell to match an equivalent GABA response (160 μM GABA) but exhibited a much smaller conductance change.

reversal potential corresponds very closely with the chloride equilibrium potential in these cells[7], suggesting that (as expected) chloride ions probably mediate both the GABA and glycine responses.

The apparent conductance changes produced by GABA and glycine were enhanced in the depolarizing direction by membrane rectification. Oocytes injected with mRNA seemed to exhibit a more pronounced rectification than the non-injected controls; perhaps the heterologous mRNA mixture can induce the cell to express a voltage-sensitive rectifying channel in its membrane (see ref. 8). This rectifier was not blocked by tetraethylammonium (20 mM) or 4-aminopyridine (10 μM).

The properties of these neutral amino acid receptors were further investigated using picrotoxinin and strychnine (both antagonists at vertebrate CNS inhibitory synapses[9,10]), and the GABA potentiators pentobarbitone[11] and chlorazepate[4], a water-soluble benzodiazepine. Strychnine, 1 μM, inhibited the responses to glycine (Fig. 3*a*) in a 'non-competitive' manner

(that is, simply reducing the maximum of the dose–response curve with no lateral shift), with little effect on membrane potential. The glycine responses were slow to recover fully, requiring up to 2 h washing. Using a larger dose (10 μM), strychnine blocked not only glycine but also GABA responses (Fig. 1*a*); however, the latter responses were easily recovered on washing. Similar effects of 'high dose' strychnine on GABA responses have been observed previously on CNS neurones[12]. Picrotoxinin (1–5 μM) reversibly depressed both GABA and glycine responses (Fig. 3*b*), suggesting that this agent lacks the specificity exhibited by strychnine. (+)Bicuculline (20 μM) showed more specificity by reversibly antagonizing GABA in preference to glycine responses. However, GABA responses compared with glycine, were selectively enhanced by 50 μM pentobarbitone (Fig. 4*a*), and also by 10 μM chlorazepate (Fig. 4*b*).

The preliminary results indicated that these GABA receptors possessed a pharmacological sensitivity that was representative of vertebrate CNS GABA receptors. However, the 'oocyte-expressed' glycine receptors appeared more sensitive to picrotoxinin than their counterparts in native neuronal membranes[13].

The heterologous mRNA also endowed the oocyte with sensitivity to the acidic amino acids L-glutamate (10 μM–1 mM), kainate (1–200 μM), L-aspartate (100 μM–2 mM) and quisqualate (10–20 μM) (Fig. 5). Sensitivity to serotonin (1 nM threshold) and β-alanine (0.3–5 mM) was also observed, but taurine (0.5 mM), *N*-methyl-D-aspartate (100 μM), L-cysteic acid (100 μM) and D-aspartate (50 μM) were inactive. Generally, the excitatory amino acids exhibited the following order of potency on a single oocyte: quisqualate ≃ kainate > glutamate ≃ L-aspartate. The relative potencies were again dependent on the

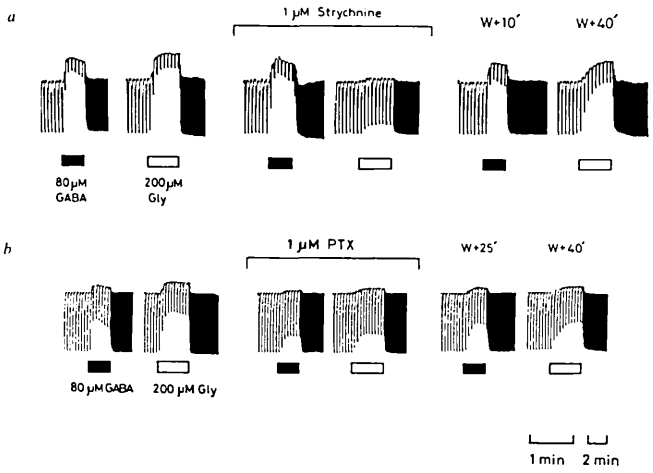

Fig. 3 The effect of the amino acid antagonists strychnine and picrotoxinin on the responses to bath-applied GABA (hatched bars) and glycine (open bars) responses obtained from two oocytes. Upward deflections of the trace indicate membrane depolarizations and downward deflections are hyperpolarizing electrotonic potentials (evoked by 15 nA (*a*) and 24 nA (*b*)). *a*, GABA responses are virtually unaltered by 1 μM strychnine, which preferentially antagonized the glycine response. An almost complete recovery from strychnine was obtained after 40 min washing (W). Single oocyte, resting potential, −47 mV. *b*, Low-dose picrotoxinin (1 μM) did not exhibit any selectivity for the GABA response as glycine responses were also reduced. Recovery for GABA responses was obtained after 25 min washing in normal Ringer, whereas glycine was still depressed after 40 min. Single oocyte, resting potential, −51 mV.

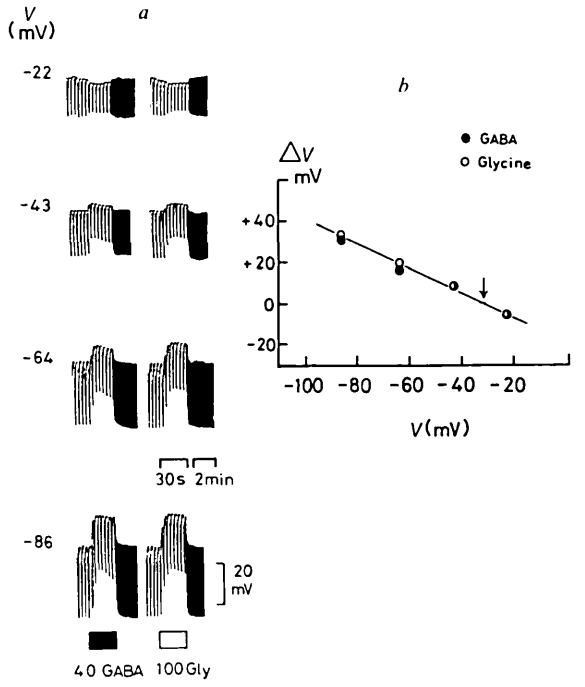

Fig. 2 Measurement of the reversal potential for the GABA- and glycine-induced membrane depolarizations in the same oocyte. *a*, 40 μM GABA and 100 μM glycine were bath applied and the membrane depolarization was measured at four holding potentials, −22, −43, −64 (normal resting potential) and −86 mV. *b*, The change in the induced membrane depolarization (Δ*V*) is plotted for GABA (●) and glycine (○) against the holding potential (*V*). Both responses had the same reversal potential, approximately −31 mV. Hyperpolarizing electrotonic potentials were evoked by −25-nA current steps.

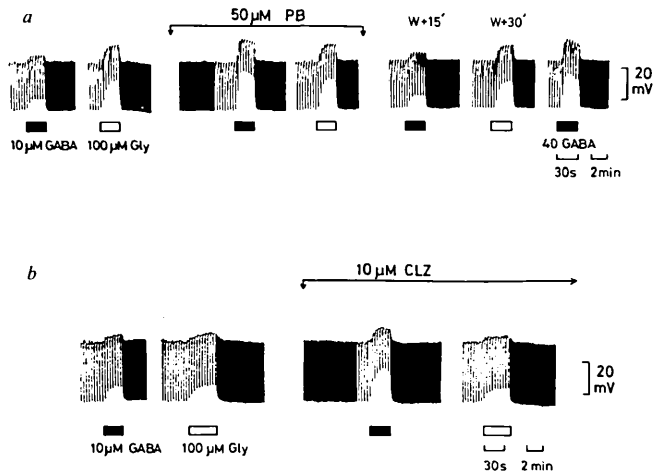

Fig. 4 Enhancement of the GABA-evoked conductance change by 50 μM pentobarbitone (PB) and chlorazepate (CLZ) in two oocytes. *a*, 10 μM GABA and 100 μM glycine responses (both submaximal) were recorded in control solution, then after 5 and 15 min in 50 μM PB, respectively, and finally after 15 and 30 min wash in control Ringer. Resting potential, −69 mV. Note that only the GABA response was enhanced, an effect which was easily reversed on washing. The 10 μM GABA response was enhanced to the level of a 40 μM GABA response in control solution as shown by the end response in this trace. *b*, Control responses to 10 μM GABA and 100 μM glycine were recorded in normal solution, and then after 7 and 14 min in 10 μM CLZ, respectively. Resting potential, −62 mV. Only the GABA response was again enhanced but on washing a recovery was not observed as noted previously[4].

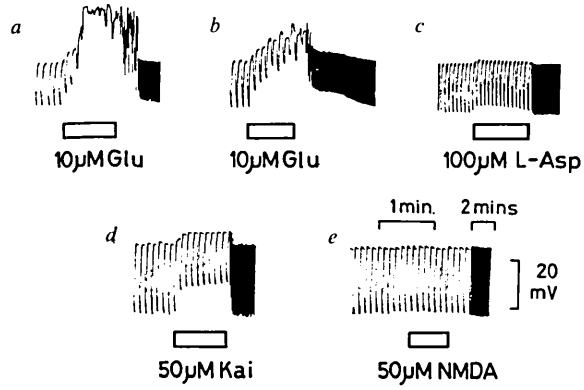

Fig. 5 Membrane conductance changes produced by bath application of excitatory amino acids in *Xenopus* oocytes injected with rat brain mRNA. Records are from four oocytes (*a–d*). *a*, Response to 10 μM L-glutamate (Glu); note the oscillatory nature of the membrane potential and conductance. Recovery was rapid on washing. The low-dose response suggests that the effect of glutamate uptake is minimal. Electrotonic potentials were elicited by −10 nA injected current; resting potential, −81 mV. *b*, Response to 10 μM L-glutamate on a different oocyte which is less sensitive to Glu but which still produces membrane potential and conductance oscillations. Current injection, −10 nA; resting potential, −84 mV. *c*, Response to 100 μM L-aspartate (L-Asp) on a different oocyte. Note only a small, fading or desensitizing response with no obvious oscillation of the membrane potential or conductance. Current injection, −20 nA; resting potential, −74 mV. *d*, Response to 50 μM kainate (Kai). Membrane conductance and potential changes exhibited no fade, desensitization or oscillation and were rapidly reversible on washing. Current injection, −22 nA; resting potential, −62 mV. *e*, Lack of response to 50 μM *N*-methyl-D-aspartate (NMDA); same oocyte as in *d*. D-aspartate (100 μM) also failed to evoke any response.

individual oocyte, quisqualate and kainate being 2–10 times more potent than glutamate and L-aspartate.

Our experiments clearly show the expression of receptor complexes to the two neutral amino acids which are integrally involved with inhibitory neurotransmission in the vertebrate CNS[10]. Whereas the role for GABA as an inhibitory neurotransmitter in mammalian brain is reasonably established, comparable evidence for glycine is lacking (compare ref. 14). It is possible that mRNA promotes the expression of glycine receptors even in the possible absence of any discrete glycinergic pathway, for example, non-synaptic receptors. A precedent for this situation does exist in the peripheral nervous system, where most neural tissues contain GABA receptors in the absence of any GABAergic pathway[15].

The GABA receptor from mammalian brain has recently been purified and found to contain several subunits, incorporating the various specific ligand sites for this receptor[16,17]. Presumably, the mRNA fraction used here contains mRNA molecules coding for each of these subunits; the subunits are subsequently assembled and integrated into the membrane with the formation of their intrinsic ionophore, adopting the correct orientation. The same must be true for the mRNAs coding for the glycine receptor subunits, which have also been isolated as a pure protein[18]. It is presently unclear whether these two receptors possess identical chloride ionophores: the evidence, both from patch clamping of cultured neuronal membranes[19] and from our translation results, is compatible with this concept, but not incontravertible. Our results suggest that the biosynthesized proteins modulating the chloride channel(s) in the receptor complexes are different, because the amino acid-evoked conductance is selectively modified by several different ligands. It was interesting that the receptor for the major excitatory neurotransmitter, glutamate, and several related, putative transmitters can also be assembled in the oocyte.

This mRNA preparation from mammalian brain provides a further example of the faithful post-translational processing at which the *X. laevis* oocyte excels. The pharmacological accessibility of this preparation makes a detailed study of amino acid receptors (both neutral and acidic) a reality without the complications presented by an intact CNS.

After submission of this manuscript, Gundersen *et al.*[20,21] reported the expression of kainate and glycine receptors in the *X. laevis* oocyte, after microinjection with human brain mRNA. Our results with kainate, glycine and rat brain mRNA are largely confirmatory to this study.

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