the gametes in the presence of antimycin, a potent inhibitor of mitochondrial electron transport and respiration. The effect of these treatments on gamete respiration was determined using Warburg manometry to estimate oxygen uptake by eggs[11] and a Teflon covered Clark electrode (Yellow Spring Instrument Co) to estimate sperm respiration[12]. Fertilization was allowed to take place in a Warburg flask at 25 °C. Eggs were placed in the main compartment in 0.5–1.0 ml of 10% Ringer solution at pH 7.4. The side arm contained 0.7 ml of the sperm suspension.

As anaerobic experiments, the flasks were flushed with nitrogen for 10 min. The contents of the flasks were maintained under nitrogen for an additional 10 min before the spermatozoa in the side arm were tipped into the main compartment containing the eggs. Control experiments carried out under aerobic conditions were made simultaneously. At the appearance of the first embryo cleavage stage in the control flask, the contents of all flasks were removed and the number of fertilized ova counted.

Table II. Anaerobic fertilization of amphibian body-cavity eggs

	Fertilization (%)	
	Anaerobic	Control
1	56 (134)	48 (100)
2	100 (21)	100 (24)
3	75 (25)	98 (54)
4	68 (30)	89 (25)
5	63 (28)	91 (28)
Mean ± SE	72 ± 7.6	85 ± 9.6

Conditions were same to those indicated in Table I.

Table III. Effect of antimycin on the fertilization of mature eggs

	Fertilization (%)	
	Antimycin	Control
1	34 (32)	85 (59)
2	29 (68)	97 (64)
3	31 (54)	98 (60)
4	33 (62)	100 (70)
5	30 (54)	92 (68)
Mean ± SE	31 ± 0.9	94 ± 2.7

Concentration of spermatozoa was 10^8 sperm/ml. Concentration of antimycin was 1.0 µg/ml. The number in parentheses indicate the number of eggs used in each experiment. Each experiment was performed on eggs and sperm obtained from different animals.

Results and discussion. As is clear from the data in Table I, oocytes from the species *Bufo arenarum* are fertilized under anaerobic conditions. The fertilization of eggs taken from the body cavity (Table II) eliminates the possibility that the gelatinous coat found on eggs taken from the ovisac prevent anoxia when a nitrogen purge alone is used. Fertilization of eggs in the presence of antimycin in concentration that completely stops respirations (Table III), eliminates the possibility that technical errors were introduced in experiments using nitrogen only.

This is the first vertebrate species in which fertilization under anaerobic conditions has been observed. However, fertilization under these conditions has been observed in invertebrates and bacteria. GUZMAN BARRON[13] reported that the germinal cells of *Nereis* were unaffected by the lack of oxygen and were able to carry out fertilization 5 h after exposure to an oxygen-free environment; and more recently, STALLIUS and CURTIS[14] showed that chromosome transfer in *E. coli* occurs at high frequency under anaerobic conditions.

The lower rate of fertilization of eggs in the presence of antimycin, compared to the experiments in which nitrogen was used, suggested that the antibiotic may have other effect on fertilization not related to an effect on mitochondrial electron transport and respiration.

PETERSON and FREUND[15] have shown that high levels of ATP are maintained in human spermatozoa even in the absence of air. A similar situation appears to exist in amphibian gametes which generate sufficient energy anaerobically not only to maintain viability, but also to carry out fertilization.

Our studies showed no difference in the cleavage rate or any other anatomical character, which would indicate morphologic abnormality when fertilization in this species is carried out anaerobically.

[11] A. LEGNAME and F. D. BARBIERI, Archos. Bioq. Quim. Farm. Tucumán *10*, 31 (1962).
[12] A. DEL RIO, A. M. ORCE REMIS, O. ROVERIS and R. VALLEJOS, Biol. Reprod. *12*, 325 (1975).
[13] E. S. GUZMAN BARRON, Biol. Bull. *62*, 46 (1932).
[14] D. R. STALLIONS and R. CURTIS, J. Bact. *111*, 294 (1972).
[15] R. PETERSON and M. FREUND, Biol. Reprod. *3*, 47 (1970).

Specialized Membrane Junctions in the Avian Cerebellum[1]

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Summary. Pentalaminar specialized membrane junctions – tight junctions – are described in the granular layer of the pigeon cerebellum. The presence of these axo-dendritique and dendrosomatic contacts suggest the existence of electrotonic coupling in the pigeon cerebellum.

Specialized junctional zones between nerve cell processes, where membranes are in close apposition, have been described in the nervous system of many animal species[3]. BENNETT, PAPPAS et al.[4] demonstrated that these low resistant junctions are the morphological counterpart of electrotonic coupling, a phenomenon which now appears to be quite frequent in invertebrates as well as vertebrates. These intercellular contacts were

[1] This work received financial support from the Instituto de Alta Cultura, Lisbon, Project No. PMC5-I.
[2] Acknowledgments. I am greatly indebted to Prof. E. G. GRAY and Prof. A. SOUSA PINTO for helpful advice and criticism. The technical assistance of Mrs. M. M. PACHECO, M. G. RODRIGUES, M. L. BRITO and Mr. BESSA NUNES is gratefully acknowledged.
[3] C. SOTELO, in *Chemistry and Brain Development* (Eds. R. PAOLETTI and A. N. DAVISON; Plenum Publishing Corporation, New York 1971), p. 239.

first regarded as tight, pentalaminar occlusions of the intercellular cleft. REVEL and KARNOVSKY[5] and KARNOVSKY[6], with the aid of lantanum and horseradish peroxidase, used as tracers, demonstrated that in liver cells and heart muscle, similar junctions have a narrow median gap, 2 to 4 nm wide, which is continuous with the intercellular space. These results were corroborated by BRIGHTMAN and REESE[7] in the central nervous system, using the same techniques as well as uranyl block staining before embedding. Seven layered gap junctions appear, therefore, as the morphological substract of electrotonic coupling. The purpose of this work is to report the finding of a large number of such low resistant junctions in the pigeon cerebellum, where, however, electrical transmission has not yet been demonstrated.

Material and methods. The present observations were confined to the cerebellar cortex of adult pigeons, fixed by immersion following the method of KANASEKI and KADOTA[8]. Diced pieces of tissue were fixed in 4% unbuffered osmium tetroxide followed by 12% unbuffered glutaraldehyde, and block stained with aqueous uranyl acetate. This fixation technique, and the subsequent steps of the procedure used here, have been described in detail in a previous paper[9].

Results and discussion. Specialized junctions were found only in the granular layer of the cerebellar cortex, and the area occupied by them seems to be very much smaller than that occupied by conventional chemical synapses. These junctions are most commonly observed between mossy fibre endings and granule cell dendrites (Figures 1–3). In a few cases, however, mixed synapses such as described by PETERS et al.[10] have been identified. This infrequent arrangement is characterized by the presence of specialized junctions immediately close to a

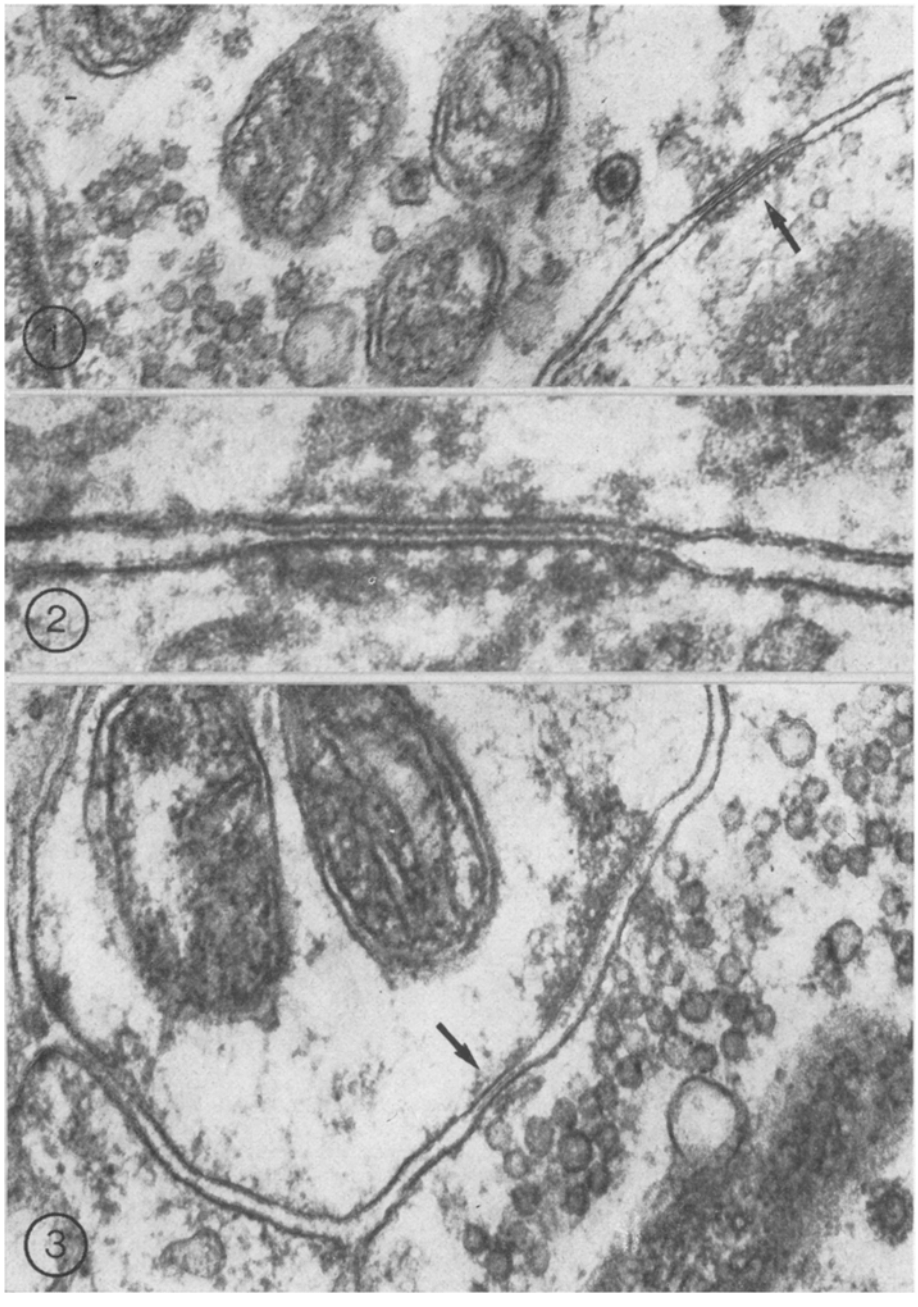

Fig. 1. Tight junction (arrow) between a mossy fibre ending and a granule cell dendrite. × 120,000.

Fig. 2. The same junction as Figure 1 at higher magnification. The external leaflets of both membranes appear fused, specially at the right side of the junction. × 300,000.

Fig. 3. Mixed synapses between a mossy fibre terminal and a granule cell dendrite. On the same synaptic interface, a classical active zone with associated vesicles and dense projections is present immediately next to a specialized junction (arrow). × 120,000

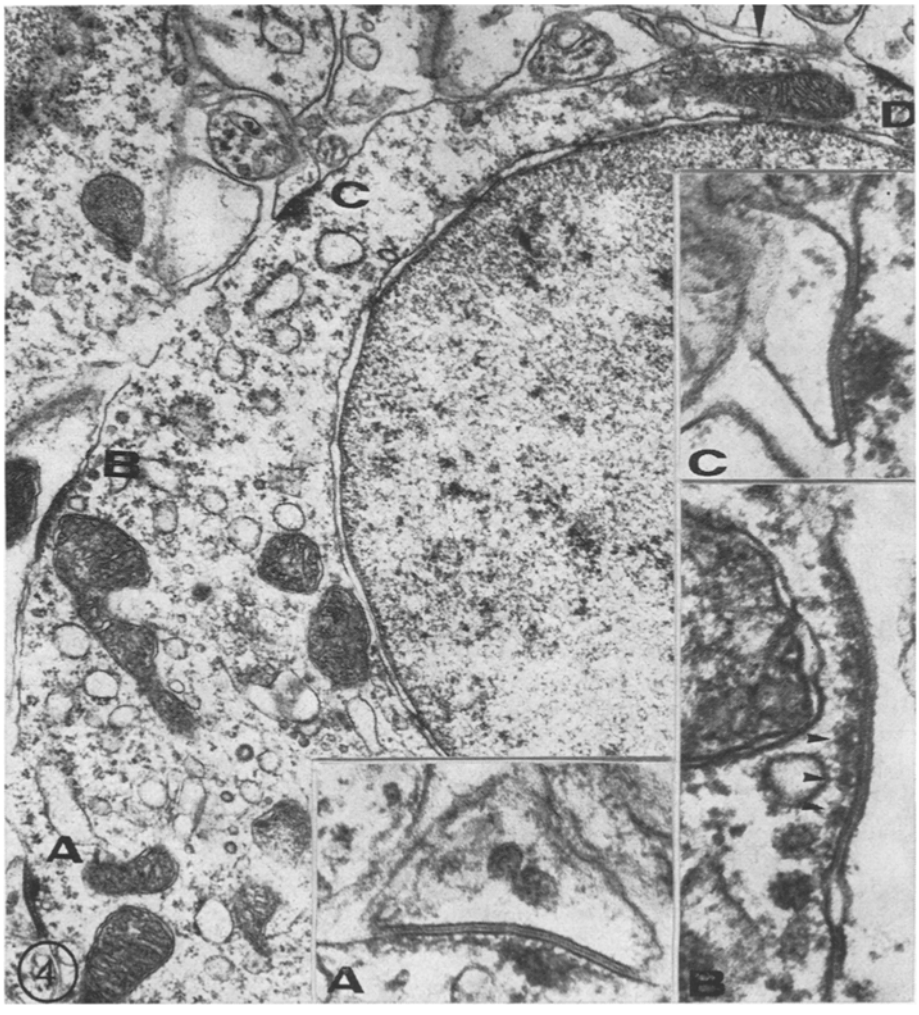

Fig. 4. 4 dendro-somatic contacts between unidentified dendrites and a granule cell body (A–D). A small attachment plate (arrow) can also be recognized. × 35,000. Insets: higher magnifications of these junctions, showing clear asymmetrical disposition of the cytoplasmatic dense material. Triangular differentiations can be seen in insets A and C. Spherical condensations are present in B (arrow-heads). × 120,000.

classical active zone (Figure 3), suggesting that chemical and electrical transmissions occur at the same synaptic interface. Some probable dendro-somatic specialized junctions have also been observed, and an example is shown in Figure 4 where unidentified dendrites establish 4 such contacts with a granule cell perykarion. Similar dendro-somatic contacts have, so far, been described only in the mormyrid electromotor nuclei[11].

The present specialized junctions could be followed only in a few consecutive sections, showing that the apposition surface is restricted in area. In both cases here described, the junctions have an overall thickness of 15 to 16 nm (Figure 2 and insets of Figure 4). Although uranyl block stain was used, a gap between the apposed membranes could not be visualized. The external leaflets of the membranes seem to be fused and to form a central dense layer with the same thickness as the inner leaflets (Figure 2). Therefore, these contacts appear as tight junctions. However, it must be emphasized that methodological differences may be a reason for the failure to visualize a gap between the junctional membranes.

Cytoplasmic dense material was found symmetrically distributed on both sides of the junctional zones, with low electron density in the axo-dendritic contacts (Figures 2 and 3), while it had an asymmetrical disposition and higher electron density on the dendro-somatic junctions (Figure 4). In some of the latter (Figure 4, A and C), the finely granular dense material assumed a triangular shape, with its base towards the perikarial membrane. A similar arrangement has been described by Sotelo and Llinás[12] in the gymnotid fish, where a triangular dendritic differentiation was found beneath a diffuse band of para-junctional dense material. In one of the present dendro-somatic contacts, the cytoplasmatic material is spherically condensed (Figure 4, B, arrow heads), and forms a structure similar to the subjunctional dense bodies described by Milhaud and Pappas[13]. However, these spherical condensations are smaller in diameter and they are situated more closely to the junctional membrane than they are in the 'post synaptic bodies' of Milhaud and Pappas. It is not known whether these different aspects of the cytoplasmatic dense material correspond to different physiological properties of the junctions.

[4] M. V. L. Bennet, in *Structure and Function of Synapses* (Eds. G. D. Pappas and D. P. Purpurea; Raven Press, New York 1972), p. 221.
[5] J. P. Revel and M. J. Karnovsky, J. Cell Biol. *33*, C7 (1967).
[6] M. J. Karnovsky, J. Cell Biol. *35*, 213 (1967).
[7] M. W. Brightman and T. S. Reese, J. Cell Biol. *40*, 648 (1969).
[8] T. Kanaseki and K. Kadota, J. Cell Biol. *42*, 202 (1969).
[9] M. Paula-Barbosa and E. G. Gray, J. Neurocytol. *3*, 471 (1974).
[10] A. Peters, S. L. Palay and H. F. de Webster, *The Cells and their Processes* (Hoeber, Harper and Row, New York 1970), p. 162.
[11] M. V. L. Bennet, G. D. Pappas, E. A. Aljure and Y. Nakajima, J. Neurophysiol. *30*, 180 (1967).
[12] C. Sotelo and R. Llinás, J. Cell Biol. *53*, 271 (1972).
[13] M. Milhaud and G. D. Pappas, J. Cell Biol. *30*, 437 (1966).

Simultaneous electrical and chemical synaptic transmission has been physiologically demonstrated by MARTIN and PILAR[14] in the chick ciliary ganglion. Subsequent ultrastructural studies[7,15] reported the existence of specialized contacts in this ganglion. HINOJOSA and ROBERTSON[16] described tight junctions in the nucleus vestibularis tangentialis of the same animal. The existence of these junctions also in the cerebellar cortex of the pigeon seems to suggest that electrotonic coupling may play an important role in the bird nervous system.

[14] A. R. MARTIN and G. PILAR, J. Physiol., Lond. *168*, 443 (1963).
[15] K. TAKAHASHI and K. HAMA, Z. Zellforsch. *67*, 174 (1965).
[16] R. HINOJOSA and J. D. ROBERTSON, J. Cell Biol. *34*, 421 (1967).

Synthesis and in vitro Cytotoxic Activity of New N-Diazoacetylglycine Derivatives

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Summary. The syntheses of various N-diazoacetylglycine derivatives are described. The results of an in vitro screening carried out on KB cells for cytotoxic activity are reported. The most active compounds are DGE, DGiBA and DGHA. A possible relationship between the activity and the liposolubility of these compounds is discussed.

N-Diazoacetylglycine amide (DGA) and some of its derivatives have shown interesting antitumour and immunosuppressive properties[2-9]. Investigations into their possible mechanisms of action have shown a broad and rather unspecific effect on purine nucleotide metabolism[10-12]. Recently some antibacterial activity[13] and a strong mutagenic activity have been demonstrated, maybe due to alkylating effect on bacterial DNA[14,15]. The pharmacological activity shown by DGA, and the fact that this substance has the same active group as found in diazoacetylserine (Azaserine) and in diazo-oxo-L-norleucine (DON), both powerful antitumour and antibacterial agents[16], prompted us to synthetize further derivatives. This communication reports the synthesis of these compounds and the results of an in vitro screening for possible cytotoxic effects.

Materials and methods. 3 synthetic ways were used for the synthesis of the diazoacetylglycine amides: A) aminolysis of diazoacetylglycine ethylester; B) aminolysis of diazoacetylglycine *p*-nitrophenylester; C) diazotization of glycylglycine amides.

A) Aminolysis of diazoacetylglycine ethylester (DGE). DGE was synthetized as reported by CURTIUS[17]. The aminolysis was carried out suspending DGE in an aqueous solution of the appropriate amine in 10-fold excess.

B) Aminolysis of diazoacetylglycine *p*-nitrophenylester (DGONP). DGONP was synthetized by diazotization, at pH 4, of glycylglycine *p*-nitrophenylester hydrobromide[18]. Yield 64%; m.p. 136–137°C dec. (Table I). The amino lysis was carried out by suspending DGONP in absolute ethanol and dropping into the cooled suspension a solution of the appropriate amine in absolute ethanol.

C) Diazotization of glycylglycine amides. A solution of the appropriate amine in acetonitrile was added to an equimolar solution of carbobenzoxyglycylglycine *p*-nitrophenylester[18] in hot acetonitrile; after 30 min refluxing followed by cooling, the expected carbobenzoxyglycylglycine amide precipitates. For the cleavage of the carbobenzoxy group, the amides were poured in small portions into a threefold amount of acetic acid saturated with HBr. The resulting glycylglycinamide hydrobromide were filtered, abundantly washed with acetone, crystal-

Table I. Chemical structure and characteristics of diazoacetyl-glycine derivatives

$R-CO-CH_2-NH-CO-CH-N_2$			M.P.[c]	Yields (%)			Recrystallization solvents
Compounds	R	Formula[b]		A	B	C	
DGE	$-O-CH_2-CH_3$	$C_6H_9N_3O_3$	106–107	—	—	—	Ethanol
DGA	$-NH_2$	$C_4H_6N_4O_2$	161–162	72	50	24	Ethanol
DGI	$-NH-NH_2$	$C_4H_7N_5O_2$	142–144	74	—	—	Abs. ethanol
DGMA[a]	$-NH-CH_3$	$C_5H_8N_4O_2$	163–164	79	65	31	Abs. ethanol
DGEA[a]	$-NH-CH_2-CH_3$	$C_6H_{10}N_4O_2$	164–166	77	60	33	Ethanol
DGPA[a]	$-NH-(CH_2)_2-CH_3$	$C_7H_{12}N_4O_2$	155–156	—	62	37	Abs. ethanol
DGiPA[a]	$-NH-CH(CH_3)-CH_3$	$C_7H_{12}N_4O_2$	153–154	—	60	35	Methanol
DGiBA[a]	$-NH-CH_2-CH(CH_3)-CH_3$	$C_8H_{14}N_4O_2$	155–156	—	60	29	Acetone
DGHA[a]	$-NH-(CH_2)_5-CH_3$	$C_{10}H_{18}N_4O_2$	146–148	—	63	30	Dioxane
DGIEA[a]	$-NH-CH_2-CH_2-OH$	$C_6H_{10}N_4O_3$	133–136	—	42	—	CH_2Cl_2/CCl_4 1/1
DGM[a]	$-N(CH_2-CH_2)_2O$	$C_8H_{12}N_4O_3$	145–148	—	56	34	Ethanol

[a] New compounds. [b] All compounds analyzed correctly for C, H, N. [c] The melting points are uncorrected. All the compounds melt with decomposition.