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.

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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.

lized from acetic acid and diazotized in the usual way.

The partition coefficients $p = C_{oct}/C_{H_2O}$ of the drugs between *n*-octanol and 10 m*M* phosphate buffer pH 7.4 were determined essentially as described by FUJITA et al.[19].

Evaluation of cytostatic and cytolytic activity. The substances were tested on KB cells (human epidermoid carcinoma of the mouth)[20] cultivated in Flow Tissue Culture Leighton Tubes. The growth medium was the Eagle Basal Medium (BME)[21], supplemented with 10% calf serum, inactivated by heating at 56 °C for 30 min. The medium was added with antibiotics (100,000 IU of Penicillin G and 100,000 µg of Streptomycin sulphate per l) and buffered with TES (3 m*M*), HEPES (3 m*M*), BES (3 m*M*) and tricine (3 m*M*). The cells used to seed were fed 24 h before testing. The cytotoxic activity was evaluated according to protocols for screening chemical agents[22], with some modifications. The cells were seeded in Leighton tubes at the rate of 5×10^4 cells per tube and incubated at 37 °C for 24 h. Then the tubes were regrouped at random and 5 of these were trypsinized and counted in a Bürker counting chamber, in order to obtain the basic value of the experiment (Baseline). To 5 of the remaining Leighton tubes, the medium was changed with 4 ml of BME, whereas to the other tubes the medium was changed by substituting it with 4 ml of BME containing the substances to be examined, at 3 dose levels at one-log intervals, previously dissolved in physiological saline and then added to the medium. DGHA was tested suspended in physiological saline as well as dissolved in DMSO. The final concentration of DMSO in BME (0.5%) was tested for non-toxicity. For each experiment positive controls were carried out with 6-mercaptopurine in doses of 0.05 and 0.5 µg/ml. The Leighton tubes, so treated, were incubated at 37 °C for 72 h, at 10° angle. After 72 h, all the cells were trypsinized and counted. The percentage of the inhibition of the growth is a function of the growth in the control tubes from which the value of the baseline has been deducted.

Results and discussion. The chemical characteristics and the yields of the single compounds are reported in Table I. Method A gives the best results, but it is suitable only for a few compounds; method B gives good yields but presents purification problems in obtaining the complete elimination of *p*-nitrophenol from the final products; method C, suitable for less water soluble compounds, presents low yields but no purification problems.

Table II reports the results of the in vitro screening of diazoacetylglycine derivatives carried out on KB cells and their log *p*-values. The positive controls, carried out with 6-mercaptopurine, under our experimental conditions, gave a growth inhibition of 30% at dose of 0.05 µg/ml and of 78% at dose of 0.5 µg/ml, according to values reported by GERAN et al.[22]. The growth inhibition percentages obtained when testing these diazoacetylglycine derivatives at doses of 10 and 100 µg/ml are very consistent for all compounds tested, whereas at dose of 1 µg/ml there is a consistent cell growth inhibition only by DGE, DGiBA and DGHA. Undoubtedly the most active compound was DGiBA, since at dose of 1 µg/ml of DGiBA and DGE the difference between the effects we have pointed out is not statistically significant. The comparison between the effects of DGHA, suspended in physiological saline and dissolved in DMSO, gave statistically significant results only at dose of 10 µg/ml, since solubility in the two different solvents at concentration of 1 µg/ml is very likely to be almost the same.

We have compared the cytotoxic effect of various diazoacetylglycine derivatives with their liposolubility, expressed as log *p* ($p = C_{oct}/C_{H_2O}$). There seems to be a fairly good correlation between cytotoxic activity and liposolubility. The only plain exception is DGHA, which, although it is about 10-fold more liposoluble than DGiBA,

Table II. Effects of N-diazoacetyl-glycine derivatives on the growth of KB cells[a]

Compounds	Drug Concentration (µg/ml) 1	10	100	Log (*p*)
DGIEA	9	14	56	— 1.349
DGA	7	22	100[b]	— 1.260
DGMA	10	22	87	— 0.873
DGI	3	28	94	— 0.762
DGEA	11	29	100[b]	— 0.620
DGM	7	18	49	— 0.480
DGiPA	2	20	100[b]	— 0.411
DGPA	5	29	100[b]	— 0.238
DGE	27	56	100[b]	— 0.082
DGiBA	22	100[b]	100[b]	0.368
DGHA (suspended in phys. saline)	16	37	100[b]	1.510
DGHA (dissolved in DMSO)	18	59	100[b]	1.510

[a] The values are % inhibition of the growth of KB cells in BME in the presence of the drug, in respect to the controls. Each value is the mean of 5 determinations. [b] Maximum cytolysis was also observed.

[1] Acknowledgment. The authors are indebted to Mr. PAOLO D'ERRICO for the elemental microanalyses.

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has lower cytotoxic activity. This finding may be explained considering on one hand the longer the side-chain, the more liposoluble the molecule, the easier the molecule gets into the cells; on the other hand the longer the side-chain is, the less its alkylating capability or, in our opinion, the lower the activation by cell biochemical mechanisms.

Therefore it may be interesting to test the alkylating capability of these diazoacetylglycine derivatives and to synthetize others of intermediate solubility between DGE and DGHA. Moreover, in our opinion, in vivo testing of these substances is indispensable, since while DGA is a good in vivo antineoplastic agent, it has given mild cytotoxic effects in vitro.

Graft-Versus-Host Reaction and Lymphoid Organs in Normally Fed and Protein-Deprived Rats

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Summary. Lymph node graft-versus-host reaction (GVHR) induced by parental splenic lymphocytes inoculated into hind foot pads of F-1 hybrid rats is correlated with the state of the thymus and the spleen of the recipients. This may explain the depression of the reaction after protracted protein deprivation. Furthermore, GVHR provokes mainly in normal rats a reduction of thymus and spleen possibly due to a T-cell transfer to the grafted area.

The graft-versus-host reaction (GVHR) induced in hybrid F-1 rats by parental spleen lymphocytes is deeply impaired if the recipients are deprived of dietary protein. Since the great majority of the cells involved in this reaction are of host origin[2,3] and seem to be formed mainly of T lymphocytes[4], one could suppose that the inhibitory effect of a protein deprived (PD) diet may be conditioned by the involution of the thymus and the decrease in the T cell population induced by this malnutrition. The findings reported here are in favour of this hypothesis.

Adult male (Sherman/Wistar) F-1 rats, derived from pathogen-free strains (C.N.R.S., Orléans), were maintained for 4 or 9 weeks on a PD diet (for its composition, see ref.[5]) while other F-1 rats were fed on a balanced diet. All the rats received, 1 week before killing, an injection into the right hind foot pad of viable spleen lymphocytes obtained from male Sherman donors (1×10^7 for well nourished recipients; 6×10^6 for PD recipients). The viability was determined by a dye (erythrosin) exclusion test[6]. 1 week later, the ratios between the weights and lymphocyte populations of ipsilateral and contralateral popliteal lymph nodes (iPLN and cPLN) were calculated[3,7]. In addition, the weights of thymus, spleen and cervical lymph nodes (not involved in the GVHR) were noted in the grafted rats, and compared with those of non-grafted weight-paired male rats which were either fed on a balanced diet or deprived of protein for 9 weeks.

Results. High iPLN/cPLN ratios were much more frequent in rats with large lymphoid organs than in those with small organs. The correlation was significant as calculated for 38 rats: $r = +0.345$ ($p < 0.05$), $+0.462$ ($p < 0.01$) and $+0.407$ ($p < 0.02$) in regard to the weights of the thymus, spleen and cervical lymph nodes respectively. However, in relation with the latter organs, the percentages of high iPLN/cPLN ratios did not decrease further between the 4 week- and the 9 week-PD groups (Table I).

The weights of the thymi were much lower in grafted rats than in non-grafted ones, fed either on a balanced diet ($p < 0.001$) or on a PD diet ($p < 0.01$). The spleen was also reduced following GVHR ($p < 0.02$) with the former diet but not with the latter one. There were no significant changes in the weight of the cervical lymph nodes.

[1] I thank Mrs. M. CL. GONZALEZ and Mr. D. SOULAS (C.N.R.S.) for their skilled technical assistance.

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Table I. Graft-versus-host reaction (GVHR) expressed as weight- and cell-ratio between ipsilateral (inoculated) and contralateral popliteal lymph nodes. Comparison with weights of lymphoid organs in normally fed or protein deprived rats

Diet	Thymus (mg)	Ratios iPLN/cPLN		Spleen (mg)	Ratios iPLN/cPLN		Cervical lymph node (mg)	Ratios iPLN/cPLN	
		Weight: > 3.0	Number of cells: > 15		Weight: > 3.0	Number of cells: > 15		Weight: > 3.0	Number of cells: > 15
Balanced	280–470	68.7% (11/16)[a]	68.7% (11/16)[a]	520–660	68.7% (11/16)[a]	68.7% (11/16)[a]	6 –19	55.0% (11/20)[a]	60.0% (12/20)[a]
Protein-free (4 weeks)	45–120	41.7% (5/12)	50.0% (6/12)	170–220	50.0% (7/14)	57.2% (8/14)	4.1– 5.5	40.0% (4/10)	40.0% (4/10)
Protein-free (9 weeks)	< 45	20.0% (2/10)	20.0% (2/10)	< 155	0 (0/8)	0 (0/8)	< 4.0	37.5% (3/8)	37.5% (3/8)

[a] Number of rats with GVHR > 3 or > 15 respectively/total number of rats belonging to the different weight groups of each lymphoid organ.