responsible for glucose intolerance. In addition, serum from patients before and after dialysis had the same effect on the in vitro activity of added insulin against rat hemidiaphragm in spite of the fact that intravenous glucose tolerance improved in every patient after treatment[1]. This speaks against a circulating insulin antagonist and is consistent with the negative effect of GSA on in vitro carbohydrate metabolism. Furthermore, improvement of glucose tolerance and recovery of sensitivity to injected insulin only after repeated dialyses[5] could not be explained by an effect of the readily dialyzable[2] compound GSA. Glucose intolerance in chronic renal failure would seem to be secondary to peripheral tissue insensitivity to the action of insulin[1,6] and/or a delayed pancreatic insulin response to glucose[6].

The effect of GSA (0.03 mg/ml) on the in-vitro response of paired rat hemidiaphragms[a] to glucose (2.0 mg/ml) and insulin (500 µU/ml)

	Basal		Insulin		I–B	
Mean	2.68[b]	2.31	5.94	6.27	3.26	3.96
± S.E.	0.22	0.16	0.22	0.27	0.18	0.28
GSA	—	+	—	+	—	+

[a] 10 animals. [b] µg/mg/h.

Zusammenfassung. Da Guanidinosuccinsäure in der Urämie akkumuliert, wurde angenommen, dass dieses Stoffwechselprodukt bei Niereninsuffizienz die bekannte Glukoseintoleranz verursachen könnte. In-vitro-Versuche am Rattenzwerchfell haben diese Hypothese jedoch nicht bestätigen können, da Guanidinosuccinsäure in der Konzentration von 3 mg/100 ml keinen Einfluss auf die basale oder insulinstimulierte Glucoseaufnahme durch dieses Gewebe zeigte.

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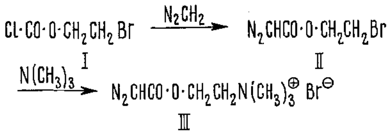
Diazoacetyl Choline Bromide

We have synthesized diazoacetyl choline bromide (III) in order to evaluate its potentialities as an affinity label for acetyl cholinesterase, choline acetyl transferase and, especially, acetyl choline receptor molecules. Diazo analogs of enzyme substrates have been shown to react with sterically favorable groups of the binding site of the enzyme either in the dark (in the presence[1–3] or absence[3–5] of cupric ions) or during photolysis[6–8]. In the dark reaction, the diazo compounds presumably attack carboxyl groups of the enzyme. Photolysis produces carbenes which may be reactive enough to insert themselves even into carbon hydrogen bonds[9]. Diazo ketones and diazo esters may, in addition, undergo a Wolff rearrangement[10–13] before producing covalent bonds with functional groups of the enzyme.

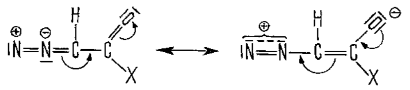
Synthesis proceeded in a straightforward manner according to the following scheme:



$$\underset{\mathrm{I}}{\mathrm{Cl\cdot CO\cdot O\cdot CH_2CH_2Br}} \xrightarrow{\mathrm{N_2CH_2}} \underset{\mathrm{II}}{\mathrm{N_2CHCO\cdot O\cdot CH_2CH_2Br}} \xrightarrow{\mathrm{N(CH_3)_3}} \underset{\mathrm{III}}{\mathrm{N_2CHCO\cdot O\cdot CH_2CH_2N(CH_3)_3^{\oplus}\ Br^{\ominus}}}$$

2-Bromoethyl chloroformate (I, product of FLUKA Ltd., Buchs, Switzerland) was treated with diazomethane to yield 2-bromoethyl diazoacetate (II). A small amount of a contaminant, presumably 2-bromoethyl chloroacetate, was removed by chromatography. Pure II was then treated with trimethylamine in toluene to yield essentially pure, crystalline diazoacetyl choline bromide (III)[14].

Hydrolysis of diazoacetyl choline bromide (III) by the enzyme acetyl cholinesterase from Electrophorus electricus at pH = 7 and 25 °C proceeds approximately 1.6×10^4 times more slowly than that of acetyl choline iodide. This phenomenon might be caused by the topochemical differences between the two molecules, but it might also be due to the inactivation of the ester linkage in esters of diazoacetic acid. It was found in our laboratory that *p*-nitrophenyl diazoacetate[8] does not behave like an active ester[15], but reacts very much more slowly with amines. An electronic mechanism is probably operative which makes it more difficult for the carbonyl C-atom to assume a partial positive charge during attack by a nucleophile. A plausible electron distribution is delineated in the following electromeric formulae:



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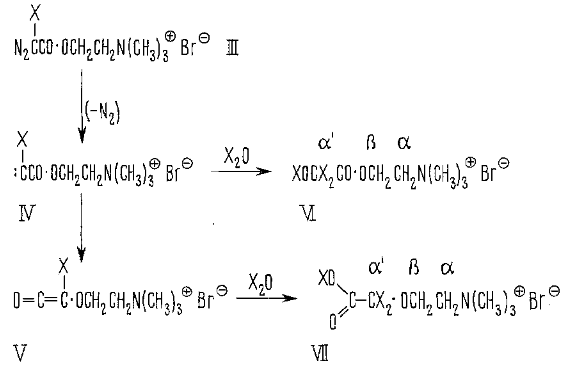
which are meant to indicate that the negative charge is distributed mainly over that portion of the molecule near the carbonyl C-atom.

Pharmacological activity of diazoacetyl choline bromide (III) is similar to that of acetyl choline bromide in a) the electroplax of *Electrophorus electricus*, b) the isolated rat diaphragm and c) preparations of central neurons[16].

Photolysis of diazoacetyl choline bromide (III) was preliminarily studied in water and deuterium oxide. Upon illumination with light from incandescent lamps (filtered to remove wavelengths below ~315 nm), nitrogen is eliminated and the light absorption due to the diazo grouping decreases accordingly (Table I). The carbene (IV) produced in this process can react either directly with the solvent or after WOLFF rearrangement[10–13] to the ketene (V):



(In these formulae, X can be either H or D, depending on the solvent; according to [13], the exchange of the methine proton against a deuteron in D_2O proceeds much more rapidly than photolysis.) The possible products of photolysis, hydroxyacetyl choline bromide (VI) and *O*-carboxymethyl choline bromide (VII), should be recognizable by their proton-NMR-spectra: a) the signals of the β-protons of VII should appear some 0.4 ppm upfield of those of VI, the difference being similar to that between choline and acetyl choline, b) in VI (X = H) the α'-protons could be coupled to the hydroxyl proton, with spin coupling being pronounced in a solvent with low exchange rate[17] (p.e. DMSO-d_6), and disappearing if the hydroxyl proton is exchanged against a deuteron, resulting in a reduction of the multiplicity at the point of the α'-protons.

The results of a preliminary NMR investigation of the mixtures of products of photolysis in H_2O and D_2O are compiled in Table II. They indicate that the carbene undergoes WOLFF rearrangement to only a small extent (~15%) during reaction with the solvent. This may be due to the strong electron-attracting properties of the quaternary nitrogen, reducing the required nucleophilicity at the choline oxygen atom.

Table I. Decrease in absorption at 251 nm of diazoacetyl choline bromide (62.5 mg in 25 ml H_2O at 4 °C) upon illumination with 2 sunlamps, distance 15 cm, in the apparatus described in the experimental part

Duration of illumination (min)	Absorption at 251 nm	Duration of illumination (min)	Absorption at 251 nm
0	1.8	220	0.66
60	1.12	280	0.49
120	0.99	340	0.32
180	0.68	510	0.08

Table II. NMR characteristics of II and III and of the unpurified mixtures of products of photolysis

Compound	Solvent (internal standard)	XO– N_2=	α' –CH_2– =CH–	$\overset{O}{\parallel}$ –C–O–	β –CH_2–	α –CH_2–	–$N(CH_3)_3$ + –Br
II	$DCCl_3$ ($HCCl_3$, 7.32)		s 4.78 (1)		t 4.45 (2)	t 3.5 (2)	–Br
III	DMSO-d_6[a] (DSS)		s 5.7 (1)		m 4.7–4.3 (2)	m 3.85–3.6 (2)	s 3.20 (9)
	D_2O (DSS)				m — 4.8–4.5	m 3.85–3.65 (2)	s 3.2 (9)
Products of photolyis in H_2O	DMSO-d_6[a] (DMSO, 2.52)	HO–[b]	d 4.15–4.00 (1.7)		m 4.65–4.35 (1.9)	m 3.80–3.60 (1.9)	s 3.15 (9)
	DMSO-d_6 + D_2O (2.52)	DO–	s 4.08 (1.7)		m 4.65–4.30 (1.9)	m 3.75–3.50 (1.9)	s 3.10 (9)
Products of photolysis in D_2O	D_2O				m — 4.80–4.45[c]	m 3.85–3.60 (1.8)	s 3.20 (9)

Chemical shifts δ in ppm with TMS, DSS, $HCCl_3$ or DMSO as internal reference; s, singlet; d, doublet; t, triplet; m, multiplet; ( ), number of protons relative to methyl protons as obtained by integration of spectra; —, superposition of solvent signals. All spectra taken at 60 MHz, except compound II at 100 MHz. [a] DMSO, dimethylsulfoxide. [b] Not observable. [c] Accompanied by very weak signals between 4.35 and 4.15 ppm with a maximum of approximately 0.25 protons.

Experimental. 2-Bromoethyl diazoacetate (*II*): A solution of 14.9 g (80 mmoles) of 2-bromoethyl chloroformate in 200 ml of diethyl ether was introduced slowly and dropwise into a quickly stirred solution of 218 mmoles of diazomethane in 680 ml of the same solvent at −10 °C. Stirring was continued for 6 h at 0 °C, and the mixture finally kept for 3 days in the dark at 4 °C. Excess diazomethane was removed with a stream of nitrogen at 0 °C, and the remaining solvent was evaporated in a rotatory evaporator at 30 °C in vacuo. The yellow fluid residue was purified by chromatography on a column of neutral alumina (activity IV) 160 cm in length with stepwise reduction of diameter from a maximum of 5 cm to a minimum of 1.5 cm[18]. Eluant was a 9:1 (v:v) mixture of *n*-hexane-benzene. Fractions were examined by IR-spectroscopy (carbonyl bands), because product and byproduct are hard to differentiate on thin-layer chromatograms (alumina and silica gel). Yield: 71% (11 g) of yellow oil.

$C_4H_5BrN_2O_2$	calc.	C 24.89	H 2.61	N 14.52
	found	24.80	2.66	14.52
		O 16.58	Br 41.41%	
		16.67	41.13	

IR-spectrum (chloroform, cm^{-1}), characteristic bands: 3130 (m), 2110 (s), 1690 (s), 1385 (s), 1350 (s), 1330 (s), 1170 (s)[19].

Diazoacetyl choline bromide (*III*): 1.028 g (5.35 mmoles) of 2-bromoethyl diazoacetate were dissolved in 1 ml of toluene and treated at −10 °C with 8 ml of a 30% solution of trimethylamine in toluene (product of FLUKA Ltd., Buchs). The ampoule was sealed and kept for 3 days in the dark at room temperature. The ampoule was then opened at −10 °C, ether was added and all solvents were removed in vacuo. The yellow, crystalline residue was dissolved in ethanol at 60 °C. Crystallization resulted in 544 mg (2.17 mmoles) of diazoacetyl choline bromide (yield 40.6%). The mother liquors contained essentially pure educt that could be converted to product in the same manner. The product was recrystallized from ethanol/diethyl ether 5:1 (v:v); mp (uncorr.) 178–179° (decomposition).

$C_7H_{14}N_3O_2Br$	calc.	C 33.34	H 5.60	N 16.67
	found	33.21	5.60	16.66
		O 12.69	Br 31.70%	
		12.56	31.66	

IR-spectrum (KBr, characteristic bands): 3070 (m), 2110 (s), 1680 (s), 1665 (s), 1365 (s), 1345 (s), 1180 (s)[19]. UV-spectrum (ethanol): $\lambda_{max} = 248$ nm ($\log \varepsilon = 4.25$); $\lambda_{max} = 370$ nm ($\log \varepsilon = 1.27$).

Hydrolysis of diazoacetyl choline bromide in the presence of acetyl cholinesterase from *Electrophorus electricus* (1000 units/mg enzyme, Worthington Biochem. Corp., USA): The reaction was followed by titration with 0.1 *N* NaOH of the acid liberated during the reaction (pH-Stat of Radiometer Ltd., Copenhagen)[20]. It was assumed that the reaction rate is proportional to the amount of enzyme present and that MICHAELIS-MENTEN conditions apply. The solutions contained 0.15 *N* NaCl, 0.04 *M* $MgCl_2$, and 0.1% albumin and were maintained at pH = 7.00 and 25 °C nitrogen atmosphere.

With acetyl choline iodide (puriss., Fluka Ltd.) at $5.6 \times 10^{-3} M$ (pS = 2.25) and 2×10^{-3} mg of enzyme, the consumption of 0.1 *N* NaOH in the linear part of the plot was 100 µl in 5.1 min, that is approximately 1.96 µmoles/min.

With diazoacetyl choline bromide at $2.5 \times 10^{-3} M$ (pS = 2.6) and 0.5 mg of enzyme, the consumption of base as above was 6 µl in 20 min, corresponding to an activity of 0.03 µmoles/min.

Photolysis was carried out in a cylindrical reaction compartment (Pyrex glass, 2.5 cm diameter) surrounded by a cooling mantel (same material, 5 cm diameter). A saturated solution of copper sulfate in water as optical filter and coolant (+4 ± 1 °C) was continuously pumped through the mantel (all radiation below 315 nm is absorbed by this filter plus the pyrex glass). Illumination with 275 Watt General Electric Sunlamps.

a) *In deuterium oxide*: 101 mg of diazoacetyl choline bromide (III) in 10 ml of solvent (c = 0.04 molar). Temperature: 4 °C. Time illuminated with 2 lamps at a distance of 15 cm each: 20 h. Absorption after illumination: 0.01 (at 251 nm). The solvent was then evaporated in vacuo in a rotatory evaporator at 30 °C. The residue was a colorless, thick oil (97 mg) that crystallized after 4 days at room temperature. IR-spectrum (liquid, no solvent added, characteristic bands): 3650–3100 (s, associated OH), 1740 (s).

b) *In water*: 100 mg of diazoacetyl choline bromide (III) in 10 ml (c = 0.04 molar). Conditions same as for deuterium oxide, however the end absorption 0.01 at 251 nm) was reached after 18 h and 15 min. Product: 89 mg of a thick colourless oil that crystallized after 4 days. IR-spectrum (liquid, no solvent, characteristic bands): 3650 to 3100 (s, associated OH), 1740 (s).

Zusammenfassung. Es wird die Synthese von Diazoacetyl-cholin-bromid beschrieben. Die Verbindung zeigt in verschiedenen biologischen Präparaten starke acetylcholin-ähnliche Wirkung; sie wird von Acetylcholinesterase etwa 1.6×10^4 mal langsamer als Acetylcholiniodid hydrolysiert. Die Photolyse gelingt mit Wellenlängen grösser als 315 nm. Das hauptsächliche Photolyseprodukt in Wasser ist Hydroxyacetyl-cholin-bromid. Es scheint – entsprechend dem stark elektronegativen Charakter der Trimethylammoniumgruppe – die WOLFF'sche Umlagerung nur in untergeordnetem Masse einzutreten. Aufgrund dieser Befunde soll die Eignung der Verbindung zur Affinitätsmarkierung von Acetylcholinesterase, Cholinacetyltransferase und von Rezeptormolekeln untersucht werden.

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[16] Private communications of a) D. NACHMANSOHN, New York, b) P. G. WASER, Zürich, and c) K. AKERT and F. A. STEINER, Zürich and Basel, whom we wish to thank for their kind collaboration. Publication of detailed results is in preparation.

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[21] This work was supported by the Swiss National Foundation for Scientific Research (grant No. 4883). We wish to thank Prof. Dr. J. NEUKOM, Institute of Agricultural Chemistry, ETH, for kindly providing us with space in his laboratories and Mr. MARIO CAVIEZEL for excellent technical assistance in the process of scaling up synthetic procedures.