SYNTHESIS AND BIOCHEMICAL PROPERTIES OF A SERIES OF SUBSTITUTED DIMETHYLAMINOETHYL ARYL(2-PYRIDYL)CARBINOL ETHERS

VRIJE UNIVERSITEIT TE AMSTERDAM

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ACADEMISCH PROEFSCHRIFT

TER VERKRIJGING VAN DE GRAAD VAN DOCTOR IN DE WIS- EN NATUURKUNDE OP GEZAG VAN DE RECTOR MAGNIFICUS MR. I. A. DIEPENHORST, HOOGLERAAR IN DE FACULTEIT DER RECHTSGELEERDHEID, IN HET OPENBAAR TE VERDEDIGEN OP VRIJDAG 2 DECEMBER 1960, DES NAMIDDAGS TE 13.30 UUR, IN HET WOESTDUINCENTRUM, WOESTDUINSTRAAT 16, TE AMSTERDAM

DOOR

PIETER ANDRIES ROUKEMA

GEBOREN TE WONS (FR.)

DRUKKERIJ WED. G. VAN SOEST N.V. - AMSTERDAM
1960

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Aan mijn ouders

Aan mijn vrouw

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INTRODUCTION

Since Bovet and Staub [1] in 1937 first synthesized a compound having antihistamine activity, a large number of compounds has been described which also counter the effect of histamine in vitro and in vivo.

Among the substances that reached the stage of clinical application was diphenhydramine (β-dimethylaminoethyl benzhydryl ether), which was described in 1944 by Rieveschl [2] and has been on the market since 1945 under the names of Benadryl and Benodine. When alkyl substituents are introduced into the phenyl groups of this compound, as has been done by Harms [3] as well as by Loew et al.[4] and Rieveschl [5], this produces a change in pharmacological properties.

De Waart [6, 7] has examined the influence of a number of the compounds obtained by Harms on the metabolism of rat brain in vitro. From his experiments he concluded that many of these compounds inhibit respiration and stimulate the metabolism of the amino acids γ-aminobutyric acid and glutamic acid. Although it is doubtful whether his interpretation of the phenomena observed is correct, the effect on amino acid metabolism remains important in itself, in view of the significance of γ-aminobutyric acid and glutamic acid in the central nervous system.

When in diphenhydramine a phenyl group is replaced by a 2-pyridyl group we obtain a compound with an antihistamine activity comparable to that of diphenhydramine. This compound and many of its derivatives have been investigated by Tilford et al.[8] and by Sperber et al.[9]. Substitution in general was found to have no favourable effect on the antihistamine activity. The sedative and spasmolytic properties, on the other hand, became more marked.

We were interested to know whether the phenyl(2-pyridyl)-carbinol ether and its derivatives would influence the metabolism of brain tissue in the same way as observed by De Waart. We therefore investigated the influence of a series of aryl(2-pyridyl)-carbinol ethers on the metabolism of slices of rat cortex.

Attempts were also made to relate the activity of our compounds to their structure. We had good hopes that the U.V. spectra would supply the necessary information on the structure (notably

the spatial arrangement). This, however, was found not to be the case. In fact, even if the spectra had been more informative, it is still questionable whether we could have reached valid conclusions about the relation between structure and activity, because in general the latter is determined by a large number of processes, such as absorption, transport, distribution and excretion of the drug. These, in turn, are governed by various factors, such as solubility, membrane potential, osmotic pressure, surface tension and the like [10, 11].

The first chapter of this thesis describes the synthesis of a series of aryl(2-pyridyl)carbinol ethers and contains some spectral data. In the second chapter it is shown how amino acids can be determined with the aid of one-dimensional paper chromatography and what factors play a role here. The third chapter reviews some aspects of the metabolic function of γ-aminobutyric acid and glutamic acid in the central nervous system. In the last chapter a description is given of the biochemical experiments and an attempt is made to interpret the results.

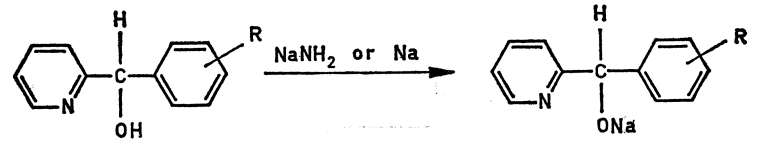
A few observations appearing in the appendix and described in chapter II and IV were statistically analysed by the late A.R. Bloemena of the Statistics Department of the Mathematical Centre in Amsterdam. He is responsible only for the interpretation of *these* observations.

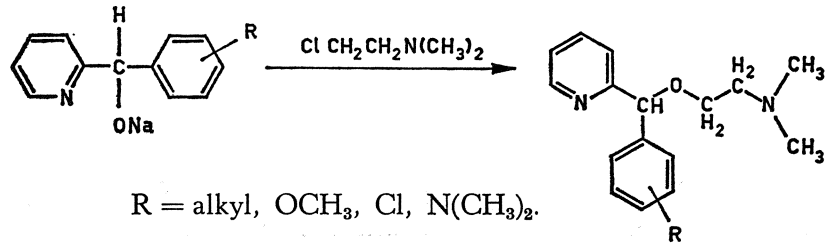
CHAPTER I

THE PREPARATION OF β-DIMETHYLAMINOETHYL ARYL(2-PYRIDYL)CARBINOL ETHERS

Although many of the methods used for the synthesis of benzhydryl ethers (see Harms [3]) might also be employed for the analogous pyridyl compounds, only two methods are given in the literature, viz:

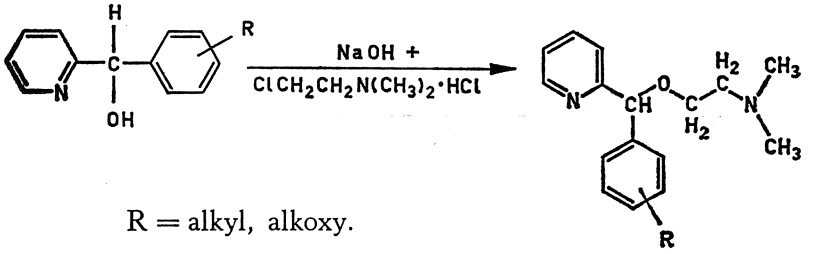
A. 1: The reaction of a substituted phenyl(2-pyridyl)carbinol with sodium amide or sodium and dimethylaminoethyl chloride [8, 9].





R = alkyl, OCH_3, Cl, $N(CH_3)_2$.

2: The reaction of a substituted phenyl(2-pyridyl)carbinol with sodium hydroxide and dimethylaminoethyl chloride hydrochloride [12].

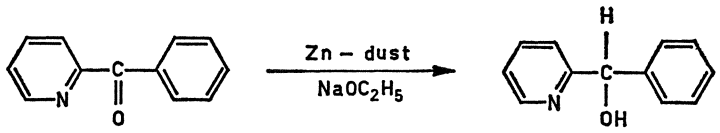


R = alkyl, alkoxy.

In principle, the former method was used, with a few modifications in the general procedure.

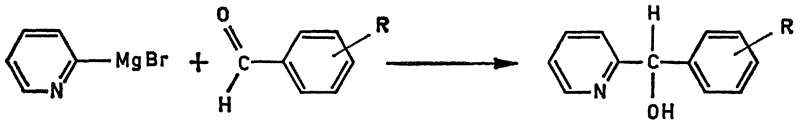
The final syntheses require the appropriate substituted phenyl-(2-pyridyl)carbinols. For the preparation of these compounds the following methods are described in the literature:

B. 1: Reduction of the corresponding ketones [13].



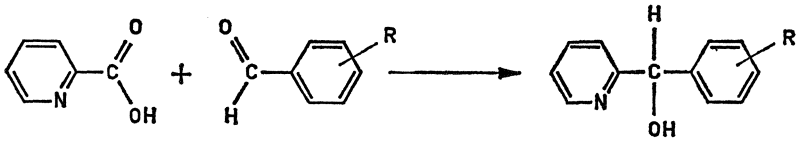
2: The reaction of an aryl aldehyde with:

(a) 2-pyridylmagnesium bromide [14, 15].



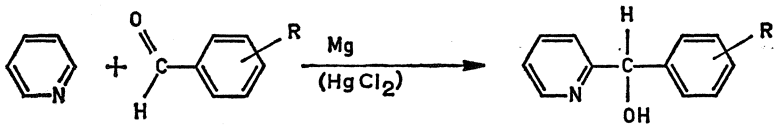
R = alkyl, alkoxy.

(b) 2-picolinic acid [9, 16].



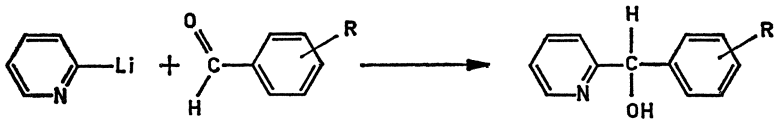
R = alkyl, OCH_3.

(c) pyridine + a catalyst [8, 17, 18].



R = alkyl.

(d) pyridyllithium [19, 20, 21].

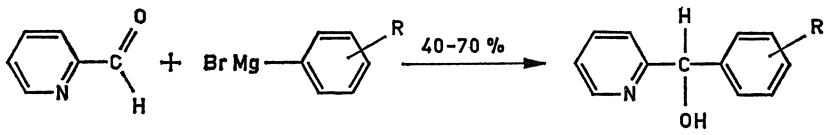


R = alkyl, OH, OCH_3, Cl.

EXPERIMENTAL PART

In 1954 Boekelheide and Linn [22] described an elegant method for the preparation of 2-pyridine aldehyde via the N-oxide of 2-picoline. At about the same time it was also obtained by the catalytic oxidation of 2-picoline [23, 24]. Since then the aldehyde has been commercially available.

As, in addition, many aryl bromides were in stock at our laboratory, or else could be obtained in good yields from the corresponding anilines via diazotization, 2-pyridine aldehyde was a suitable starting material for the synthesis of the carbinols in question. Thus we arrived at the following method, by which all the carbinols can be obtained:



R = alkyl.

Although the reaction of 2-pyridyllithium with an aryl aldehyde may be preferable because of the yield (70—90%), the advantage of our method is that the experimental conditions are far less critical and that fewer steps are required to obtain the result aimed at.

Procedure

A quantity of 0.55 mole of magnesium turnings is placed in a 2-litre three-necked flask, provided with a dropping-funnel, a reflux condenser and a stirrer. To this 0.05 mole of the aryl

bromide in 30 ml of anhydrous ether is added. After the reaction has been started by gentle heating of the flask, 0.45 mole of the bromide in 150 ml anhydrous ether is added dropwise—with stirring—at such a rate that the ether continues to reflux. If the reaction is too violent, cooling is applied. At the end of the reaction, which takes one to two hours, refluxing is continued for another 30 to 60 minutes on a water bath. The water bath is then replaced by an ice bath, the reaction mixture being cooled to 0 °C. At this temperature 0.5 mole of 2-pyridine aldehyde in 200 ml of anhydrous ether is added in one hour, with brisk stirring (note 1). A yellowish brown precipitate is so formed. At the end the reaction mixture is refluxed for half an hour on a water bath, cooled to 0 °C and decomposed with an icecold solution of ammonium chloride (60 g in 200 ml water, see note 2).

The ether layer is separated from the water layer, the latter extracted twice with ether and then the combined ether layers washed twice with water. Next, the ether layer is extracted three times with 50 ml of 10% hydrochloric acid (note 3). The reddish brown extract is neutralized with sodium bicarbonate and then extracted three times with ether (note 4).

After washing with water and drying with anhydrous potassium carbonate the etherial solution is evaporated and the carbinol crystallizes. If it dissolves too readily in ether, it is recrystallized from ether — petroleum ether. The yield varies from 40 to 70% based on aryl bromide.

Notes

1. The 2-pyridine aldehyde was obtained from Dr. F. Raschig, Ludwigshafen am Rhein and was distilled just before use; b.p. = 57 °C (8 mm); Boekelheide and Linn [22]: b.p. = 62—3 °C (13—4 mm).

2. If the two layers do not separate well, more ammonium chloride is added in the first instance. If this does not produce the desired effect, the poor solubility of the carbinol in ether is usually the cause. In that case benzene can be added and, if necessary, heating be applied until the intermediate layer has disappeared.

3. In this way any diaryl compound formed as well as unreacted aryl bromide are eliminated.

4. In cases where the carbinol is poorly soluble in ether, the extraction is carried out with benzene, after which the carbinol, if it cannot be crystallized from benzene, is at once distilled at a pressure of about 10 mm.

TABLE I

Aryl(2-pyridyl)carbinols*

No.	Substituent in the phenyl nucleus	Yield %	m.p. °C	m.p. of HCl salt °C	b.p. °C (mm)	Analysis, %					
						$C_{calc.}$	C_{found}	$H_{calc.}$	H_{found}	$N_{calc.}$	N_{found}
I	none	47	73	182-4	163(9)	77.81	77.7	5.99	6.0	7.56	7.7
II	2-methyl	48	60	179-80	178-80(9)	78.36	78.4	6.58	6.6	7.03	7.1
III	2-ethyl	66	76-7			78.84	79.3	7.09	7.1	6.57	6.5
IV	2-isopropyl	62	77			79.26	79.1	7.54	7.6	6.16	6.3
V	2-tert-butyl	51	111			79.62	79.9	7.94	7.9	5.81	5.9
VI	3-methyl	52	102	180-1		78.36	77.9	6.58	6.5	7.03	7.0
VII	4-methyl	51	96	178-80	178-80(9)	78.36	78.5	6.58	6.5	7.03	7.0
VIII	2,3-dimethyl	63	114-5		160-2(2)	78.84	78.9	7.09	7.1	6.57	6.5
IX	2,4-dimethyl	46	57	178-9		78.84	79.0	7.09	7.1	6.57	6.5
X	2,5-dimethyl	50	85-6		170-2(6)	78.84	78.5	7.09	7.1	6.57	6.6
XI	2,6-dimethyl	43	63	192-4	175-6(7)	78.84	79.1	7.09	7.2	6.57	6.4
XII	2,6-diethyl	67	42		140-5(2)	79.62	79.5	7.94	7.9	5.81	5.9
XIII	2.6-diisopropyl	47	102			80.25	80.4	8.65	8.6	5.20	5.3
XIV	3,4-dimethyl	48	86-7		178-80(8)	78.84	78.9	7.09	7.2	6.57	6.5
XV	3,5-dimethyl	44	92			78.84	78.7	7.09	7.1	6.57	6.8
XVI	2,4,6-trimethyl	40	65	188-90		79.26	79.0	7.54	7.5	6.16	6.2
XVII	2,3,5,6-tetramethyl	20	104	187-8		69.24	69.3	7.21	7.3	5.04	4.9**

* The U.V. Spectra of these compounds are given in Table III, p. 23.

** Analysis of HCl salt.

Compounds I, II, III, IV, VII, IX, XI and XVI are readily soluble in ether; compounds V, VI, VIII, X, XII, XIII, XIV, XV and XVII moderately to poorly.

I. Cislak [26]: m.p. = 72.5 °C; Tilford [8]: m.p. = 76—8 °C, m.p. of HCl salt = 182—4 °C; Sperber [9]: b.p. = 133—8 °C (1 mm). Wibaut [27] mentions the synthesis of phenyl(3-pyridyl)carbinol by the same method as was used by us. As far as we know, this is the only place in the literature where this method is referred to.

II. The same melting point was obtained when this compound was prepared from 2-pyridyllithium and 2-tolylaldehyde.

V. Part of the Grignard compound was converted into the acid by pouring out on solid CO_2; yield 85%; m.p. = 68 °C. The carbinol was crystallized from benzene.

VII. Tilford[12]: b.p. = 134—8 °C (0.3 mm); m.p. of HCl salt = 178—9 °C.

VIII. The carbinol was recrystallized from benzene.

XI and XIV-XVII incl. Upon extraction with 10% hydrochloric acid the hydrochloride precipitated. This salt was filtered off, dissolved in water and made alkaline with sodium bicarbonate. Next, the carbinol was extracted with ether or benzene and worked up further.

XII. As the carbinol is highly soluble in ether, it was first purified by distillation, then crystallized from ether in an ice-salt bath.

XIII. The Grignard compound is poorly soluble in ether. After one fourth part of the bromide had been added, the reaction mixture was diluted with four parts of anhydrous benzene, after which the rest of the bromide in benzene was added dropwise. Under these conditions the Grignard compound did not precipitate. In the reaction with 2-pyridine aldehyde no cooling in an ice-salt bath was applied.
As in the case of V, the acid was prepared. Yield 53%, m.p. = 81 °C.

The β-dimethylaminoethyl aryl(2-pyridyl)carbinol ethers were obtained by method A_1.

Procedure

In a three-necked bottle, provided with a stirrer, reflux condenser and dropping-funnel, 0.140 mole of carbinol is dissolved in 200 ml of anhydrous toluene, after which 15 ml of toluene is distilled off. The solution is then cooled to 0° with an ice-salt bath and 5.7 g (0.146 mole) of sodium amide is added. The mixture is then slowly heated to 100 °C while being stirred quietly. In the meantime a violent generation of ammonia occurs. The original solution acquires a deep blue to reddish brown colour (note 1).

The reaction mixture so obtained is heated at 100 °C for four hours. The evolution of ammonia has then ceased in any case. Now another 5.7 g (0.146 mole) of sodium amide is added and then, at 0 °C, 20.3 g (0.141 mole) of dimethylaminoethyl chloride hydrochloride (note 2), after which ammonia is again generated. The temperature is raised to 100 °C with stirring (in a period of about one hour) and the reaction mixture is kept at this temperature for 14 to 16 hours. It is then cooled to room temperature and through the dropping-funnel 100 ml of water is added. The toluene layer is separated and washed once with water, dried with anhydrous potassium carbonate and distilled.

After the percentage of ether in the distillate has been determined by titration (note 3), it is dissolved in anhydrous ethyl acetate and a calculated amount of alcoholic hydrochloric acid solution is added, required to form the mono-HCl salt. The ether then crystallizes readily in all cases (note 4).

Notes

1. The colour of the reaction mixture is reddish brown for the 2-tert-butyl- and all diorthoalkyl-substituted carbinols. It comes nearer to the reddish brown according as the size of the alkyl substituent increases. The violence of the ammonia production decreases in the same direction.

2. The hydrochloride of dimethylaminoethyl chloride can be obtained as described in Org. Synthesis [30] from dimethylamino-

ethanol and thionyl chloride. Yield 90%, m.p. = 200—2 °C. According to Sperber [9, 31] originally the base was liberated from this compound and added dropwise at 0 °C.
According to Eisleb [32], however, aminoethylhalides readily react with themselves to form piperazine derivatives, especially under the influence of an elevated temperature and hydroxyl ions. In the presence of sodium amide, however, the tertiary alkyl halides are remarkably stable and at 100 °C they do not react with sodium amide and ammonia or, to any marked extent, with themselves. This is why we used the hydrochloride plus an equivalent quantity of sodium amide instead of the free amine (for a similar reaction see Huttrer [33]).

3. It is also possible to neutralize against Congo red at the end of the reaction in order to separate the ether and the carbinol. In practice this method appeared to be unsatisfactory, because the ethers were difficult to crystallize. Instead of crystals, an oil was obtained in all cases. After distillation of the product, which should have consisted of the ether only, it was found still to contain 10—40% of the carbinol. This was determined by titrating a sample of the distillate with 0.1 N hydrochloric acid in 50% alcohol, the end point of the titration being determined with the aid of a pH-meter. As for the ether the slope of the titration curve at the equivalence point is steep and, in addition, the final pH of the ether differs by about three units from that of the carbinol, this determination can be carried out with sufficient accuracy.

4. Several ethers are hygroscopic, this property being more pronounced according as the purity is lower.

TABLE II

β-dimethylaminoethyl aryl(2-pyridyl)carbinol ethers*

No.	Substituent in the phenyl nucleus	Yield %	m.p. of mono HCl salt °C	b.p. °C (mm)	Analysis, %					
					$C_{calc.}$	C_{found}	$H_{calc.}$	H_{found}	$N_{calc.}$	N_{found}
XVIII	none	60	102-4	155-8(3)						
XIX	2-methyl	51	114-5		66.54	66.5	7.56	7.7	9.13	9.0
XX	2-ethyl	35	180-2	160-5(3)	67.38	66.7	7.85	7.9	8.73	8.5
XXI	2-isopropyl	30	173-4	165-70(2)	68.15	68.1	8.13	8.3	8.37	8.3
XXII	2-tert-butyl	41	186-7	155-60(1)	68.84	68.9	8.38	8.4	8.03	7.9
XXIII	3-methyl	67	103-5	142-6(0.03)	66.54	66.4	7.56	7.6	9.13	9.0
XXIV	4-methyl	43	122-3	133-6(0.01)	66.54	67.2	7.56	7.5	9.13	9.3
XXV	2,3-dimethyl	63	164-7	158-60(3)	67.38	67.5	7.85	7.9	8.73	8.7
XXVI	2,4-dimethyl	40	113-5	170-5(4)	67.38	67.6	7.85	8.0	8.73	8.4
XXVII	2,5-dimethyl	78	140-2	150-5(1)	67.38	67.5	7.85	7.9	8.73	8.9
XXVIII	2,6-dimethyl	35	146-9	166-7(3)	67.38	67.7	7.85	7.9	8.73	8.5
XXIX	2,6-diethyl	30	151	172-4(2)	68.84	68.5	8.38	8.5	8.03	7.9
XXX	2,6-diisopropyl	46	156-7	160-8(2)	70.09	70.3	8.83	8.7	7.43	7.3
XXXI	3,4-dimethyl	45	126-8	160-4(1)	67.38	67.1	7.85	7.9	8.73	8.8
XXXII	3,5-dimethyl	66	139-41	150-3(1)	67.38	67.5	7.85	7.9	8.73	8.9
XXXIII	2,4,6-trimethyl	42	173-4	167-74(2)	68.15	68.3	8.13	7.9	8.37	8.4
XXXIV	2,3,5,6-tetramethyl	48	197	175-80(1)	68.84	68.9	8.38	8.5	8.03	7.6

* Mono HCl salts. The U.V. spectra of these compounds are given in Table IV, p. 24.

The mono HCl salts of compounds No. XVIII, XIX, XXIV, XXVI and XXVIII are hygroscopic.

XVIII. In 1952, Harms and Nauta [34] described the following new mode of preparation for benzhydryl ethers:

$$(C_6H_5)_2CHCl + HOCH_2CH_2N(CH_3)_2 \xrightarrow{90\ ^\circ C} \left[(C_6H_5)_2CH\,\underset{\displaystyle CH_3}{\overset{\displaystyle CH_3}{\overset{|}{\underset{|}{N^+}}}}\,CH_2CH_2OH\right] Cl^-$$

$$\xrightarrow[\text{rearrangement}]{170\text{—}180\ ^\circ C} (C_6H_5)_2CHOCH_2CH_2N(CH_3)_2 \,.\, HCl.$$

In a similar way we have attempted to prepare the corresponding phenyl(2-pyridyl)carbinol ether. The phenyl(2-pyridyl)methyl chloride was obtained in a yield of 78% by the method indicated by Hamlin c.s., b.p. = 112—8 °C (0.02 mm), n_D^{20} = 1.5948. Hamlin [35]: yield 90%, b.p. = 126—31 °C (0.3 mm), $n_D^{25.5}$ = 1.5927. After addition of an equivalent amount of dimethylaminoethanol the temperature was slowly raised to 180 °C. At 70—80 °C an exothermal reaction could clearly be observed. At the same time the reaction mixture became viscous. A product was isolated that melted at 157—8 °C after crystallization from alcohol. The same product was obtained in 90% yield when the reaction was allowed to take place at room temperature for 24 hours. By analogy with Harms and Nauta's findings [34] this should be the quaternary ammonium salt. When this product, or the original reaction mixture, is heated to a higher temperature, another exothermal reaction occurs at 160—70 °C. When the reaction is complete the product is taken up in water, made alkaline with sodium carbonate, extracted with benzene and, after drying, distilled. From the distillate the ether was obtained in a yield of 27%; m.p. of the mono-HCl salt = 102—4 °C. The melting point of a mixture with an authentic sample showed no depression.
Tilford [8]: b.p. = 147—51 °C (0.3 mm); m.p. of mono-HCl salt = 103—105 °C; Sperber [9]: b.p. = 158—62 °C (1.5 mm).

XXII. m.p. of free base = 50—51 °C.

XXIII. Sperber [9]: b.p. = 155—9 °C (0.5 mm).

XXIV. Sperber [9]: b.p. = 156—60 °C (1 mm); Tilford [12]: b.p. = 144—50 °C (0.2 mm).

XXXIV. m.p. of free base = 45—6 °C.
Analysis: C = 76.88%, H = 9.03%, N = 8.97% (calc.)
C = 76.6%, H = 9.1%, N = 9.0 (found)

Spectra

The spectra of compounds I—XXXIV (p. 23 and 24) were measured in 95% alcohol with a Unicam Spectrophotometer S.P. 500. The general picture they show is represented in fig. I.

All the carbinols were oxidized to form the corresponding ketones with chromium trioxide in glacial acetic acid [36]. Their spectra will be published elsewhere.

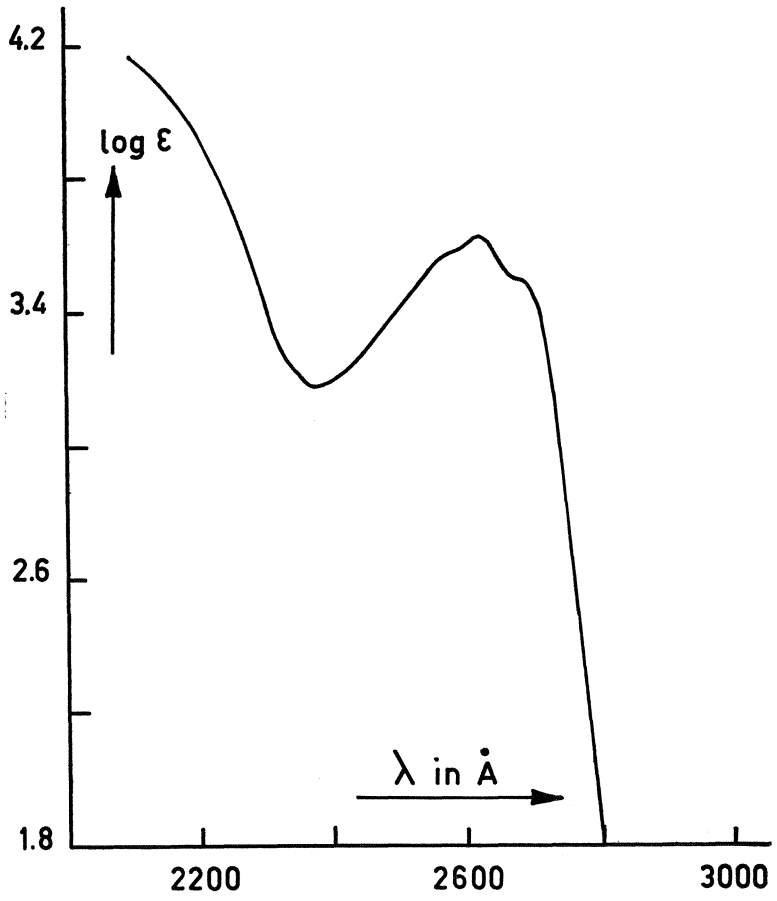


Fig. 1

TABLE III

U.V. absorption spectra of the aryl(2-pyridyl)carbinols

No.	Substituent in the phenyl nucleus	Maxima						λ (Å) at log $\varepsilon = 3.8$
		λ (Å)	ε	λ (Å)	ε	λ (Å)	ε	
I	none	2680[1])	3110	2615	4210	2580[2])	3980	2225
II	2-methyl	2685[1])	3300	2625	4390	2580[2])	3940	2235
III	2-ethyl	2690	3290	2625	4390	2580[1])	3970	2245
IV	2-isopropyl	2690	3290	2625	4420	2580[1])	4000	2235
V	2-tert-butyl	2680[1])	3200	2615	4330	2575[1])	3950	2245
VI	3-methyl	2690[1])	3380	2625	4510	2580[2])	4050	2240
VII	4-methyl	2690[1])	3240	2625	4550	2580[2])	4090	2280
VIII	2,3-dimethyl	2680[1])	3370	2625	4610	2580[2])	4080	2250
IX	2,4-dimethyl	2680[2])	3530	2625	4660	2580[2])	4150	2300
X	2,5-dimethyl	2690	3660	2625	4650	2580[1])	4040	2265
XI	2,6-dimethyl	2680[1])	3280	2625	4300	2580[1])	3790	2250
XII	2,6-diethyl	2685	3300	2630	4420	2580[1])	3860	2255
XIII	2,6-diisopropyl	2690	3300	2635	4470	2590	3950	2255
XIV	3,4-dimethyl	2680[2])	3730	2625	4890	2580[2])	4390	2295
XV	3,5-dimethyl	2690[2])	3450	2625	4750	2580[2])	4210	2285
XVI	2,4,6-trimethyl	2685[1])	3310	2630	4430	2585[1])	3950	2310
XVII	2,3,5,6-tetramethyl	2695	3650	2635	4670	2585[1])	4040	2295

[1]) shoulder

[2]) inflexion

TABLE IV

U.V. absorption spectra of the aryl(2-pyridyl)carbinol ethers

No.	Substituent in the phenyl nucleus	Maxima						λ (Å) at $\log \varepsilon = 3.8$
		λ (Å)	ε	λ (Å)	ε	λ (Å)	ε	
XVIII	none	2680	3470	2620	4620	2580	4200	2230
XIX	2-methyl	2680	3880	2620	5020	2580	4380	2235
XX	2-ethyl	2690	3290	2630	4330	2580	3950	2225
XXI	2-isopropyl	2685	3280	2630	4340	2580	3970	2225
XXII	2-tert-butyl	2690	4310	2630	5710	2580	4940	2260
XXIII	3-methyl	2680	3800	2620	4970	2580	4380	2245
XXIV	4-methyl	2690	3830	2625	5250	2580	4650	2310
XXV	2,3-dimethyl	2690	4060	2625	5280	2580	4580	2250
XXVI	2,4-dimethyl	2680	4130	2625	5390	2580	4710	2300
XXVII	2,5-dimethyl	2690	3990	2650	5070	2580	4330	2260
XXVIII	2,6-dimethyl	2690	4130	2625	5370	2580	4470	2245
XXIX	2,6-diethyl	2700	4180	2630	5580	2580	4590	2255
XXX	2,6-diisopropyl	2700	4260	2635	5610	2580	4640	2250
XXXI	3,4-dimethyl	2680	4170	2625	5440	2580	4760	2300
XXXII	3,5-dimethyl	2680	3920	2625	5250	2580	4550	2290
XXXIII	2,4,6-trimethyl	2690	4060	2625	5510	2580	4590	2305
XXXIV	2,3,5,6-tetramethyl	2700	4510	2635	5750	2580	4780	2290

CHAPTER II

THE DETERMINATION OF AMINO ACIDS BY ASCENDING ONE-DIMENSIONAL PAPER CHROMATOGRAPHY

Introduction

In the biochemical investigation of our compounds we were interested among other things in their influence on the metabolism of amino acids in brain tissue. In order to be able to assess this influence we had to determine the amino acids quantitatively.

To this end we used ascending one-dimensional paper chromatography. An advantage over the two-dimensional method is that, if desired, mixtures of known composition can be chromatographed on the same strip as the mixture under test, under exactly the same conditions. A disadvantage is that no solvent is known in which all amino acids can be effectively separated from each other. One device by which this drawback can be obviated is the use of different solvents, so that on at least one chromatogram each amino acid is completely separated from all the others and can be determined quantitatively [37]. Besides, these solvents can be buffered at different pH values [38]. Another very useful method is that described by Grassmann [39], in which the amino acids are first separated into various groups by means of paper electrophoresis. In the subsequent one-dimensional chromatography little or no overlap of the amino acid bands within a given group is observed.

As was found by De Waart [6] and, about simultaneously, by other investigators [40, 41, 42], a good separation of the various amino acids can also be obtained by allowing the solvent to ascend three or more times. This method produces sharp bands and, for amino acids having an R_f value lower than 0.5, a better separation than after one ascent. As we were primarily interested in the amino acids γ-aminobutyric acid and glutamic acid, and since these amino acids can be readily separated by the method introduced into our laboratory by De Waart, this method was adopted.

Procedure

In the ascending one-dimensional paper chromatography we used butanol — glacial acetic acid — water (12 : 3 : 5) as the solvent and Schleicher u. Schüll 2043^{B} Mgl. filter paper. The dimensions of the strips were 7 × 20 cm. At most eight of these were hanged simultaneously in the tank. The temperature was 20 ± 2 °C. The solvent was renewed at least once a week, this being dependent on the frequency of use, which causes the composition to change: the partition of the three components in the paper is not the same as that in the solvent.

The height of ascent was 10 cm above the starting line of the amino acids. The solvent was allowed to rise three times and after each time (duration about two hours) the strips were dried for 10 minutes in a drying oven at 80 °C.

After the third time the strips were sprayed twice with a solution of 1 g of ninhydrin in 500 ml of acetone, about 5 ml being applied to each strip. When the chromatograms are heated for 10 minutes at 80 °C to produce the colour given by the ninhydrin reaction, an interfering background may occur if ammonia is present. To prevent this, a Petri dish containing concentrated sulphuric acid is placed in the drying oven [40].

In general 24 strips were required for one experiment. All possible precautions were taken to ensure that for all these strips the test conditions were as nearly the same as practicable. Any neglect in this respect lowered the repeatability of the determination.

The amino acid solution is applied with the aid of a perspex wedge, 0.06 ml being spread over the full width (5 cm) of the sharp edge by means of a micro-pipette. The strip is placed on a glass plate and, after application of the solution, is dried at once under a hair-drier, the temperature being about 70 °C.

After development of the colour the strips are first soaked with a mixture of α-bromonaphthalene and paraffin oil (1 : 6), which has the same n_D as cellulose [43]; then they are blotted between filter paper and measured in a Zeiss extinction recorder No. II, the strips being passed through a narrow light beam at a constant rate. As the strips are 7 cm wide and the Zeiss extinction recorder can take a width of only 4 cm, a width of 1.5 cm had to be cut off the strips on either side, only the middle part being measured. As during chromatography the amino acid bands usually travel some-

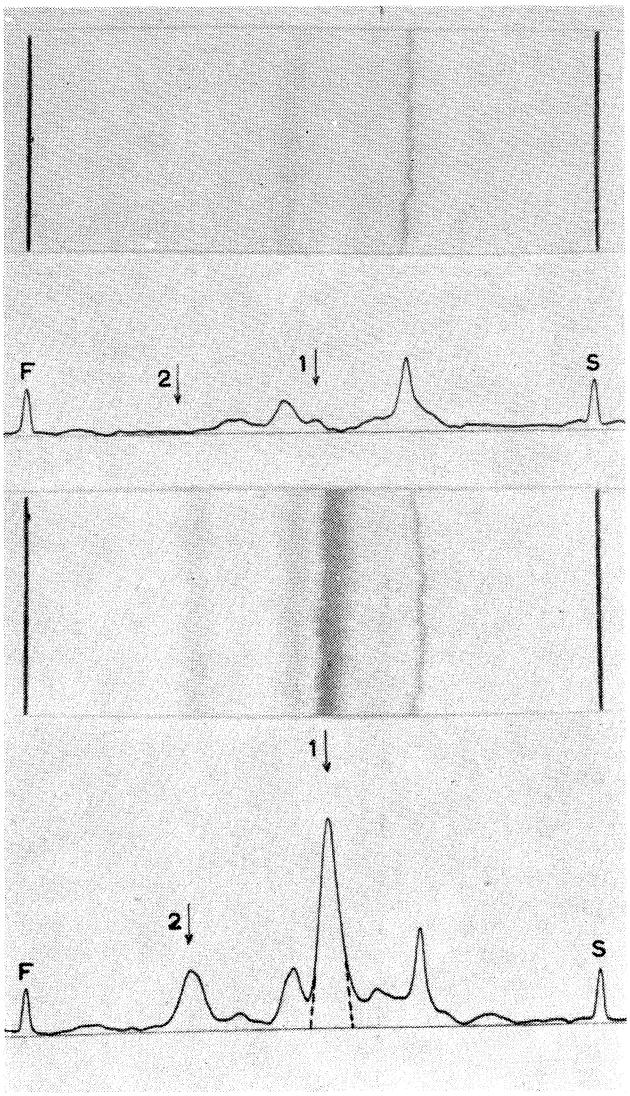


Fig. 2

Paper chromatograms and corresponding extinction curves of amino acids (ninhydrin colouring) in the incubation liquid of rat brain slices after metabolizing for one hour aerobically.
Upper half: strip of the blank with curve; lower half: 0.002 M. orphenadrine added; S is start, F is finish; band 1: glutamic acid; band 2: γ-amino butyric acid.

what faster in the middle than at the sides of the strip, the advantage of reducing their width is that the parts of the bands actually measured are straighter.

The extinction curves of the amino acid to be determined are integrated and the corresponding amount of amino acid is determined from a standard curve. Since the extinction measured against the distance x along the chromatogram proceeds almost linearly up to the maximum (see fig. 2 and [39, 44, 45]) we can calculate the surface area A of the extinction curve from $\frac{1}{2} E_{max} . x$.

If we define c as the concentration of the amino acid in the original solution, a similar relation as exists between A and c will also hold for A and the quantity of dye on the paper, if there exists a linear relation between c and the amount of dye on the paper. From experiments carried out by us (see further on) it appeared that this condition was fulfilled.

Results

In order to find the relation between A and c, a standard curve has to be plotted. We found that if 1.25—20 γ of γ-aminobutyric acid (GABA) and glutamic acid (Glu) in phosphate buffer was applied per strip, the relation $\log A = a \log c + b$ holds. This can also be written as $A = b' c^a$. For $a = 1$ therefore $A = b'c$. In this case we should find a linear relation between A and c. Under our conditions, however, *a* is smaller than unity.

As in the present investigation amino acids had to be determined in a 'homogenate' of rat cortex in buffer and in buffer in which slices of rat cortex had been incubated, it had to be ascertained whether under these conditions the same standard curve was valid. To this end GABA and Glu were added to (1) phosphate buffer, (2) buffer in which slices of tissue had been incubated for 40 minutes and (3) homogenate in buffer, such that the concentration of the amino acids added was 20, 10, 7.5, 5, 2.5 and 1.25 γ per 0.06 ml. For each concentration four strips were made and the surface area was determined of the extinction curve obtained after running a chromatogram and developing the colour. This was repeated on five successive days. The values of A obtained over these five days were averaged for a given concentration and for the average values of all concentrations a standard curve

was plotted (fig. 3 and 4, curve I). For (2) and (3) we have to take into account the contribution of the amino acids already present.

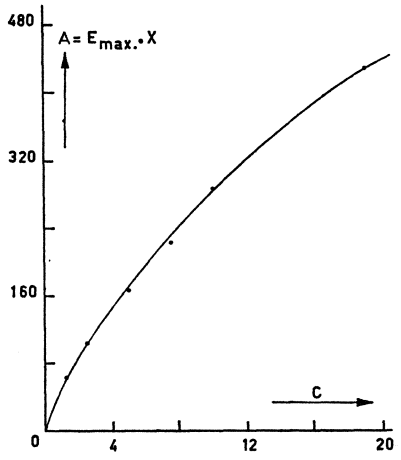


Fig. 3

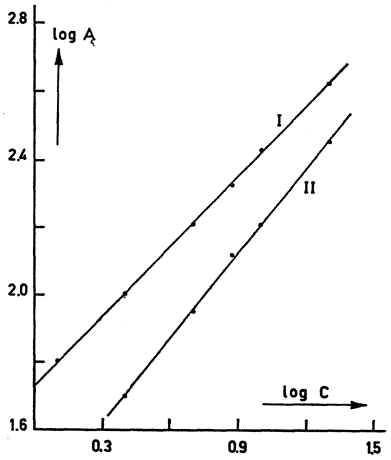


Fig. 4

The observations (1) have been analysed by the Statistics Department of the Mathematical Centre in Amsterdam. Their results are described in the appendix. The following conclusions have been taken from this appendix.

1. The assumption of a linear relation between the logarithm of c and the logarithm of A can very well serve as a working hypothesis. Upon application of a statistical test the hypothesis of linearity could not be rejected. From the observations on each day a standard line can be calculated. There appeared to be no *systematical* difference between the lines on different days.

2. The best estimate of the line calculated from all observations with $c \geqslant 2.5\ \gamma$ is for

   GABA: $A = 0.671 \log c + 1.745$
   Glu: $A = 0.681 \log c + 1.741$

Under all the conditions investigated by us we obtained the same standard curve, provided always that the base line was invariably

drawn in the same manner. Redfield [46] draws it through those parts of the curve where no amino acid is present; we drew it invariably through the parts before the start and after the finish.

A similar relation as for GABA and Glu was also found for alanine, aspartic acid and glutamine. Constants a and b have different values here (table V).

The R_f value (table V) for a given amino acid was the same under all conditions.

TABLE V

Amino acid	$\log A = a \log c + b$		$R_f \pm$ s.d.
	a	b	
γ-Aminobutyric acid	0.671	1.745	0.66 ± 0.02
Glutamic acid	0.688	1.735	0.45 ± 0.02
Alanine	0.63	1.93	0.51 ± 0.02
Glutamine	0.76	1.58	0.32 ± 0.01
Aspartic acid	0.69	1.57	0.41 ± 0.02

Discussion

In addition to the relation $\log A = a \log c + b$, found by us, in the literature many other relations have been found between a quantity relating to the coloured spot (A, E_{max}, x, spot area) and the concentration of the amino acid. Some relationships involving E_{max} are the following:

$$E_{max} \cdot x = b.c \text{ [39]} \qquad E_{max} = b.c \text{ [47]}$$

$$1/E_{max} \cdot x = \frac{b}{a} \cdot \frac{1}{c} + \frac{1}{a} \text{ [48, 49]}$$

and $E_{max} = a \log c$ [44, 50, 51, 52].

Relationships connected with spot area are:

$$A_{spot} = k \log c \text{ [45]} \quad \text{and} \quad \log x = k \log c \text{ [6, 45]}.$$

These relations have been found not only for amino acids but also for proteins and sugars and appear not to be bound to any one colouring reaction [39, 43, 44, 47—54].

It is difficult to say how so many different relationships have been arrived at. No doubt several factors are involved; for instance, the nature of the measuring equipment, the structure of the filter paper, the type of light filter, the nature of the solvent, the colour development and the shape of the spot measured.

What always strikes us in the literature is that so many authors mention the applicability, or otherwise, of Beer's Law in extinction determinations on paper [39, 43, 49, 55, 56]. Most of them realize that it does not apply. Yet a linear relation is sometimes found between E and c [39, 43, 47]. We therefore wondered if it would be possible to indicate why we failed to obtain this relation. Various possibilities were examined:

(1) the amount of dye formed is not directly proportional to the concentration of the amino acid;

(2) the deviation is due to the light not being monochromatic;

(3) the non-linear relation is connected with the structure of the paper.

re (1). If we denote the amount of dye formed per unit $-NH_2$ of an amino acid the *colour yield,* it is at once clear from the literature that it is dependent on various factors.

The time stated in the literature for the colouring reaction varies from 24 hours to 3 minutes and the corresponding temperature from 20 °C to 110 °C. We developed the colour for 10 minutes at 80 °C in a drying oven. As the colour yield might be dependent on the time, we ascertained whether 20 instead of 10 minutes' colour development produced any increase. This proved not to be the case.

The amount of ninhydrin, too, might have some influence. For, it can be calculated that for an amount of 20 γ GABA and Glu per strip the excess of ninhydrin is only 15- and 20-fold, respectively, whereas for 1.25 γ per strip this is 230 and 320-fold.
H. Meyer [58] states that for comparable quantities in solution (20 γ) about a 75-fold excess is used. Therefore, part of the strips was sprayed with a quantity of ninhydrin five times as large as usual. For none of the concentrations of 1.25 to 20 γ per strip did we observe an increase in colour yield.

The maximum colour yield is also dependent on the pH. Meyer [58] found the optimum pH in solution to be 6.18; Moore and Stein [59] pH = 5.5. In the former case, Meyer, as well as Troll and Cannon [60], obtained a 100% colour yield for all α-amino acids, if only the right solvent is chosen (95% alcohol, phenol-pyridine). For paper this is certainly not true. According to Grassmann [39] the colour yield varies with the amino acid. Our results are in agreement with this finding (table V). For a given amino acid, however, the colour yield is the same for all concentrations. For, if we elute the colour from the paper with a certain volume of 75% acetone-water and we measure the extinction in the Unicam spectrophotometer S.P.500 at λ = 578 mμ, we repeatedly find a linear relation between E and c (fig. 5).

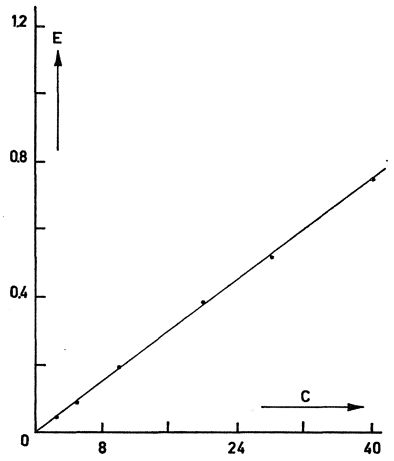


Fig. 5

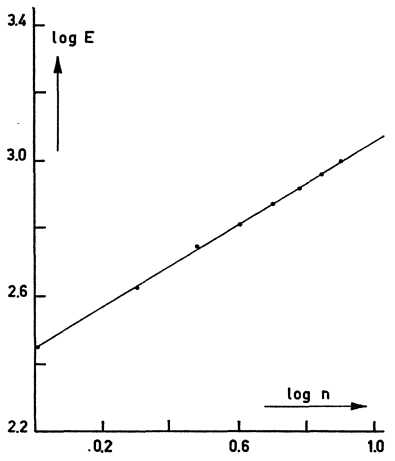


Fig. 6

When measuring in solution (95% alcohol, acetone-water) one finds for the dye obtained upon reacting ninhydrin with an amino acid two maxima [58], one at λ = 410 mμ and one at λ = 578 mμ. On paper only the maximum at 578 mμ appears to have shifted, namely to a lower wavelength. For GABA, Glu and $GluNH_2$ we found this maximum at 545, 548 and 543 mμ, respectively. Besides, the band is much wider than in solution. The filter used in these measurements was Zeiss filter FE_{54} (λ max = 525 mμ).

re (2). In order to examine the influence of the light source the dye on the strips was measured with different filters, viz. Zeiss filter FE_{54}, a Schott interference filter (λ max = approx. 550 mμ) and with a combination of Schott filters OG_1 and VG_6 (λ max = approx. 550 mμ). The dye was then eluted and measured once again in 1-mm cells (1) in the Zeiss extinction recorder and (2) in the Unicam spectrophotometer. Irrespective of the filter used, in the Zeiss apparatus on paper we invariably found the relation $\log A = a \log c + b$, if $c = 1—20\ \gamma$ per 0.06 ml (fig. 3, p. 28).

In the Unicam spectrophotometer, at $\lambda = 578$ mμ, however, the relation $E = k.c$ (fig. 5, p. 31) obtained, whereas it did not when the extinction of the eluted dye was determined in the Zeiss apparatus. There was a departure from linearity in that the extinction was too low for higher concentrations. This is an indication that the use of non-monochromatic light may indeed produce a deviation from linearity, also when the dye is measured on paper.

That the relation between A and c, when the dye is measured on paper, is independent of the filter used was also known from the literature [43, 50]. However, Grassmann [43] finds that $A = E_{max} . x = k.c$, whereas McFarren and James [50] find that $E_{max} = k \log c$. This is an indication suggesting that the relation found is governed by still other factors.

re (3). (a) Grassmann states that, if the relation $A = k.c$ is to be obtained, it is essential to make the strips perfectly transparent. Otherwise, light scattering will inevitably cause deviations. Complete soaking is accomplished quickest in vacuum. Otherwise it takes hours before all air has been displaced from the strip. Although Crook [49] had fulfilled this condition, he obtained for proteins, contrary to Grassmann's findings, no linear relation between $A = E_{max} . x$ and c, but between $1/E_{max}$ and $1/c$.

The picture we obtained for strips made fully transparent (not blotted between filter paper after soaking in vacuum), did not comply with Grassmann's relation, although approximating it. For, in our case, where $\log A = a \log c + b$ or $A = b' c^a$, *a* should be equal to unity. Under the conditions used by us we found $a = 0.68$, under those of Grassmann we found $a = 0.85$ (see fig. 4, curve II, p. 28).

(b) The lack of uniformity of the paper also plays a role. From a photograph taken by us of a strip (magnification 780 ×), it

was clear that the distribution of the dye in the paper is very inhomogeneous. Jencks [55], among others, came to the same conclusion for proteins.

Grassmann has derived a formula for the case where a fraction α of the paper is not coloured and the rest uniformly coloured, namely

$$E_r = -\log\left[(1-\alpha) \cdot 10^{-\frac{1}{(1-\alpha)} \cdot E_o} + \alpha\right],$$

where E_r = measured extinction.
E_o = calculated extinction at a homogeneous distribution.

The calculation is based on the assumption that the intensity of the light falling on the strip is distributed uniformly over the total slit width. If 20% of the strip contains no dye at all ($\alpha = 0.2$), we find for $E_o = 0.2$ an error of 5%, for $E_o = 0.4$ one of 14% and for $E_o = 0.6$ one of 23%. In practice, however, the error will not increase so strongly with E_o, because we must take into account the fact that α decreases according as E_o increases. Furthermore, α will also depend on the intensity of the band being measured, which in turn will be different for a given concentration of the amino acid according to the solvent used and the width over which the amino acid is applied. This might also partly explain why, for instance, Grassmann finds a linear relation and we do not.

(c) Light scattering and the non-uniform distribution of the dye in the paper will therefore both affect the extinction value we ultimately measure. Light scattering will give an extinction that is too high, inhomogenity one that is too low. What in the end we are concerned with is the effect of these two factors. In a strip containing no dye the two factors can be examined.

If we measure the extinction of the blank paper, we find under our experimental conditions that the extinction increases with the layer thickness, n, according to the equation $\log E = a' \log n + b'$. This relationship was found by placing 1 or more (maximum: 9) soaked strips on top of each other and measuring E in the strip colorimeter with respect to air (see table VI and fig. 6, p. 31).

TABLE VI

layer thickness (n)	1	2	3	4	5	6	7	8	9
extinction (E)	0.280	0.452	0.560	0.650	0.740	0.825	0.905	0.983	1.065

Each extinction value is the average of five observations.

It is seen that, when the paper contains no dye, the extinction increases logarithmically with the logarithm of the layer thickness (see fig. 6).

The question now arises whether this is also the case when the paper contains dye. It is certain that the dye is absorbed on the paper, in any case partly. To the extent to which it is not adsorbed on the fibre it will reduce the inhomogenity (α becomes smaller). The adsorbed part will have the same pattern as the paper fibre and consequently give rise to light absorption as well as to light scattering. Although it cannot be said to what extent the relation between the extinction and the layer thickness of the paper ($\log E = a' \log n + b'$) is connected with the relation between the extinction of the dye on the paper and its concentration, we are inclined to say that there is some connection, because in both cases the relationship is determined by the same factors.

From the above discussion it will be clear that several factors determine the relation ultimately obtained. It is certain that in our case the colour yield was invariably the same for all concentrations, so that this quantity cannot account for the relation found by us. Although the light filter used also has some influence, this cannot be the determining factor, because several authors have found [43, 50] the relation between E and c to be independent of the filter used. Our findings were in agreement with this. Finally, there is the structure of the paper. The inhomogeneity of the paper and its light scattering are probably the major factors determining the relation found to exist between E and c.

The reader who wishes to obtain an idea of the accuracy of our experiments is referred to the appendix from the Mathematical Centre at Amsterdam. The quantity of amino acid generally determined with the aid of paper chromatography is between 2.5 γ and 40 γ per strip. The accuracy usually stated is 2—5%, if the amino acid is determined against a standard.

CHAPTER III

SOME ASPECTS OF THE METABOLISM OF γ-AMINOBUTYRIC ACID AND L-GLUTAMIC ACID IN THE CENTRAL NERVOUS SYSTEM

Although the metabolism of carbohydrates is of vital importance for the brain, in mammals only 5% of the dry weight consists of carbohydrates, as against 40% of amino acids and proteins. Of the amino acids, especially γ-aminobutyric acid (GABA), L-glutamic acid (Glu), L-glutamine ($GluNH_2$), L-aspartic acid, its N-acetyl derivative and taurine occur in relatively high concentrations [62].

Amino acids are usually determined by paper or column chromatography. After separation of the amino acids they can be coloured with, for instance, ninhydrin and then be determined quantitatively [72, 76]. Only those amino acids which are present in the tissue free or in an easily extractable form are determined in this way. The part retained in the cytoplasma or the nucleoplasma by absorption, for instance on the surface of proteins, escapes detection.

Hydrolysis with 6 N HCl does not affect the quantity of GABA found [69]. It must therefore be present in a readily extractable form. The values obtained by the various investigators, however, show a considerable spread. GABA was first demonstrated to be present in the brain of mammals by Roberts and Frankel [69] and, independently, by Awapara [70]. The literature values reported for GABA in rat brain vary from about 20 to 80 γ per 100 mg [63, 64, 66, 68, 70]. The values for Glu range from 120 to 170 γ per 100 mg. One of the factors accounting for the spread is that the L-glutamic acid decarboxylase (GAD) is still active after dissection, so that GABA can still be formed from Glu. Since, owing to the low concentration of oxygen present, oxidative processes will proceed only slowly, GABA will accumulate. No doubt the method of determination and the strain of rats used also play a part.

An accurate, specific, spectrophotometric method for the determination of GABA was recently introduced by Scott and Jacobi [71], who use a bacterial enzyme. Outside the central nervous system GABA occurs only in small quantities [72]. It is unique for the

central nervous system as regards its relatively high concentration there. Of the GABA and Glu occurring in the central nervous system the greater part (84 and 86%, respectively) is present in the grey matter.

The concentration of the amino acids is generally much lower in the blood than in the brain. For all amino acids together these two concentrations differ by a factor of about six, for Glu + $GluNH_2$ by a factor of about fifteen and for GABA by a still larger factor [62]. So there exists a concentration gradient between the blood and the brain. The blood-brain barrier is relatively impermeable to GABA and Glu. After intravenous injection of Glu-U-C^{14}, Tower [77] observed an intensive exchange, but no increase in the brain. The same was found by Roberts et al.[75] for GABA.

As the turnover of GABA and Glu in the brain is large and the blood-brain barrier is relatively impermeable to these amino acids, the question arises how they are formed in the central nervous system. From experiments with mice that were given glucose-U-C^{14}, Roberts et al.[67] concluded that about 10% of the Glu in the brain is supplied direct from the blood. The rest is produced in the brain. By decarboxylation with GAD, GABA is formed from Glu. On comparison of the amounts of GABA and GAD in various parts of the brain in various animals there appears to be a linear relation between the amount of GABA present and the activity of GAD. The fact that the line representing the linear relationship can be extrapolated to the origin supports the hypothesis that GAD is the principal, if not the only, enzyme responsible for GABA formation in the brain [72].

Both in vivo and in vitro indications have been obtained that glucose is the main source of Glu. Dawson [92], studying insulin-induced hypoglycaemia and using fluoroacetate, which reduces glycolysis and blocks the citric acid cycle, observed a drop of the Glu level in the brain. Further, Busch [93] three minutes after intravenous injection of glucose-U-C^{14} in rats found 53% of the total C^{14} activity of the brain in the glutamate fraction. Beloff-Chain and co-workers [94] obtained a similar picture in experiments in vitro with rat cortex after one hour's metabolism. Waelsch [95] allowed sections of rat cortex in which hardly any glutamate was left to metabolize for one hour. Only when glucose was present as the substrate was the original level of Glu + $GluNH_2$ reached again.

This is in agreement with the results obtained by Krebs et al.[96]. They also found that oxygen is indispensable, which was confirmed by experiments of Tsukada et al.[97]. Tower[98] found that the Glu concentration did not reach its normal level even when glycolysis was prevented by the presence of 2-deoxyglucose.

The conclusion is therefore justified that glucose is the ultimate source of Glu and that for its formation it is essential that glycolysis takes place and that the citric acid cycle can operate.

Indications that GABA is formed from Glu and that the metabolism of GABA proceeds via the citric acid cycle are the following: Upon intracerebral administration of Glu-U-C^{14} to mice, GABA is rapidly formed[99]. Tower[77], who carried out in vitro experiments with cat cortex, observed the same thing when slices of it were metabolized with Glu-U-C^{14}. When GABA-U-C^{14} is used as the substrate a large part of the C^{14} activity is found back in CO_2. Tsukada et al.[100] demonstrated the same phenomenon with GABA labelled with C^{14} in the carboxyl group. Roberts and co-workers[99], 2 minutes after intracerebral injection of GABA-U-C^{14}, demonstrated the presence of radioactive succinic acid, aspartic acid and $GluNH_2$ in rat brain. Upon intraperitoneal administration of the GABA, 2% of the radioactivity was found in the urine in succinic acid and also some in α-ketoglutaric acid.

From α-ketoglutaric acid the citric acid cycle can proceed in two different ways[80]. This is shown schematically in fig. 7.

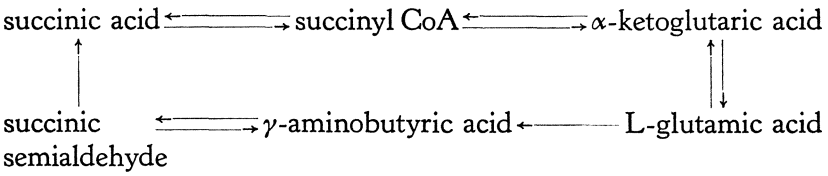


fig. 7

(1) via succinyl CoA to succinic acid;

(2) via a *shunt*, α-ketoglutaric acid first being converted into Glu by reductive amination. Normally the conditions are such that reductive amination occurs rather than the reverse reaction[78, 79].

The reaction by which Glu is converted into GABA proceeds with the enzyme GAD, which is specific for the decarboxylation of Glu [69].

$$\underset{\text{L-glutamic acid}}{HOOC.CH_2CH_2CH(NH_2)COOH} \xrightarrow[B_6 \text{ co-enzyme}]{GAD} \underset{\gamma\text{-aminobutyric acid}}{HOOC.CH_2CH_2CH_2NH_2} + CO_2$$

Roberts [69] finds this reaction to be irreversible. This is concluded from there being no fixation of $C^{14}O_2$ in the manometric experiments by which the GAD activity is determined. Koppelman et al.[81] have succeeded in determining an equilibrium constant for this reaction, using isotopes. This also suggests that the reaction is virtually irreversible.

The GABA-α-ketoglutaric acid transaminase (GABA-T), which catalyses the transamination of GABA with α-ketoglutaric acid, is, like GAD, found mainly in the grey matter, but, unlike GAD, also in other tissues [82, 83].

$$\underset{\gamma\text{-aminobutyric acid}}{HOOC.CH_2CH_2CH_2NH_2} + \underset{\alpha\text{-ketoglutaric acid}}{HOOC.CH_2CH_2\overset{O}{\overset{\|}{C}}.COOH} \xrightleftharpoons[B_6 \text{ co-enzyme}]{GABA\text{-}T} \underset{\text{succinic semi-aldehyde}}{HOOC.CH_2CH_2CHO} + \underset{\text{L-glutamic acid}}{HOOC.CH_2CH_2CH(NH_2)COOH}$$

Glu, from which GABA is formed by decarboxylation, is thus found in this reaction to be resynthesized, succinic semialdehyde being irreversibly oxidized to succinic acid under the influence of a dehydrogenase [84].

$$\underset{\text{succinic semi-aldehyde}}{HOOC.CH_2CH_2CHO} + DPN^+ + H_2O \xrightarrow{\text{dehydrogenase}} \underset{\text{succinic acid}}{HOOC.CH_2CH_2COOH} + DPNH + H^+$$

It will be clear from the above that Glu, GABA and succinic semialdehyde, unlike many other amino acids (Krebs [89]), can support the oxidative metabolism [85, 86, 87] and can therefore temporarily replace glucose as substrate.

McKann et al.[88] obtained indications from experiments in vitro that more than 40% of the oxygen consumption proceeds via the shunt. This goes to show that Glu and GABA occupy a strategic position among the amino acids.

Although GABA may also undergo transamination reactions in other tissues, according to Roberts [72] it is improbable that the reactions involving GABA will normally play an important part in other tissues, because the GAD occurs almost exclusively in the grey matter. As moreover the reactions with GAD and succinic semialdehyde dehydrogenase are virtually irreversible, this will also apply to the metabolism of GABA and therefore there exists only in the central nervous system an irreversible shunt around the α-ketoglutaric acid oxydase system.

In the foregoing some important aspects of the metabolism of Glu and GABA have been dealt with. We have in no way aimed at completeness. Thus, for Glu other transamination reactions and the equilibrium with $GluNH_2$ have been left out of consideration, for GABA the transamidation with arginine and the formation of GABA choline, etc.

The metabolism of Glu and GABA can be influenced by various factors both in vivo and in vitro. How our compounds do this in vitro is described in the next chapter.

CHAPTER IV

THE METABOLISM OF RAT CORTEX IN VITRO UNDER THE INFLUENCE OF PHENYL(2-PYRIDYL)CARBINOL ETHERS

As was remarked in the introduction, we were interested to know whether our compounds would have a similar effect on the metabolism of rat cortex as had been found by De Waart [6, 7] for a series of alkyl-substituted benzhydryl ethers and by Ernsting et al. [101] for a number of psycho-drugs.

In the first instance we examined, as did the above-mentioned authors, the influence on oxygen consumption and the changes in the amino acid pattern, especially of GABA and Glu. Oxygen uptake was determined by the Warburg technique; for the determination of the amino acids we used the chromatographic method described in chapter II. In order to gain an impression of oxygen consumption as a function of time, we measured it after 20, 40 and 60 minutes' metabolism. The amino acids were in general determined:
(1) at once after the rat had been sacrificed;
(2) in the incubation medium before and after one hour's metabolism;
(3) in the tissue after one hour's metabolism.

Thus a picture was obtained of the influence of the compounds under test on the metabolism of minced rat cortex.

Experimental

Experiments carried out by Tower [102] and Stern [103] among others have shown that brain tissue, after incubation, releases part of the amino acids. The amount of amino acids passing into the medium increases with the incubation time.

In our experiments the tissue was placed in the first flask 10 minutes after the rats had been sacrificed. The filling of the twelve flasks used in each experiment was completed about 18—20 minutes later. The metabolism started after about another 20 minutes, so that the incubation time for the different portions of tissue varied from 20 to 40 minutes. The quantities of GABA and Glu, however, had already reached their maxima 20 minutes after the

tissue was placed in the flasks and were still at this level after 40 minutes, so that under our experimental conditions the medium in all the flasks contained equal amounts of these amino acids.

As we used two rats for each experiment, the cortex of both animals was minced in one operation. In order to have a check on the "uniformity" of the tissue, equal portions (80–100 mg) of it were incubated, GABA and Glu in the medium being determined after 40 minutes. Of the incubation medium from each flask four strips were made. Calculations carried out by the Mathematical Centre in Amsterdam (see appendix) showed that the differences between the strips from one flask far exceeded the spread due to a possible lack of uniformity of the tissue from the various flasks.

In view of this conclusion it was decided for the determination of amino acids in the incubation medium to make only one strip from each flask, each time taking four flasks of tissue as a control group and four flasks as a group for the compound under test. If after one hour's metabolism the amino acids in the tissue were also determined, the tissue from the four flasks of one group was homogenized together in distilled water and, after centrifuging, four strips were made of the supernatant.

For the calculation of the oxygen consumption, too, invariably the average of four flasks was taken. Table VII shows the oxygen consumption after 20, 40 and 60 minutes' metabolism of four flasks in which the tissue (80—100 mg) was incubated at the time t, t + 6, t + 12 and t + 18 minutes. Each figure is the average of the observations on eight different days. It is seen that, within the time interval investigated, oxygen uptake is independent of the time at which the tissue is incubated. It is also clear that oxygen consumption increases practically linearly with the time of metabolism.

TABLE VII

	oxygen uptake (in μl) per 100 mg of tissue after an pre-incubation time of				
metabolized for	10 min. (1)	16 min. (2)	22 min. (3)	28 min. (4)	av. of 1-4 (5)
20 minutes	0.561	0.564	0.570	0.545	0.557
40 minutes	1.112	1.109	1.137	1.126	1.121
60 minutes	1.607	1.630	1.666	1.664	1.642

In addition to the twelve manometers with connected flasks for the process of metabolism, in each experiment two thermobarometers were used. The tissue, which had been minced with a razor-blade, was incubated in 2 ml of buffer of the following composition:

per litre of distilled water 19.5 mM of $Na_2HPO_4 . 2H_2O$,
8.5 mM of KH_2PO_4, 120 mM of NaCl and 1 mM of $MgSO_4 . 7H_2O$.

The time elapsed between the dissection of the cortex and the beginning of metabolism was in general 40—50 minutes. The tissue was weighed with an accuracy of 1 mg. D-glucose was used as the substrate at a final concentration of 0.02 mole. This was present in the side arm, with or without the compound to be tested, in 0.5 ml of buffer. The final concentration of the compound under test was 0.002 mole. The centre cup contained 0.2 ml of 10% KOH with filter paper projecting about 5 mm above the edge. After 10 minutes' equilibration the contents of the side arm were added to the main flask and this system was equilibrated for another 4 minutes. Next, the manometers were read, the cocks closed and metabolism was allowed to take place for 1 hour at 37 °C. At the end of this period the pH of the incubation medium was invariably determined with pH-paper. After one hour's metabolism the pH had dropped by 0.1 to 0.2 unit.

Results and discussion

Before metabolism was started, an average of 10 (8—13) γ of GABA per 100 mg of cortex had leaked from the tissue. For Glu this figure is 114 (106—127) γ per 100 mg of cortex. Immediately after dissection we find in the cortex for GABA 22 (17—27) γ per 100 mg and for Glu 177 (139—198) γ per 100 mg. The proportion of amino acids already present in the medium before metabolism is lower for GABA than for Glu. This suggests that the manner in which these amino acids are retained in the tissue is different. This is evidently true also for other amino acids. Certain amino acids which after chromatography of a homogenate give clearly visible bands can hardly be detected in the medium prior to metabolism.

The value found by us for Glu in rat cortex is in agreement with that stated by Berl and Waelsch [104], namely 170 (152—183) γ per 100 mg. So is the value for GABA, namely 20.6 (15.4—27.8) γ per

100 mg. Roberts [105] reports 26 (24—27) γ per 100 mg for GABA. That we found a lower value may be because we did not wash the tissue after centrifuging.

In addition to the influence of compounds XVIII to XXXIV inclusive on the metabolism of rat cortex, we also examined that of orphenadrine (XXXV) as a reference compound. The results of this part of the investigation are given in tables VIII and IX (p. 44 and 45).

Table VIII leads to the following conclusions:

(1) Contrary to what was found in the control experiments, the consumption of oxygen under the influence of our compounds does not increase linearly with time (see also p. 41).
(2) In general, the medium contains more GABA and Glu than in the control experiments, the difference being greater according as the alkyl substituent in the phenyl nucleus becomes larger or the number of substituents increases.
(3) The quantity found may be considerably larger than that present after dissection.

It follows from table IX that:

(4) According as GABA and Glu in the medium increase, the quantities in the tissue decrease, this decrease being greater according as GABA and Glu in the medium increase more strongly.
(5) In the control experiment the levels of GABA and Glu for medium and cortex are about the same as after dissection, for GABA 23 (21—27) γ per 100 mg, for Glu 159 (138—182) γ per 100 mg.
(6) In the control experiments virtually all the GABA and Glu is resorbed by the tissue after metabolism.

To be able to explain the effects observed, we should realize that in an isolated piece of tissue, which has moreover been cut into small fragments, several functions are disturbed, for instance because the supply of oxygen and substrate and the removal of waste material have ceased. Thus, it is known that after dissection the amounts of phosphocreatine and ATP in the brain decrease rapidly. If, however, sections of the brain metabolize with glucose and oxygen, the level of both substances is restorted to 70% of its previous value within 10 minutes [106—109].

TABLE VIII

No.	Sustituent in the phenyl nucleus	% 0_2 inhibition compared with the control				γ amino acid in the medium after one hour's metabolizing per 100 mg tissue				No. of experiments
						GABA level		Glu level		
		1st 20 min.	2nd 20 min.	3rd 20 min.	after 60 min.	inhibitor added	minus control	inhibitor added	minus control	
XVIII	none	14	25	25	21	1	0	30	10	3
XIX	2-methyl	9	18	19	15	1	0	34	17	3
XXIII	3-methyl	6	12	16	12	2	1	29	12	3
XXIV	4-methyl	5	7	12	8	3	2	37	14	2
XXV	2,3-dimethyl	17	24	25	21	8	7	62	43	2
XXVI	2,4-dimethyl	3	10	13	9	7	6	54	30	2
XXVII	2,5-dimethyl	16	15	17	17	4	3	48	30	2
XXVIII	2,6-dimethyl	−3	3	6	2	7	6	40	29	2
XXXI	3,4-dimethyl	14	16	17	16	6	5	67	47	2
XXXII	3,5-dimethyl	2	9	11	7	6	5	63	45	2
XX	2-ethyl	20	17	24	21	2	1	50	28	2
XXI	2-isopropyl	9	20	27	19	9	8	87	71	2
XXII	2-tert-butyl	7	28	39	25	27	26	232	211	3
XXXIII	2,4,6-trimethyl	6	14	15	11	26	25	224	201	3
XXXIV	2,3,5,6-tetramethyl	10	30	36	24	34	33	241	226	3
XXIX	2,6-diethyl	−2	15	33	16	20	19	223	201	3
XXX	2,6-diisopropyl	61	80	88	76	27	25	266	250	3
XXXV	orphenadrine	48	57	69	58	30	28	261	237	3

Each figure is the mean of the observations on two or three different days.
All values have been rounded off to the nearest integer.

TABLE IX

No.	Substituent in the phenyl nucleus	GABA level (γ/100 mg tissue)						Glu level (γ/100 mg tissue)					
		inhibitor added			control			inhibitor added			control		
		medium	tissue	total	medium	tissue	total	medium	tissue	total	medium	tissue	total
XVIII	none	1	20	21	1	20	21	31	158	189	21	131	152
XIX	2-methyl	1	21	22	2	20	22	40	142	186	23	141	164
XXIII	3-methyl	3	22	25	1	20	21	21	166	187	16	143	159
XIV	4-methyl	3	28	31	1	29	30	39	140	179	28	154	182
XXV	2,3-dimethyl	11	20	31	2	24	26	80	151	231	14	124	138
XXVII	2,5-dimethyl	4	25	30	2	21	23	59	139	197	23	127	150
XXVIII	2,6-dimethyl	10	20	30	2	25	27	43	143	186	19	151	170
XXXII	3,5-dimethyl	5	23	28	2	21	23	59	149	208	23	127	150
XXII	2-tert-butyl	32	8	40	2	24	26	235	41	276	16	143	159
XXXIII	2,4,6-trimethyl	24	11	35	1	29	30	229	79	308	28	154	182
XXXIV	2,3,5,6-tetramethyl	38	2	40	2	25	27	238	50	288	19	151	170
XXIX	2,6-diethyl	22	4	26	2	20	22	286	34	320	23	140	164
XXX	2,6-diisopropyl	26	1	27	1	20	21	273	13	286	21	131	152
XXXV	orphenadrine	26	4	30	1	20	21	269	32	301	14	124	138

All values have been rounded off to the nearest integer.

Similarly, Glu and $GluNH_2$ passing into the medium before metabolism can only be resorbed and maintained on the right level by metabolizing with oxygen and glucose as the substrate. The assimilation of these amino acids, therefore, is determined by the same factors as the concentration of ATP and phosphocreatine. For, in all cases energy is required for uptake and synthesis.

The same applies to the resorption of potassium. Even if oxygen and glucose are present in the medium, 50—70% passes from the tissue into the medium. Not until metabolism occurs is the potassium resorbed. Fluorides, which prevent glycolysis, and 2,4-dinitrophenol, which uncouples oxidative phosphorylation, oppose resorption. Thus, here, too, the key lies in the energy that is obtained by the oxidation of glucose and is required for the resorption of potassium in the tissue against the concentration gradient (Pappius and Elliot [110], Krebs et al.[111] and Eggleston et al.[96]. According to Pappius and Elliot, the resorption of Glu and potassium occurs in about equimolar quantities.

In view of the foregoing, we can explain the influence of our compounds on the metabolism of rat cortex as follows:

After the tissue has been incubated, an equilibrium is rapidly reached, a considerable proportion of the amino acids being present in the medium (see p. 42). As soon as metabolism starts, a variety of processes that were inhibited by the medium being poor in oxygen and substrate get going again. One of these is the production of energy required for the resorption of the amino acids. For the control tissue the conditions are obviously favourable for resorption. After one hour's metabolism the level of GABA and Glu has practically been restored. In view of the results obtained by Pappius and Elliot [110] the same may be assumed to hold for potassium. During metabolism not only resorption of amino acids occurs in the tissue, but also synthesis and decomposition. As soon as the original levels of GABA and Glu in the tissue have been restored, there will be an equilibrium between synthesis and decomposition on the one hand and between the resorption and the release of the amino acids, on the other. The latter equilibrium exists also for potassium. Krebs et al. [112] demonstrated with the aid of K^{42}, that 5% is exchanged per minute.

Most of our compounds inhibit oxidative metabolism. The result will be a decreased synthesis of ATP and other energy-rich

compounds. Consequently, less energy is available for the resorption of the amino acids. Whether resorption occurs and the degree to which it takes place will be dependent on the concentration of the drug and the inhibition of oxygen uptake. Thus, for orphenadrine and chloropromazine Ernsting et al.[101] found that up to a concentration of 5×10^{-4} and 1.7×10^{-4} mole, respectively, the same resorption of GABA and Glu occurs as in the control experiments. At these concentrations the inhibition of oxygen uptake amounts to 10% and 30% respectively. It is only at higher concentrations that more GABA and Glu are present in the medium than in the control. In the case of orphenadrine the quantity in the medium no longer increases when oxygen inhibition increases from 35 to 83%. Obviously, a limit has been attained here. The concentrations of GABA and Glu, however, are higher than after dissection of the cortex. This was also found by us (see tables VIII and IX). The obvious explanation is that in this case the synthesized amino acids are prevented from decomposing because they pass into the medium. In this case an equilibrium is attained at which practically all the GABA and Glu are present in the medium, hardly any being left in the tissue.

Indeed, for several of our compounds (XXII, XXIX, XXX, XXXIII and XXXIV) we find, after one hour's metabolizing, more GABA and Glu in the medium than after dissection in the cortex. As with these compounds the inhibition of oxygen uptake increases with the time, the equilibrium conditions, at which the optimum quantity of amino acids is present in the medium, need not yet have been reached after one hour's metabolism. In order to obtain information on this score, we would have to analyse the medium and the tissue for amino acids after, say, 20, 40, 60 and 120 minutes.

It is noteworthy that there exists a certain relation between the structure of our compounds and the amount of amino acids present in the incubation medium after the metabolism. As was already remarked above, more amino acids are present in the medium according as the alkyl substituents become larger and their number increases.

Apparently, the quantity found is dependent not so much on the position of the alkyl groups, but rather on their number (cf. for example XIX with XXIII and XXIV, and XXVIII with XXV, XXVI, XXVII, XXXI and XXXII) and on the size (cf. XIX with XX, XXI and XXII, and XXVIII with XXIX and XXX).

Although there is no clear relation with oxygen inhibition, we do find that the compounds under whose influence the largest amount of amino acids is present in the medium are among those with which the inhibition is greatest during the last 20 minutes of metabolism (33—88%). Compounds XXXIII forms an exception.

If Glu is not resorbed, the same applies to potassium [110]. According to Kini and Quastel [112], a lowered potassium level in the tissue will result in the synthesis of acetyl co-enzyme A from pyruvic acid and co-enzyme A being inhibited. This in turn results in a reduced synthesis of citric acid from oxalacetic acid and co-enzyme A. What was found by De Waart [6] for orphenadrine and other alkylsubstituted benzhydryl ethers is in agreement with the above.

Before the beginning of metabolism and also during metabolism, no potassium being resorbed (and no sodium being given off) by the cell, the cell membrane will be in a state of depolarization. If the supply of energy does not get going, this state will be maintained. The possibility that our compounds have a depolarizing influence cannot a priori be ruled out. A corresponding decrease in permeability might result in oxidative metabolism being inhibited. On the other hand, it is equally well possible that our compounds primarily interfere with oxidative metabolism and produce a state of depolarization as a secondary effect.

The overall picture we obtain is complex. The energy supply may be interfered with, for instance, by oxidative phosphorylation being uncoupled. As our compounds give rise to an inhibition of oxygen uptake, it is conceivable that they block the citric acid cycle. This hypothesis can be checked by looking at the isolated enzyme systems. As in particular the high concentration of Glu under the influence of some of the compounds tested is a salient point, it seemed desirable to ascertain whether the enzyme systems determining the Glu level are affected. These include various transaminases, glutaminase, L-glutamic acid dehydrogenase (GSDH) and L-glutamic acid decarboxylase (GAD).

We have examined the influence of our compounds on GAD activity with a bacteria enzyme of the strain Escherichia Coli*), and with homogenized rat cortex.

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*) This enzyme was obtained from the Nutricial Biochemical Corporation.

As a measure of the activity of the bacterial enzyme we chose CO_2 production. This was determined in the Warburg apparatus. We worked at the optimum pH, which was found to lie between 4.6 and 4.7. The incubation medium was 0.1 mole of acetate buffer.

The Warburg vessel contained 0.64 mg of GAD in 0.5 ml of buffer, 200 γ of pyridoxal phosphate in 0.2 ml of buffer and 0.5 ml of buffer with or without the compound under test in a final concentration of 10^{-3} mole and the side arm contained 0.1 mole of Glu in 0.5 ml of buffer, all previously adjusted at a pH of 4.7. As with the bacterial enzyme the co-enzyme pyridoxal phosphate is very strongly bound [113] and CO_2 production is almost equally large without a surplus, it may also be omitted. Although we are aware of the fact that in this case CO_2 and GABA production do not increase linearly with the time and consequently the percentage of inhibition found is dependent on the time at which the reaction is stopped, yet it is possible to compare a number of compounds with each other as long as all these compounds are tested for the same period of time. The reaction was carried out under nitrogen and was stopped after 20 minutes by the addition of 0.3 ml of concentrated sulphuric acid, which was present in another side arm. The reaction temperature was 37 °C. None of the compounds examined by us (XVIII—XXXV incl.) produced a significant change in enzyme activity. Only with hydroxylamine, which was also tested, did we find inhibitions of 97% and 90% at final concentrations of 10^{-3} and 10^{-4} mole, respectively. The effect could be reversed by the addition of pyridoxal phosphate in equimolar quantities. [114]

The method for the determination of the GAD activity of homogenized tissue, described by Roberts and Frankel [115], was suitable for examining the influence of our compound on the GAD from rat cortex. If a reasonable CO_2 production is to be measured, about 500 mg of homogenate is required per flask. In order to reduce this quantity we did not determine the CO_2 production but the quantity of GABA formed, using the chromatographic method described in chapter II.

In each test a flask contained 1 ml of homogenate in buffer (100 mg of cortex per ml) and 1 ml of buffer with or without the compound under test in a final concentration of 2×10^{-3} mole. The side arm contained 0.1 mole of Glu in 0.5 ml of buffer. The

potassium phosphate buffer had a pH of 6.2. All these media were previously adjusted at a pH of 6.2, except the Glu (pH = 6.8). Thus the reaction was carried out at pH = 6.4—6.5. After 15 minutes' equilibration the reaction was allowed to take place under the same conditions as with the bacterial enzyme, i.e. without the addition of pyridoxal phosphate. This was done in order to approximate as closely as possible the conditions prevailing during the metabolism of rat cortex, another reason being that the inhibition of the GAD could be observed more readily. In the case of competitive inhibition with pyridoxal phosphate, the effect should be reversible with an excess of pyridoxal phosphate. The same applies, for instance, to a strong inhibitor like hydroxylamine [17, 18].

After 40 minutes the cocks were opened and the reaction was stopped. This was done by placing the manometers with the flasks on the associated stand, to which an adjustable tank of boiling water had been connected in such a way that the flasks came to rest in the water. The quantity of GABA produced was determined chromatographically, a correction being applied for the GABA already present at the beginning of the reaction. In these experiments, just as in those with the bacterial enzyme, we observed no influence of our compounds on enzyme activity. Hydroxylamine at a concentration of 10^{-3} mole caused 100% inhibition. As the experiments with GSDH are still in progress and the other enzyme systems determining the level of Glu have not been examined, there is no point in speculations about their behaviour.

Summary.

1. All our compounds more or less inhibit oxidative metabolism. At the same time energy production is reduced.
2. As a result, the resorption of GABA and Glu is either prevented or decreased, the concentration of these amino acids being higher in the incubation medium than in the reference. The total amount of GABA and Glu may even be higher than after dissection.
3. The increase becomes larger with the number and the size of the alkyl substituents and need not necessarily be due to an increased synthesis of GABA and Glu.
4. None of our compounds affects the activity of GAD.

SUMMARY

Starting from alkyl-substituted phenyl(2-pyridyl)carbinols, a series of β-dimethylaminoethyl aryl(2-pyridyl)carbinol ethers has been synthesized. The influence of these compounds on the metabolism of rat cortex was examined in vitro. Attention was paid to the influence on oxygen consumption and the changes in the amino acid pattern, especially of GABA and Glu.

The amino acids were determined by ascending one-dimensional paper chromatography. The relation between the concentration (c) of the amino acid and the area (A) of the curve obtained after measurement of the coloured chromatogram in a strip colorimeter can be represented by the equation $\log A = a \log c + b$.

Several factors that may govern the relation obtained have been examined. The structure of the paper undoubtedly plays an important part.

Our compounds in general inhibit oxidative metabolism. Besides, it was observed that shifts occur in the amino acid pattern: after one hour's metabolism a considerable proportion of the amino acids may be present in the incubation medium, while the total amount of amino acids may be considerably larger than in the blank. The effect of our compounds on shifts in the amino acids is greater according as the alkyl substituents become larger and their number increases. These phenomena may be explained on the basis of the assumption that the supply of energy is interfered with.

It was hoped that the U.V. spectra of our compounds would provide sufficient information on spatial arrangement, which could then be correlated with the effects observed. This appeared not to be the case.

Some of our experimental results were analysed by the Statistics Department of the Mathematical Centre, Amsterdam.

SAMENVATTING

Uitgaande van alkylgesubstitueerde phenyl(2-pyridyl)carbinolen werd een serie β-dimethylaminoaethyl aryl(2-pyridyl)carbinol aethers gesynthetiseerd. De invloed van deze verbindingen op de stofwisseling van rattencortex werd in vitro onderzocht. Nagegaan werd de invloed op het zuurstofverbruik en de veranderingen in het aminozuurpatroon, speciaal van GABA en Glu.

Voor de bepaling van de aminozuren werd gebruik gemaakt van stijgende, ééndimensionale papierchromatografie. Het verband tussen de concentratie (c) van het aminozuur en het oppervlak (O) van de curve, die verkregen werd na doormeten van het gekleurde chromatogram in een strip-colorimeter kan weergegeven worden door de vergelijking $\log O = a \log c + b$.

Verschillende factoren, die bepalend kunnen zijn voor de gevonden relatie, werden onderzocht. Ongetwijfeld speelt de structuur van het papier een belangrijke rol.

Onze verbindingen remmen in het algemeen het oxydatieve metabolisme. Bovendien werd gevonden, dat er verschuivingen optreden in het aminozuurpatroon in die zin, dat een belangrijk deel van de aminozuren zich na één uur stofwisselen in het incubatiemedium kan bevinden, terwijl het totaal aan aminozuren belangrijk hoger kan zijn dan bij de contrôle. Het effect van onze verbindingen op de aminozuurverschuivingen is des te groter, naarmate de alkylsubstituenten groter worden en het aantal toeneemt. Een verklaring van het geheel is mogelijk, op grond van de veronderstelling, dat de energievoorziening gestoord is.

Gehoopt werd, dat de U.V.-spectra van onze verbindingen voldoende informatie zouden geven over de ruimtelijke bouw, zodat wij deze zouden kunnen correleren met de gevonden effecten. Dit bleek niet het geval te zijn.

Enkele van onze waarnemingen werden geanalyseerd door de Statistische Afdeling van het Mathematisch Centrum te Amsterdam.

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ACKNOWLEDGEMENT

My sincere thanks are due to —

Professor Dr H. K. Oosterhuis for the many instructive discussions and for his confidence,

the late Ir A. R. Bloemena of the Mathematical Centre, Amsterdam, for his disinterested contribution to this thesis in the form of a statistical analysis of part of the observations,
the Ministry of Education for the financial grant added,
the Management of the N.V. Koninklijke Pharmaceutische Fabrieken, v/h Brocades-Stheeman & Pharmacia, Amsterdam, for the opportunity I was given to get this thesis ready for the press,
my parents, for the sacrifices they made to enable me to study, and my wife for her lasting support,
Drs R. Ph van Hoeven, for the many times I could call on his knowledge of biochemistry,
Mr C. Steendijk, for his share in the biochemical experiments,
Mr A. Pomper, for drawing the figures.

Finally, I wish to thank all who in some way or another, showed their interest or lent their assistence in the realization of this thesis.

LEVENSLOOP VAN DE SCHRIJVER

De auteur van dit proefschrift werd op 23 november 1927 te Wons (Fr.) geboren. Na verloop van tijd volgde hij het L.O., M.U.L.O. en M.O., het laatste aan de Christelijke H.B.S. (Afd. B) te Leeuwarden.

In 1946 werd hij als student ingeschreven aan de Vrije Universiteit te Amsterdam en begon de voorbereiding op het candidaatsexamen e in de faculteit der Wis- en Natuurkunde. Na het candidaatsexamen in maart 1952 volgde een onderbreking van twee jaren voor het vervullen van de militaire dienstplicht. In april 1954 werd de studie hervat. Als hoofdvak werd gekozen organische chemie uitgebreid en als bijvak farmacologie. Voordat het doctoraalexamen in juli 1958 werd afgelegd, werd reeds begonnen met het bewerken van een proefschrift.

Mede als gevolg van het gekozen promotieonderwerp richtte zijn belangstelling zich steeds meer op de biochemie. In deze sector aanvaardde hij per 1 juli 1960 een functie bij de N.V. Koninklijke Pharmaceutische Fabrieken, v/h Brocades-Stheeman en Pharmacia te Amsterdam.

APPENDIX

Statistical analysis of observations on γ-aminobutyric acid and L-glutamic acid

BY

A. R. BLOEMENA

A chromatographic determination of γ-aminobutyric acid (GABA) or L-glutamic acid (Glu) results in an observation of an area. This observation has to be referred to a calibration curve in order to obtain the observed value for GABA or Glu, respectively. The problem to be dealt with is to establish these calibration curves.

Statistical evaluation of the accuracy of a method of analysis becomes very much simpler if the calibration curve happens to be a straight line. Plotting a set of areas measured at known concentrations of GABA and Glu, respectively, against the concentrations, using linear scales in both directions, showed that the points clustered fairly closely around a line that was definitely not a straight one. Moreover the spread of the points along the line increased with the concentration. However, when the logarithms of the measured areas were plotted against the logarithms of the concentrations, the points clustered around a straight line, while an increase in spread along the line was no longer observed. At this point it was decided to carry out a small experiment to verify these indications and to procure the necessary data for the calculation of the calibration lines. On each of 5 days 4 observations at each of 6 levels of the concentrations (viz. 1.25; 2.5; 5.0; 7.5; 10 and 20 γ) of GABA and Glu were made. These observations are recorded in Table 1; their logarithms (to the base 10) are plotted in figure 1 for GABA, in figure 2 for Glu. The logarithms of the observations will be called "measurements".

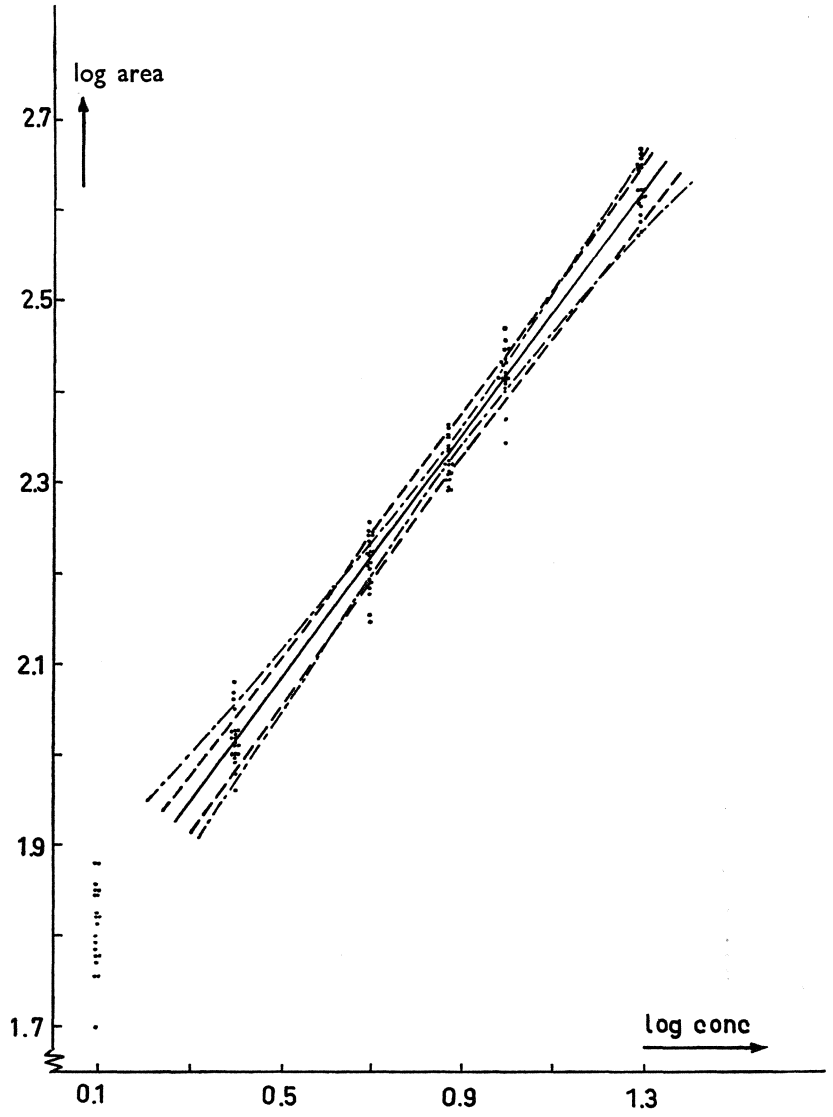
From each of the thirty sets of four measurements an estimate of the variance has been computed. For a given level of the concentrations the five estimates thus obtained were pooled to give the results of Table II.

Report S 277 of the Statistics Department of the Mathematical Centre, Amsterdam.

TABLE I

Observations

Date	1.25 γ		2.5 γ		5 γ		7.5 γ		10 γ		20 γ	
	GABA	Glu	GABA	Glu	GABA	Glu	GABA	Glu	GABA	Glu	GABA	Glu
6-10-'59	61	71	104	108	165	187	200	225	233	273	392	430
	71	63	115	112	174	185	195	220	263	269	400	440
	60	61	106	95	171	165	215	220	259	247	441	453
	50	53	117	112	156	162	217	207	259	263	410	403
7-10-'59	76	74	112	113	152	155	228	228	278	242	416	442
	63	68	99	102	167	167	210	217	259	253	417	472
	70	76	120	103	160	172	196	209	279	273	443	466
	59	55	103	102	162	169	204	224	270	270	403	402
26-1-'60	70	65	106	105	142	142	195	203	257	274	462	433
	57	57	95	98	170	173	208	216	270	261	453	405
	66	62	91	106	166	166	224	210	260	253	410	412
	76	67	104	101	176	161	208	231	268	257	442	418
1-2-'60	67	65	98	93	180	172	218	210	273	286	406	394
	60	50	100	100	174	178	223	222	286	251	374	414
	71	66	102	80	167	168	230	222	219	258	439	414
	72	69	100	111	161	180	216	210	255	276	445	420
2-2-'60	62	62	106	107	175	181	203	211	250	262	372	458
	57	60	105	108	155	165	208	207	252	252	385	405
	66	51	100	95	150	162	199	227	294	263	416	402
	65	68	102	102	140	155	202	205	259	257	459	445

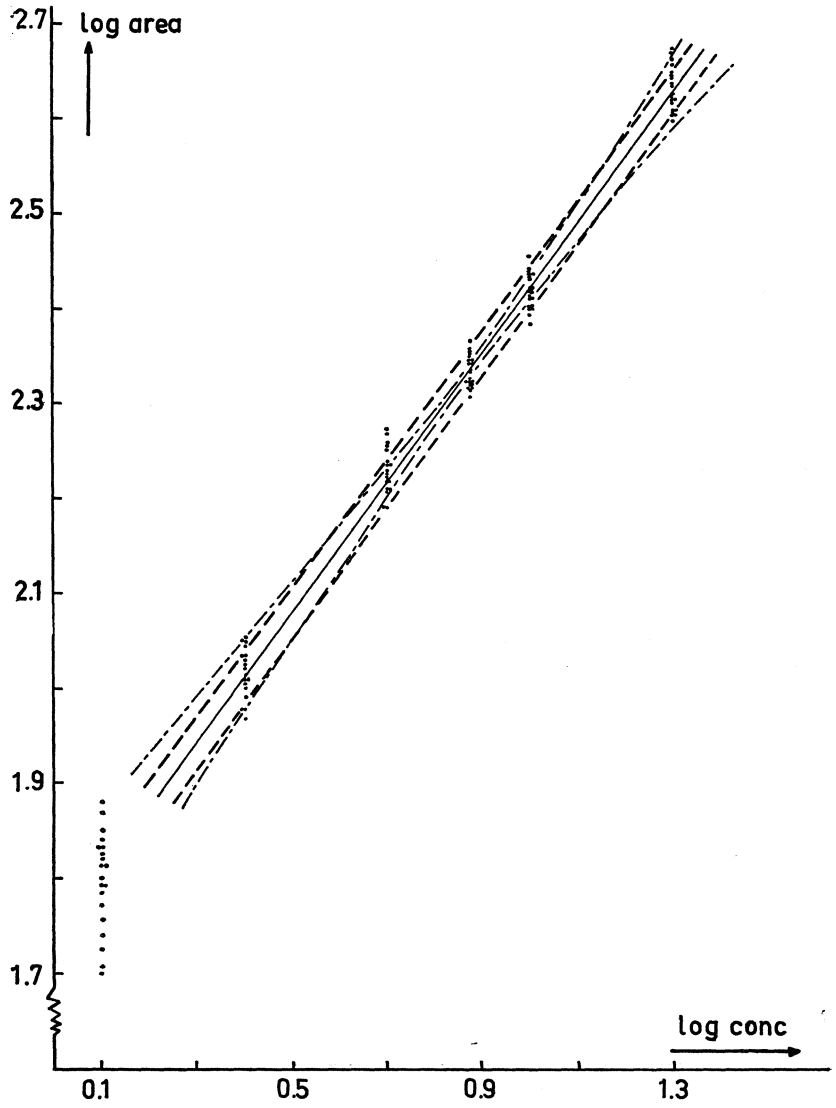


GABA Fig. 1

——————— regression line;

— · — · — · — · limits of the 90% confidence belt for the true line;

— — — — — — limits of the 90% tolerance interval for 90% of the population, to be used for the mean of four replicate measurements.



Glu Fig. 2

——————— regression line;

— · — · — · — · limits of the 90% confidence belt for the true line;

— — — — — — limits of the 90% tolerance interval for 90% of the population, to be used for the mean of four replicate measurements.

TABLE II

Estimates of the variance between replicate-measurements

Multiply entries by 10^{-4}

level of concentration	1.25 γ	2.5 γ	5 γ	7.5 γ	10 γ	20 γ
GABA	19	5	8	3	8	7
Glu	24	10	6	3	3	4

The hypothesis that the variances are the same for all concentrations was tested by means of BARTLETT's test (cf. A. HALD (1952), 291), assuming that the distributions involved are normal, and was rejected (P = 0.007 and 7 . 10^{-5}). Upon inspection of the figures given in table II it is fairly obvious that this rejection is due to the greater variance at the low levels of the concentration. In the following part of this report the observations at levels $\geqslant 2.5\ \gamma$ have been used only, assuming that the variance for these levels is independent of the concentration itself.

Let $\underline{y}_{ijk}$ be the k-th measurement on concentration level x_j on the i-day (k = 1, . ., 5, j = 1, . ., 5, i = 1, . . ., 5). The examination of the properties of the regression line started from the model

$$\underline{y}_{ijk} = \alpha_i + \beta_i x_j + \underline{\varepsilon}_{ijk},$$

where $\underline{\varepsilon}_{ijk}$ are the error components, α_i and β_i are unknown constants which are still allowed to depend on the day on which the measurements are made. $\underline{\varepsilon}_{ijk}$ will be assumed to have variance σ^2, independent of i, j and k, and all ε's are assumed to be independent observations of normal distributions.

First the hypothesis that the regression lines $\alpha_i + \beta_i x_j$ are linear was examined. The test for this hypothesis is a slight generalization of the one described in e.g. F. S. ACTON (1959), chapter 3. The result of this test (P $\approx$ 0.30 and 0.30) indicates that there is no reason to reject the hypothesis of straight lines. Next — assuming the lines to be straight — the usual test from the analysis of covariance, cf. F. S. ACTON (1959), page 77, was applied to test the hypothesis

$$\alpha_1 = \cdots = \alpha_5 \quad \text{and} \quad \beta_1 = \cdots = \beta_5,$$

which means that only one line is involved. Again the result of the test ($P \approx 0.10$ and > 0.50) forms no reason to reject this hypothesis. It appears reasonable from these considerations based on an evaluation of a fairly large amount of data to assume as a working hypothesis the existence of *one* straight regression line. The best estimate of the line for GABA and Glu is

GABA: $^{10}\log \text{area} = 1.745 + 0.671 \cdot {}^{10}\log \text{concentration}$

Glu: $^{10}\log \text{area} = 1.741 + 0.681 \cdot {}^{10}\log \text{concentration}.$

These lines, which are drawn in figures 1 and 2 are *estimates* of the true line. Statistical theory gives also a mean to determine a confidence belt within which the true line will lie with a given level of confidence (cf. F. S. ACTON (1959), 41*). The belt indicated in figure 1 and 2 by its limits is the one for confidence level 0.90.

A different question is to outline a belt within which, say, 90% of all future measurements will lie. Basing results on a *sample* of measurements this question cannot be answered in this strict form. What can be done is to outline a belt within which 90% of all future observations will lie with a given confidence level, say, 0.90, cf. F. S. ACTON (1959), 49. This confidence level thus refers to the fact that owing to sampling errors in the measurements on which this so-called tolerance interval is to be based, the true percentage of future observations included may not be 90%, but lower. The limits of this tolerance interval, calculated for GABA are drawn in figure 3 in heavy lines. The practical use of this interval is as follows. Every time a GABA analysis is made one obtains a measurement. Plot this measurement along the vertical scale, draw through this point a horizontal line. Record the value c_1 and c_2 on the horizontal scale at which the horizontal line intersects the limits of the intervals. Compute $\gamma_1 =$ antilog c_1 and $\gamma_2 =$ antilog c_2. The statement that the true, unknown concentrations lies between γ_1 and γ_2 will on the average be wrong in one out of ten times this procedure is followed. The "one out of ten" part of this will be on the "safe" side with 0.90 confidence.

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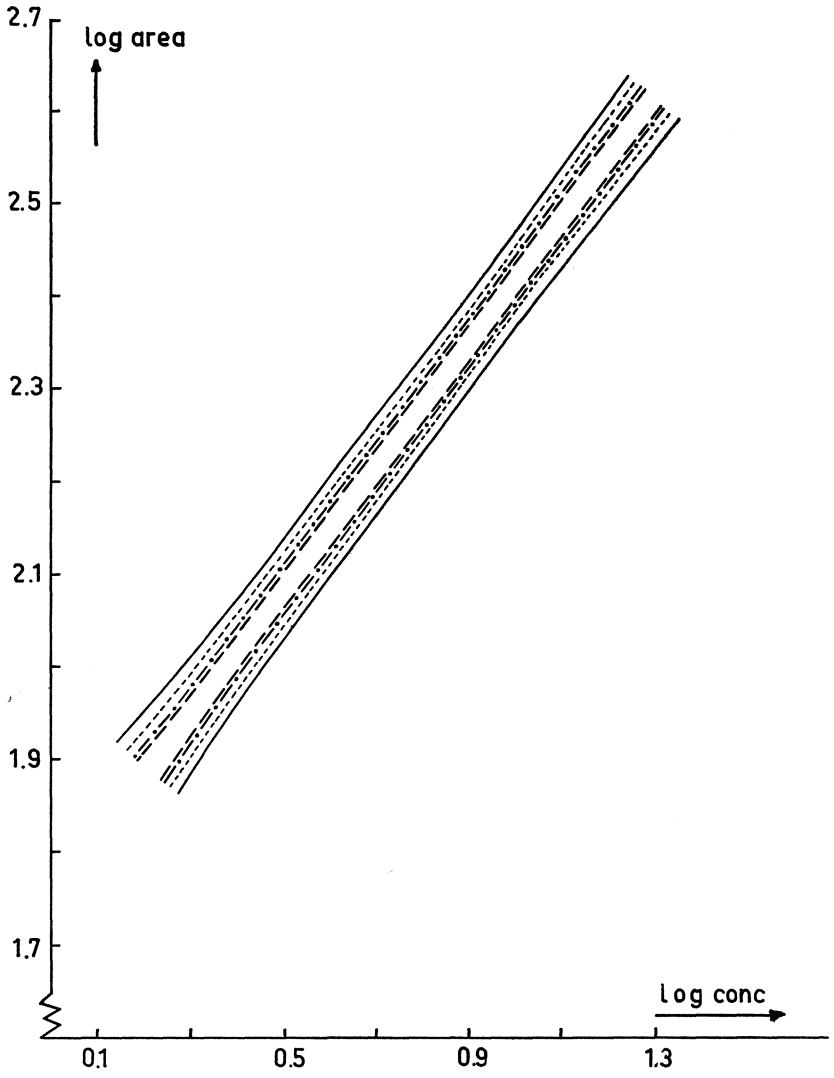
* ACTON's formulae on page 41 are both in error. In both cases the denominator should be multiplied by 2.



GABA Fig. 3

90% tolerance intervals for 90% of the population for use with:

one measurement ————————

the mean of two measurements - - - - - - - - - - - -

,, ,, ,, four ,, · — · — · — · —

,, ,, ,, six ,, — — — — — —

One might wonder whether more than one observation on every unknown concentration increases the accuracy of the statements obtainable with this calibration procedure. In figure 3 therefore four 90% tolerance intervals have been drawn, all with the same level of confidence, but to be used, respectively, for

1 measurement on an unknown concentration,
the mean of 2 measurements* on an unknown concentration,
the mean of 4 ,, ,, ,, ,, ,, ,
the mean of 6 ,, ,, ,, ,, ,, .

As is clear from figure 3, the determination based on the mean of four measurements per unknown concentration gives accurate results which cannot be improved upon to any marked extent without increasing the number of observations substantially.

The tolerance intervals to be used for means of four replicate measurements are drawn in figures 1 and 2.

The GABA- and Glu-analyses have been carried out on the liquids obtained from sliced brain tissue. The brain tissue was suspected of being heterogeneous. In taking a small sample of this tissue and analysing its liquids one therefore introduces a sampling error. A small experiment was carried out to establish the influence of this sampling error on the final result. On each of four days four parts of the sliced brain tissue were taken and each part was analysed for Glu, which had leaked from the tissue, four times. Each of the four days a different batch of the tissue was used. The observations are recorded in table III.

Let $\underline{y}_{ijk}$ be the k-th measurement (= logarithm of the observation) on the j-th part on day i, *having a weight*. Let the logarithm of the weight of the j-th part on day i be x_{ij}, then the analysis proceeded from the model

$$\underline{y}_{ijk} = \mu + \beta x_{ij} + \underline{\varepsilon}_i + \underline{\varepsilon}_{ij} + \underline{\varepsilon}_{ijk},$$

where

μ is a grand average.
βx_{ij} is a component introduced to take account of the varying weight of the parts of the tissue on each day.

* In the terminology used the arithmetic mean of two measurements is the geometric mean of two observations.

$\underline{\varepsilon}_i$ is the contribution to the measurement on the i-th day not accounted for by μ and βx_{ij}. In view of the procedure followed $\underline{\varepsilon}_i$ has been assumed to be a random variable.

$\underline{\varepsilon}_{ij}$ is the contribution to the measurements of the j part on the i-th day not accounted for by μ, βx_{ij} and $\underline{\varepsilon}_i$. Large values (in the absolute sense) of $\underline{\varepsilon}_{ij}$ point to heterogeneity of the sliced tissue.

$\underline{\varepsilon}_{ijk}$ is the contribution to the measurement y_{ijk} not accounted for by μ, βx_{ij}, $\underline{\varepsilon}_i$, $\underline{\varepsilon}_{ij}$.
This $\underline{\varepsilon}_{ijk}$ is the component connected with the accuracy of the Glu-measurement.

The usual analysis of variance assumptions has been used, viz. that all ε's are distributed independently with means zero and variances

$$\left.\begin{array}{lll} \text{var } \underline{\varepsilon}_i = \sigma_d^2 & (\textit{d}\text{ays}) & \\ \text{var } \underline{\varepsilon}_{ij} = \sigma_h^2 & (\textit{h}\text{eterogeneity}) & \\ \text{var } \underline{\varepsilon}_{ijk} = \sigma_s^2 & (\textit{s}\text{trips}) & \end{array}\right\} \text{for all i, j, k.}$$

As we are concerned with estimating these variances rather than testing hypothesis about them, we need not assume normal distributions.

TABLE III
Observations (Glu)

	weight (mg)	observations (mm^2)			
1st day	98,0	115,	115,	140,	131
	99,0	130,	117,	135,	135
	96,5	126,	114,	126,	150
	104,0	132,	156,	120,	133
2nd day	96,5	130,	132,	123,	121
	103,0	125,	131,	143,	142
	97,0	127,	123,	123,	135
	103,0	117,	123,	123,	132
3rd day	98,0	130,	140,	120,	133
	96,5	140,	133,	147,	136
	97,0	123,	123,	125,	114
	96,5	154,	147,	138,	125
4th day	103,0	127,	132,	127,	143
	98,0	121,	138,	130,	128
	104,5	138,	150,	138,	144
	98,5	144,	121,	143,	110

Details about the estimation procedure are omitted; they can be found in any handbook on analysis of variance. We only record the result, adding between brackets the corresponding results if not the transformed, but the original data are analysed.

The estimates s_d^2, s_h^2 and s_s^2 of σ_d^2, σ_h^2, σ_s^2 are

$$s_d^2 = 0.16 \times 10^{-5} \ (0.40),$$
$$s_h^2 = 12.6 \times 10^{-5} \ (12.0),$$
$$s_s^2 = 106 \times 10^{-5} \ (97.6).$$

In any case σ_s^2 dominates heavily, while σ_d^2 is extremely small. To see what consequences these variances have we consider two cases. If we follow the procedure of taking four samples of the brain tissue and analysing each sample once, the estimated standard deviation of the mean of four measurements is

$$\sqrt{\frac{12.6 + 106}{4} \times 10^{-5}} = 0.017 \ (5.2)$$

while if we take one sample of the brain tissue and analyses this sample four times this estimated standard deviation is

$$\sqrt{\left(12.6 + \frac{106}{4}\right) \times 10^{-5}} = 0.020 \ (6.0)$$

which is slightly higher.

References

F. S. Acton (1959): Analysis of straight line data, Wiley, New York.
A. Hald (1952): Probability theory and its engineering applications, Wiley, New York.

STELLINGEN

I.

De veronderstelling van WISS et al., dat het door hen bepaalde U.V. spectrum van het primaire oxydatieproduct van 3-hydroxyanthranilzuur alleen aan dit product is toe te schrijven, is onvoldoende gemotiveerd.

O. WISS, H. SIMMER, H. PETERS, Hoppe-Seyler's Z. physiol. Chem. **304** 221 (1956).
O. WISS, G. BELLINGDORF, Hoppe-Seyler's Z. physiol. Chem. **306** 145 (1957).

II.

GULBIN et al. concluderen uit de ontledingssnelheid van cyclische carbonaten onder invloed van basische katalysatoren, tot de optimale reactieomstandigheden voor de vorming van 2-oxazolidonen uit deze cyclische carbonaten en gesubstitueerde isocyanaten.

Mede in het licht van de onderzoekingen van TSUZUKI et al. kan deze conclusie niet verantwoord worden geacht.

K. GULBINS, G. BENZING, R. MAYSENHÖLDER, K. HAMANN, Ber. **93** 1975 (1960).
R. TSUZUKI, K. ICHIKAWA, M. KASE, J. Org. Chem. **25** 1009 (1960).

III.

De veronderstelling van HIROTA et al., dat de toestand van het aan het oppervlak van SiO_2 en Al_2O_3 geadsorbeerde mierenzuur bij de door deze oxyden gekatalyseerde ontleding verschilt, is aanvechtbaar, omdat de structuur van de geadsorbeerde toestand bij kamertemperatuur werd bepaald, terwijl de ontleding plaats vindt bij ca. 300 °C.

K. HIROTA, F. FUEKI, K. SHINDO, Y. NAKAI, Bull. Chem. Soc. Japan **32** 1261 (1959).

IV.

Bij de papierchromatische bepaling van aequivalente hoeveelheden van verschillende aminozuren wordt na reactie met ninhydrine niet in alle gevallen dezelfde kleuropbrengst verkregen. Dit is slechts gedeeltelijk te verklaren uit het verschil in structuur der aminozuren.

Dit proefschrift, hoofdstuk II.
W. GRASSMANN, K. HANNIG, M. PLÖCKL, Hoppe-Seyler's Z. physiol. Chem. **299** 258 (1955).

V.

Het berekenen van gecorrigeerde ijkcurven voor aminozuren, zoals dit is gedaan door HERRMANN en RATHS, is overbodig en bovendien minder ,,ideaal'' dan deze onderzoekers veronderstellen.

J. HERRMANN, J. RATHS, Pharmazie **11** 582 (1956).

VI.

De door BOSE en VYAYVARGIYA gegeven verklaring voor de verlaging van het serotoninegehalte in de hersenen van met reserpine behandelde ratten, is aanvechtbaar.

B. C. BOSE, R. VYAYVARGIYA, Arch. intern. pharmacodynamie **127** 27 (1960).

VII.

De door SCHNITGER en GROSS ontworpen coagulometer is niet geschikt voor een nauwkeurige bepaling van de bloedstollingstijd.

H. SCHNITGER, R. GROSS, Klin. Wochschr. **32** 1011 (1954).

VIII.

Bij het onderwijs in de analytische chemie zou het aanbeveling verdienen, om de fundamentele begrippen, die betrekking hebben op de titratie van zuren, basen en amphoteren te behandelen op basis van pH-fractie curven*), daar op deze wijze de genoemde typen beter vanuit één gezichtspunt kunnen worden bekeken dan met de gangbare titratiecurven.

*) H. NETTER, Theoretische Biochemie, pag. 165, 181; Springer Verlag, Berlin, Göttingen, Heidelberg, (1960).
P. HÖSLI, Arzneimittel Forsch. **10** 66 (1960).

IX.

De wijze, waarop KINI en QUASTEL de veranderingen interpreteren, die optreden in het aminozuurpatroon van in coupes gesneden schors van rattehersenen na stofwisseling in een medium, dat 100 in plaats van 5 mM KCl per liter bevat, is niet verantwoord.

M. M. KINI, J. H. QUASTEL, Nature **184** 252 (1959).

X.

De hypothese van DI SABATO, dat 2,4-dinitrophenol de zwelling van mitochondrieën tegengaat, doordat het zich bindt aan de eiwitten van deze celbestanddelen, is niet te handhaven.

G. DI SABATO, Exp. Cell Research **16** 441 (1959).

XI.

Het in de organisch chemische litteratuur algemeen gebruikelijke vermelden van de destillatietemperatuur bij een bepaalde druk als „kookpunt bij een bepaalde druk" is voor identificatie- en vergelijkingsdoeleinden onvoldoende en dient vervangen te worden door het vermelden van een kookpunt, dat in een gestandaardiseerd kookpuntapparaat is bepaald.

XII.

De bewering van BROWN, dat "The American pharmaceutical industry is second to none in the world in both the quality and quantity of its research results . . .", geeft een geflatteerd beeld van de bestaande situatie, daar het grootste deel van het fundamentele onderzoek ten behoeve van de ontwikkeling van nieuwe geneesmiddelen plaats vindt buiten de Verenigde Staten.

H. C. BROWN, Chem. Eng. News van 4 juli 1960, pag. 42.