Biochemical Pharmacology, Vol. 25, pp. 625–630. Pergamon Press 1976. Printed in Great Britain.

CHANGES IN ACTIVITY OF RAT BRUSH BORDER ENZYMES INCUBATED WITH A HOMOLOGOUS SERIES OF ALIPHATIC ALCOHOLS

SALVADOR MITJAVILA, CHRISTIANE LACOMBE and GEORGES CARRERA

Groupe de recherches sur la toxicologie des aliments et des Boissons—INSERM U-87
2, rue François Magendie 31400 Toulouse, France

(*Received* 10 *June* 1975: *Accepted* 17 *September* 1975)

Abstract—The effects of some aliphatic alcohols on the activity of several brush border enzymes have been investigated. At the concentrations used we found strong inhibition of $(Na^+ + K^+)$-APTase, and Mg^{2+}-APTase while alkaline phosphatase and arylamidase activity was little influenced. The half-maximal ATPase inhibitory concentrations of alcohols were closely correlated with the length of the carbon chain and the interaction with the enzyme appeared to be hydrophobic. The change in free energy due to addition of one CH_2 group is estimated at -620 cal for $(Na^+ + K^+)$-ATPase and -730 cal for Mg^{2+}-ATPase. The relationship between intestinal pharmacological activity of the alcohols and active transport is discussed.

Because of their characteristic physicochemical properties, aliphatic alcohols are especially active on biochemical mechanisms localized in the lipoprotein structures in membranes and on hydrophobic sites of enzymes. Because of this, the pharmacological effects observed with these alcohols are not specific and vary with the preparation used [1, 2, 3, 4, 5].

Aside from the interest in the effects of these alcohols on specific membrane systems, studies on the digestive epithelium are important from a toxicological point of view because following voluntary or involuntary ingestion, their effects are seen primarily on the membranous structures involved in metabolite absorption.

The natural presence of some of these alcohols in fermented drinks poses a problem in alimentary toxicology, the consequences of which are as yet little known [6]. In addition, little work has been done on the effects of aliphatic alcohols on the digestive epithelium. Hill [7] studied the effects of these alcohols on the absorption of various trace elements in isolated segments of chicken duodenum. Many studies are limited to ethanol; the effect on intestinal transport mechanisms are reviewed in 'the biology of alcoholism' [8, 9, 10]. Tran *et al.* [11], using isolated enterocytes, showed that ethanol inhibits metabolic activity, and Carrera *et al.* [12] demonstrated a diminution in vitamin B_{12} absorption.

Enzymes in the brush border of enterocytes play a very important role in hydrolysis and the transport of metabolites from the intestinal lumen to the blood.

In addition, the ATPases of the brush border have been implicated in the maintenance of ionic equilibrium and in active absorption. Israel and Kalant [13] have shown that at concentrations considered toxic for the animal, ethanol inhibits active Na transport in frog skin. More recently, Israel *et al.* [14] found an inhibition of ATPases localized in microsomes of nerve cells by short chain alcohols.

In this paper, we report experiments on the activity of several enzymes in the brush border of enterocytes incubated in the presence of aliphatic alcohols.

METHODS

Brush borders were prepared from the small intestines of male Sprague–Dawley rats weighing 250 g. Activities of various enzymes present in the preparations as well as the effects of aliphatic alcohols were measured.

Isolation of brush borders. The method used was that developed by Mitjavila *et al.* [15]. Starting with a homogenate of isolated rat intestinal cells, and after several centrifugations, the subcellular fraction is treated with glass wool to expel most of the DNA. In these conditions a fraction is obtained consisting essentially of brush borders in EDTA–Tris buffer at pH 7·4. The volume was adjusted to obtain a final protein concentration of approximately 1 mg/ml. Proteins were measured by the method of Lowry *et al.* [16].

Determination of enzymatic activities. $(Na^+ + K^+)$-ATPase, Mg^{2+}-ATPase, alkaline phosphatase and arylamidase activities were measured. All incubations were done at 37°. The incubation medium contained 0·5 ml of buffer and 0·1 ml of enzyme (brush border preparation) in a final volume of 1 ml. The effects of eight *n*-alcohols, from methanol to octanol, (purchased from Fluka and redistilled) were measured. Each alcohol was tested at three different concentrations. Addition of substrate marked the beginning of the 15 min incubation period. Control enzymatic activity was measured in the same conditions, the alcohol being replaced by an equivalent volume of water. The details for each enzyme assay are given in the tables. The results are expressed as specific activity (μmoles hydrolysed substrate/mg protein/15 min) and as per cent of the control activity.

Table 1. Effect of alcohols on ATPases activities in the brush border of rat enterocytes

Alcohol	M	Mg^{2+}-ATPase activity Sp. act.	%	$(Na^+ + K^+)$-ATPase activity Sp. act.	%
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Methanol	1	5·80 ± 1·17	94	2·00 ± 0·20†	69
	2	5·20 ± 1·13	84	0·54 ± 0·14‡	19
	4	2·30 ± 0·47*	37	0·30 ± 0·12‡	10
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Ethanol	0·6	5·50 ± 1·02	89	0·83 ± 0·22‡	29
	1·2	3·60 ± 0·85*	58	0·34 ± 0·17‡	12
	2·4	1·03 ± 0·10‡	17	0·17 ± 0·08‡	6
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Propanol	0·2	5·70 ± 1·19	92	0·94 ± 0·34‡	32
	0·4	3·80 ± 0·94*	61	0·32 ± 0·19‡	11
	0·6	2·50 ± 0·25†	40	0·30 ± 0·24‡	10
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Butanol	0·06	6·02 ± 0·85	97	1·28 ± 0·10‡	44
	0·12	3·85 ± 0·70	62	0·50 ± 0·16‡	17
	0·24	1·15 ± 0·20*	19	0·33 ± 0·10‡	11
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Pentanol	0·02	6·25 ± 0·85	101	1·60 ± 0·60*	55
	0·04	4·25 ± 0·89	69	1·00 ± 0·40†	34
	0·06	1·78 ± 0·20*	29	0·37 ± 0·11‡	13
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Hexanol	0·006	5·07 ± 1·03	82	2·20 ± 0·20*	76
	0·012	3·38 ± 0·74*	55	1·40 ± 0·10‡	48
	0·024	1·33 ± 0·20†	21	0·24 ± 0·05‡	8
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Heptanol	0·002	5·33 ± 1·20	86	1·90 ± 0·30*	66
	0·004	3·60 ± 0·90	58	1·50 ± 0·30†	52
	0·006	1·52 ± 0·20†	25	0·37 ± 0·03‡	13
	0	6·20 ± 1·11	100	2·90 ± 0·30	100
Octanol	0·0006	4·45 ± 0·85*	72	1·90 ± 0·20*	66
	0·0012	2·90 ± 0·50†	47	1·70 ± 0·10‡	59
	0·0024	1·35 ± 0·28‡	22	0·38 ± 0·13‡	13

Incubation were carried out as described in Methods. For total ATPases the reaction mixture contained 30 μmoles Tris (pH 7·4); 7·5 μmoles $MgCl_2$; 120 μmoles NaCl; 20 μmoles KCl; and 5 μmoles ATP(disodium ATP, Boehringer Mannheim GmbH). After stopping the reaction with 2·5 N perchloric acid, the quantity of inorganic phosphate liberated was measured [17]. Mg^{2+}-ATPase was assayed in the presence of 1 μmole phlorizin. The difference between the two measurements (total ATPase–Mg^{2+}-ATPase) was that of $(Na^+ + K^+)$-ATPase. The results are expressed as sp. act. (μmoles phosphate/mg protein/15 min.) and as per cent of the control activity. Each value represents the mean ± S.E.M. of four different brush border preparations. Difference statistically significant in relation to control: $*P < 0{\cdot}05$; $\dagger P < 0{\cdot}01$; $\ddagger P < 0{\cdot}001$.

RESULTS

Effect on the ATPases. In the concentration range used, the eight alcohols investigated have an inhibitory effect on ATPase activities (Table 1).

$(Na^+ + K^+)$-ATPase is specially sensitive to the action of alcohols; the activity decreases very rapidly in a significant manner, and at the highest concentration the inhibition is nearly complete (90–95%).

At the same concentration range, the effect on Mg^{2+}-ATPase is less important. At the lowest concentration only octanol produces a significant decrease and in no case was complete inhibition observed.

Effect on alkaline phosphatase and arylamidase. Concerning alkaline phosphatase (Table 2), only methanol, ethanol, and propanol. used at the same concentration range as previously, caused a significant decrease in enzyme activity. From butanol on, doses effective on ATPase activities have no effects on alkaline phosphatase.

Arylamidase (Table 3) is more resistent to the alcohols studied; only 2·4 M ethanol produces a significant inhibition. Nevertheless considering the regression between enzymatic activity and alcohol concentration we observed that, for ethanol and propanol, the decrease with rising concentrations is statistically significant ($P < 0{\cdot}01$ and $P < 0{\cdot}05$ respectively).

Equitoxic concentration. The concentration of alcohol which causes a 50 per cent inhibition of enzymatic activity (I_{50}) was used as a criterion for comparison of the different alcohols. This concentration is estimated from ATPase inhibition curves calculated from results in Table 1. The mean values are obtained, expressed in mM alcohol/l of incubation medium, and presented in Table 4. It can be seen that the I_{50} concentration diminishes considerably as the length of

Table 2. Effect of alcohols on alkaline phosphatase activity in the brush border of rat enterocytes

Alcohol	M	Sp. act.	%	Alcohol	M	Sp. act.	%
Methanol	0	31·1 ± 2·1	100	Pentanol	0	31·1 ± 2·1	100
	1	25·9 ± 1·1*	83		0·02	28·9 ± 0·8	92
	2	21·9 ± 1·1†	70		0·04	29·9 ± 1·4	96
	4	16·9 ± 1·1‡	53		0·06	28·7 ± 0·9	92
Ethanol	0	31·1 ± 2·1	100	Hexanol	0	31·1 ± 2·1	100
	0·6	24·1 ± 1·2†	77		0·006	29·2 ± 0·9	93
	1·2	21·1 ± 1·4‡	67		0·012	28·0 ± 0·5	90
	2·4	14·0 ± 1·4‡	45		0·024	27·6 ± 0·7	88
Propanol	0	31·1 ± 2·1	100	Heptanol	0	31·1 ± 2·1	100
	0·2	26·8 ± 1·2	86		0·002	28·7 ± 0·6	92
	0·4	24·7 ± 0·8†	79		0·004	29·3 ± 0·9	94
	0·6	22·8 ± 1·3†	73		0·006	28·2 ± 0·9	90
Butanol	0	31·1 ± 2·1	100	Octanol	0	31·1 ± 2·1	100
	0·06	30·8 ± 2·1	99		0·0006	30·3 ± 1·4	97
	0·12	28·5 ± 1·2	91		0·0012	30·6 ± 2·1	98
	0·24	26·7 ± 1·3	85		0·0024	28·3 ± 1·5	90

Incubation were carried out as described in Methods. According to the method of Bessey and Lowry *p*-nitrophenyl phosphate disodium salt, (Boehringer Mannheim GmbH) was used as substrate in a glycine buffer at pH 9·1 [18]. After stopping the reaction with 0·02 N NaOH. the *p*-nitrophenol released was measured by colorimetry at 405 nm. The results are expressed as sp. act. (μmoles *p*-nitrophenol/mg protein/15 min.) and as per cent of control activity. Each value represents the mean ± S.E.M. of four different brush border preparations. Difference statistically significant in relation to control: $*P < 0·5$; $†P < 0·01$; $‡P < 0·001$.

the chain increases; this is very clear for the two ATPases studied. A similar value could not be calculated for alkaline phosphatase or arylamidase because insufficient inhibition was obtained.

In the same table are included for each alcohol (for the determination of correlation coefficient) several physicochemical constants such as chain length, boiling point and partition coefficient which are themselves related to liposolubility of these alcohols.

Relationship between physicochemical properties and enzymatic inhibition

Solubility of alcohols in lipids is only an expression of the chemical potential (thermodynamic activity) which can be determined in aqueous solution using coefficients of thermodynamic activity of the alcohols. We have calculated the regression between thermodynamic activity (y), corresponding to I_{50} (Table 4), and the number of carbon atoms in the alcohol (x)

Table 3. Effect of alcohols on arylamidase activity in the brush border of rat enterocytes

Alcohol	M	Sp. act.	%	Alcohol	M	Sp. act.	%
Methanol	0	2·74 ± 0·18	100	Pentanol	0	2·74 ± 0·18	100
	1	2·75 ± 0·30	100		0·02	2·78 ± 0·14	101
	2	2·49 ± 0·30	90·9		0·04	2·66 ± 0·17	97·1
	4	2·36 ± 0·27	86·1		0·06	2·55 ± 0·21	93·1
Ethanol	0	2·74 ± 0·18	100	Hexanol	0	2·74 ± 0·18	100
	0·6	2·42 ± 0·22	88·3		0·006	2·76 ± 0·16	101
	1·2	2·24 ± 0·24	81·8		0·012	2·66 ± 0·29	97·1
	2·4	1·67 ± 0·35*	60·9		0·024	2·61 ± 0·24	101
Propanol	0	2·74 ± 0·18	100	Heptanol	0	2·74 ± 0·18	100
	0·2	2·46 ± 0·21	89·8		0·002	2·90 ± 0·33	107
	0·4	2·28 ± 0·23	83·2		0·004	3·03 ± 0·30	111
	0·6	2·05 ± 0·25	74·8		0·006	3·00 ± 0·45	111
Butanol	0	2·74 ± 0·18	100	Octanol	0	2·74 ± 0·18	100
	0·06	2·45 ± 0·36	89·4		0·0006	2·80 ± 0·19	104
	0·12	2·46 ± 0·27	89·8		0·0012	2·73 ± 0·13	100
	0·24	2·21 ± 0·33	80·7		0·0024	2·73 ± 0·13	100

Incubations were carried out as described in methods. Leucine *p*-nitroaniline (Boehringer Mannheim GmbH) was used as substrate in a 0·1 M phosphate buffer at pH 7·2 [19]. The enzyme was inhibited by heating and the *p*-nitroaniline released was determined by colorimetry at 405 nm. The results are expressed as sp. act. (μmoles *p*-nitroaniline/mg protein/15 min.) and as per cent of control activity. Each value represents the mean ± S.E.M. of four different brush border preparations. Differences statistically significant in relation to control: $*P < 0·05$.

Table 4. Various forms of I_{50} expression and values of some physical constants as related to liposolubility of aliphatic alcohols

Alcohol	Chain length	Boiling point*	α†	$(Na^+ + K^+)$-ATPase			Mg^{2+}-ATPase		
				I_{50} (mM)‡	log I_{50}	A§	I_{50} (mM)‡	log I_{50}	A§
Methanol	1	64·96	0·0095	1350	3·130	0·038	3362	3·527	0·096
Ethanol	2	78·50	0·035	438	2·642	0·029	1425	3·154	0·099
Propanol	3	97·10	0·155	153	2·185	0·040	450	2·654	0·119
Butanol	4	117·50	0·630	52·5	1·720	0·050	153·7	2·187	0·148
Pentanol	5	137·30	2·300	24·5	1.389	0·095	48·5	1·685	0·187
Hexanol	6	158·00	7·500	9·5	0·978	0·155	12·9	1·110	0·209
Heptanol	7	177·00	22·000	3·3	0·518	0·208	4·1	0·612	0·263
Octanol	8	194·50	59·000	1·18	0·072	0·260	1·15	0·061	0·255

* Boiling point in C degrees, at 760 mm [20].
† α = Partition coefficient water triolein [21].
‡ I_{50} = Alcohol concentration which produces a 50% inhibition of enzymatic activity. This value is calculated from the results in Table 1 and represents the mean of four experiments.
§ A: Thermodynamic activity of I_{50} = activity coefficient [22] × molecular fraction of I_{50}.

and obtained the following equations:

$(Na^+ + K^+)$-ATPase: $y = 0{\cdot}0338\ x - 0{\cdot}0428$
$(F = 117)\ (p < 0{\cdot}001)$

Mg^{2+}-ATPase: $y = 0{\cdot}0266\ x + 0{\cdot}0520$
$(F = 70)\ (p < 0{\cdot}001)$

The slopes of these equations are not zero. The regressions are very significant, showing that there is a relation between chain length and thermodynamic activity of I_{50}. One can conclude that the thermodynamic activity necessary to obtain an identical inhibition rises with the chain length of the alcohol.

This led us to consider the relationship between the I_{50} concentration and several physical constants characteristic of these alcohols. In Table 5 are presented the regressions; the significance of the relation between the two variables is given by the F-test of regression. The results show that the highest F-values are obtained with the logarithm of I_{50} and that between the parameters considered, the representation of log I_{50} as a function of the number of carbon atoms gives the most suitable values for regression as well as linearity (F of the regression and F of deviation from linear regression).

Combination of alcohol molecules with ATPases. Free energy involved in enzyme inhibition

Results obtained so far demonstrate a close relation between the hydrophobic nature of aliphatic alcohols and inhibition of the ATPases. Calculation of the free energy involved during transfer of a methyl group from the alcohols, solubilized in water, to the active sites of the enzyme could be helpful in understanding the hydrophobic nature of the active sites. This free energy is calculated with the following equation:

$$\Delta F = -(RT \ln b/N)$$

in which b represents the slope of the regression line and N the number of carbon atoms. From the values in Table 5, for the regression log I_{50}/N we found -620 cal for $(Na^+ + K^+)$-ATPase and -730 cal for Mg^{2+}-ATPase which demonstrates the more hydrophobic nature of the second enzyme.

Table 5. Relationship between I_{50} concentration and some physico-chemical parameters. Analysis of variance of the regression and linearity.

	Y/x	Straight line equation $Y = ax + b$	F of the regression $df = 1$ and 24	F of deviation from linear regression $df = 6$ and 24
$(Na^+ + K^+)$-ATPase	I_{50}/chain length	$Y = -143{\cdot}69x + 900{\cdot}46$	867‡	87·7‡
log	I_{50}/chain length	$Y = -0{\cdot}4291x + 3{\cdot}4980$	2379·7‡	0·64
log	I_{50}/boiling point	$Y = -0{\cdot}0224x + 4{\cdot}4367$	2368‡	2·45
log	log I_{50}/log α	$Y = -0{\cdot}7783x + 1{\cdot}5537$	2373‡	1·6
Mg^{2+}-APT-ase	I_{50}/chain length	$Y = -381{\cdot}55x + 2399{\cdot}20$	585‡	74‡
log	I_{50}/chain length	$Y = -0{\cdot}5035x + 4{\cdot}1335$	6553·1‡	2·8*
log	I_{50}/boiling point	$Y = -0{\cdot}0263x + 5{\cdot}2361$	6554‡	4·23†
log	I_{50}/log α	$Y = -0{\cdot}9100x + 1{\cdot}8520$	6490‡	13·2‡

α = Partition coefficient water–triolein [21].
The mean numeric values of the different parameters used in this calculation are presented in Table 4.
Statistically significant: *$P < 0{\cdot}05$; †$P < 0{\cdot}01$; ‡$P < 0{\cdot}001$. The significance of the regression and linearity are calculated according to Schwartz [23]. When the deviation from linear regression is not significant, the hypothesis of linearity can be retained.

DISCUSSION

The activity of various enzyme systems presents in the brush border of enterocytes is influenced very differently by the presence of aliphatic alcohols in the incubation medium. Results show that at concentrations active on the ATPases, the alcohols tested (beginning with butanol) have no effect on arylamidase and alkaline phosphatase. On the other hand, methanol, ethanol and propanol partially inhibit these enzymes. Inhibitions found with alkaline phosphatase and arylamidase are perhaps related to the moderate polarity of the first in the series of alcohols which could form clathrates with the polar groups of the proteins. Along this line, Hill *et al.* [24] found that in the case of leucine aminopeptidase prepared from porcine kidney, hydrophobic interactions play a very small role in the inhibition caused by aliphatic alcohols.

With regard to the ATPases, we have demonstrated a relationship between alcohol chain length and the thermodynamic activity of the I_{50} concentration. The thermodynamic activity necessary to obtain an identical effect rises with chain length. According to the Ferguson principle [25], in a homologous series, equal effect should be achieved with equal thermodynamic activity. But Brink and Posternak [3] reported that this principle does not apply to certain biological preparations.

Our results also show that interactions between the active sites of the ATPases and the alcohols are largely hydrophobic according to the theoretical work of Butler [22]. The existence of a linear relation between log I_{50} and the number of carbon atoms in the alcohol shows that there is an interaction between the alcohols and the non-polar sites on the enzyme. This linear relation agrees with the results of Rang [4] showing that as the length of the carbon chain rises arithmetically, the concentration necessary to obtain a given effect falls logarithmically. The less significant results obtained with other physical constants (boiling point, partition coefficient) show that the values for the coefficients used in these calculations, themselves obtained in well-defined experimental conditions, are not strictly applicable to complex membrane structures like the brush border.

The transfer of an alcohol in aqueous solution to a lipid phase or a hydrophobic site of an enzyme involves forces of association between molecules of solvent and solute. Using activity coefficients of aliphatic alcohols, Butler *et al.* [22] calculated that the free energy arising from the addition of each methyl group is approximately 800 cal. Presently, the value of 830 cal of free energy on transfer of each methyl group in a non-polar substance dissolved in water to a non-polar solvent is calculated [26]. The transfer of an alcohol to the hydrophobic site of an enzyme is thus an endothermic process; the free energy values for solubilization less than 830 cal indicate an incomplete dehydration of the methyl groups of the alcohol and show that the sites are not completely hydrophobic. This is the case for $(Na^+ + K^+)$-ATPase in enterocyte brush border for which we found a ΔF of -620 cal. This value is comparable to the figure of -600 cal obtained by Hegyvary [27] studying the inhibition of $(Na^+ + K^+)$-ATPase of guinea-pig kidney by various organic solvents. Similar results have been found for other enzymes. Mitsuda *et al.* [28] found a free energy value of -650 cal for each methyl group in the inhibition of lipoxygenase and Tang [29] found a value of -560 cal for pepsin under similar conditions.

For Mg^{2+}-ATPase, we found a free energy value of -730 cal which indicates the existence of more lipophilic sites.

From a pharmacological point of view, the concentration of ethanol necessary to inhibit *in vitro* the activity of the brush border ATPases is not high, relative to the concentration which can be found in the digestive tract after the ingestion of alcohol [30]. On one hand several authors have shown an inhibition of active transport of amino acid *in vitro* by ethanol and other alcohols [31] and *in vivo* by ethanol [30]. On the other hand $(Na^+ + K^+)$-ATPase is necessary for the greatest active amino acid and sugar transport [32]. According to Israel *et al.* [13, 14] the depression of amino acid intestinal transport linked to $(Na^+ + K^+)$-ATPase activity is conceivable. This work shows that the ATPases of the brush border are inhibited by alcohols. This poses the problem of pharmacological activity of fermented drinks containing ethanol and low concentrations of high alcohols sufficient for inhibiting ATPases.

Acknowledgement—This work was carried out at INSERM U-87. The authors would like to acknowledge their gratitude for the facilities provided by Director Dr. R. Derache.

REFERENCES

1. G. W. Taylor, *J. Cell. comp. Physiol.* **4,** 329 (1933).
2. M. G. Larrabee and J. M. Posternak, *J. Neurophysiol.* **15,** 91 (1952).
3. F. Brink and J. M. Posternak, *J. Cell. comp. Physiol.* **32,** 211 (1948).
4. H. P. Rang, *Br. J. Pharmac.* **15,** 185 (1960).
5. R. Lindbohm and H. Wallgren, *Acta pharmac. Tox.* **19,** 53 (1962).
6. D. Gaillard, S. Mitjavila and R. Derache, *Rev. alcoolisme* **15,** (3), 199 (1969).
7. H. G. Hill, *Fedn. Proc.* **28,** 300 (1969).
8. H. Kalant in *The Biology of Alcoholism* (Eds. Kissin and Begleiter) Vol 1, p. 40, Plenum Press, New York–London (1971).
9. J. M. Orten and V. M. Sadesat in *The Biology of Alcoholism* (Eds. Kissin and Begleiter) Vol 1, p. 232, Plenum Press, New York–London (1971).
10. S. W. French, in *The Biology of Alcoholism* (Eds. Kissin and Begleiter) Vol 1, p. 448, Plenum Press, New York–London (1971).
11. Tran Van Tuan, M. T. Mitjavila and R. Derache, *C.R. Soc. Biol.* **165,** 2011 (1971).
12. G. Carrera, S. Mitjavila and R. Derache, *Biol. gastroenterol.* **7,** 43 (1974).
13. Y. Israel and H. Kalant, *Nature, Lond.* **200,** 476 (1963).
14. Y. Israel, H. Kalant and A. E. Le Blanc, *Biochem. J.* **100,** 27 (1966).
15. M. T. Mitjavila, S. Mitjavila, N. Gas and R. Derache, *Toxic appl. Pharmac.* **34,** 72 (1975).
16. O. H. Lowry, M. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193,** 265 (1951).
17. C. Fiske and J. Subbarow, *J. biol. Chem.* **66,** 375 (1926).
18. O. A. Bessey, O. H. Lowry and M. J. Brock, *J. biol. Chem.* **164,** 321 (1946).
19. W. Josch and U. C. Dubach, *Clin. chim. Acta* **15,** 325 (1967).
20. *Handbook of chemistry and physics*, The Chemical Rubber Co., 45th ed, table C, Cleveland (1964).

21. B. A. Lindenberg, *J. Chim. phys.* **48,** 350 (1951).
22. J. A. V. Butler, C. N. Ramchandani anl D. W. Thomson, *J. chem. Soc.* **952,** 280 (1935).
23. D. Schwartz, *Méthodes statistiques à l'usage des médecins et des biologistes*, (Ed. Flammarion) p. 228, Paris (1963).
24. R. L. Hill and E. L. Smith, *J. biol. Chem.* **224,** 209 (1957).
25. J. Ferguson, *Proc. R. Soc. B*, **127,** 387 (1939).
26. D. A. Haydon and F. A. Taylor, *Phil. Trans. R. Soc. London A* **252,** 225 (1960).
27. C. Hegyvary, *Biochim. biophys. Acta.* **311,** 272 (1973).
28. H. Mitsuda, K. Yasumoto and A. Yamamoto, *Archs Biochem. Biophys.* **118,** 664 (1967).
29. J. Tang, *J. biol. Chem.* **240,** 3810 (1965).
30. Y. Israel, I. Salazar and E. Rosenmann, *J. Nutr.* **96,** 499 (1968).
31. T. Chang, J. Lewis and A. J. Glasko, *Biochim. biophys. Acta.* **135,** 1000 (1967).
32. S. G. Schultz and P. F. Curran, *Physiol Rev.* **50,** 637 (1970).