groups. Under these circumstances changes in glutaminase and asparaginase activities of the enzyme were found to be similar in character. Inactivation of GA-ase by WK is also accompanied by spectral changes characteristic of interaction of the reagent with the accessory peak, with a maximum in the 340 nm region and with reduction of the ratio A_{280}/A_{250} [6, 9].

Investigation of the effect of two chemical agents on GA-ase thus revealed that 2,3-butandione, which modifies arginine residues in proteins, does not affect activity of the enzyme. The enzyme is completely inactivated in the presence of WK. Data obtained by a study of the effect of concentration of the modifying agent, pH of the medium, competitive inhibitors, and certain other substances, on the degree of inactivation suggest that carboxyl groups, essential for substrate binding, are present in the active center of glutamin-(asparagin)ase.

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MECHANISM OF ACTION OF KETAMINE DERIVATIVE ANESTHETICS ON PHOSPHOLIPID MEMBRANES

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Cell membranes have for a comparatively long time been considered to be the site of action of anesthetics. As regards ketamine derivatives, which are powerful anesthetics of the latest generation, no systematic studies of the mechanism of their action on membranes have yet been undertaken, and the available data on this matter are few in number and contradictory in nature. Some workers consider that ketamines "liquefy" the lipid phase of membranes [8], whereas others consider that they make it more "rigid" [10]. Finally, information has been obtained to the effect that ketamine, which has a positive charge at neutral pH values, modifies the surface potential of the ganglioside films [7].

In the investigation described below the effect of the ketamine derivatives Calypsol and Ketalar on viscosity and surface charge was studied on model phospholipid membranes (liposomes).

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TABLE 1. Conditions of Measurement of Fluorescence of Probes

Probe	Wavelength of excitation, nm	Wavelength of fluorescence, nm	Optical half-width of monochromator slit, nm	
			excitation	fluorescence
Pyrene	320	372 (monomers)	4	0,5
		470 (excimers)	4	0,5
DMCH	430	520	4	4
ANS	370	470	4	5
DSM	470	570	4	10

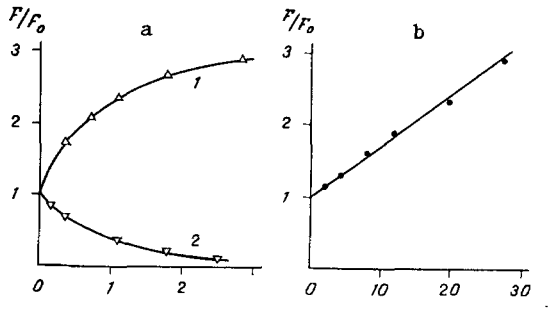

Fig. 1. Effect of Calypsol (a) and CTAB (b) on intensity of fluorescence (F) of ANS^- (a, 1 and b) and DSM^+ (2) probes in liposome suspension. F_0) Intensity of fluorescence of probes in absence of Calypsol or CTAB. Concentraton of phosphatidylcholine 256 μM, of ANS 58 μM, of DSM 8 μM. Abscissa: a) concentration of Calypsol (in mM), b) concentration of CTAB (in μM).

EXPERIMENTAL METHOD

Liposomes were obtained from egg lecithin (produced by Khar'kov Bacterial Preparations Factory), and injected in the form of its ethanol solution [6] in 10 mM Tris-HCl (pH 7.4).

Aqueous solutions of Calypsol and Ketalar (50 mg/ml) were obtained from Zdravije (Yugoslavia) and Gedeon Richter (Hungary), respectively. The probes used were 1-anilinonaphthalene-8-sulfonate (ANS) and pyrene (Sigma, USA), 4-n-dimethylaminostyrylmethylpyridinium (DSM), synthesized by G. Ya. Dubur, et al. [2], and 4-dimethylaminochalcone (DMCH), synthesized at the Monokristall Scientific Production Combine (Khar'kov), and described in [9]. 5-Doxylstearate (Sigma) was used as the spin probe. The cetyltrimethylammonium bromide (CTAB), Tris, and KCl were of Soviet origin.

Fluorescence was recorded on an MPF-4 spectrofluorometer (Hitachi, Japan) in a flat quartz cuvette with thickness of the optical layer of 0.1 cm, determined along the bisectrix of the right angle formed by the optical axes of the monochromators. The spectral conditions of measurement of fluorescence of the probes are given in Table 1.

Polarization of fluorescence of DMCH in the liposomes was measured with the aid of polarization filters and calculated by the formula:

$$P = \frac{F_I - F_{II}}{F_I + F_{II}},$$

where P denotes the degree of polarization, F_{II} the intensity of fluorescence of the solution with parallel transmission planes of the exciting and analyzing filters; F_I the same, with the planes arranged perpendicularly. EPR spectra were measured on an E-4 spectrometer (Varion, USA) under conditions described previously [5].

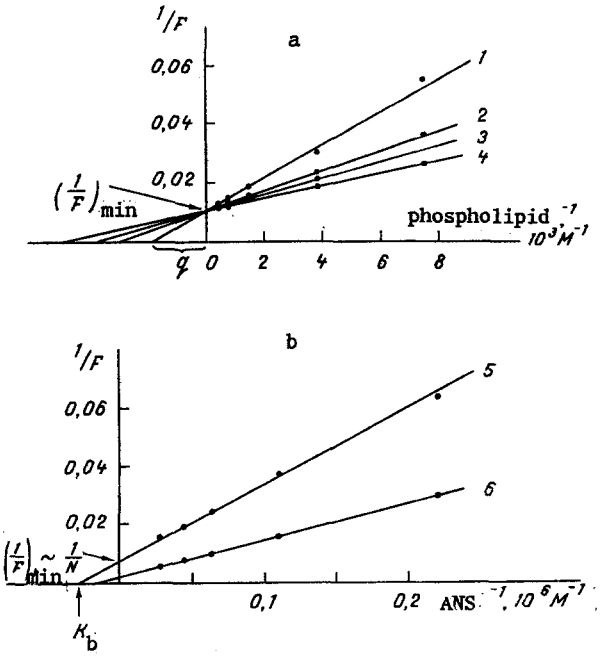

Fig. 2. Titration of ANS solution (11.5 μM) by liposomes (a) and solution of liposomes (256 μM phospholipid) by ANS probe (b) in absence and presence of Calypsol. Concentration of Calypsol in experiments: 1, 5) 0 mM; 2) 0.36 mM; 3) 1.08 mM; 4) 2.5 mM; 6) 1.8 mM. Value of $(1/F)_{min}$ in a is directly proportional to 1/Q (Q denotes quantum yield of fluorescence of probe, directly proportional in b to 1/N. Segment q in Fig. 2a is directly proportional to concentration of bound probe (r): $q \sim r \sim N \cdot K_b$.

All experiments were carried out at a temperature of 20-23°C in 10 mM Tris-HCl (pH 7.4).

EXPERIMENTAL RESULTS

The effect of ketamine on the viscosity of the lipid membrane was estimated by three methods: 1) based on the degree of excimerization of pyrene [1]. With pyrene in a concentration of 21 μM and liposomes in a concentration of 0.5 g/liter, the F_{470}/F_{372} ratio was 0.45 ± 0.02 in the absence of Calypsol and 0.46 ± 0.02 in the presence of 3.6 mM Calypsol; 2) based on the degree of polarization of fluorescence of DMCH [1]. With DMCH in a concentration of 40 μM and liposomes in a concentration of 2 g/liter the degree of polarization in the absence and in the presence of 3.6 mM Calypsol did not differ significantly; 3) based on the parameter of orderliness (S) [3] of 5-doxylstearate. With liposomes in a concentration of 2 g/liter and the probe in a concentration of 10^{-4} M the value of S was 0.56 ± 0.05, and it was not significantly changed in the presence of Calypsol.

Thus none of the approaches used revealed any effect of ketamine on viscosity of the lipid membrane.

It will be clear from Fig. 1a that in the presence of Calypsol the intensity of fluorescence of the anion-probe in the liposome suspension was increased, whereas the intensity of fluorescence of the cation-probe DSM was reduced. The cause of the fluorescence effects observed may have been changes in the quantum yield of fluorescence due to the fact that Calypsol quenches fluorescence of DSM and potentiates fluorescence of ANS, or changes in the concentration of membrane-bound probes.

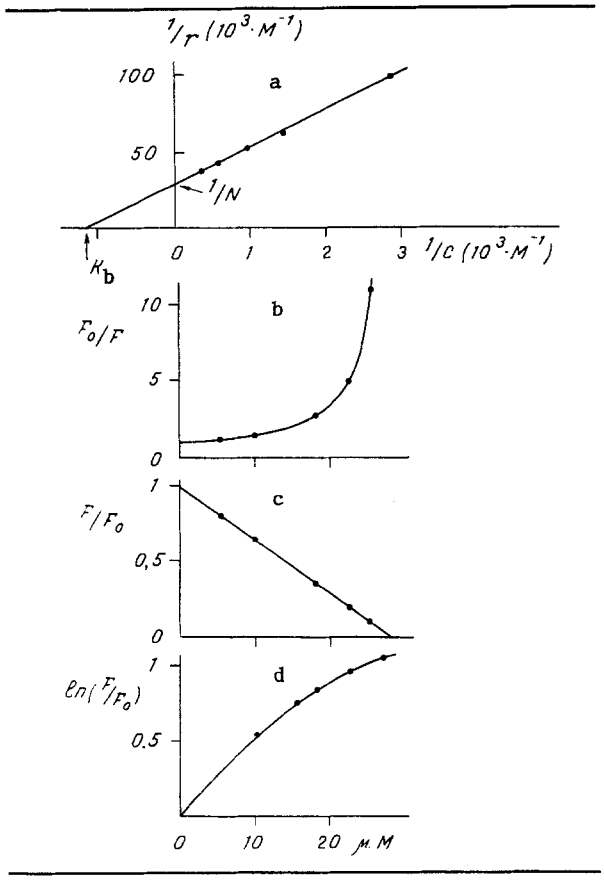

Fig. 3. Determination of binding parameters of Calypsol (a) and dependence of fluorescence of DSM (b, c) and ANS (d) probe on concentration of membrane-bound Calypsol (r). C) Concentration of free Calypsol (C = a − r, where a is the total concentration). F_0 and F have the same meanings as in Fig. 1.

In the case of ANS it can be shown directly that ketamine did not affect the quantum yield of fluorescence of the probe in liposomes, but increased the concentration of membrane-bound molecules (Fig. 2). This result seems trivial if it is considered that ketamine is positively charged [7] while ANS is negatively charged. However, it must be pointed out that the increase in concentration of bound ANS (r) took place only on account of a marked increase (fourfold) in the number of binding sites (N): from 16.5 μM in "intact" liposomes to 67 μM in liposomes in the presence of 1.8 mM Calypsol (values of N were calcualted on the basis of data given in Fig. 2), whereas the binding constant (K_b) of ANS in the liposomes underwent opposite changes, paradoxical from the point of view of charge interaction: in the presence of the anesthetic it was reduced by half – from $2.6 \cdot 10^4$ to $1.4 \cdot 10^4$ M^{-1} (Fig. 2b). The net effect of the action of Calypsol was an increase in the concentration of bound molecules (r) of ANS, since $r \sim N \cdot K_b$.

The other ketamine derivative, namely Ketalar, has the same action both qualitatively and quantitatively on parameters of binding of ANS as Calypsol, in the same concentrations.

It was impossible to carry out such a detailed analysis for another probe (DSM) because of the low value of its K_b with liposomes [2] and the consequent technical difficulties arising in determination of the binding parameters. However, it will be clear that since dependence of the decrease in the intensity of fluorescence of DSM on the concentration of bound Calypsol is not linear (Fig. 3b), this process is determined by the fact that quenching of the excited DSM molecules by Calypsol molecules is not constant [1]. Another mechanism of quenching of fluorescence, namely nonradiative transfer of energy, likewise is impossible in this case, because Calypsol does not absorb light in the region of wavelengths over 330 nm. Only one possible explanation of the reduction of fluorescence of DSM

in the presence of Calypsol remains: a decrease in concentration of membrane-bound molecules of the probe due to direct competition between the molecules of these cations (Fig. 3c).

Ketamine derivatives in a suspension of phosphatidylcholine liposomes at neutral pH and in solution with low ionic strength thus reduce the concentration of membrane-bound molecules of the cationic probe DSM but increase the concentration of bound molecules of the anionic probe ANS. Hence it follows that ketamine interacts with the lipid membrane, giving its surface an additional positive charge. The charge nature of the effect of ketamine is confirmed also by the fact that in a solution of high ionic strength (200 mM KCl) this effect is not exhibited.

To determine the quantity of membrane bound cation (ketamine) the method of "calibration" titration of the (membrane + ANS^-) complex with another cation (CTAB; Fig. 1b) was used, which is considered to be completely bound with liposomes. It was assumed that equal concentrations of bound CTAB and Calypsol (r) induce an equal increase in the number of bound ANS molecules. A similar method of estimating the concentration of the bound preparation was used previously in [4], which quenching of the fluorescence of DSM by CTAB was used as "calibration." However, we preferred another probe, namely ANS, because in this case the causes of the change in quantity of the bound probe can be analyzed. The binding parameters of Calypsol were determined between Klotz coordinates (Fig. 3a). It was found that $K_b = 1.2 \cdot 10^3$ M^{-1} and N = 34.5 μM with the lipid in a concentration of 256 μM, i.e., on average there is one binding site for every 7.5 lipid molecules.

It will be clear from Fig. 3c that with a concentration of bound Calypsol of 28.5 μM the DMS probe ceased to bind with the membrane. If the stoichiometry of competition between ketamine and DSM is 1:1, it follows that 28.5 μM is also the value of r for DSM. The molar fractions of binding sites of the two cations in the lipid were thus closely similar: 1:7.5 for ketamine and 1:9 for the DSM probe. This is not surprising, for the two molecules have an identical charge and are similar in size.

As regards K_b for DSM, it is evidently less than K_b for ketamine, and its value is $1.2 \cdot 10^3$ M^{-1}.

Ketamine thus does not affect the mobility of different fluorescent probes or of the spin probe in liposomal membranes and, consequently, the anesthetic has no effect on the "viscosity" of the lipid layer. By interacting with the membrane, ketamine creates an additional positive charge on its surface, and this leads to an increase in binding of the anionic probe and to a decrease in binding of the cationic probe. In this way ketamine modifies the membrane binding sites of the anionic probe (as is shown by the decrease in K_b of ANS), while at the same time increasing sharply the number of binding sites.

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