The effect of barbiturate on the activity of Mg^{++}-dependent ATPase in erythrocytes

Erythrocytes	Concentration of barbiturate	No. of cases	Mg^{++}-dep. ATPase		s	t	P
			Control	+Barbiturate			
A	10 mM	5	113.3	252.4	32.26	4.31	<0.02
A	100 mM	4	115.9	0.0	29.65	3.91	<0.05
AS	10 mM	5	1272.5	1183.0	35.71	2.51	>0.05
AS	100 mM	4	1311.2	621.5	100.95	6.83	<0.01

A, Erythrocytes hemolysed by 4 mM ATP. AS, Erythrocytes hemolysed by 4 mM ATP in 0.025% saponin.

cytes under given conditions after a 1 h incubation. For more details see our previous paper[2]. Barbital (5,5-diethylbarbituric acid) supplied by SPOFA was used.

Results and discussion. The activity of Mg^{++}-dependent ATPase in erythrocytes hemolysed by 4 mM ATP was measured after the treatment with barbiturate at various concentrations. The mode of action of barbiturate is 2-staged. At the first stage it stimulates the enzyme, whereas at the second the enzyme activity decreases with increasing barbiturate concentration and at 100 mM barbiturate concentration no activity was observed in any of the four experiments (Figure, Table). When the enzyme activity was stimulated by saponin, it decreased following the treatment with barbiturate. This decrease was not statistically significant at 10 mM concentration of barbiturate, but it grew with increasing concentration of barbiturate and was greatest at the highest concentration used, i.e. at 100 mM (Figure, Table). Pair-t-test was used for statistical evaluation of the results.

An increase in the activity of Mg^{++}-dependent ATPase in erythrocyte membrane induced by barbiturate can be accounted for by the interference of barbiturate with some membrane components, thus affecting the latency of the enzyme. The inhibiting action of barbiturate may be due to the blockade of some groups in the enzyme molecule.

It is interesting that many substances interfering with nerve action also influence the activity of Mg^{++}-dependent ATPase and some of them even affect shape of erythrocytes. Braash and Rogausch[5] detected the formation of crenated erythrocyte following the injection of barbiturate Nembutal in animals; the changes were more pronounced in rabbit erythrocytes than in dogs. Whether this is due to accidental coincidence or to a certain regularity will be a subject of further study.

Zusammenfassung. Die Aktivität der Mg^{++}-abhängigen ATPase in der Membran menschlicher Erythrozyten ändert sich in Anwesenheit von Barbiturat (Barbital der Firma Spofa). 10 mM Barbiturat stimuliert das Enzym, während 100 mM zu einem Verlust der enzymatischen Aktivität führt. Bei Erythrozyten, deren Enzym mit Saponin stimuliert wurde, ist der Einfluss von 10 mM Barbiturat statistisch nicht signifikant. 100 mM Barbiturat hat eine hemmende Wirkung.

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[5] D. Braash and H. Rogausch, Pflügers Arch. *323*, 41 (1971).

Interaction of Polyene Antibiotics and Serum Lipoproteins

We have shown previously that the aromatic heptaene levorine and the non-aromatic heptaene amphotericin B interact with cholesterol in vitro forming a sterol-polyene complex[1]. Since cholesterol is present as lipoprotein complexes in blood serum, it seemed to be of interest to study the binding of polyene antibiotics by serum lipoproteins. In this study we have investigated the interaction of levorine and amphotericin B with lipoproteins (LP) of the rabbit serum.

Table I. The binding of levorine and amphotericine B by normal and hypercholesterolemic rabbit serum

Antibiotic	Concentration of antibiotic (mg/ml)	Binding by serum (%)	
		normal	hyper-cholesterolemic
Levorine	0.5	57	83
Amphotericin B	1.0	0	53

Materials and methods. The sodium salts of levorine and amphotericin B with the specific biological activity of 30,000 U/mg and 600 U/mg respectively were used in this study. The activity of antibiotics was defined by the method of the diffusion into agar with the test-organism *Torula utilis*[2]. We judged the interaction of antibiotics with LP on the basis of the loss of the polyene biological activity in the presence of LP in comparison with the control experiment (antibiotic in the phosphate buffer, buffer, pH 7.2).

Normal rabbit serum containing 150 mg/100 ml of β-LP, hypercholesterolemic serum containing 2,000 mg/100 ml of β-LP, a 2% solution of β-LP and a 0.4% solution of α-LP were used in the experiments. The β-LP were isolated by Cornwell and Kruger's[3] procedure from the rabbit hypercholesterolemic serum. The concentration of β-LP in the solution was brought to the concentration

[1] A. N. Klimov, A. A. Nikiforova, E. D. Etingof, Antibiotics *3*, 243 (1971), in Russian.

[2] V. S. Dmitrieva and S. M. Semenov, *Microbiological Control of the Activity of Antibiotic Preparations* (Medicine, Moscow 1965), in Russian.

[3] D. Y. Cornwell and F. A. Kruger, J. Lipid Res. *2*, 110 (1961).

Table II. The binding of levorine and amphotericin B (in %) by serum lipoproteins (average data of 3 experiments)

Antibiotic	2.0% solution of β-LP	Solution of delipidated β-LP	0.4% solution of α-LP	Solution of delipidated α-LP
Levorine	87 (83–91)	69 (59–80)	31 (25–37)	10 (7–15)
Amphotericin B	61 (50–72	0	17 (14–20)	0

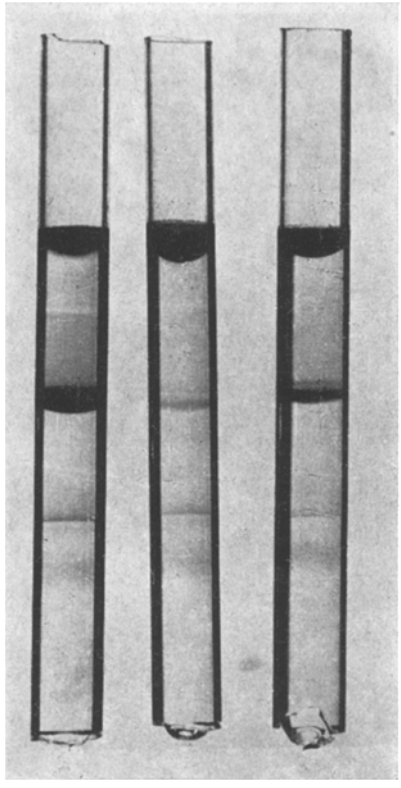

Fig. 1. Separation of LP of normal human serum by disk electrophoresis method in the presence of polyene antibiotics 1. Serum; 2. Serum + levorine; 3. Serum + amphoterecin B.

in the primary serum (2,000 mg/100 ml). α-LP were isolated using BURSTEIN's and MORFIN's[4] procedure and their concentration in the solution also was brought to the concentration in the primary serum (400 mg/100 ml). Delipidation of LP was carried out according to the WINDMUELLER and LEVY[5] method. The disc electrophoresis in polyacrylamide gel was carried out according to the method of WOLLENWEBER and KAHLKE[6] with minor modification. The paper electrophoresis was performed according to the universally adopted method in the veronal buffer, pH 8.6.

The weight ratio of polyene/LP in the experiments with the electrophoretic separation was 2:1. When the paper electrophoresis was used to study the binding of antibiotics by LP, some electrophoregrams were applied to the Petri dishes with the *Torula utilis* culture. The presence of antibiotic was judged by the occurrence of the zone of inhibition of growth of this culture.

For the quantitative estimation of antibiotic, the paper electrophoregram was cut into equal pieces corresponding to the origin and the bands of α- and β-LP. In some experiments the antibiotic was extracted from these strips with 90° ethanol. The concentration of polyene was defined spectrophotometrically[7] and by the diffusion into agar[2].

[4] M. BURSTEIN and R. MORFIN, Life Sci. *8*, 345 (1969).
[5] H. Y. WINDMUELLER and R. J. LEVY, J. biol. Chem. *243*, 4878 (1968).
[6] J. WOLLENWEBER and W. KAHLKE, Clin. chim. Acta *29*, 411 (1970).
[7] A. A. NEVINSKY and E. D. ETINGOF, Antibiotics *6*, 541 (1971). in Russian.

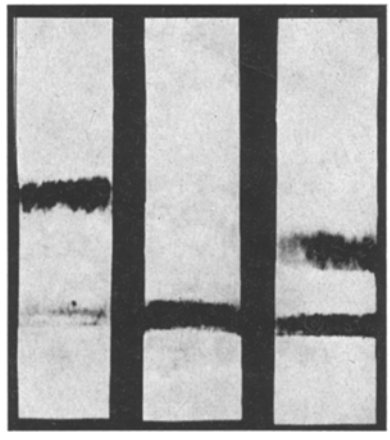

Fig. 2. Separation of LP of rabbit hypercholesterolemic serum by paper electrophoresis in the presence of polyene antibiotics (previous staining of lipids with sudan black). 1. Serum; 2. Serum + levorine; 3. Serum + amphotericin B.

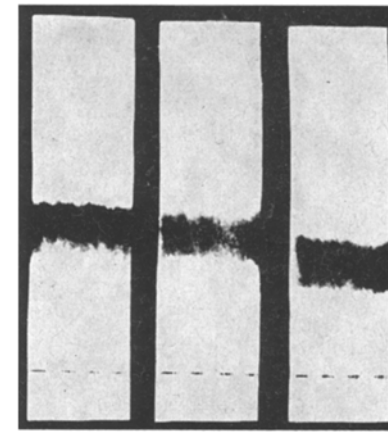

Fig. 3. Separation of LP of rabbit hypercholesterolemic serum by paper electrophoresis in the presence of polyene antibiotics (subsequent staining with oil red 0. 1. Serum; 2. Serum + levorine; 3. Serum + amphotericin B.

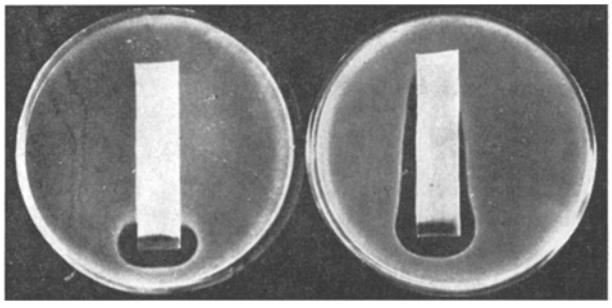

Fig. 4. Zones of inhibition of growth on the agar surface after covering it with sections of electrophoregrams. Left, levorine alone. right, complex of β-LP-levorine.

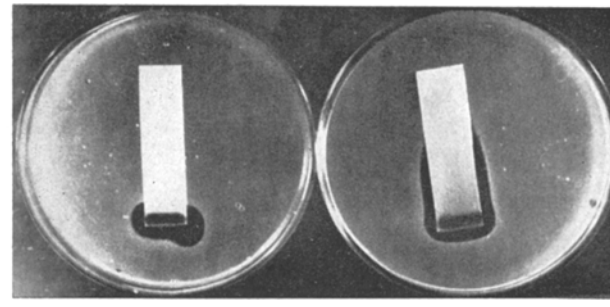

Fig. 5. Zones of inhibition of growth on the agar surface after placing theron sections of electrophroegrams. Left, amphotericin B alone; right, complex β-LP-amphotericin B.

Results and discussion. The data on the study of binding of levorine and amphotericin B by normal and hypercholesterolemic serum are shown in Table I.

As it is apparent from Table I, the normal rabbit serum binds a considerable amount of the aromatic antibiotic levorine, but does not bind the non-aromatic antibiotic amphoreticin B. In the presence of hypercholesterolemic serum, not only the binding of the aromatic antibiotic increases, but also marked fixation of non-aromatic antibiotic becomes evident.

Furthermore we undertook an investigation of the interaction of levorine and amphotericin B with LP isolated from serum. It is seen from Table II that the binding of amphotericin B by both α- and β-LP is weaker that the binding of levorine.

The 2% solution of native β-LP bound an average of 87% of levorine and 61% of amphotetricin B. Delipidation of β-LP caused only a slight decrease of the percentage of levorine binding but completely deprived the β-LP of their ability to bind amphotericin B. Approximately the same peculiarity is noted in case of delipidation of α-LP. As a result of delipidation of LP they lose their cholesterol almost completely, which is likely to explain the decrease of levorine binding and the loss of the ability to bind amphotericin B. Proceeding from Table II, one comes to the conclusion that the presence of lipids (cholesterol) plays the major part in the amphotericin B binding by serum LP, while the levorine binding is brought about by both protein and cholesterol components of LP. Also consideration must be given to the fact that free cholesterol, as well as its esters, possess the ability to bind levorine. As to amphotericin B it can be bound only by the free cholesterol[8]. It is known that the LP contain both free and esterified cholesterol. This also contributes to a stronger levorine binding by LP.

When studing the binding of polyene antibiotics by serum LP by means of disk electrophoresis and also using paper electrophoresis (previous staining for lipids with sudan black), it was found that in the presence of levorine the stained bands of LP were almost completely absent, and in the presence of amphotericin B the intensity of their staining became considerably lessened (Figures 1 and 2). This phenomenon could be explained by either decoloration of the sudan black by polyene antibiotics, by the absence of electromobility of LP-polyene complex or by the displacement of sudan black from LP by polyene antibiotics. Purposely designed experiments showed that the decoloration did not happen on mixing the polyene and sudan black solutions. As to the electrophoretic mobility of the LP-polyene complex, the data of the paper electrophoresis with subsequent staining of the LP bands with oil red 0 proved that the changes of mobility of the complexes were minor: only a slight retardation of the mobility of LP-levorine complex, and somewhat more marked retardation of the mobility of LP-amphotericin B complex were observed (Figure 3) .It is characteristic that the subsequent staining of electrophoregrams with oil red 0 resulted in the appearance of stained bands in the place of the LP-polyene complex. It is explained by the fact that during 3 h processing of the electrophoregram with alcohol solution of oil red 0, extraction of antibiotic from the complex takes place which liberates the functional groups in the LP particles. These groups are able to interact with the stain.

So there is reason to believe that due to the high affinitiy of polyene for LP, the antibiotics levorine and amphotericin B displace the sudan black, the specific stain for the lipids, from the binding with LP.

Another proof of the interaction between polyene antibiotics and LP is the detection of the antibiotic activity in the bands corresponding to α- and β-LP when making the paper electrophoresis of the latter in the presence of polyene. In the experimental samples (LP + polyene) the antibiotics are revealed over the whole length of the electrophoregram (Figures 4 and 5), while in the control samples (polyene in the phosphate buffer) the antibiotics were detected only at the origin. The quantitative estimation of antibiotics in the electrophoretic bands gave the following results: at the separation of the mixture of LP + polyene somewhat less than half the added levorine can be detected at the start, about a quarter of added levorine can be detected in the band of β-LP, almost the same amount – in the band of α-LP and the trace amount in the area between the origin and the band of β-LP. In the control experiment (levorine in the phosphate buffer) the total antibiotic is detected at the origin.

ЫВОВДЫ. Полиеновые антибиотики леворин и амфотерицин ь взаимодействуют с липопротеинами плазмы крови с образованием комплекса "липопротеин-полиен".

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[8] A. N. Klimov and A. A. Nikiforova, Antibiotics *4*, 325 (1971), (in Russian).