Biochimica et Biophysica Acta, 363 (1974) 182–189

BBA 76720

LIPOSOME MODEL EXPERIMENT FOR THE STUDY OF ASSUMED MEMBRANE DAMAGE IN PORPHYRIA CUTANEA TARDA

F. KÓSZÓ, CS. SIKLÓSI and N. SIMON

Department of Dermatology and Venereal Diseases, University Medical School, Szeged (Hungary)

(Received February 8th, 1974)

SUMMARY

In the early stages of toxic porphyria induced by organic chloro compounds, the amount of coproporphyrins firstly rises. In this phase of the disease, the porphyrins are secreted from the liver via the bile duct; in contrast, in "porphyria cutanea tarda" and the porphyria of the cutanea tarda type resulting from the action of hexachlorobenzene, the various examinations show a diffuse porphyrin accumulation in the entire liver, extending also to the interstice. Accordingly, in hepatic cutaneous porphyrias the liver cells secrete porphyrin not only into the bile capillaries, but also into the intercellular substance; this, however, assumes the pathological change of the cell-membrane permeability.

A model experiment was performed to test this hypothesis. A liposome membrane was prepared from phospholipid extracted from egg yolk. The permeability of this to glycine was compared with the permeability of a similar membrane which had been modified with hexachlorobenzene. The permeabilities to glycine of membranes "poisoned" with two different hexachlorobenzene concentrations at two temperatures (6 and 38 °C) were clearly larger (for the same diffusion mechanism) than that of the control, proving the correctness of the hypothesis.

It must be noted, however, that the development of porphyria cutanea tarda is probably not the consequence of only the one aetiological factor, cell-membrane lesion.

INTRODUCTION

The porphyrinogenic nature of hexachlorobenzene has been recognized since the description of "Turkish" porphyria [1]. Rimington and Ziegler [2] showed that practically all chlorinated benzene derivatives are able to induce in rats a similar pathological process corresponding to the biochemistry of porphyria cutanea tarda symptomatica. Other authors [3–5] have reported similar results in connection with various polychlorinated biphenyls and the analogous bromo compounds.

Each of these compounds was apolar, with no functional groups, and with very low reactivity.

Examinations have likewise already been made of the effects on the porphyrin

metabolism of a number of organic chloro compounds also containing hydrophilic groups, but these exhibited substantial differences from the former group. They gave rise merely to toxic coproporphyria lasting for several days, or possibly a few weeks [6].

In rats poisoned with hexachlorobenzene, porphyrin fluorescence can first be observed in the bile duct (Siklósi, Cs., unpublished results). The findings are similar in the case of coproporphyria following Lindane and 2,4-dichlorophenoxyacetic acid poisoning [6]. In the early stages of toxic porphyria caused by either hexachlorobenzene or the other organic chloro compounds, firstly the amount of coproporphyrins increases, but at this time porphyrin fluorescence can not be detected elsewhere than in the bile duct; this is in contrast with porphyria cutanea tarda, and with the porphyrias of the cutanea tarda type developing later on after the action of hexachlorobenzene, where the entire liver surface fluoresces a vivid red colour, while in native sections a fluorescence microscope reveals a diffuse porphyrin accumulation, extending even to the interstice [7].

The described phenomenon indicates that the porphyrins are secreted from healthy liver via the bile, while in hepatic cutaneous porphyrias the liver cells secrete porphyrin not only into the bile capillaries, but also into the intercellular substance. It follows automatically from this assumption that in hepatic cutaneous porphyrias there is a pathological change in the permeability of the cell membranes. To test our hypothesis, permeability experiments were carried out on a model liposome membrane "poisoned" with hexachlorobenzene. The results of these are reported in the present paper.

MATERIALS AND METHODS

Materials

For the preparation of the liposome system the phospholipid was extracted from egg yolk by a slightly modified version of the method of Dawson [8]. Acetone was used to precipitate the phospholipids from the crude lipid extract dissolved in light petroleum. The precipitate was purified by redissolving it in light petroleum and re-precipitation with acetone. The precipitate thereby obtained was washed three times with acetone to remove the bulk of the carotinoid impurities. The composition of the preparation was studied by a two-dimensional thin-layer chromatographic method [9]. The running was carried out on 20 cm × 20 cm Kieselgel G plates, 0.25 mm in thickness, with 65 : 35 : 5 and 35 : 65 : 5 mixtures of chloroform–methanol–7 M ammonia as solvent. The phospholipid preparation contained mainly choline and ethanolamine phosphatides; sphingomyelin and phosphatidyl inositol could be detected only in traces. The ratio of the two main fractions was about 65:35. The evaluation was performed densitometrically (Zeiss, Type ERI-65) after one-dimensional running and carbonification. Prior to use, the phospholipid was stored at −20 °C at a concentration of 0.1 g/ml in chloroform solution in sealed 10 ml ampoules.

Sephadex G-50 Fine gel (Pharmacia, Uppsala) was used for gel filtration. The other chemicals employed during the experiments were Reanal products of pro analysis purity.

Preparation of liposome system

The liposome system was prepared in the following way, on the basis of the methods of Finer et al. [10] and Erdei et al. [11]. 10 ml of a 0.1 g/ml phospholipid solution in chloroform were freed from solvent in a round-bottomed flask with a rotary vacuum evaporator. The lipid film adhering to the wall of the vessel was emulsified with 10 ml of an aqueous 0.1 M glycine solution. The pH of the glycine solution was previously adjusted to 7.2 with 0.1 M sodium hydroxide, and the solution was stabilized with 0.02 % sodium azide. The phospholipid emulsion, cooled with ice-water, was treated for 45 min with an MSE ultrasonic apparatus (Type 150 W, 21 kcycles/s, 6.5 μm amplitude) at a frequency of 50 kHz and at maximum amplitude. The disintegration converted the milk-like emulsion to a weakly opalescent, but transparent, fairly homogeneously distributed and very stable liposome system, which was finally centrifuged at 2 °C for 30 min at 10 000 $\times g$ to remove the titanium particles entering the system from the sonic head during the ultrasonic treatment. The glycine remaining in addition to the liposomes was removed by medium-exchange via gel-filtration. The liposome suspension thus obtained was regarded as the normal (control) system.

In the preparation of the poisoned membrane, 0.1 or 1.0 ml of a $3.05 \cdot 10^{-4}$ g/ml solution of hexachlorobenzene in chloroform was added to 10 ml of the 0.1 g/ml solution of the phospholipid in chloroform. The system prepared in the above manner contains liposomes 124 Å in radius on average, and with a bilayer structure [11]. If these are regarded as spherical, if the surface requirements of one phospholipid molecule are 50–60 Å^2, and if the average molecular weight of the phospholipids is taken as 757, then on average 7000 phospholipid molecules can be reckoned with per liposome. On the basis of these data, there are 0.56 hexachlorobenzene molecules on average per liposome in the more weakly poisoned system (in the case of the addition of 0.1 ml hexachlorobenzene solution), while on the stronger poisoning (addition of 1.0 ml hexachlorobenzene solution), there are 5–6 hexachlorobenzene molecules on average per liposome. The complete incorporation of the added hexachlorobenzene into the liposome membrane was ensured by the insolubility of hexachlorobenzene in water, and by the interaction of the hexachlorobenzene and the lipophilic molecular moieties of the amphipathic phospholipid. Subsequently, all operations with the poisoned systems were carried out in parallel with the control.

Permeability experiment

The centrifugation after the ultrasound treatment was followed by medium-exchange of the sample under examination. The glycine remaining in addition to the liposomes was removed from an 8 ml sample by gel-filtration on a Sephadex G-50 Fine gel column, 2 cm in diameter and 50 cm long, with a throughout rate of 45 ml/h. An isosmotic phosphate buffer solution (pH = 7.2) stabilized with 0.02 % sodium azide was employed as eluent solution. In the course of the gel-filtration the volume of the liposome suspension increased by a factor of three. In order to follow the outward diffusion of the glycine enclosed in the liposomes, moving towards the medium, aliquots were taken at appropriate times after the first gel-filtration (daily in the experimental series performed at 6 ± 0.5 °C, and at roughly hourly intervals for the experiments at 38 ± 0.5 °C) and the media were exchanged by gel-filtration. The glycine content of the medium was determined spectrophotometrically [12].

RESULTS

All the data used to evaluate the measurements are the averages of the results obtained from three different ultrasound treatments. The first membrane-diffusion experiment was performed at 6±0.5 °C. The increase with time of the glycine concentration of the medium exceeded the level of the control in both hexachlorobenzene-poisoned systems (Fig. 1). It can also be observed that with the increase of the hexachlorobenzene content of the liposomes the permeability of the membrane to glycine is also higher.

The van 't Hoff differential method, well known in reaction kinetics, was applied to the concentration vs time graph obtained with the single initial concentration, to compare the mechanisms of the diffusion processes in the control and poisoned systems.

The differential quotient of the curve was determined graphically at the appropriate points. A double logarithmic plot according to the equation $\log(-dc/dt) =$

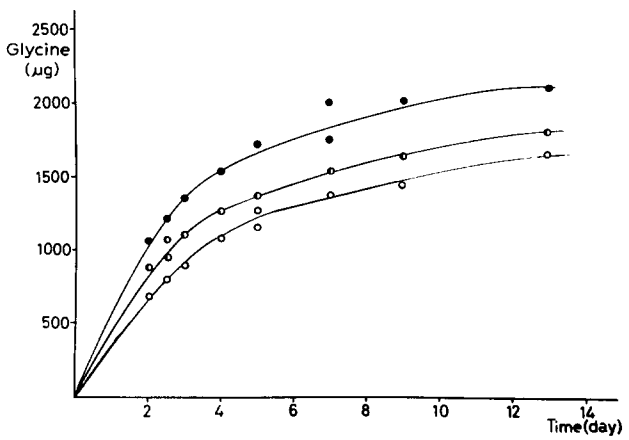

Fig. 1. Time dependence of amount of glycine diffusing from 10 ml liposomes at 6 °C. ○, control system (without hexachlorobenzene); ◐, $3.05 \cdot 10^{-3}$ wt% hexachlorobenzene (on dry phospholipid); ●, $3.05 \cdot 10^{-2}$ wt% hexachlorobenzene (on dry phospholipid).

TABLE I

RATE CONSTANTS

System	Rate constant ($mol^{-3} \cdot l \cdot min^{-1}$)	
	at 6±0.5 °C	at 38±0.5 °C
Control	$4.24 \cdot 10^{-7}$	$2.14 \cdot 10^{-5}$
Poisoned ($3.05 \cdot 10^{-3}$ wt% hexachlorobenzene on dry phospholipid)	$6.87 \cdot 10^{-7}$	
Poisoned ($3.05 \cdot 10^{-2}$ wt% hexachlorobenzene on dry phospholipid)	$9.12 \cdot 10^{-7}$	$2.24 \cdot 10^{-5}$

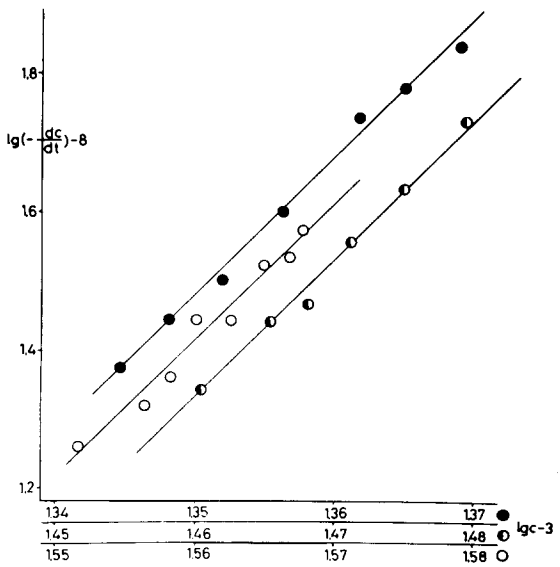

Fig. 2. Application of van 't Hoff differential method for a single initial glycine concentration at 6 °C. (Concentration in mole · l^{-1}, and time in min.) ○, control system; ◐, $3.05 \cdot 10^{-3}$ wt% hexachlorobenzene (on dry phospholipid); ●, $3.05 \cdot 10^{-2}$ wt% hexachlorobenzene (on dry phospholipid). The identical slopes for the control and hexachlorobenzene-poisoned systems confirm the same mechanism for the diffusion processes in the different systems.

$\log k + n \log c$ (where k is the rate constant, and c is the concentration of glycine enclosed in the liposome at time t) than gave a straight line for each system. These had the same slope, as an indication that the diffusion processes in the control and the poisoned systems can be characterized by the same reaction order (Fig. 2). The rate constants for the various parameters are listed in Table I.

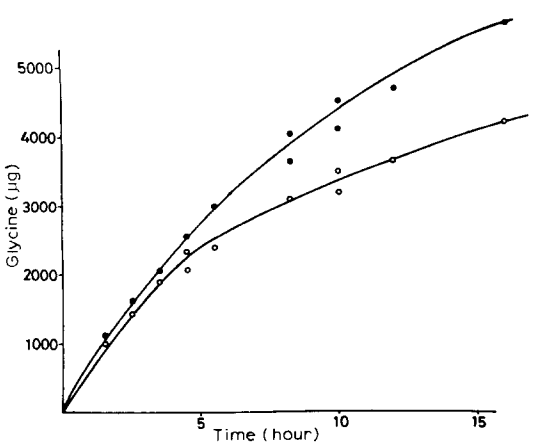

Fig. 3. Time dependence of amount of glycine diffusing out from 10 ml liposome at 38 °C. ○, control system (without hexachlorobenzene); ●, $3.05 \cdot 10^{-2}$ wt% hexachlorobenzene (on dry phospholipid).

In accordance with expectations, when the experiments were carried out at 38 ± 0.5 °C, the process was faster in both the control and the poisoned system. Just as in the case of incubation at 6 °C, however, the glycine penetration was more considerable in the poisoned system (Fig. 3).

The activation energy calculated from the temperature dependence of the rate constants was 20 % less for the system poisoned with the higher hexachlorobenzene concentration than for the control (1.40 and 1.75 kcal/mole, respectively).

DISCUSSION

Following the work of Bangham [13], many groups have carried out model experiments with artificially made phospholipid bilayer or multilayer systems. In the course of these, investigations have been made into the permeability (mainly to ions) of phospholipid membranes modified with various components [14, 15]. A few papers have been published which deal with the penetration of non-ionic substances [16, 17]. Increasing attention has recently been paid to the compositions of the components making up the model membrane [15, 17–22].

Gas-chromatographic investigations were made by Vos et al. [23] with regard to the tissular accumulation following hexachlorobenzene poisoning in animal experiments. Their results indicated that when a diet containing 5 ppm hexachlorobenzene was administered for 90 days the hexachlorobenzene content of the liver attained even 16.7 ppm. Calculated for 5 % tissular lipid, this means a content of 334 ppm. In our own experiments a study was made of the permeabilities to glycine of liposome membranes poisoned with two different hexachlorobenzene concentrations, in comparison with an identically treated control not containing hexachlorobenzene. The hexachlorobenzene concentration selected was 30.5 or 305 ppm, referred to the lipid; this corresponded approximately to the concentration conditions used by Vos et al. At the same time the "Turkish" porphyria was brought about by the prolonged consumption of dressed seed-corn containing hexachlorobenzene in 0.2 %. In our estimation the hexachlorobenzene concentrations selected attain at least the lower limit of the accumulation probable here.

In our experiments only the initial stage of the diffusion process was evaluated, where the factors disturbing the observations, such as an appreciable membrane potential, solvent diffusion, etc., had not developed to any considerable extent.

The role of the phospholipids in lipoprotein membranes is recognized, and we feel that our model experiments have succeeded in proving that hexachlorobenzene leads to a significant increase of the permeability of lipoprotein membranes to water-soluble compounds of low molecular weight. As a strongly lipophilic compound, hexachlorobenzene can be conceived as exerting such an effect by being incorporated into the phospholipid, thereby causing a change there in the strictly controlled orientation of the amphipathic molecules. This orientation change may be sufficient reason for the penetration of hydrophilic substances in the immediate vicinity of the resulting defect sites. Such substances are 5-aminolaevulinic acid, porphobilinogen and uroporphyrin, suitable intracellular concentrations of which are necessary for the initiation of the feed-back and product-inhibition mechanisms involved in the regulation of the process of biosynthesis, and for the establishment of a state of physiological equilibrium. It follows of necessity from such considerations that if the

equilibrium state is disturbed by the loss of any of the precursors, then there will be an increase in the intensity of the processes preceding the development of that precursor.

It must not be forgotten, however, that the phospholipids are not the only components of the membranes in the living organism. The membrane permeability may well be affected simply by the proportion of these other components; in other words, it is highly probable that, besides the orientation disturbances confirmed in the present model experiments, an essential role may be played by the variation of the composition of the components making up the membrane.

Glycine is a good representative of the water-soluble haem-precursors, which presumably have no energy-dependent active retention. Just because of the absence of such active mechanisms, which in most cases are ATP-dependent, the liposome system differs particularly from the cell membranes, but for just this reason too it is convenient to use it to study passive transport conditions without the influence of other, disturbing effects. We consider that any other compound of similar molecular structure, which has a molecular size commensurable with those of the haem-precursors (5-aminolaevulinic acid, porphobilinogen, etc.), will behave similarly in the model system examined here. For this reason, glycine, the solubility of which is also suitable, is just as convenient for the study of the passive transport processes as are the labile haem-precursors.

Our experiments were aimed at clarifying the pathomechanism of "Turkish" porphyria. However, as regards its laboratory characteristics, this disease corresponds fully to porphyria cutanea tarda symptomatica, and displays considerable similarity even clinically; it can reasonably be assumed, therefore, that a very significant role may be played in its development by the change of the membrane structure. Nevertheless, it must be noted that various other causal factors too may be involved in the development of porphyria cutanea tarda.

REFERENCES

1 Cam, C. (1958) Saglik Derg. 32, 215–216
2 Rimington, C. and Ziegler, G. (1963) Biochem. Pharmacol. 12, 1387–1397
3 Vos, J. G. and Koeman, J. H. (1970) Toxicol. Appl. Pharmacol. 17, 656–668
4 Vos, J. G. and Beems, R. B. (1971) Toxicol. Appl. Pharmacol. 19, 617–633
5 Strik, J. J. T. W. A. (1973) Hoppe-Seyler's Z. Physiol. Chem. 354, 13
6 Simon, N. and Siklósi, Cs. Berufsdermatosen, in the press
7 Szabó, É., Berkó, Gy., Husz, S. and Simon, N. (1973) Acta Morphol. Acad. Sci. Hung. 21, 155–163
8 Dawson, R. M. C. (1963) Biochem. J. 88, 414–423
9 Skidmore, W. D. and Entenman, C. (1962) J. Lipid Res. 3, 471–475
10 Finer, E. G., Flook, A. G. and Hauser, H. (1972) Biochim. Biophys. Acta 260, 49–58
11 Erdei, L., Joó, F., Csorba, I. and Fajszi, Cs. (1974) Acta Biochim. Biophys., Acad. Sci. Hung. 9, 121–129
12 Sardesai, V. M. and Provido, H. S. (1970) Clin. Chim. Acta 29, 67–71
13 Bangham, A. D. (1968) in Progress in Biophysics and Molecular Biology (Butler, J. A. V. and Noble, D., eds), Vol. 18, pp. 31–95 Pergamon Press, Oxford
14 Petkau, A. and Chelack, W. S. (1972) Biochim. Biophys. Acta 255, 161–166
15 Gutknecht, J. and Tosteson, D. C. (1970) J. Gen. Physiol. 55, 359–374
16 Cohen, B. E. and Bangham, A. D. (1972) Nature 236, 173–174
17 Demel, R. A., Bruckdorfer, K. R. and van Deenen, L. L. M. (1972) Biochim. Biophys. Acta 255, 321–330

18 Li-Fu Chen, Lund, D. B. and Richardson, T. (1971) Biochim. Biophys. Acta 225, 89–95
19 De Gier, J., Mandersloot, J. G. and van Deenen, L. L. M. (1968) Biochim. Biophys. Acta 150, 666–675
20 Demel, R. A., Geurts Van Kessel, W. S. M. and van Deenen, L. L. M. (1972) Biochim. Biophys. Acta 266, 26–40
21 Lelievre, J. and Rich, G. T. (1973) Biochim. Biophys. Acta 298, 15–26
22 Horton, A. W., Schuff, A. R. and McClure, D. W. (1973) Biochim. Biophys. Acta 318, 225–234
23 Vos, J. G., van der Maas, H. L., Musch, A. and Ram, E. (1971) Toxicol. Appl. Pharmacol. 18, 944–957