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IONIC TRANSPORT THROUGH MODEL MEMBRANES

III. CONTRASTING EFFECTS OF TESTOSTERONE AND 5-α-DIHYDROTESTOSTERONE. DEPENDENCE ON TEMPERATURE AND BOUND WATER

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SUMMARY

1. The transfer of the dyestuff probe, methyl orange, from an aqueous phase into phospholipid–cholesterol micelles in an adjacent organic phase was found to be affected by testosterone quite differently than by dihydrotestosterone. The kinetics of transport of the probe proved extremely dependent in the presence of dihydrotestosterone upon the extent of prehydration of the organic phase. Rates were measured at controlled temperatures from 19 to 45 °C.

2. For the system, lecithin–cholesterol–testosterone at selected concentrations, high rates of transfer of methyl orange were observed between 23 and 35 °C. Below 22 and above 36 °C, the transport rates were reduced to those observed with lecithin alone.

3. For the corresponding system containing dihydrotestosterone the transition at 35 °C was essentially the reverse of that for the system containing testosterone. Low rates were observed below 35 °C, elevated rates above this temperature.

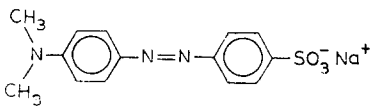
INTRODUCTION

In previous papers, we have reported that the rate of ionic transport in a physical system developed as a model of the membrane-cytoplasm interface proved to be sensitive to the inclusion of cholesterol, steroid hormones, and cocarcinogenic *n*-alkanes [1, 2]. The model involved the transfer of a water-soluble ionic probe, Orange IV, from a buffered aqueous KCl solution into an adjacent hydrocarbon phase containing micelles of membrane lipids, with an essential requirement of unsaturated phospholipids, either natural (egg phosphatidylcholine) or synthetic lecithin (1-oleyl-2-hexadecyl ether).

However, under the experimental conditions employed, little difference was observed in the effects of the two most active androgens, testosterone and 5-α-dihydrotestosterone. Although the basic cellular mechanisms of their biological

activity remain obscure, it is apparent that these two steroids affect the rate of growth and the secretory capacity of various male organs to differing extents [3, 4].

Prehydration of the organic phase micelles before contact with the dyestuff probe led to a modified transport system differentially susceptible to the effects of these androgens. Replacement of Orange IV by the more water-soluble methyl orange yielded a more suitable probe, one that could be readily transferred from the lipid micelles to a second aqueous phase. This azo dye is derived from sulfonation from the classical liver carcinogen, butter yellow.



The differences between the effects of the two androgens on transport kinetics have been measured at controlled temperatures over the range 19–45 °C. It will be shown that transitions occur at approximately 23 and 33 °C, in opposite directions for the two steroids.

MATERIALS AND METHODS

Egg phosphatidylcholine, General Biochemicals, single spot by thin-layer chromatography. Cholesterol, Merck, triply recrystallized from methanol. Testosterone and dihydrotestosterone, Schwartz-Mann. Methyl orange, sodium *p*-(*p*-dimethylaminophenylazo)-benzene sulfonate, Matheson, Coleman and Bell, recrystallized twice from doubly distilled water and once from 95 % ethanol. Potassium 2-*p*-toluidinylnaphthalene-6-sulfonate, Sigma Chemical, recrystallized twice from 2 % KOH aq., sample courtesy of Dr G. Witz, New York University Institute of Environmental Medicine.

Aqueous phase (solvent for dyestuff probes), 134 mM KCl, 4 mM phosphate buffer (MCB BX1635-5, pH 7 concentrate, 12.3 ml diluted in 1 l doubly distilled (glass) water). Solvent for lipids, *cis*- and *trans*-decalin, Matheson, Coleman and Bell, practical grade, chromatographed on activated silica gel to remove impurities, especially those responsible for ultraviolet absorbance at 260–280 nm (e.g. tetralin).

Experimental procedure

4 ml of the colloidal solutions of 0.3 mM egg phosphatidylcholine plus selected steroids in decalin were allowed to equilibrate and prehydrate by standing for 2 h over 2 ml of the buffered KCl solution in a 50-ml round-bottomed flask and then shaken on a Burrell wrist-action shaker (at a maximum setting) for $\frac{1}{2}$ h before contacting the dye solutions. Temperatures were controlled within about 0.5 °C by immersion of the mixing flasks in large constant temperature baths.

The azo dye in the buffered KCl solution (40 μM in the total aqueous phase) is then added and shaken with an equal volume of the equilibrated solution of lipids in decalin for specified periods of time, and finally rinsed into centrifuge tubes using 2 ml of fresh decalin followed by 2 ml of fresh buffered KCl aq. The emulsions are broken by centrifugation at 1700 × *g* and the absorbance of the clarified phases determined in a Beckman DB spectrophotometer.

The actual recovery of dyestuff in the clarified aqueous and organic phases is calculated on the basis of its absorbance at the maximum in the region of 410–465 nm (315–325 nm in the case of toluidinylnaphthalene sulfonate) and the volume of each phase recovered. The distribution of dyestuff is reported as the percent of the original quantity that has disappeared from the aqueous phase after specified periods of mixing, the percent found in the organic phase, and the percent remaining at the interfaces (by difference). The rate of disappearance of the dyestuff probe from the aqueous phase is also referred to as the rate of interfacial adsorption, since this is the first step in the transport process. In the absence of phospholipid in the organic phase, it may be the only step.

RESULTS

Attempts to dissolve the crystalline anionic dyestuff probes directly into the dry colloidal solution of egg phosphatidyl choline in decalin have been largely unsuccessful. Hence, in the model transport system hydration of the lipid micelles at the interface and in the bulk organic phase was assumed to be a necessary initial step in the transfer of the water-soluble dyes from the aqueous to the organic phase.

However the modification of the experimental procedure to prehydrate the lipid solutions prior to the introduction of the aqueous dye solutions has had somewhat unexpected effects on the rates when certain steroids were involved, particularly the most active androgen, 5-α-dihydrotestosterone [5]. In Fig. 1 are shown the effects of the prehydration procedure at 26 °C on the rate of transport of methyl orange into mixed micelles of egg lecithin, cholesterol, and androgens. Transport

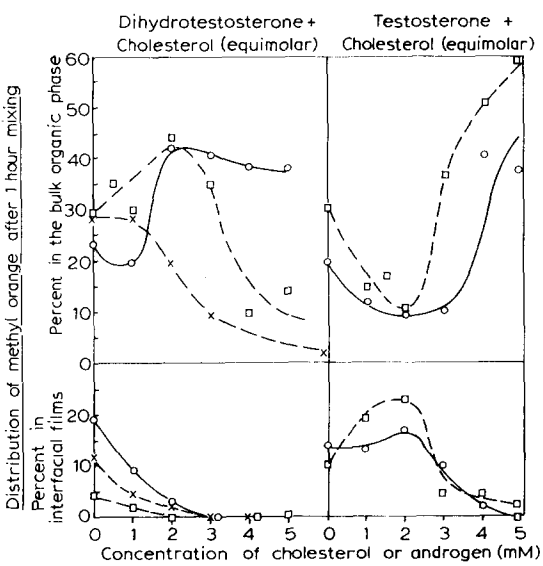


Fig. 1. Effects of prehydration of the organic phase on the rate of transport of methyl orange at 26 °C. ○—○, 0.3 mM egg lecithin, ◦ mM cholesterol, × mM androgen; □ - - □, same system saturated with water prior to mixing with dye solution; ◦ - - ×, 0.3 mM egg lecithin, × mM cholesterol prehydrated.

rates were reduced by prehydration of micelles containing dihydrotestosterone above 3 mM, but not much altered in the presence of testosterone, which gives the opposite effect above 3 mM, i.e. high rates, whether prehydrated or not.

The reduction in the apparent transport rate by testosterone at 1–2 mM, as estimated by the appearance of the dyestuff probe in the organic phase, is actually associated with an increased retention of the probe in interfacial films. Dihydrotestosterone gives this effect at 1 mM, but to a much lesser extent, especially when prehydrated. Note that the mechanism of retardation above 3 mM of the dihydro derivative is fundamentally different than at 1 mM. It does not involve increased retention of dye in interfacial films, but instead is a retardation of interfacial adsorption from the aqueous phase. That this particular retardation is not seen unless the prehydration procedure is used demonstrates that the saturation of the lipid micelles with water is not a simple initial phase that facilitates the solubilization of the anionic dyestuff, indeed that it may have the opposite effect depending upon the particular steroids involved.

Retardation by 5 mM dihydrotestosterone is comparable to that for the cholesterol–lecithin system in the absence of androgen. In the previous paper [2], it was shown that 5 mM cholesterol reduced the initial rate of dye adsorption but increased the ultimate negative free energy of transfer to bulk phase micelles. An equilibrium study on the prehydrated system containing 0.3 mM egg lecithin, 5 mM cholesterol, and 5 mM dihydrotestosterone gives similar results, i.e. $>60\,\%$ of the methyl orange in the bulk organic phase after 24 h mixing.

Reference to our earlier use of Orange IV as the dyestuff probe would demonstrate that the rates of transfer of methyl orange across the interface are much lower. The preference shown by methyl orange for the aqueous phase reflects its greater water solubility (methyl Orange, 1.2 mM, Orange IV, 0.08 mM in buffered KCl at 25 °C). In contrast to the tenacity of the lipid micelles for Orange IV previously reported [1], the transport of methyl orange is rapidly reversed by shaking the organic phase with fresh KCl aq. (unless the lipid composition is 0.3 mM egg lecithin plus 5 mM cholesterol, and androgens are absent).

A limited investigation has been made of the effects on transport kinetics of other structural changes in the dyestuff probe. In particular the arylamino-substituted fluorescent probe [6], potassium 2-*p*-toluidinylnaphthalene-6-sulfonate, is transported by egg lecithin at rates comparable to Orange IV. Further, the apparent effects of cholesterol plus the androgenic steroids on the rate of transport of this probe were generally paralled to those for Orange IV [2]. Technical difficulties were encountered with the use of this dye. Its absorption maximum at 320 nm is subject to considerable interference in this model system, especially in the presence of the androgens. However, in view of the frequent use of the fluorescent arylaminonaphthalene derivatives as probes of micellar or membrane structure (see, for example, ref. 8), it is of interest to note that their transport is affected by steroids like that of the azo dye probes.

All of the results presented to this point were obtained at 25–26 °C. In view of the dramatic differences in transport rates associated with the incorporation of 5 mM testosterone or dihydrotestosterone, the obvious question was raised about the differences that might obtain at physiologic temperatures.

Referring again to Fig. 1, which shows the effects of the two androgens on transport kinetics and equilibria at 26 °C, the following differences at 38 °C may be

cited. As would be expected, in general equilibrium is achieved much more rapidly at the higher temperature (in less than $\frac{1}{2}$ h for all systems except those containing 5 mM testosterone). For the basic system without steroids, the organic/aqueous distribution of methyl orange is reduced slightly. At 38 °C, dihydrotestosterone up to 5 mM (plus equimolar cholesterol) has no significant effect. It will be seen in the subsequent details of the temperature dependence over the range 19–45 °C that this androgen has its main influence on the distribution below 35 °C.

In contrast the unsaturated androgen, testosterone, still has significant effects at 38 °C (in combination with equimolar cholesterol). At $\leqslant$ 3 mM of the hormone there is the usual marked retention of the dyestuff probe in interfacial films. Only at 5 mM is the equilibrium transfer of methyl orange to lipid micelles in the organic phase enhanced by this steroid, and to a much lesser extent than at 26 °C (47 versus 77 %).

In Fig. 2 is presented the transport in 1 h of methyl orange to prehydrated micelles of 0.3 mM egg lecithin plus 5 mM of a single steroid in the bulk organic phase at temperatures from 19–45 °C. In contrast to the previously reported [2] dramatic effects of testosterone on the transport of Orange IV at 25 °C by lecithin in this model system it has almost no effect on the rate of transport of methyl orange without the addition of cholesterol.

In contrast 5 mM dihydrotestosterone enhances the rate at temperatures up to 35 °C. The marked retention of methyl orange in interfacial films of egg lecithin in the absence of steroid (see Fig. 3) is completely eliminated by inclusion of 5 mM of any of the single steroids.

The subtle dependence of the micellar organization on the structure of the androgen is apparent from the results in Fig. 3 on the effects of equimolar combinations of the steroids. In the range 23–30 °C, testosterone, which had no effect on the lecithin micelle by itself, now enhances transport of the ionic probe when it is in combination with cholesterol, whereas in contrast, dihydrotestosterone plus cholesterol retards transport up to 30 °C.

The modification of lecithin micellar structure by combinations of cholesterol

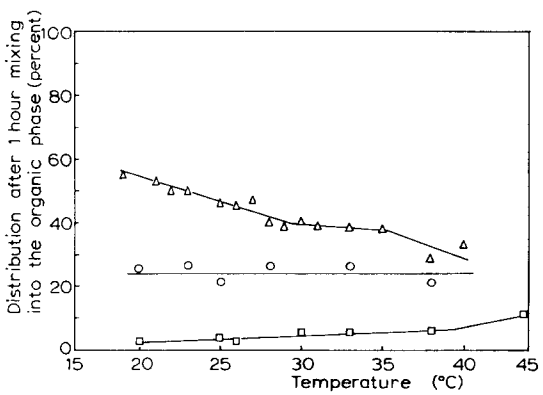


Fig. 2. Temperature dependence of rate of transport of methyl orange. △ – △, 0.3 mM egg lecithin, 5 mM dihydrotestosterone; ○ – ○, 0.3 mM egg lecithin, 5 mM testosterone; □ – □, 0.3 mM egg lecithin, 5 mM cholesterol.

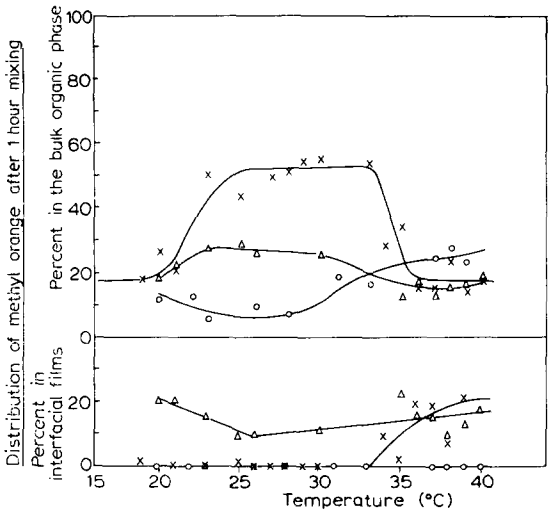


Fig. 3. Temperature dependence of rate of transport of methyl orange. △ — △, 0.3 mM egg lecithin (no steroids); × — ×, 0.3 mM egg lecithin, 5 mM cholesterol, 5 mM testosterone; ○ — ○, 0.3 mM egg lecithin, 5 mM cholesterol, 5 mM dihydrotestosterone.

with either androgen produces dramatic transitions in the 30–35 °C range (Fig. 3). The kinetic changes for testosterone plus cholesterol with increasing temperature generally reflect a much greater retention of the dyestuff probe in interfacial films as well as a decreased rate of adsorption from the aqueous phase. However, in the case of dihydrotestosterone plus cholesterol, a greatly increased rate of disappearance from the aqueous phase is involved with a continued high efficiency of conversion to bulk phase micelles.

It should be emphasized that the large differences in transport rates for the various systems of Fig. 3 are dependent upon the degree of unsaturation of the lecithin and freedom from autoxidation products. The iodine number of our phospholipid (General Biochemicals) has ranged from 57 to 59. A limited study of the effect of other egg lecithins having lower iodine numbers indicates that the differences in rates at 26 °C for the various steroid combinations of Fig. 3 tend to be much reduced.

DISCUSSION

During the past decade a number of techniques have been developed to probe the structure of membranes or model systems. The phospholipid-mediated transport system of the present research utilizes two of the main types of molecular structures that have been used as probes.

It has already been pointed out that toluidinylnaphthalene sulfonate, one of the aromatic sulfonates used by others [6–8] as conformation-sensitive fluorescent probes, has been used as the transported ion in our model system. Its behavior therein is very similar to that of the anionic azo dye, Orange IV.

In the consideration of interaction "sites" for these aromatic sulfonates in phospholipid films, it is of interest to note the results of the fluorescence studies of

Sackmann and Träuble [7]. They added 8-anilino-1-naphthalene sulfonate to an aqueous dispersion of dipalmitoyllecithin and found that the quantum yield for the fluorescence was $Q = 0.08$, characteristic of a local environment with a dielectric constant of about 40. This would place the probe within the structured water bound to the ionic headgroups of the phospholipid.

From our experiments on the effect of prehydration of systems containing dihydrotestosterone, water in the saturated organic phase may remove an appreciable fraction of the interaction sites for adsorption of the dyestuff probe, methyl orange, to the interfacial lipid films. It seems likely that these sites are the closely associated cluster of hydrogen-bonded water molecules bound to the lipid headgroups. But what the orientation of the difunctional androgens is in such films has not been established to date.

A second important type of compound used as a probe of the orientation of steroids in lipid vesicles is a paramagnetic nitroxide derivative of the androgen dihydrotestosterone [7, 9]. In the interpretation of the results of ESR studies of aqueous dispersions of lipid mixtures containing these "spin labels", it is usually assumed that the OH group of the androgen is anchored in the aqueous interface. However the striking differences in the rate of interfacial adsorption of the aromatic sulfonate, methyl orange, depending upon the extent of prehydration of the lipid micelles that contain dihydrotestosterone (see Fig. 1), suggest that this androgen particularly may not be anchored to the interface with the bulk aqueous phase. In contrast to the more polar testosterone, the dihydro-derivative may be readily withdrawn from the interface and associated with structured water bound within the phospholipid vesicle or micelle.

It would appear that comparative ESR studies of nitroxide derivatives of testosterone versus dihydrotestosterone would provide useful information on differences in conformation as a function of the degree of dispersion of the different lipid mixtures in water. It might be expected that the sonication procedure used to prepare the dispersions would thoroughly hydrate the phospholipid vesicles.

But why does prehydration affect the transport properties of systems containing dihydrotestosterone so much more than it does those containing testosterone? It may be relevant to note that, although the solubilities of these two steroids are similar in a liquid hydrocarbon, such as decalin, their solubilities in a hydrogen-bonding solvent, such as 95 % ethanol, differ by a factor of 3 (testosterone being much more soluble). Perhaps in involving a much greater contribution of the enolic configuration to the equilibrium structure of the unsaturated ketone, there may well be an enthalpy of hydration of this steroid that strongly favors its location at the aqueous interface (more than making up any entropy loss associated with loss of a degree of freedom). Thus there may well be a large difference in the free energy of hydration of testosterone at the aqueous interface as compared to hydration by water molecules in the hydrocarbon phase, solvated by decalin or in phospholipid micelles.

At this stage of limited knowledge of the mechanisms of the biological activities of these androgens, one would be speculating idly about the relevance of these differences in transport kinetics. Note that the concentrations in the bulk organic phases are not to be compared with physiologic concentrations. Only the fraction of androgen in the lecithin micelles and films is significant. Since cholesterol also forms micelles in decalin, it has been difficult to assess the effects of the steroids on the size and shape

of the lecithin micelles by light scattering, which provides only a weight average molecular weight, M_w [10]. We are looking forward to some new developments in gel permeation chromatography which may make it possible to fractionate these colloidal systems in hydrocarbon solvents. It will then be possible to analyze the isolated lecithin micelles for their content of steroids.

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REFERENCES

1 Horton, A. W. and McClure, D. W. (1971) Biochim. Biophys. Acta 225, 248–253
2 Horton, A. W., Schuff, A. R. and McClure, D. W. (1973) Biochim. Biophys. Acta 318, 225–234
3 Mann, T., Rowson, L. E. A., Baronos, S. and Karaginannidis, A. (1971) J. Endocrinol. 51, 707–718
4 Baulieu, E., Lasnitzki, I. and Robel, P. (1968) Biochem. Biophys. Res. Commun. 32, 575–577
5 Liao, S., Liang, T., Fang, S., Castañeda, E. and Shao, T. (1973) J. Biol. Chem. 248, 6154–6162
6 Witz, G. and Van Duuren, B. L. (1973) J. Phys. Chem. 77, 648–651
7 Sackmann, E. and Träuble, H. (1972) J. Am. Chem. Soc. 94, 4482–4510
8 Wallach, D. F. H., Ferber, E., Selin, D., Weidekamn, E. and Fischer, H. (1970) Biochim. Biophys. Acta 203, 67–76
9 Hubbell, W. F. and McConnell, A. M. (1971) J. Am. Chem. Soc. 93, 314–326
10 Stacey, K. A. (1956) Light-scattering in Physical Chemistry, p. 2, Butterworths, London