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EFFECT OF HYDROCARBON CHAIN LENGTH ON RENAL TRANSPORT OF MONOSUBSTITUTED SULFAMYL BENZOIC ACID DERIVATIVES AND PROBENECID

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Abstract—The characteristics of renal transport of a homologous series of hydrocarbon derivatives of sulfamyl benzoic acid were examined in cortical slices of rabbit kidney. The length of the hydrocarbon substituent varied from C_1 to C_5, and the compounds differed from the previously examined probenecid series [5] in having only one hydrocarbon substituent, instead of two, at the sulfamyl group. Transport rate, maximal accumulation and affinity of the monosubstituted compounds for transport were enhanced by an increase in the length of the hydrocarbon substituent. In comparison with the probenecid series less binding by tissue constituents under anaerobic conditions was observed. Competition experiments indicated that the monosubstituted compounds were transported by the same transport system as that of PAH. Octanoate and fumarate exerted a biphasic effect on accumulation (i.e. stimulation at low and inhibition at high metabolite concentrations), as in the case of other organic anions. In comparison with probenecid the monosubstituted compounds were more susceptible to inhibition by PAH, fumarate, and octanoate. Nevertheless, pentylsulfamyl benzoic acid exerted a somewhat more powerful inhibitory effect on the accumulation of phenol red and PAH than did probenecid. The monosubstituted compounds were less hydrophobic than probenecid as evidenced by less extractability from an aqueous phase into an organic liquid (1,2-dichloroethane). It is concluded that for the sulfamyl benzoic acid derivatives moderate hydrophobicity results not only in a higher affinity, but also in an enhanced turnover rate of the carrier system for organic anions.

Probenecid (di-n-propyl sulfamyl benzoic acid) is a substrate of the organic anion transport system in kidney [4]. The drug is therapeutically valuable because of a strong inhibitory potency on the excretion of other organic anions and a low urinary excretion [2–3], resulting from reabsorption by non-ionic diffusion [4]. In a previous communication we reported our data on the active transport of homologous sulfamylbenzoic acid derivatives of probenecid as studied in the kidney slice system [5]. The hydrocarbon length of the compounds varied from C_1 to C_4. The affinity of the compounds for the transport system was found to increase as a function of the hydrocarbon length. Higher accumulation ratios were obtained with probenecid $[C_3H_7]_2$-R than with any of the other compounds, at a medium concentration of 0.04 mM. Thus the results suggested that the chemical structure of probenecid possesses optimal properties for acquisition of high transport rates. But the results were not quite conclusive in this regard, since no extensive studies were performed on accumulation at different medium concentrations, due to a shortage of the radiochemically labelled compounds.

The above mentioned data are also of interest in connection with the role of hydrophobic interactions for the renal transport of organic anions. Forster and colleagues [6–8] in their studies on uptake of phenolsulfophthalein dyes by fish tubules noted an inverse relationship between affinity and maximal rates of transport, i.e. compounds with a strong inhibitory potency are sluggishly transported. These results have been confirmed by studies in the mammalian kidney [9–13]. The compounds with a high affinity for the transport system are also characterized by a pronounced hydrophobicity [13]. The dye data in conjunction with the results obtained in the probenecid series are consonant with the view that the presence of substituents, leading to a moderate increase in hydrophobicity, promotes active transport rates, while a higher degree of hydrophobicity results in inhibition of the turnover of the carrier system. In the present communication we report the behaviour of a homologous series of sulfamyl-benzoic acid derivatives which in contrast to the probenecid series is only monosubstituted with hydrocarbon. The length of the hydrocarbon chain was varied from one to five carbon atoms. The compounds were found to be moderately hydrophobic as tested by partitioning between an aqueous and organic liquid phase. With this series of compounds we have been able to show that an increase in hydrocarbon length resulted in a distinct increase, not only in the affinity for the transport system, but also of maximal rates of transport. In addition, the transport process for these compounds was compared with that of other organic anions.

METHODS

Kidney slices and homogenate experiments. The experimental procedures were essentially the same as those recently described by us [5]. In short, kidneys were excised from anesthetized and exsanguinated rabbits, and cortical slices were prepared with a razor blade. The slices were incubated in media containing 145 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM sodium phosphate buffer (pH 7.40) and 0.04 mM of one of the radioactive derivatives of sulfamyl benzoic acid (CH_3-R, C_2H_5-R, C_3H_7-R, C_4H_9-R, or C_5H_{11}-R), together with the non-labelled form of the compound in some experiments (Fig. 2) to attain the desired medium concentration. Metabolites (acetate, fumarate and octanoate) and other substrates of the organic anions transport system (*p*-aminohippurate, phenol red) were added to the incubation media as described in the legends to the figures. All incubations were carried out for 2 hr at 25° in a 100% O_2 atmosphere, except in some of the experiments plotted in Fig. 1, where the experiments were also performed under 100% N_2 and for different time intervals. After the incubation the slices were blotted on a piece of filter paper, weighed and dissolved by treatment with 1 N NaOH at 80° for 20 min. A sample of the medium (1 ml) was deproteinized by addition of 4 ml 5% (w/v) trichloroacetic acid.

Binding of radioactive derivatives of sulfamyl benzoate to kidney cortical homogenates was examined by an ultrafiltration technique [14]. A 20% (w/v) homogenate of renal cortex was prepared in a solution containing 0.25 M sucrose, 0.033 M sodium phosphate buffer (pH 7.4) and 0.1 mM of the radioactive compound. Binding was determined from comparison of the radioactive contents in the ultrafiltrate with that of a reference solution in which the volume occupied by the tissue in the sample had been replaced by homogenization solution [13].

Distribution of sulfamyl benzoic acid derivatives between 1,2-dichloroethane and aqueous phases. Monosubstituted sulfamyl benzoic acid derivatives and probenecid were dissolved in 0.033 M sodium phosphate buffer solutions (pH range 1.8–7.0) at a final concentration of 15 mg/l. Equal volumes (5 ml) of the 0.033 M phosphate solutions and 1,2-dichloroethane were mixed and vigorously agitated for 2 hr at 25°. The amount of derivative remaining in the 1,2-dichloroethane phase was determined from measurement of light absorption at 244 nm on a Zeiss PMQ II spectrophotometer.

Preparation of sulfamyl benzoic acid derivatives. [^{35}S]Chlorosulfonyl benzoic acid was prepared according to the method of Meerwin *et al.* [15] as modified by us [5]. Equimolar amounts of the sulfonylchloride and the appropriate alkylamine (methyl-, ethyl-, n-propyl-, n-butyl- and n-pentylamine) were mixed to produce 4-(alkyl) sulfamyl benzoic acids. The precipitated product was collected on filter paper and washed twice with water before drying *in vacuo*. The product was further purified by thin layer chromatography using aluminium sheets coated with silica gel. The material was dissolved in methanol and applied in a narrow band on the plates. A mixture of light petroleum–diethyl ether–acetic acid (50:50:2, by vol.) was used for development of the plates. The main component after chromatography, located by an ultraviolet lamp, was cut out from the plate and eluted with methanol. The eluate was centrifuged and finally the methanol was evaporated by flushing the solution with oxygen-free nitrogen. The purity of the products was checked by melting point determinations. The following melting points were found; values in parentheses are melting points taken from Itaya *et al.* [16]: 4-methyl, 243° (246°); 4-ethyl, 220° (220.5°); 4-propyl, 217° (211.5°); 4-butyl, 218° (216°), and 4-pentyl sulfamyl benzoic acid, 220.5° (208.5°). The molecular weights of these compounds are as follows: 4-methyl (215), 4-ethyl (229), 4-propyl (243), 4-butyl (257), and 4-pentyl (271). The corresponding "cold" compounds were prepared in large scale in a similar way, except that they were purified on short silica gel 60 columns using the same solvent system as for thin layer chromatography.

Analytical procedures. The radioactive content of the NaOH digested solution of renal tissue and the trichloroacetic acid filtrates of the medium was measured in a liquid scintillation counter as previously described [5]. The amount of PAH and phenol red was estimated chemically after extraction from the renal tissue and medium as reported earlier [17]. Inulin space and dry weight were estimated in separate experiments as described in [13].

Calculation of tubular accumulation. The uptake of sulfamyl benzoic acid derivatives, PAH and phenol red in the renal tubule was calculated as the difference between the content of these compounds in the whole slices and that present in the inulin space. This difference was then divided by the tubule water content (wet weight of tissue minus inulin space minus dry weight) to obtain the tubular concentration of the compounds from which the accumulation was calculated by division with the medium concentration after the incubation.

RESULTS

Tubular accumulation of monosubstituted sulfamyl benzoate. Figure 1 shows the uptake of monosubstituted sulfamyl benzoates by renal cortical slices as a function of time after addition of the compounds to the incubation medium at concentration of 0.04 mM. Uptake occurs rapidly during the first 15 min, but equilibrium is not approached until after incubation for 2 hr, as has been found for other organic anions [17]. The rate of uptake is increased with the length of the hydrocarbon substituent. All the compounds are accumulated by an active mechanism as evidenced by a much higher rate of uptake under aerobic than under anaerobic conditions. Slight anaerobic accumulation, presumably as a consequence of binding to tissue constituents [1, 5], is observed for C_4H_9-R and C_5H_{11}-R (shown as unfilled symbols in Fig. 1), while the final tubule concentration of the compounds with the smaller hydrocarbon substituent did not differ from that of the medium (not shown). The existence of binding to tissue constituents was confirmed in kidney tissue homogenate experiments which indicated a binding for C_4H_9-R of 8.7 ± 1.7% (5 experiments) and for C_5H_{11}-R of 10.2 ± 2.1% (5 experiments) at 0.1 mM total concentrations of the compound in a 20% homogenate. Binding of CH_3-R and C_2H_5-R was not statistically different from zero, while C_3H_7-R was bound to an intermediate extent (5.1 ± 2.3%, 5 experiments).

Figure 2 shows the amount of sulfamyl benzoate derivative accumulated at different medium concentrations after an incubation period of 2 hr. It is seen that, in agreement with the results of Fig. 1, the accumulated amount increases with the length of the hydrocarbon substituent at all medium concentrations above 100 μM. Only in the case of CH_3-R is there any

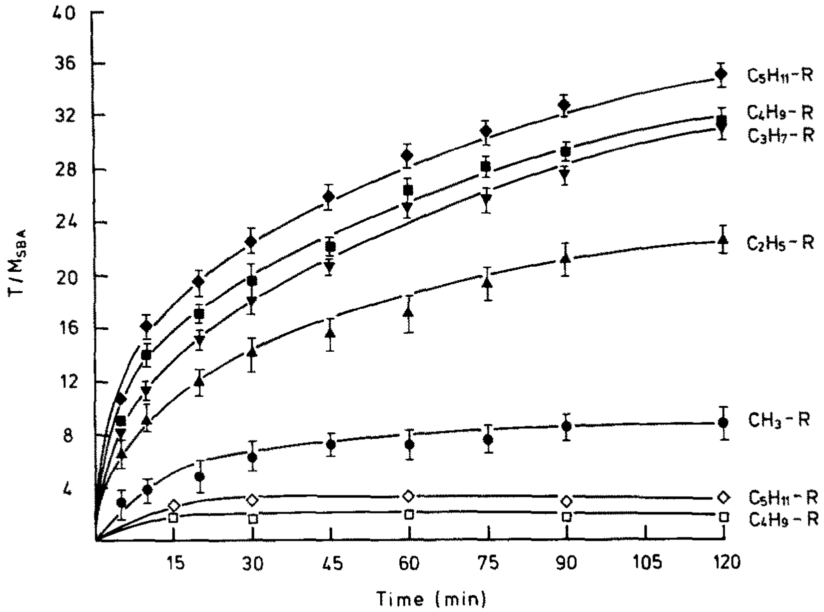

Fig. 1. Uptake of CH_3-R (●——●), C_2H_5-R (▲——▲), C_3H_7-R (▼——▼) C_4H_9-R (■——■), and C_5H_{11}-R (♦——♦) in the presence of acetate (10 mM), under aerobic conditions. Corresponding open symbols represent accumulation of C_4H_9-R and C_5H_{11}-R under anaerobic conditions. R denotes sulfamyl benzoic acid moiety. The initial concentrations of sulfamyl benzoic acid analogues were 0.04 mM. Ordinate: T/M_{SBA} ratio between the concentration of sulfamyl benzoic acid analogues in the tubule water and medium. Abscissa: time of incubation. Note: the same symbols have been used in this paper to indicate the renal accumulation of these compounds. Fig. 1 and subsequent figures in this paper show mean values of four to six experiments. Vertical bars indicate ± S.E.

appreciable rise in the accumulated amount above this medium concentrations.

Figure 3 shows that the accumulation of the sulfamyl benzoate derivatives is severely reduced at high PAH concentrations, being complete in the case of CH_3-R at 5 mM medium PAH. Half-maximal reductions of accumulation of the monosubstituted derivatives occur at a medium concentration of around 0.5 mM. By contrast previous data [5], as depicted by the broken line, indicate less efficient inhibition of probenecid accumulation by PAH. It is noteworthy that the inhibitory effect of unlabelled PAH on the accumulation of $[^3H]$-PAH follows a similar curve to that of the monosubstituted derivatives.

Figure 4 shows that probenecid drastically reduces the accumulation of the monosubstituted derivatives. In comparison with PAH, inhibition by probenecid oc-

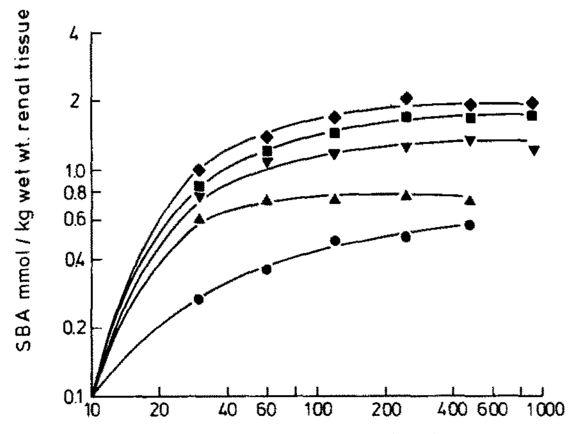

Fig. 2. Uptake of sulfamyl benzoic acid analogues in slices from rabbit kidney cortex at various concentrations of these compounds in the medium under aerobic conditions. Acetate (10 mM) was added as metabolic substrate to the incubation medium. For further details see Fig. 1.

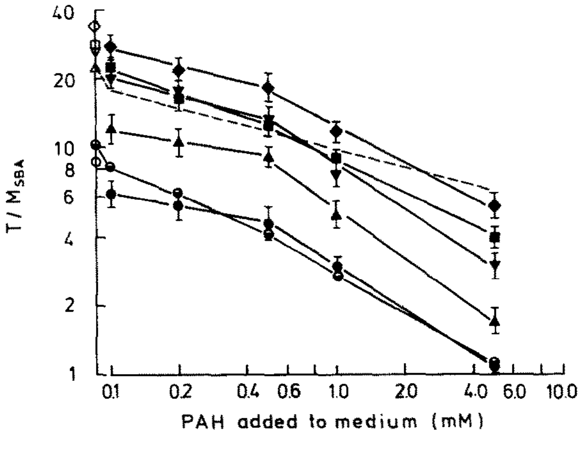

Fig. 3. Effect of increasing concentrations of PAH on the aerobic accumulation of sulfamyl benzoic acid analogues and $[^3H]$-PAH (⊖——⊖), in the presence of acetate (10 mM). The initial concentration of sulfamyl benzoic acid analogues and labelled PAH (specific activity 289 mCi/mmole) was 0.04 mM. The broken line shows the accumulation of probenecid under similar conditions. For further details see Fig. 1.

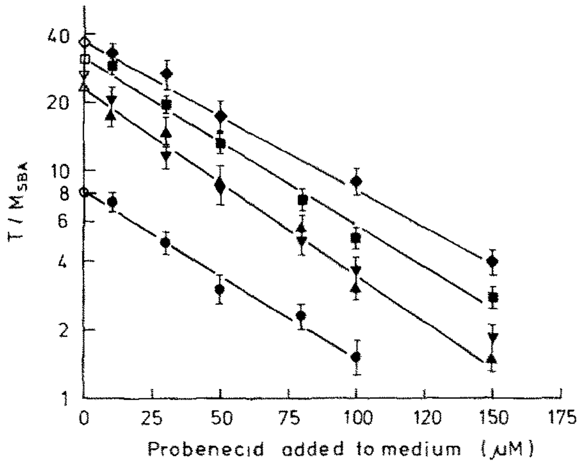

Fig. 4. Influence of probenecid on the aerobic accumulation of sulfamyl benzoic acid analogues in the presence of acetate (10 mM). The initial concentration of sulfamyl benzoic acid analogues was 0.04 mM.

curs at lower medium concentrations, half-maximal reduction of accumulation requiring approx. 50 μM probenecid.

Effect of alkylsubstituted sulfamyl benzoate on PAH and phenol red accumulation. The results depicted in Fig. 5A and B indicate that alkylsubstituted sulfamyl benzoate compounds have a strongly inhibitory effect on PAH and phenol red accumulation, in agreement with their high affinity for transport. Phenol red accumulation is somewhat less affected than that of PAH, in agreement with other studies which indicate a higher affinity of the dye than of PAH for the transport system. The inhibitory effect increases with the length of the hydrocarbon substituent. Virtually complete inhibition of PAH accumulation is observed by high concentrations of the C_{2-5} compounds. It is of interest to note that in comparison with C_5H_{11}-R, the previous data on probenecid [5] indicate less inhibition of the accumulation of phenol red and PAH. This occurs in spite of the more pronounced inhibitory effect of PAH on the accumulation of C_5H_{11}-R than on probenecid (Fig. 3). The possible significance of this finding is considered in the Discussion.

Effect of metabolites on accumulation of sulfamyl benzoate derivatives. Accumulation of organic anions exhibits typical patterns of stimulation and inhibition by various metabolites (17). Fig. 6A and B show that 0.5 mM octanoate and 2 mM fumarate produce peak stimulation of sulfamyl benzoate derivatives, while higher metabolite concentrations have an inhibitory effect. A biphasic action of these metabolites on renal accumulation of PAH and phenol red has previously been noted [17]. However, as shown by the broken lines, probenecid is less amenable to the inhibitory effect of the metabolites than are the monosubstituted derivatives.

As in the case of other organic anions acetate only has a stimulatory effect on the accumulation of sulfamyl benzoate compounds. The accumulation in the presence of 10 mM acetate is similar to peak values obtained in the presence of fumarate and octanoate as can be seen by comparison of Fig. 6A and B with Fig. 1.

Extractability of sulfamyl benzoic acid compounds from an aqueous to an organic liquid phase. We have used extractability from an aqueous into an organic liquid phase as a relative measure of the hydrophobicity of the sulfamyl benzoic acid compounds. 1,2-Dichloroethane was used as the organic liquid phase rather than octanol (which was employed previously in a similar study on the hydrophobicity of phenolsulfophthalein dyes [13]) because of the better solubility of the sulfamyl benzoic acid compounds in the former solvent. Figure 7 shows that CH_3-R is slightly extracted from the aqueous medium, while C_5H_{11}-R and probenecid are almost quantitatively transferred to the organic phase at low pH. The reduction of extractability at pH 5–7, which is due to the decreased concentration of non-ionized sulfamyl benzoic acid, occurs at higher pH values for probenecid than for C_5H_{11}-R. According to spectrophotometric measurements there is no detecta-

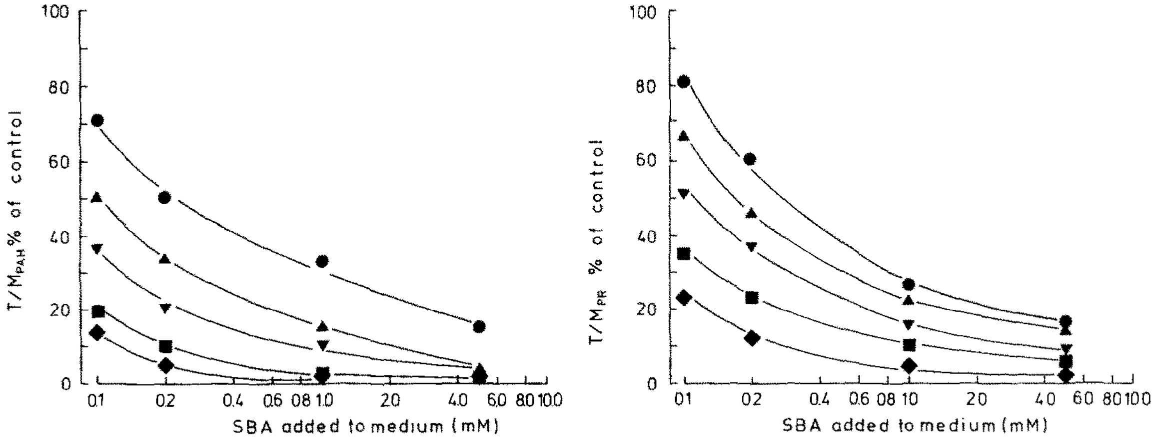

Fig. 5. Left (A) and right (B) panels demonstrate the effect of sulfamyl benzoic acid analogues on the aerobic accumulation of PAH ((T/M_{PAH}) and phenol red (T/M_{PR}) in kidney cortical slices in the presence of acetate (10 mM). Accumulation of PAH and phenol red in the absence of inhibitor has been taken as 100 per cent. The initial concentration of PAH and phenol red was 0.04 mM. Each point denotes the mean value of 4 experiments. For further details see Fig. 1.

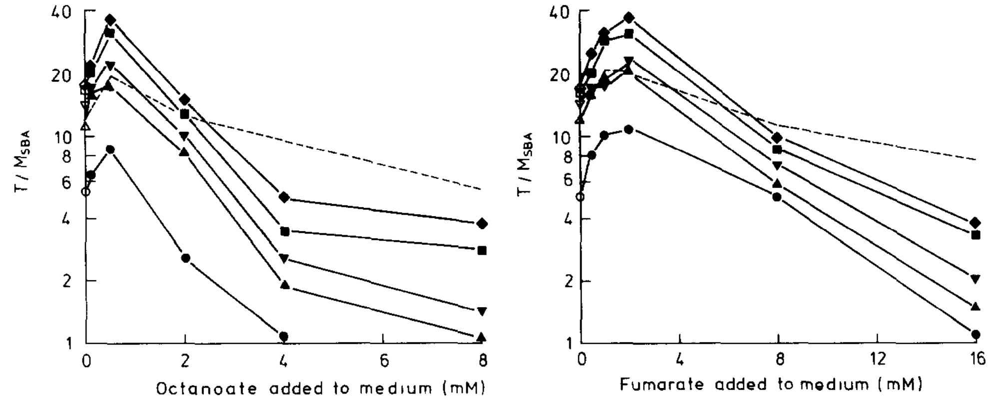

Fig. 6. Left (A) and right (B) panels demonstrate the effect of octanoate and fumarate on the aerobic accumulation of sulfamyl benzoic acid analogues. Acetate was not added to the incubation medium in these experiments. Accumulation ratio of these compounds in the absence of octanoate and fumarate has been taken as 100 per cent. The initial concentration of sulfamyl benzoic acid analogues was 0.04 mM. Each point denotes the mean value of 4 experiments. The interrupted line shows the accumulation of probenecid under similar conditions. For further details see Fig. 1.

ble difference of pK of the carboxylate group among various derivatives (p$K_{app} \simeq 3.3$ according to Ref. 4). It can therefore be presumed that probenecid exhibits a higher degree of hydrophobicity than C_5H_{11}-R, consistent with the presence of fewer hydrocarbon atoms in the latter compound.

DISCUSSION

The results reported in this paper on the renal accumulation of monosubstituted sulfamyl benzoate compounds indicate that the affinity of the compounds for transport is enhanced by the increase of hydrophobicity, in analogy with previous findings on probenecid analogues [5] and phenolsulfophthalein dyes [13]. A novel feature is the demonstration of an increase of uptake rates and maximal capacity for accumulation by an increase of the hydrocarbon length. For phenolsulfophthalein dyes an increase in hydrophobic properties, as reflected by higher partition coefficients between octanol and water as well as stronger binding to lipid, serum albumin and cellular membranes, is accompanied by a reduction of transport rates [13]. In the probenecid series the transport rate of $[C_4H_9]_2$-R is less than for probenecid ($[C_3H_7]_2$-R), but in contrast transport rates of $[CH_3]_2$-R and $[C_2H_5]_2$-R were found to be less than that of probenecid at a medium concentration of 0.04 mM [5]. Taken as a whole the results therefore suggest that moderate hydrophobicity results in an enhanced interaction and turnover of the compounds by the transport system, while transport rates are reduced for compounds with strong hydrophobic interactions, perhaps as a consequence of slower dislodgment from the transport system [13]. In agreement with this view the hydrophobicity of the monosubstituted sulfamyl benzoate derivatives was found to be less than that of probenecid. Furthermore, binding of the monosubstituted compounds to renal tissue constituents was less than observed for phenolsulfophthalein dyes [13] and probenecid [5].

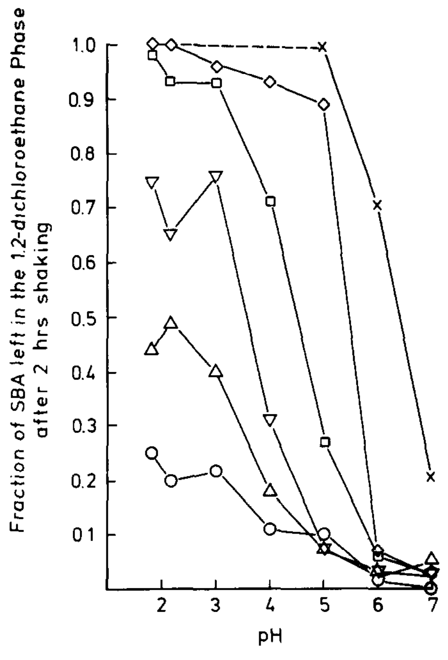

Fig. 7. Distribution of sulfamyl benzoic acid analogues in 1,2-dichloroethane and aqueous phases at various pH. Each point denotes the mean value of 3 experiments.

Transport of the monosubstituted sulfamyl benzoate compounds appears to occur exclusively by the same transport mechanism as that of PAH as evidenced by complete inhibition of PAH accumulation by the C_{2-5} derivatives (Fig. 5A) and of CH_3-R by high concentrations of PAH (Fig. 3). However, the present study reveals some quantitative differences in the transport characteristics of monosubstituted sulfamyl benzoate and probenecid. High medium concentrations of PAH, octanoate and fumarate had a less inhibitory effect on probenecid accumulation than on the accumulation of the monosubstituted derivatives. The possibility that this could reflect a stronger affinity of probenecid for the transport system is not substantiated by the observation that C_5H_{11}-R has a stronger inhibitory effect than probenecid on phenol red and PAH accumulation.

To account for this difference it may be assumed that part of the binding site for probenecid falls outside that of other organic anions, perhaps as a consequence of increased hydrophobic interactions. In that case probenecid may be displaced by other organic anions in a strictly competitive manner. An alternative possibility is to postulate an additional transport system for probenecid, which is not inhibited by PAH. Barany [18] has reported that iodipamide, a good substrate of the organic anion transport system in liver cells, is accumulated in kidney slices, both by a hippurate sensitive and insensitive mechanism. In a subsequent study Bárány [19] compared the inhibitory effect of probenecid and many alkylated derivatives of sulfamyl benzoate on the uptake of hippurate and iodipamide. In general strongly hydrophobic derivatives were reported preferentially to inhibited transport of iodipamide which would suggest interaction with another transport system that for PAH. However, a scrutiny of Bárány's results (Fig. 1 of Ref. 19) reveals that both CH_3-R, C_2H_5-R and probenecid preferentially inhibited accumulation of hippurate. We conclude that on the basis of these results there is no basis for assuming different transport systems for renal accumulation of probenecid.

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