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due to the transitions overlap. The enthalpies of these transitions, therefore, could not be determined with the calorimeter. Undecane and tridecane undergo solid–solid transitions at temperatures much lower than their melting points. The separate recorder peaks corresponding to melting and solid–solid transitions are clearly distinguishable, and it is possible to independently use data for both the liquid–solid and solid–solid transitions to calculate hydrocarbon binding. It was found that whereas only 0.88 mmoles of $C_{11}H_{24}$/g casein were bound against freezing, 4.2 mmoles $C_{11}H_{24}$/g casein were bound against the solid–solid transition; and whereas only 1.08 mmoles $C_{13}H_{28}$/g casein were bound against freezing, 2.2 mmoles $C_{13}H_{28}$/g casein were bound against the solid–solid transition. Apparently fractions of the undecane and tridecane, which are free to be transformed from the liquid to the first solid state, retain sufficient mobility in solid forms to become bound under the influence of the casein surface as the temperature of the system is lowered, and then do not undergo the solid–solid transition.

The significantly greater binding of the aromatic compounds compared with aliphatic compounds may be due to the ability of these compounds to stack on the surface of casein particles or may result from more specific interactions with the macromolecular surface. Strong binding to specific hydrophobic binding sites was demonstrated (2) in studies of the adsorption of benzene vapor onto the surface of solvent-transfer dried casein.

A variety of experimental techniques have been used to study hydrophobic ligand binding to a number of proteins (11); however, in almost every instance a mixed system was employed, consisting of protein and ligand both dispersed in an aqueous medium. In the present study we have demonstrated calorimetrically that neutral hydrocarbons may be bound in a structured, unfreezable form by bovine casein in the absence of water.

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TABLE I

BINDING OF HYDROCARBONS TO CASEIN

Compound	Mmoles bound/g casein[a]
Aliphatic	
octane	1.97 ± 0.17
decane	1.92 ± 0.09
dodecane	1.60 ± 0.17
tetradecane	1.10 ± 0.15
hexadecane	0.99 ± 0.14
undecane	0.88 ± 0.10
tridecane	1.08 ± 0.13
cyclohexane	4.31 ± 0.36
Aromatic	
benzene	8.44 ± 0.30
p-xylene	4.69 ± 0.26
mesitylene	3.29 ± 0.24

[a] The values given are the averages of eight determinations for each compound. The limits indicated are the average deviations from the means.

Aside from binding differences between the types of compounds, distinctions were noted between the members of each group. Even numbered homologous carbon compounds from octane through hexadecane showed a steady decrease in the millimoles of bound hydrocarbon per gram casein, as chain length increased. Though the differences in binding between successive members of this series are small, relative to the experimental errors, a trend throughout the series is evident. The mean values indicate that only half as many moles of hexadecane as of octane are bound to the protein. Conversely, the limited data for odd numbered compounds, i.e., undecane and tridecane, suggest that binding increases with chain length. Ring substitution decreased binding among the aromatic compounds, cf. *p*-xylene and mesitylene.

Though some of the hydrocarbon did not solidify in each case, no melting point depressions were observed with the fusible fractions. This behavior is different from that of aqueous protein dispersions (6–8); water is associated with the proteins in several forms, including water that does not freeze and water that freezes at a lower temperature. Such depression may be due either to solubilization of salts associated with the protein or to entrapment of water in capillaries within the solid protein (8).

The existence of unfreezable hydrocarbon in the presence of casein suggests an oriented structure at the protein interface; however, this phenomenon may not be the same as that of hydrophobic bonding (4). Hydrophobic bonding occurs strictly in aqueous media where, due to entropy effects, the hydrophobic segments of a protein molecule, together with hydrophobic ligands, are mutually forced into cavities in the water (9). Here we are reporting a binding of hydrocarbons to a protein in the absence of water. This does not involve hydrophobic bonding in the usual sense (4), but rather an orientation of hydrocarbon at the interface with the casein particles. Referring to the apparent steric control of binding of the even numbered alkanes, the data in Table I show decreased binding with increased chain length of these compounds on a molar basis; however, if these same data are considered in terms of the mass of bound hydrocarbon there is less variation; e.g., 225 mg of octane and 225 mg of hexadecane are bound per gram casein. Similarly, the effects of ring substitution in the aromatic series may be associated, at least to some degree, with steric factors at the protein–hydrocarbon interface, rather than with any differences in strength of binding.

The suggested differences in the effects of chain length on binding of the even and odd *n*-paraffins can be associated with the known differences in the crystal structure and phase behavior of these two series of compounds (10). There are not sufficient data for the odd numbered compounds to assess binding trends in the series. Only undecane and tridecane were studied and the value for bound tridecane may actually be in line with the even numbered aliphatics. Short chain compounds, $<C_8$, melt at temperatures too low for operation of the Perkin Elmer DSC-1B. Nonane could not be studied, as it normally melts at 219.7°K and undergoes a solid-solid transition at 217.2°K. The interval between these temperatures is small, and the peaks on the recorder tracings

form of "bound water" which will not freeze into ice even at cryogenic temperatures (6).

If benzene adsorption on the organic solvent treated casein is formally analogous to water adsorption on freeze-dried casein, the possibility exists that casein dispersed in the liquid benzene will bind some of the benzene in a structured form and thereby prevent its transition to a solid state on cooling. We have extended our low-temperature studies with the differential scanning calorimeter (6) and have demonstrated the existence of such unfreezable benzene. In addition to benzene, we also studied the binding of several other hydrocarbons. Compounds were selected to consider any influences of aromaticity and chain length, in a homologous series of compounds, on association with casein.

EXPERIMENTAL [2]

Casein preparation and solvent exchange. Casein was isolated from raw skim milk by centrifugation at 78,400 g for 20 min in the Spinco Model L preparative ultracentrifuge. The casein pellets were washed twice by dispersing in distilled water with a tissue grinder and recentrifuging. The washed casein was redispersed in a limited amount of water (90 g casein/liter) and dehydrated according to the solvent replacement method used in our earlier studies (1). The transfer sequence was from water to methanol and then either directly to the aromatic hydrocarbon under study or to hexane and then to the aliphatic compound studied. As was done earlier, the various casein dispersions were subjected to ultrasonic oscillation to ensure uniform particle size distribution and to prevent aggregation into large particles during solvent replacement.

The alkanes studied were cyclohexane, undecane, tridecane, and the even carbon numbered linear alkanes from octane through hexadecane. The aromatic compounds were benzene, *p*-xylene, and mesitylene. Benzene (spectral grade), hexane (99% mole pure), and cyclohexane (99% mole pure), (all from Fisher Chemical Co.) were dried with CaH_2 before use. Absolute methanol was used as obtained. Mesitylene, *p*-xylene, octane, undecane, and tetradecane (all from J. T. Baker Co.) were redistilled and dried with CaH_2. Decane, dodecane, hexadecane, and tridecane (all from Matheson, Coleman, Bell) were also redistilled and dried with CaH_2.

Binding measurements. "Bound hydrocarbon" was determined with the Perkin Elmer Model DSC-1B differential scanning calorimeter using a procedure similar to that employed to measure unfreezable water in aqueous protein dispersions (6). Samples weighing 10–14 mg and containing approximately 30% casein in the organic solvent were hermetically sealed in aluminum capsules, placed in the calorimeter cell, cooled rapidly with liquid nitrogen to solidify the free solvent, and then heated at a programmed rate of 2.5°C/min to a temperature high enough to melt the hydrocarbon. The mass of hydrocarbon melted was determined from the energy absorbed during melting, as calculated from the DSC thermograms in comparison with pure hydrocarbons as standards. Total hydrocarbon content was determined, after the calorimeter scans, by puncturing each capsule lid to form a small pinhole and heating the sample under vacuum at 60–80°C to constant weight. The difference between the total and free hydrocarbon contents was taken as a measure of the unfreezable solvent associated with the protein.

RESULTS AND DISCUSSION

Data for binding of the various hydrocarbons are listed in Table I. Clearly, substantial quantities of all the solvents are held in a sufficiently immobilized form by casein to prevent transitions to solid states. Definite distinctions, however, exist between the types of compounds; aromatic compounds are much more extensively bound than are linear alkanes. Cyclohexane binding was intermediate between these two groups.

[2] Mention of brand or firm names does not constitute an endorsement by the Department of Agriculture over others of a similar nature not mentioned.

Hydrocarbon Binding by Particles of Bovine Casein

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Casein, isolated from milk by high-speed centrifugation, was washed with water and dehydrated by serial transfer through liquids of decreasing polarity to provide a system consisting of protein dispersed in anhydrous, liquid hydrocarbon. Heats of fusion of solvents associated with casein were measured to determine how much hydrocarbon is free and can be frozen and what fraction is bound to the protein and immobilized. Ring substitution decreased binding in the aromatic compounds studied, i.e.: 8.4 mmoles benzene, 4.7 mmoles *p*-xylene, and 3.3 mmoles mesitylene bound per gram casein. Binding of even *n*-paraffins was significantly less than that of the aromatic compounds and decreased with increasing chain length, i.e.: 2.0 mmoles C_8H_{18}, 1.9 mmoles $C_{10}H_{22}$, 1.6 mmoles $C_{12}H_{26}$, 1.1 mmoles $C_{14}H_{30}$, and 1.0 mmoles $C_{16}H_{34}$ bound per gram casein. The observation of unfreezable hydrocarbon indicates that some solvents, commonly considered as hydrophobic, attain preferred orientation at the protein–solvent interface.

INTRODUCTION

During investigations of the fundamental aspects of milk dehydration, we observed that the adsorption properties of the dried solids are related to the mode of water removal. Casein dehydrated by serial transfer through liquids of decreasing polarity with the final solvent removed *in vacuo* adsorbed more nitrogen (1) and benzene (2) than lyophilized casein. Multitemperature adsorption data (2) showed that benzene is strongly bound to solvent-transfer dried casein at low surface coverage, presumably through specific hydrophobic binding sites. As more benzene is adsorbed, the isosteric heat of adsorption decreases and the adsorbate is held more loosely. Lyophilized casein, however, adsorbs benzene less strongly at all levels of surface coverage.

The effects of solvent replacement on adsorption probably represent changes in protein configuration occurring during dehydration. The apolar amino acid residues of the casein (3) which are normally in the interior of the macromolecular complex with minimum contact with the aqueous environment, according to theories of hydrophobic bonding (4) may become separated and exposed when water is replaced with a less polar solvent. This rearrangement could provide surface sites for specific interaction with benzene at lower relative pressures and allow weaker adsorption at higher relative pressures with multilayer formation.

An analogous interpretation has been made of water adsorption by proteins (5). It is held that proteins sorb water by binding water molecules to specific hydrophilic sites at low relative pressure and by condensation or multimolecular adsorption at high vapor pressures. As the saturation pressure is approached the protein becomes as extensively hydrated as in solution. The water associated with proteins, dispersed in an aqueous system, constitutes a

[1] Research conducted while Dairy Products Laboratory was located in Washington, DC.