

Influence of Dehydration Method on the Adsorption of Benzene Vapor by Dried Casein

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Washed casein micelles were dehydrated by serial transfer through solvents of decreasing polarity followed by drying *in vacuo*. The product adsorbed approximately three times as much benzene as lyophilized casein for all P/P_0 values at 24 and 30°C. Adsorbed C_6H_6 molecules are very mobile on the surfaces of the lyophilized particles, $-q_{st} = 8.7$ kcal/mole over the entire isotherm. However, much of the benzene is in a more tightly bound adsorbed state on solvent transfer dehydrated casein, with $-q_{st}$ gradually decreasing from a high of 20 kcal/mole to a constant value near 8 kcal/mole. Both types of casein adsorbed more C_6H_6 than anticipated from BET surface areas calculated from low temperature N_2 adsorption data. The results suggest that the apolar amino acid residues of the casein subunits, which are normally squeezed into cavities in water according to theories of hydrophobic bonding, may be separated when water is replaced with a less polar solvent. Ultimately a porous dried product is thus obtained with localized binding sites for benzene, rather than hydrophobic regions in the macromolecule.

INTRODUCTION

Research on water removal from biological substances has led us into studies of the effects of dehydration on the physical surface features of the dried materials. We have reported (1) substantial increases in the BET surface area of casein micelles dried by serial transfer through liquids of decreasing polarity with the final solvent removed *in vacuo* over that observed with lyophilized micelles.

When dispersed in water, casein micelles are highly solvated (2,3) porous structures penetrable even by such large molecules as carboxypeptidase A (MW 40,000) (4). The solvent replacement technique as opposed to lyophilization successfully prevented the collapse of the swollen hydrated structures responsible for micelle porosity.

Though the solid materials obtained with

these drying methods adsorbed very different amounts of N_2 at -195°C , their water vapor sorption properties at or near ambient temperature are nearly identical. It is therefore of interest to study the adsorption by these materials of a different adsorbate at ambient temperature which would not interact with the protein in the same manner as H_2O . In the present work, we have studied the C_6H_6 vapor adsorption properties of washed casein micelles dried either by lyophilization or solvent transfer procedures using benzene as the last solvent before drying *in vacuo*.

Using the same hydrocarbon as both solvent and adsorbate may then allow some understanding of the nature of the solvent replacement drying process including possible protein-hydrocarbon associations. Such hydrophobic interactions may be expected with casein as it has been calculated by Hill and Wake (5) that the caseins rank among the most hydrophobic proteins of those tabulated by Bigelow (6). Furthermore, it is known from sequence studies

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that at least the major subunits of the casein micelle contain substantial segments or patches of apolar amino acids (7-10) which lead to the stabilization or self-association of the subunits (11-13) and to a degree the entire casein micelle through hydrophobic forces.

Numerous studies have been reported on the interactions of hydrophobic materials (14) with proteins, however in almost every instance a mixed solvent system composed of water and the hydrophobic component was used and only limited work has been completed (15) on the binding of hydrocarbons to proteins in the absence of water as in the present investigation.

Data on the absorption of hydrocarbons by proteins should also provide necessary information pertaining to the often encountered difficulties (16-18) in removing the last traces of organic solvents used in processing proteinaceous or cellulosic materials.

MATERIALS AND METHODS²

Casein preparation and dehydration. Casein was isolated from raw skimmilk by high speed centrifugation at 44,330 g for one hour in the Spinco Model L preparative ultracentrifuge. The casein pellets were washed twice by dispersing in distilled water, using a Ten Broeck tissue grinder and recentrifuging. The washed fully hydrated casein micelles were then dried, either by lyophilization or by the solvent replacement method used in our earlier studies (1) which was adapted from that used by Neihof, Thompson, and Deitz (19) in their studies of bacterial spores. The transfer sequence used in the present work was from H₂O to anhydrous CH₃OH and then to C₆H₆. The casein was kept dispersed in C₆H₆ until used in the adsorption measurements. Spectroscopic grade benzene used both as solvent and adsorbate was first treated with CaH₂ to remove any traces of moisture.

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.

Adsorption Measurements

Benzene vapor adsorption was measured gravimetrically at 24, 30, and 35°C using the Cahn RG recording electrobalance coupled with a Honeywell 1 mV recorder. The balance was mounted in the vacuum bottle supplied by the Cahn Instrument Co. for weighing in controlled atmospheres. The bottle was incorporated into an all glass custom-made adsorption apparatus equipped with pumps for sample outgassing and suitable devices for controlling and monitoring benzene pressure. The ground glass seal on the vacuum bottle was greased with Apiezon T and the apparatus was fabricated with high vacuum teflon stopcocks to hold stopcock grease at a minimum. The entire apparatus was kept in a room maintained at 37-39°C to prevent benzene condensation on the walls of the apparatus at higher P/P_0 levels of the 35°C isotherm.

Samples were placed on aluminum sample pans and nichrome stirrups as supplied by the balance manufacturer. They were suspended from the balance beam into a hangdown tube thermostated at either 24, 30, or 35°C with a constant temperature water bath rated at $\pm 0.02^\circ\text{C}$. A chromel-alumel thermocouple was placed inside the tube in the vicinity of the sample pan and was wired through the balance control unit with temperature readout obtained with a Leeds and Northrup millivolt potentiometer.

Benzene was kept in a reservoir sealed to the system and maintained at a temperature lower than that of the sample so that during the adsorption experiments the benzene pressure in the apparatus was equal to the vapor pressure of benzene at the temperature of the reservoir. Dissolved gases were removed from the benzene prior to adsorption by pumping through several freeze-thaw cycles. Pressure in the system during adsorption was monitored with a conventional U-tube mercury manometer. The mercury was covered with a layer of Dow Corning Silicone Oil diffusion pump fluid to retard mercury evaporation and thus prevent condensation of mercury on the

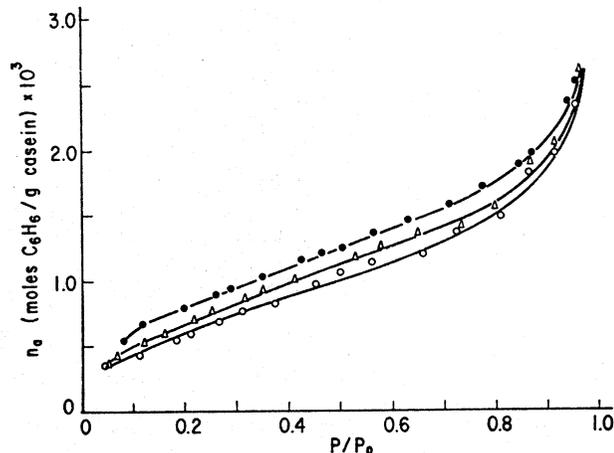


FIG. 1. Benzene vapor adsorption on solvent transfer dehydrated casein, ● 24°C, △ 30°C, and ○ 35°C.

balance and/or sample. Observed values for benzene vapor pressure at all reservoir temperatures were in good agreement with values calculated from the Antoine Equation using the constants given in the A.P.I. Research Project 44 of the National Bureau of Standards.

Prior to measuring C_6H_6 adsorption by the casein blank runs were made to obtain appropriate correction factors for adsorption on the balance pans and other components for each adsorption isotherm temperature.

Lyophilized casein samples weighing 10–12 mg were taken and degassed at ambient temperature (37–39°C), at 10^{-6} Torr, for a minimum of 24 hr before beginning adsorption. Larger samples of solvent exchanged casein, wet with C_6H_6 , were taken to yield 10–12 mg, dry weight, after degassing at 10^{-6} Torr. These samples were heated to 50°C during outgassing to avoid condensation of mercury from the diffusion pump and McLeod gauge. After degassing, C_6H_6 vapors were admitted to the system before lowering the sample temperature to that of the adsorption isotherm. The system was then kept at constant pressure until equilibrium after which the C_6H_6 reservoir temperature was raised for the next point on the isotherm. The pressure increments taken were $0.05 P_0$. The sample was considered to be at adsorption equilibrium when the weight remained constant within $\pm 4 \mu g$ for at least

5 hr. This represents constant weight within $\pm 0.04\%$. During the adsorption runs the system was held at each pressure for a minimum of 24 hr.

RESULTS

Adsorption data for benzene on the solvent exchange dehydrated casein are presented graphically as the isotherms of Fig. 1. At all three temperatures, sigmoid type II isotherms were obtained with a normal inverse relationship between temperature and the quantity of vapor adsorbed. Monolayer values computed, using the BET (20) equation from the data in the BET range, $0.05 \leq P/P_0 \leq 0.35$, were 7.2×10^{-4} , 6.1×10^{-4} , and 5.7×10^{-4} moles C_6H_6/g casein at 24, 30, and 35°C, and the BET C values were 21.3, 20.4, and 22.2, respectively.

The adsorption data obtained with lyophilized casein at 24°C and 30°C are given in the isotherm of Fig. 2, which is also a sigmoid type II isotherm, though substantially linear up to $P/P_0 \approx 0.5$. At all relative pressures the lyophilized casein adsorbed less C_6H_6 than the solvent treated casein. The monolayer value computed from the BET equation is 2.87×10^{-4} moles C_6H_6/g casein and the C value is 3.0. When the data are plotted against relative pressure as in Fig. 2, the adsorption appears temperature independent. This may

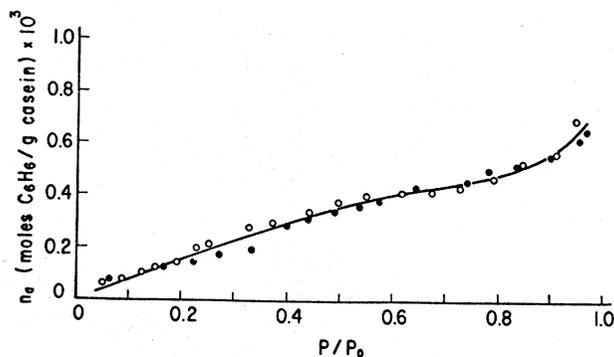


FIG. 2. Benzene vapor adsorption on lyophilized casein ● 24°C and ○ 30°C.

actually show a slight positive thermal coefficient of adsorption up to $0.6 P_0$. There is, however, an inverse temperature dependence when the adsorption values are plotted as a function of pressure, thus permitting calculation of the isosteric heat of adsorption, q_{st} , using the integrated form of the Clapeyron-Clausius equation. A constant value of -8.7 kcal/mole benzene was obtained irrespective of the extent of surface coverage.

Isosteric heat values computed from the absorption data in Fig. 1 for C_6H_6 solvent exchanged casein are shown graphically in Fig. 3. The values for $-q_{st}$ fall off from a high of 20 kcal/mole at 0.5 mmole adsorbed C_6H_6/g casein to a constant value of 8.5 kcal/mole

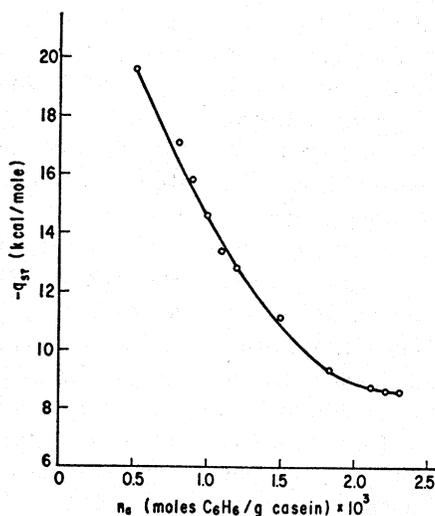


FIG. 3. Variation in isosteric heat of adsorption of benzene with increasing surface coverage (mmoles C_6H_6/g casein) on solvent transfer dehydrated casein.

after 2.1 mmole C_6H_6/g casein are adsorbed. These results indicate a somewhat stronger interaction than due to ordinary condensation forces even beyond the first adsorbed layer as $-q_{st}$ does not approach the heat of vaporization of liquid benzene until after the adsorption of three times as much C_6H_6 as required for a BET monolayer. In contrast $-q_{st}$ for lyophilized casein was close to the heat of vaporization of benzene at all levels of C_6H_6 adsorption measured.

Benzene adsorption was not completely reversible with both types of casein. Residual adsorbed C_6H_6 after 1-5 days evacuation at $37-39^\circ C$ was 1.2×10^{-4} mole C_6H_6/g casein for the solvent exchanged material and 1.5×10^{-4} mole/g lyophilized casein. These results do suggest the possibility that some benzene may not have been removed during the initial outgassing of the exchange dried casein.

DISCUSSION

Dehydration of casein by solvent transfer and lyophilization clearly results in solid materials which interact with benzene in different ways. This is apparent from the isosteric heat values which indicate a stronger interaction of C_6H_6 with the solvent treated casein than with the freeze-dried protein. Calculations (21) of residence times, τ for adsorbed molecules using the Frenkel equation (22)

$$\tau = \tau_0 e^{-\Delta H_0/RT}$$

where τ_0 is of the order of 10^{-13} sec are also

useful in examining the C_6H_6 -casein interactions. At lower benzene coverages, on solvent dehydrated casein $\Delta H_a \approx 20$ kcal/mole, hence $\tau \approx 100$ sec. and benzene adsorption approaches the behavior expected in chemisorption. At higher C_6H_6 coverages or at all P/P_0 values with lyophilized casein, ΔH_a approaches 8 kcal/mole and $\tau \approx 10^{-8}$ sec. This difference in residence time of over ten orders of magnitude implies a distinction in the adsorbate between a tightly bound and a very loose state.

This distinction between the caseins is also supported by the BET C values which indicate much weaker interactions for C_6H_6 with lyophilized casein. Actually the application of the BET equation to the lyophilized casein data for monolayer calculations is questionable because of the shape of the isotherm and the unusual relation between temperature and adsorption.

Differences may also be pointed out between the N_2 and C_6H_6 adsorption properties of the caseins. The same sample of lyophilized casein used in this study displayed a BET surface area from N_2 adsorption measurements of 3.2 m²/g with a C value of 45. N_2 adsorption was not measured with the particular preparation of solvent exchange dehydrated casein used in this study; however, other samples (1) from similar preparations exhibited areas of 71.0 and 65.5 m²/g with a C value of 45. Surface area values calculated from the C_6H_6 data for solvent dehydrated and freeze-dried caseins are 144 m²/g and 69 m²/g using the value of 40 \AA^2 /molecule for the benzene ring parallel to the surface and 118 and 56 m²/g using the value of 32.7 \AA^2 /molecule for the freely rotating absorption model or the benzene ring normal to the surface (23). Obviously much more area is available for C_6H_6 adsorption than N_2 adsorption with either casein irrespective of the orientation of the adsorbed C_6H_6 molecule. Furthermore, there is a much greater difference between the quantities of N_2 adsorbed by the two types of dried casein than between the quantities of C_6H_6 adsorbed.

These effects on adsorption must be related to differences in the nature of the drying

methods as they affect the casein micelles. Casein, the major protein fraction in bovine milk, exists in a unique micellar form as a calcium phosphate complex of the various monomeric casein subunits. These colloidal casein particles have intrigued many investigators who have proposed a number of different models for the micelle structure, based primarily on chemical evidence (24-30). The involvement of lyophobic forces in maintaining structural integrity is common to all models though they differ in major details of the actual protein subunit associations. Some ionic bonding does occur between α_{s1} and κ -casein; however, the stability of the casein micelle is nevertheless due in part to hydrophobic interactions. As discussed by Kauzman (31) hydrophobic interactions in proteins result from changes in solvent (water) structure due to the presence of apolar amino acids. By repelling the apolar residues, i.e., forcing them out of the water and into the interior of the protein molecule, a small quantity of stabilization entropy is gained per residue transferred. If the water is replaced with a less polar solvent, such repulsions no longer will occur and the apolar residues need not be forced into cavities in the solvent. Such a process may result in gross reordering or changing of protein conformation with the apolar sites becoming widely distributed. The net result would then be a less compact material after drying by removal of the solvent under vacuum. Binding sites available to benzene after solvent transfer drying, therefore, need not be squeezed into patches but may be present at different locations on the porous surface of the dried casein particles.

Absorption of benzene in solvent dehydrated casein will, therefore, proceed first through localized binding sites with higher energies, on the order of 20 kcal/mole, and then as more C_6H_6 is adsorbed the binding energy will be decreased as is normal with physisorption.

The freeze-dried casein micelle will, however, be a more compact solid with apolar sites aggregated into hydrophobic regions. Drying removes the water of hydration in-

volved in the electrostatic repulsion necessary for micelle stabilization in preventing coagulation and precipitation which would occur through additional hydrophobic binding between subunits. The drying process will, therefore, result in the absence of any highly localized benzene binding sites in the cavities of a porous system but rather hydrophobic regions either buried in the interior of the collapsed micelle or available as patches on the surface. Under these circumstances adsorbed benzene will be in a highly mobile state on the surface of the lyophilized casein as deduced from thermodynamic treatment of our data.

The increased adsorption of benzene relative to that predicted from the BET nitrogen area may be related to thermal expansion of the casein. Though no measurements have been made of the appropriate thermal expansion factors for dehydrated caseins, inferences may be drawn from data we have published on the effect of temperature on the permeability of dried milk powder particles to helium and nitrogen gases (32). The density of milk powder measured by helium displacement was 1.26 g/cm³ at 25.1°C, but only 0.47 g/cm³ at -195.7°C implying contraction of the particles as the temperature is lowered with concomitant closing of the pores. Though the milk powders were not dried the same way as the caseins in the present study, the earlier data at least indicate a likelihood for thermal expansion of dried casein particles as milk powders contain approximately 21% casein. Thus heating from 77.4 to 300 K may open the porous structure and permit benzene to enter deep into the dehydrated casein and interact with appropriate hydrophobic sites.

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