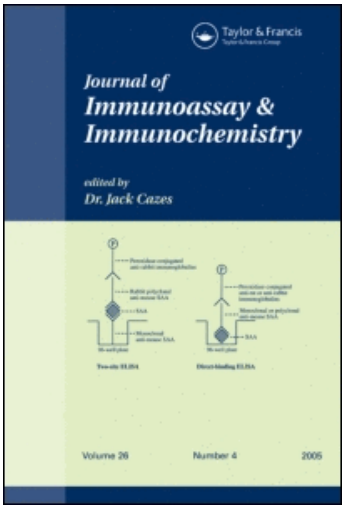

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Charcoal as a Phase Separating Agent in Ligand Assays: Mechanism of Action and the Effect of Dextran and Various Proteins on the Adsorption of Small Molecules

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CHARCOAL AS A PHASE SEPARATING AGENT IN LIGAND ASSAYS: MECHANISM OF ACTION AND THE EFFECT OF DEXTRAN AND VARIOUS PROTEINS ON THE ADSORPTION OF SMALL MOLECULES.

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Key words: charcoal adsorption, phase separation, protein coating, dextran coating, ligand assay.

ABSTRACT

In ligand assays, charcoal mixed with dextran is sometimes used to separate free from bound ligand. This is done with the view that dextran "coats" the charcoal and produces a sieve effect on its surface. To further elucidate the mechanism of differential adsorption of small molecules to charcoal, studies were made on the interaction between a number of commonly measured ligands and charcoal, either unmixed or mixed with dextran, albumin, immunoglobulin (IgG) or insulin. "Coating" with dextran was shown not to have any effect on subsequent adsorption of the various ligands. However, coating charcoal with albumin, IgG or insulin did have an effect and even augmented subsequent adsorption of small molecules under certain conditions. It is concluded that the use of "dextran coating" of charcoal to give a sieve effect for the separation of small molecules from large ones is unnecessary and has no basis in fact. This is not the case for coating charcoal with proteins.

INTRODUCTION

The separation of free from bound ligand is an essential step in assay techniques based on the preferential binding of an analyte to a specific binding molecule. Charcoal has been used extensively

for this purpose by virtue of its ability to separate mixtures of small molecules in an aqueous medium (1, 2). This separating ability is due to the differential adsorption of small molecules to activated charcoal particles. In an attempt to improve the ability of charcoal to separate molecular mixtures, some authors have recommended the mixing of charcoal with various agents, such as serum proteins or dextran, with the aim of producing a sieve effect on the surface of the particles (2, 3, 4, 5). To further elucidate the mechanism of differential molecular adsorption by charcoal and the effect of "coating", we studied the interaction between a number of commonly measured hormones and charcoal, both without and in the presence of dextran, albumin, immunoglobulin (IgG) and a polypeptide (insulin).

MATERIALS

Suspensions of activated charcoal (Norit A decolourizing carbon, Baker Chemical Co., Phillipsburg, New Jersey, USA) were freshly prepared every day at a concentration of 0.1 percent (W/V). Dextran preparations of mean molecular weights 10, 20, 40, 70, 110, 150, 250, 500 and 2000 x 10^3 daltons (Pharmacia Fine Chemicals, Uppsala, Sweden) were made up daily in concentrations specified under methods. All other reagents were obtained and prepared as previously described by us (6).

METHODS

The experiments were modifications of commonly used ligand assay techniques (6). Throughout all experiments the assay medium used was 0.01 M sodium phosphate buffered saline (PBS), pH 7.4,

unless otherwise indicated. Suspensions of charcoal were freshly prepared each day and constantly stirred magnetically during use. For the measurement of radioactivity a triple headed well counter was used.

The first set of experiments was to determine the effect of the amount of charcoal added, incubation time, incubation temperature and medium used on the adsorption of various radiolabelled molecules to activated charcoal. Two hundred microlitres of a 0.1 percent (W/V) charcoal suspension in PBS was added to a series of six 12 x 75 ml polystyrene assay tubes with conically shaped bottoms. After incubation the tubes were centrifuged at 2000 g for ten minutes and the supernatants decanted and discarded. Two ml of PBS buffer was then added to each of the charcoal pellets in the tubes and the charcoal resuspended by vortexing. Then, while vortexing, 200 microlitres of a radiolabelled-hormone-containing solution was added rapidly to each tube. The tubes were then incubated at either room temperature or $4^{o}C$ for a varying period of time. They were then again centrifuged at 2000 g for ten minutes and the supernatants discarded. The radioactivity adsorbed to the charcoal in each tube, as well as the total in 200 microlitres of the radiolabelled-hormone-containing solution, was then measured in a gamma counter.

The experiment was repeated using a different incubation medium (normal saline), and varying incubation time, temperature and volume of charcoal solution added to each tube.

The experiments designed to assess the effect of "coating" charcoal with dextran, various proteins or a polypeptide were done

by putting two ml of solutions of the various molecular weight dextrans, immunoglobulin G (IgG), bovine serum albumin (BSA) or a polypeptide (insulin) in assay tubes in duplicate. Two hundred microlitres of a 0.1 percent charcoal suspension was then added to each tube and the tubes vortexed to ensure adequate mixing. The mixtures were then allowed to stand at room temperature for thirty minutes after which they were centrifuged at 2000 g for ten minutes and the supernatant discarded. Two ml of PBS was then added to each of the tubes containing the pellet. They were then vortexed to resuspend the "coated" charcoal mixture in the pellet. Then, while vortexing, 200 microlitres of the radiolabelled-hormone-containing solution was added to each tube. After incubation at room temperature for varying periods of time (see below) they were again centrifuged for ten minutes at 2000 g and the supernatants discarded. The radioactivity in each tube was then counted as described above. Variations in the individual experiments are described in the results.

In all experiments the radioactivity adhering to plain charcoal was expressed as the percent precipitated (B_o) of the total radioactivity added (T). The radioactivity precipitated with the charcoal in the presence of other molecules such as dextran, proteins or insulin, was expressed as a ratio (B/B_o) of the amount precipitated (B) to the amount precipitated under identical conditions in the presence of charcoal only (B_o).

RESULTS

The adsorption of various small molecules to charcoal was affected by the amount of charcoal, incubation time, and the med-

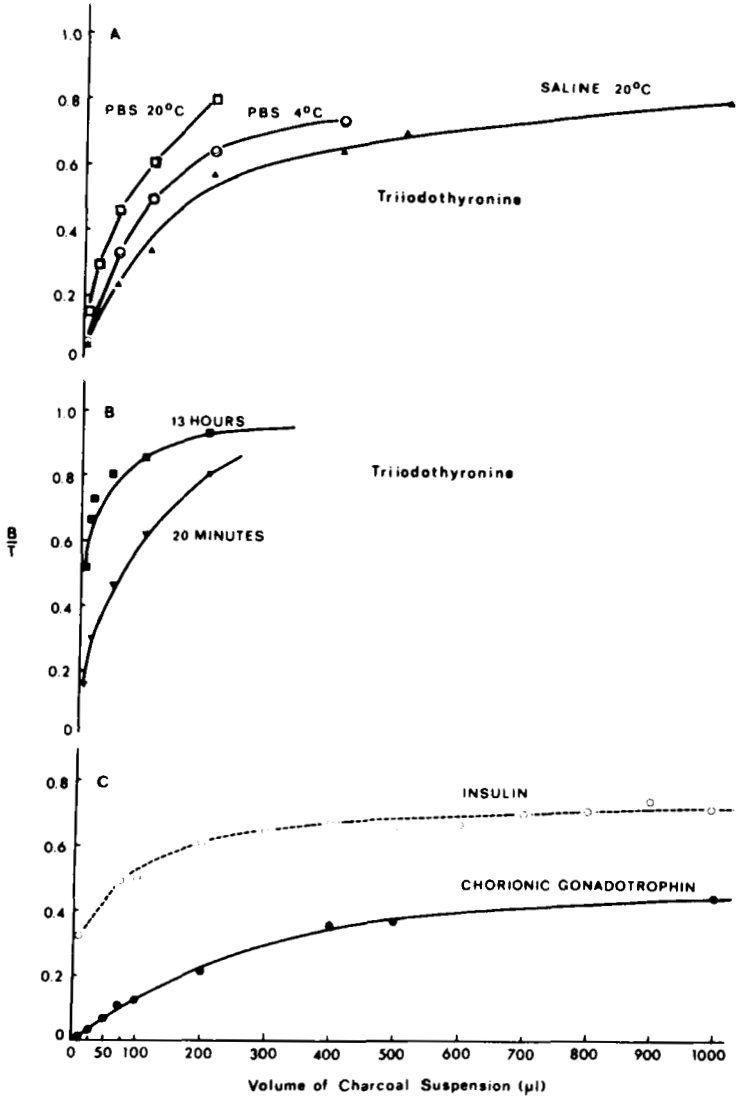

FIGURE 1. Effect of varying the amount of charcoal, incubation time, temperature, reaction medium or type of molecule on the adsorption of various small molecules to charcoal. The results are expressed as the fraction (B/T) of the total radiolabelled hormone (T) added that was adsorbed to the charcoal (B). It is apparent that for all hormones tested a larger fraction (B/T) was adsorbed as more of it was added. A rise in incubation temperature increased adsorption while for identical amounts of charcoal added more radiolabelled triiodothyronine was adsorbed in phosphate buffered saline (PBS) than in normal saline (panel A). Increasing incubation time had the same effect (panel B). Adsorption also varied with the type of molecular species added (panel C).

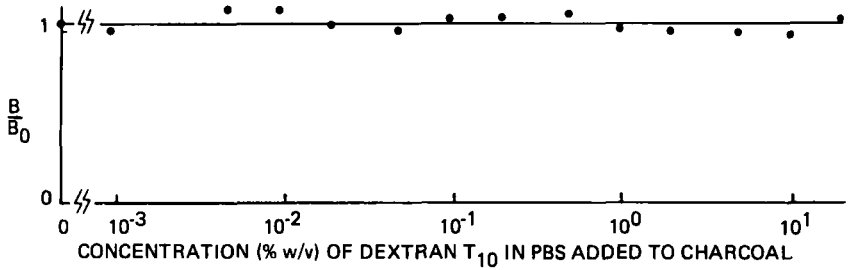

FIGURE 2. Adsorption of triiodothyronine by a constant amount of charcoal "pre-coated" with increasing amounts of dextran T_{10}. The results are expressed as the ratio (B/Bo) of the amount of radiolabelled triiodothyronine bound by charcoal "pre-coated" with dextran (B) to that bound when no dextran was added (B_o). "Pre-coating" of charcoal with dextran T_{10} had no effect on the adsorption of the hormone.

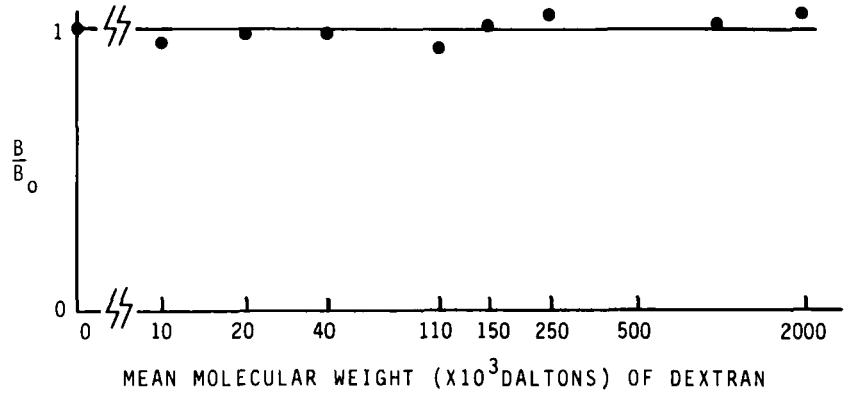

FIGURE 3. Adsorption of radiolabelled triiodothyronine by a constant amount of charcoal "pre-coated" with a constant amount (1 percent W/V) of various dextrans of increasing mean molecular weights. "Pre-coating" with the dextrans had no effect on the adsorption of the hormone to charcoal.

ium used (Figure 1). For all subsequent experiments PBS was used and the amount of charcoal added was kept constant while incubation time was varied depending on whether maximum adsorption of the radiolabelled hormones was desired or not (see discussion).

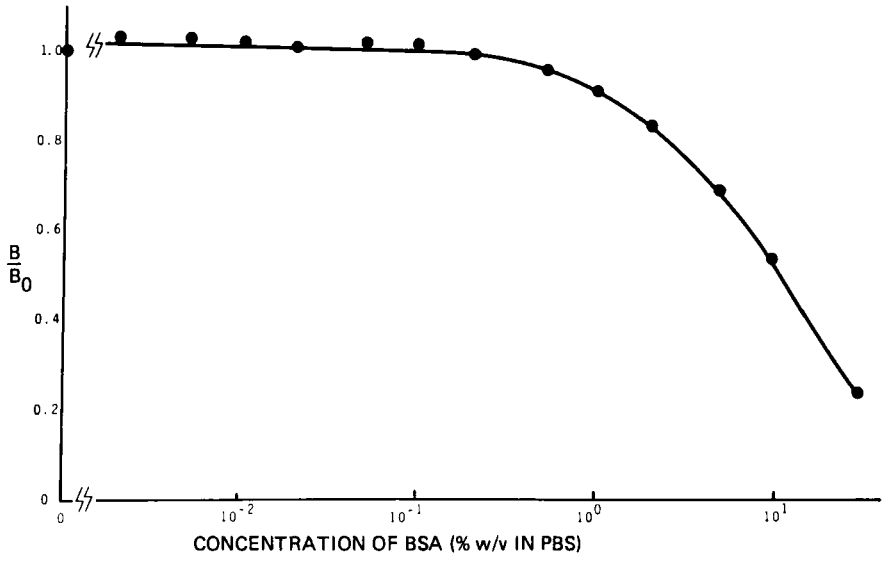

FIGURE 4. Adsorption of radiolabelled triiodothyronine by a constant amount of charcoal pre-coated with increasing amounts of bovine serum albumin (BSA). Adsorption was measured after 30 minutes of incubation. A gradual decrease in the adsorption of the hormone occurred when the BSA concentration exceeded 0.1 percent (W/V).

Figure 1 also demonstrates that adsorption to charcoal varied among different hormones. All the remaining experiments were done to assess the effect of adding dextran, proteins or a polypeptide to the charcoal on the adsorption of small molecules to charcoal ("pre-coating"). Figure 2 shows the effect of adding increasing amounts of dextran with an average molecular weight of 10,000 daltons to the charcoal prior to the adsorption of triiodothyronine. Dextran had no effect on the adsorption of the hormone. Adding a constant amount (1 percent W/V) of dextrans of increasing molecular weight also had no effect on the adsorption of radiolabelled triiodothyronine to charcoal (Figure 3).

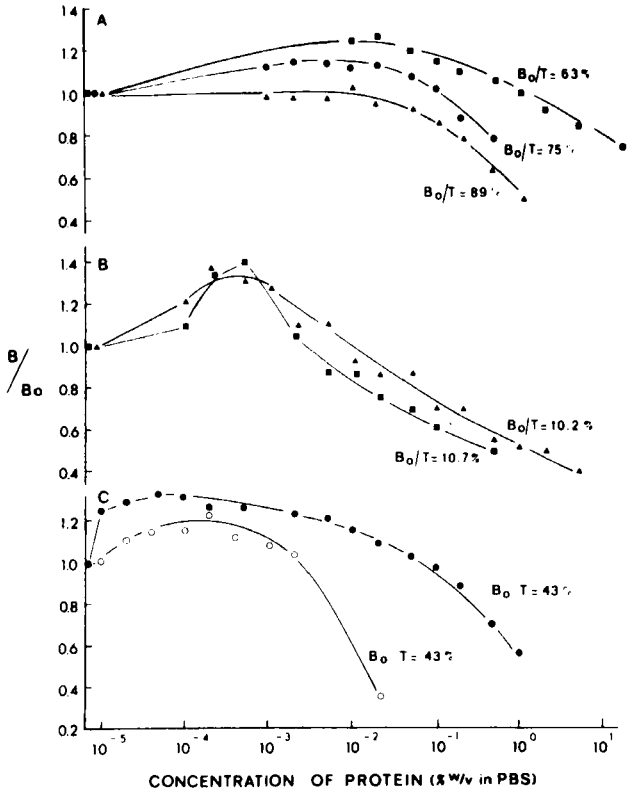

FIGURE 5. The effect of pre-coating charcoal with various proteins or a polypeptide on the adsorption of various radiolabelled hormones. Panel A shows the adsorption of triiodothyronine by a constant amount of charcoal pre-coated with increasing amounts of immunoglobulin (IgG). Three identical experiments were performed with the exception of the incubation time which was varied from 15 min (■) and 30 min (●) to 45 min (▲). The results showed increased adsorption of the hormone to charcoal by itself (B_O/T) as the incubation time was increased. However, with shorter incubation times (■) and (●) pre-coating charcoal with IgG caused an initial increase in adsorption of the hormone. As the IgG concentration was increased further it decreased the adsorption of triiodothyronine. Panel B shows the adsorption of human chorionic gonadotrophin by a constant amount of charcoal pre-coated with increasing amounts of either BSA (▲) or IgG (■). The incubation time was reduced so as to obtain a low degree of adsorption of the hormone to charcoal by itself ($B_O/T < 11\%$). Pre-coating with either protein caused an initial increase in the adsorption of the hormone after which it decreased to levels below those of uncoated charcoal. Panel C shows the adsorption of radiolabelled insulin by a constant amount of charcoal pre-coated with increasing amounts of either IgG (●) or insulin (O). Incubation times were adjusted to obtain submaximal adsorption of insulin to charcoal by itself. The pre-coating of charcoal with either IgG or insulin caused an initial increase in adsorption of the radiolabelled insulin. This was followed by a decrease in adsorption.

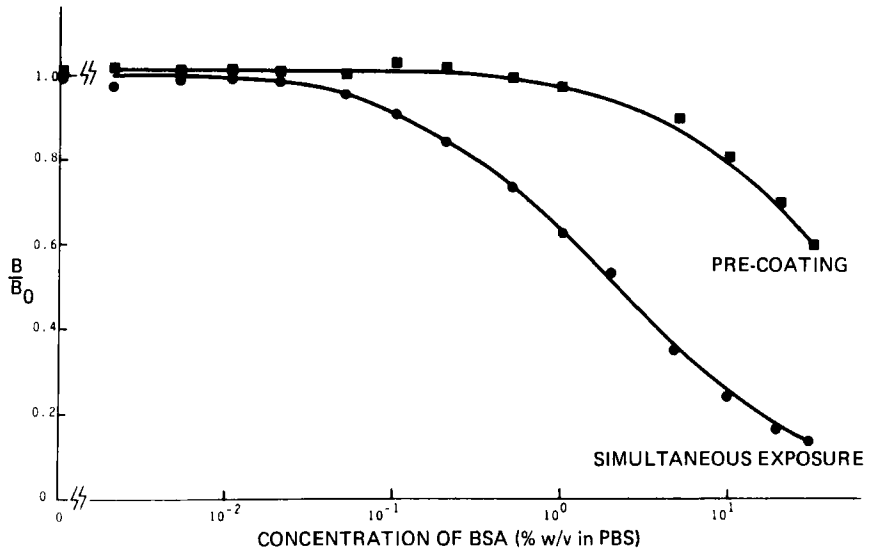

FIGURE 6. Adsorption of radiolabelled triiodothyronine to charcoal by itself (B_o) and in the presence of increasing amounts of albumin. The latter was added either 30 min prior to the addition of the hormone (pre-coating;) or at the same time (●). Incubation time was 30 min to ensure that adsorption of the hormone would be maximal. The results show that simultaneous addition of the hormone and albumin to the charcoal caused a reduction in the adsorption of the hormone at a lower concentration of albumin.

The mixing of bovine serum albumin with the charcoal (Figure 4) before the radiolabelled triiodothyronine was added decreased adsorption of the hormone markedly when the concentration of the BSA exceeded 0.1 percent (W/V). Figure 5 shows the effect of precoating charcoal with IgG, BSA or pork insulin on the adsorption of small molecules if the experiment was terminated before adsorption of the radiolabelled hormone was complete. Under these conditions all three substances used to coat the charcoal caused an increase in the fraction ($B/_{Bo}$) at low adsorbate concentration

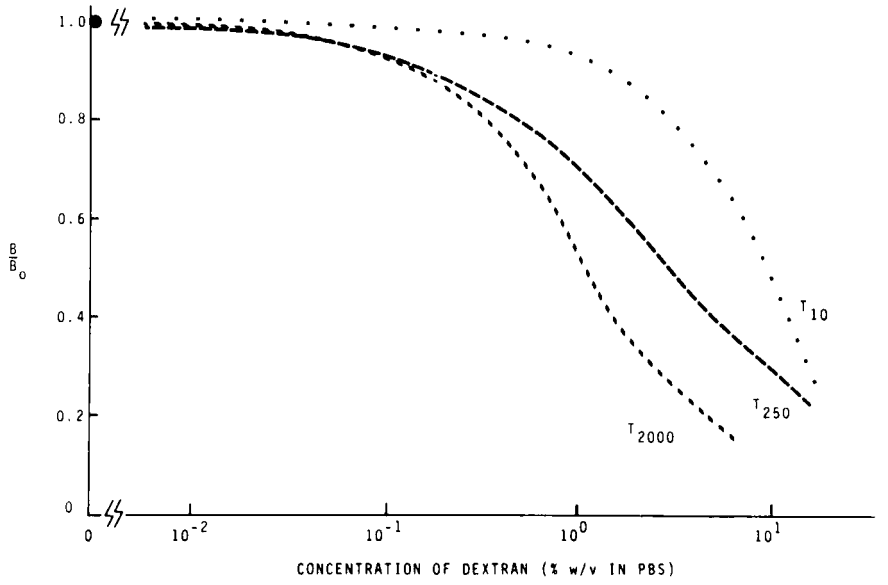

FIGURE 7. Adsorption of radiolabelled insulin to charcoal in the presence of increasing amounts of dextrans of varying molecular weights. The experiment differs from that described in figures 2 and 3 in that the charcoal-dextran mixture was not centrifuged prior to the addition of the radiolabelled hormone. The graphs show that as more dextran was added there was a gradual decrease in the adsorption of radiolabelled insulin by the charcoal. It also shows that the larger molecular weight dextrans (T_{250} and T_{2000}) had a greater effect on reducing insulin adsorption to charcoal.

while at higher concentrations a decrease in adsorption occurred. Figure 6 shows the effect of adding BSA and labelled triiodothyronine simultaneously upon adsorption of the latter. At low concentrations of BSA there was no difference from pre-coating on the adsorption of the hormone. As the concentration of the BSA increased, adsorption decreased more with simultaneous exposure. Figure 7 shows the effect of much higher concentrations of dextran than are normally used for phase separation on adsorp-

tion of labelled insulin to charcoal. This experiment differs from that described above (Figures 2 & 3) in that the dextran-charcoal mixture was not centrifuged prior to the addition of radiolabelled insulin. A decrease in adsorption of insulin to charcoal occurred at dextran concentrations exceeding 0.1 percent (W/V). For the same percent W/V concentration of dextran the larger molecular weight varieties had a greater effect on decreasing adsorption of the radiolabelled hormone.

In summary, "pre-coating" with dextran had no effect while protein pre-coating generally caused a decrease in the adsorption of hormones to charcoal. However, coating the charcoal very lightly with proteins and using a short incubation time caused a paradoxical increase in adsorption of the hormones.

DISCUSSION

The effect of variations in temperature, time, incubation medium, molecular species to be adsorbed and amount of charcoal added (Figure 1) are similar to those reported by others and were used by us primarily to chose optimum conditions for the "coating" experiments (4, 5, 7, 8, 9, 10). The "pre-coating" of dextran on charcoal had no effect on the adsorption of hormones. However, dextran present in high concentration simultaneously with the labelled hormones decreased adsorption of hormones to charcoal (Figure 7). This effect was greater with the larger molecular weight dextrans suggesting the decrease in adsorption was due to an increase in viscosity of the medium. At high concentrations BSA had the same effect with simultaneous exposure (Figure 6).

Interestingly, the decrease in adsorption was greater when the BSA was added at the same time as the hormone than when added 30 minutes earlier (pre-coated) and left in solution. Presumably impairment of adsorption of the radiolabelled hormone to charcoal was less as viscosity, and hence impedance to molecular movement, decreased due to the greater adsorption of the albumin molecules themselves if added before the hormone. At lower concentrations of coating proteins the adsorption of hormones to charcoal was found to be increased if the charcoal was pre-coated with BSA, IgG or insulin (Figure 5) and if incubation of the radiolabelled hormone with the protein-coated charcoal was stopped before the hormone could be completely adsorbed. No such behaviour was observed if charcoal was "pre-coated" with dextran. The results obtained by us cannot be explained on the basis of the theory postulated by Herbert et al (3) that proteins or dextran are adsorbed to the surface of charcoal, thereby forming a "molecular sieve" allowing only small molecules, such as free but not bound ligand, to be adsorbed.

Activated charcoal is used widely throughout industry to separate components from mixtures. Its mechanism of action has been extensively investigated and has been shown to be primarily a volume effect (11). In the manufacture of activated carbon, the raw material is first carbonized and then "activated". This results in the development of a pore system (Figure 8). Micropore widths are typically 0.5 to 0.8 nanometers (nm) but can be selected to be no more than 0.5 nm. In strongly activated carbons, the

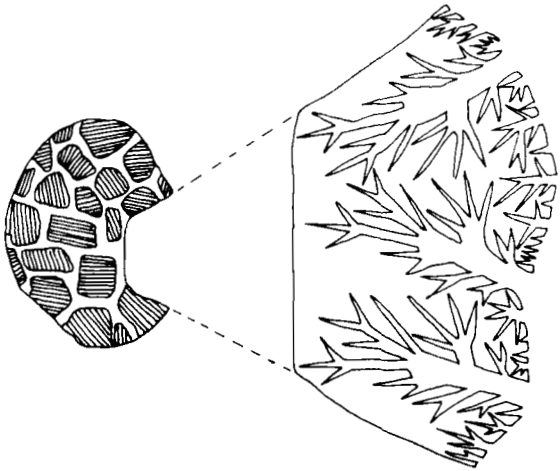

FIGURE 8. The structure of an activated charcoal particle. The enlarged section shows a vast network of micropores which vary widely in diameter. The size of the micropores and their frequency distribution determine the adsorption characteristics of that particular charcoal particle - see text.

distribution of pore size shifts towards larger micropores. The adsorption and separating properties of charcoal are directly related to its micropore structure. Important factors affecting adsorption are micropore size, shape, distribution and accessibility. Equally important are ligand geometry, distribution, polarity and availability. Environmental factors affecting adsorption are concentration, electrical charge, temperature, solvent choice, pH, thermal conductivity of the system as a whole, and the presence of other competing molecules (11). The small molecules in a mixture readily enter the micropores while larger ones are excluded. Because of their random movement, the molecules then either escape from the pores or penetrate further into the interior of the charcoal into micropores of decreasing diameter. In these they bounce

off the walls of the pores losing kinetic energy and hence their ability to escape from the micropores. The "trapping" of small molecules in the interior of the charcoal particles explains both the essentially irreversible nature of adsorption of small molecules to charcoal as well as the heat produced in the process (11).

Large pores play a key role in adsorption by small pores in that molecules are transported through the larger into the small ones. Obstruction of passage through a large pore then impedes adsorption by small pores beyond and forms the basis for the use of "coated" charcoal. There is some adsorption on exposed surfaces but interaction energies are about 1.7 times larger in micropores than on a single flat surface of the same chemical nature (11). Small molecules readily enter the micropores and are also able to egress. The ability of molecules to penetrate the pores in a function of their size and shape as well as the size of the micropores. Accordingly the frequency distribution of various pore sizes greatly affects the ability of a particular batch of charcoal to differentially adsorb molecules from a mixture. Using a formula developed by Dubinin and Radushkevich (11) it is possible to accurately predict the total volume of adsorbate for micropores if their size distribution is known.

The results we obtained are readily explained using the above mechanism of charcoal action on small molecules. Once charcoal is activated, the distribution of micropore width and shape can be altered by adsorption of various molecules. Subsequent adsorption of more of that or another molecular species is

then determined by the new distribution. In this way large pores can be made smaller with the result that, under the right circumstances, adsorption of small molecules can be increased by virtue of the increased total number of small pores (Figure 5). This is contrary to what is expected to happen with surface adsorption and is due to the fact that charcoal adsorption is predominantly a volume phenomenon. With adsorption of large amounts of one type of molecule the total volume available for subsequent adsorption of another is decreased. Thus, in order to see the anomalous increase in adsorption by pre-coated charcoal, the pre-coating must be of the appropriate amount and in appropriate distribution. Allowing pre-coating with too high a concentration of coating material, or allowing too long a contact time with this coating material, decreases total available pores.

We failed to see any effect on adsorption with dextran "pre-coating" as we saw with proteins. This suggests that dextran did not effectively adsorb to the charcoal whereas the proteins did. In Norit A decolourizing carbon approximately 40% by volume of the pores are less than 10 A in diameter (micropores), 20% are between 10 - 1000 A (transitional pores) and 40% are greater than 1000 A (macropores) (11). We suggest that dextran does not alter pore size distribution because of its inability to enter even the larger pores. Dextran is a linear polymer of isomaltose with some branching (12). Unlike dextran, albumin and IgG are relatively globular. For small molecules shape is less important. Dextran of molecular weight 10,000 - 2,000,000 daltons is a very long

molecule and would be expected to have trouble "weaving" its way into the pores of Norit A charcoal whereas smaller molecules would not. The fact that, even at very high dextran concentrations and prolonged exposure of the charcoal to the dextran, no effect on subsequent uptake of the other molecules was seen implies that the dextran used was not effectively adsorbed to the charcoal to block entry by other molecules.

It should be pointed out that in publications (1, 4, 5, 6, 7) reporting experiments that show a "dextran-coating" sieve effect on charcoal adsorption, the charcoal-dextran preparations used were actually suspensions of charcoal in dextran solutions. The only dextran effects really tested were those that impeded intimate contact of molecules with charcoal by mass action or physical hinderance to movement in solution. The "coated" charcoal was not separated from dextran solution prior to adding compounds for adsorption to eliminate the effects of the dextran solution itself. In addition the amounts of dextran used in all these reports was 10% of the amount of charcoal used (the concentration of charcoal used ranged from 3 to 5 percent W/V). The ratio of dextran to charcoal used in the experiments reported here attains much higher values so that more dextran coating should have occurred, yet no evidence for coating is seen.

The experiments done showed marked dependency of charcoal adsorption on medium, temperature, exposure time, and even on the age of the preparations used. Repetition of an experiment under the supposed same conditions gave quantitative results varying by

as much as 20% from one day to the next in some cases. This was found to happen when the room temperature of the laboratory varied greatly and exposure times were kept down to only a few minutes. The most difficult quantitative results to reproduce were the augmented adsorptions by pre-coated charcoal. This is understandable since two adsorption steps were involved, each of which critically depended upon conditions used.

The amount adsorbed in a given (small) interval of time is also directly dependent on the rate of adsorption which in turn is directly dependent on the availability of adequate pores for the molecule being adsorbed. Only a significant increase of availability of such pores can result in a significant increase in the rate of adsorption and thereby an increase in adsorption in a given time. It was then essential, in order to demonstrate a significant adsorption augmentation by pre-coated charcoal, that exposure times and amounts of charcoal used be small - precisely those conditions (along with other factors such as temperature) small changes in which produce significant changes in adsorption and therefore make reproducibility errors large. The small amounts of charcoal used meant that more than usual care had to be exercised in carrying out these experiments. Conditions which allowed near maximum adsorption by uncoated charcoal cannot allow more adsorption by pre-coated charcoal since there is little more compound left to be adsorbed or there is little room left in the charcoal for further adsorption.

Our observations are not consistent with the creation of a sieve mechanism on the surface of charcoal as proposed by Herbert

et al (3). However, the mechanism of action of activated charcoal as detailed above not only explains the results obtained by us but also those of other workers. Ekins (10), Palimieri et al (1) and Binoux and Odell (7) failed to find that dextran conferred additional selectivity on charcoal's ability to separate molecular mixtures. These authors suggested that the pre-coating of charcoal with plasma proteins has the effect of reducing the total number of available adsorption sites and has the same affect as reducing the total amount of charcoal present. We suggest that the known structure and characteristics of activated charcoal provide an alternate explanation of its mechanism of separating molecular mixtures. Unlike the above theories it explains not only all our observations but also those reported by the authors quoted above.

We conclude that the differential adsorption of various hormones to charcoal is not affected by "pre-coating" the charcoal with dextran. At high concentrations of dextran in the medium there is interference with the adsorption of the hormones which is likely due to the increased viscosity of the medium produced by the dextran. Proteins do have an effect and at high concentrations decrease the adsorption of hormones to charcoal. However, at low concentrations of protein, provided incubation time is kept short so as to prevent maximal adsorption of hormones, there is an increase in the adsorption of the hormones to charcoal. These results can be explained on the basis of the known mechanism of action of activated charcoal on small molecules.

As dextran does not appear to affect that action it is probably unnecessary to add it to the charcoal when using it to separate molecular mixtures.

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