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Title: INTERACTION BETWEEN PARALYTIC SHELLFISH POISON AND CLAM MELANIN AND PROTEIN

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Interactions between paralytic shellfish poison (PSP) and components of the butter clam (Saxidomus giganteus) were studied to determine which component was involved in the binding and retention of PSP by the butter clam, and to characterize the nature and strength of the interaction. A simple and accurate assay for PSP in buffer and salt solutions was developed for this study using the Folin-Ciocalteau phenol reagent.

Equilibrium dialysis binding experiments with PSP and bovine serum albumin or fibrinogen indicated that proteins did not interact significantly with PSP. Binding experiments with PSP and a butter clam siphon homogenate indicated an apparent electrostatic interaction between PSP and some component of the clam siphon homogenate, which was later identified as melanin. Synthetic and natural clam melanin interacted strongly with PSP, and the interaction was

similar in nature to a cation exchange reaction.

The interaction between PSP and melanin was rapid, reaching an equilibrium within one hour at 25° C. The interaction was strongly influenced by pH; maximum binding took place at pH 6.0 to 8.0, but at pH 2.0 to 3.0 there was essentially no binding. The interaction was reversible and rapid desorption of bound PSP took place at pH 3.0. The interaction was also affected by the presence of competing cations; tri-, di-, and monovalent cations, in that order of effectiveness, interfered with the binding and caused desorption of bound PSP.

The anatomical distribution of melanin in butter clams paralleled the distribution of PSP, and PSP was apparently bound to melanin in toxic butter clam siphons. Butter clams, which retain PSP for longer periods than do other bivalve mollusks, had a higher melanin content in the interior walls of their siphons than the other mollusks studied.

The close relationship between the distribution of PSP and of melanin in butter clams together with the observed electrostatic interaction between PSP and melanin suggest that melanin may play an important role in the retention of PSP in butter clams. The reversible nature of the PSP-melanin interaction in the presence of competing cations suggests that polyvalent salt solutions may be used in the detoxification of toxic butter clams.

Interaction Between Paralytic Shellfish Poison and Clam Melanin and Protein

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INTERACTION BETWEEN PARALYTIC SHELLFISH POISON AND CLAM MELANIN AND PROTEIN

INTRODUCTION

Paralytic shellfish poisoning (PSP) has been known since ancient times. Hudson (as cited by Meyer, Sommer, and Schoenholtz, 1928, p. 368) related that "from time immemorial" it has been the custom among certain coastal tribes of Alaskan Indians, particularly the Poma,

> to place sentries on watch for Kal-ko-o (mussel poison). Luminescence of the waves, which appeared rarely and then only during very hot weather, caused shellfish to be forbidden for two days; those eating shellfish caught at such times suffered sickness and death.

PSP outbreaks occur almost worldwide in a sporatic and unpredictable manner (Schantz, 1969a), contributing to an anxiety toward all shellfish and hindering the utilization of many shellfish.

Because PSP is not readily destroyed by heat processing or removed by washing of the shellfish, it presents a unique public health problem for commercial packers of shellfish (Schantz, 1969a). The shellfish industry of Alaska has been all but destroyed (Lehman, 1966), and more than half of the coastline of British Columbia has been closed to shellfish harvesting because of PSP (Bourne, 1969).

The primary source of PSP in bivalve mollusks has been satisfactorily established as marine dinoflagellates (Sommer et al.,

1937). Butter clams (Saxidomus giganteus Deshayes) may accumulate PSP by ingesting the causative dinoflagellate Gonyaulax catenella (Schantz et al., 1966), and can retain high levels of PSP in their siphons for as long as two years (Quayle, 1969). The process by which shellfish bind PSP without harm to themselves has not been determined, although the binding is believed to be electrostatic in nature (Schantz, 1969b). A knowledge of the process by which shellfish bind PSP in their own systems may suggest a means of removing PSP from shellfish without destroying their commercial value (Schantz, 1969a).

The primary objective of this investigation was to determine the mechanism by which butter clams bind and retain PSP, and to characterize the nature and strength of the interaction. Interactions between PSP and protein and melanin were studied to determine which component of the butter clam siphon was involved in the binding and could have been responsible for the retention of PSP in butter clams. The possibility of removing PSP from toxic clams by reversing the PSP binding was also investigated.

REVIEW OF LITERATURE

Epidemiology of PSP

The first recorded death on the North American Continent attributed to paralytic shellfish poisoning (PSP) occurred on June 15, 1793, on the central coast of British Columbia. Captain George Vancouver (as cited by Quayle, 1969) described one death and four illnesses resulting from the consumption of toxic mussels. The origin of PSP, however, was not discovered until 1937 when Sommer et al.(1937) demonstrated that PSP was present in the dinoflagellate Gonyaulax catenella, and that PSP was passed directly to mussels (Mytilus californianus Conrad) which became toxic after feeding on that dinoflagellate.

Since that time, additional species of the genus Gonyaulax including G. acatenella along the coast of British Columbia (Prakash and Taylor, 1966), G. tamarensis along the north Atlantic coast (Prakash, 1967), and G. polyedra along the Southern California coast (Schradie and Bliss, 1962) have been shown to be the sources of toxins that are very similar, if not identical, to PSP produced by G. catenella.

Filter feeding mollusks such as clams and mussels may accumulate PSP by ingesting toxic dinoflagellates (Ray, 1970). The poison

causes no observable disturbance of the natural life processes of the mollusk but is lethal to persons unfortunate enough to eat the mollusks during periods when they are toxic (Schantz, 1963). The amount of PSP accumulated by shellfish depends upon the number of toxic dinoflagellates in the water and the amount of water filtered by the shellfish. One small mussel weighing about 50 g may contain as much as 20,000 to 30,000 mouse units of PSP (10 human lethal doses), and butter clams have been found to contain over 8.6 mg of PSP (30-40 human lethal doses) per 100 g of meat (Schantz, 1969a; Quayle, 1969). The lethal dose for man is not known but has been estimated to be 0.2 to 0.3 mg (Schantz, 1969a).

In most species of bivalve mollusks, the toxin loss following a bloom of toxic dinoflagellates is rapid: nonsiphonate forms including the bay mussel (Mytilus edulis Linné) and the Pacific oyster (Crassostrea gigas Thunberg) lose the toxin within a few days, and siphonate forms including the native littleneck clam (Protothaca staminea Conrad), the Manila clam (Venerupis japonica Deshayes), and the horse clam (Tresus capax Gould) lose the toxin within a few weeks. Butter clams, however, may retain PSP for as long as four years (Quayle, 1969).

PSP is not evenly distributed throughout the tissues of toxic shellfish, but accumulates in specific locations. In mussels, the tissues of the hepatopancreas or dark gland apparently bind the toxin

and the hepatopancreas may contain 95% or more of the toxin. In butter clams, PSP apparently moves from the hepatopancreas to the siphon where it is bound and retained (Schantz, 1969a). Quayle (1969) found that the black tips of butter clam siphons may contain more than 17 mg of PSP per 100 g, and there is a reduction in toxicity from the distal to the proximal portion of the siphon.

Biochemistry of PSP

Isolation and Purification of PSP

Procedures for the isolation and purification of PSP from clams and mussels (Schantz et al., 1957), and from G. catenella cultures (Burke et al., 1960; Schantz et al., 1966) have been described in detail. All purification procedures are based on the selective adsorption and elution of PSP on cation exchange resins. The optimum pH for adsorption of PSP onto Amberlite XE-64 cation exchange resin was about 5.2. At higher pH values some of the PSP was either destroyed or washed off the column by the buffer. The poison can be adsorbed at a pH as low as 4.5, but as the adsorption progresses the pH of the solution may decrease enough to cause partial elution of the poison (McFarren et al., 1958). Adsorption of PSP onto cation exchange resins is also affected by high salt concentrations in the PSP solution (Burke et al., 1960).

PSP has been eluted from cation exchange resins with 0.1 M acetic acid (Schantz et al., 1957), 0.5 N trichloroacetic acid (Burke et al., 1960), and 0.3 M acetic acid (Schantz et al., 1966). Further purification of the PSP extract involved chromatography on acid-washed alumina, and conversion to the hydrochloride salt which could be dehydrated to a pure white amorphous material (Schantz et al., 1957). Principal evidence for the purity of PSP prepared by the above procedure was obtained, using both PSP and a dihydro derivative of PSP, from countercurrent distribution experiments and paper chromatography indicating a purity of 99-100% (Mold et al., 1957).

Structure and Properties of PSP

Elemental analyses, pH titrations, and diffusion coefficient determinations have indicated that PSP, as the dihydrochloride, has a molecular weight of 372 and molecular formula of $C_{10}H_{17}N_7O_4 \cdot 2HCl$ (Schantz et al., 1957; Schantz et al., 1966). Sequential oxidation of PSP with hydrogen peroxide and reduction with phosphorus and hydrogen iodide converted PSP to a degradation product with a molecular formula of $C_9H_{12}N_6O_2$ and identified as 2-imino-8-amino-6-methyl-3-β-carboxyethyl purine (Rapoport, as cited by Mosher, 1966). Based on his investigations, Rapoport proposed a substituted tetrahydropurine structure for PSP (Figure 1) and named the substance "Saxitoxin" (Rapoport et al., as cited by Russell, 1967;

Schantz, 1969a). This proposed structure of PSP, with a molecular formula of $C_{10}H_{15}N_7O_3$, does not account for one oxygen and two hydrogen atoms and is no longer believed to be correct (Rapoport, as cited by Schantz, 1969b). Some properties of PSP are summarized in Table 1.

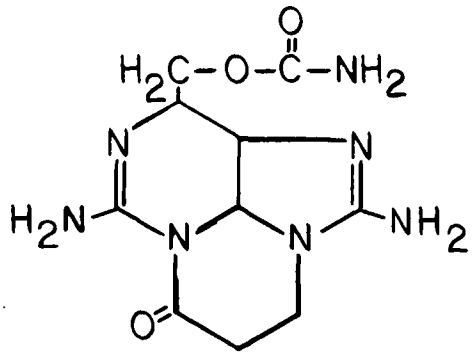

Figure 1. Proposed structure for PSP (Rapoport et al., as cited by Russell, 1967).

PSP Assays

Mouse Bioassay

Sommer and Meyer (1937) developed the first bioassay for PSP by establishing the mean death time for mice injected with an LD_{50} dose of PSP, and constructing a dose vs. death time curve. Toxicity was expressed in mouse units (M. U.), and one mouse unit was defined as the amount of toxin that caused death of a 20 g mouse in 15 min when injected intraperitoneally. Correction factors for

Table 1. Some chemical properties of PSP.[a/]

Molecular formula	$C_{10}H_{17}N_7O_4 \cdot 2HCl$
Molecular weight	372
N content	26.1
Diffusion coefficient	4.9×10^{-6} cm/sec
Absorption in ultraviolet and visible	None
pKa	8.3; 11.5
Specific optical rotation	+130° ± 5
Solubility	Soluble in H_2O; slightly soluble in EtOH and MeOH; insoluble in lipid solvents
Form	Two tautomers
Sakaguchi test	Negative
Benedict-Behre test	Positive
Jaffe test	Positive
Weber test	Positive
Ring structure	Present
Aromatic structure	None
Carbonyl groups	None
Reduction with H_2	Nontoxic dihydro derivative

[a/] Compiled from data presented by Mold et al. (1957), Schantz (1960), Schantz et al. (1961), and Schantz et al. (1966).

weight differences in mice were also determined. The availability of purified PSP (Schantz et al., 1957; Mold et al., 1957) made it possible to use reference standards for bioassays, to correct for differences in tolerances among various mice strains, and to convert mouse units into absolute weight of pure toxin (Schantz et al., 1958). Although the bioassay may underestimate the PSP content of shellfish low toxicity (400 M. U. /100 g) by as much as 60% (McFarren et al., 1957), and has an accuracy of only ± 15% (McFarren et al., 1958), the bioassay is generally accepted as the best available means of detecting PSP in shellfish (McFarren, 1971).

Chemical Assay

A chemical assay for PSP based on the Jaffe test has been developed (McFarren et al., 1958; McFarren et al., 1959). An acid extract from toxic shellfish was adsorbed onto an ion exchange resin, and PSP was eluted from the column with 0. 5 N trichloroacetic acid (McFarren et al., 1958). The eluted PSP was measured colorimetrically after reacting the PSP with picric acid in an alkaline solution to form a red-colored compound, and extracting the colored solution with a pyridine-ethyl acetate mixture to remove unreacted picric acid (McFarren et al., 1959). Although this assay has an accuracy of ± 31.9 μg/100 g of shellfish (ibid.), it is unlikely that the chemical assay will replace the mouse bioassay for general use

because of the complex nature of the procedure and the difficulties caused by interfering substances in the samples (Quayle, 1969).

Serological Assay

Serological assays for PSP have been developed involving passive haemagglutination and bentonite flocculation (Johnson and Mulberry, 1966; Johnson et al., 1964). PSP was conjugated to protein by formaldehyde condensation, and antibodies against the PSP-protein conjugate were obtained from rabbits (Johnson et al., 1964). In the passive haemagglutination test, a sample containing PSP was reacted with rabbit antibodies, and the residual antibodies were titrated with a tanned sheep red blood cell-PSP antigen preparation. This test was about ten times as sensitive as the mouse bioassay, but the antigen preparation was unstable and had to be prepared daily. In the bentonite flocculation inhibition method, PSP antigen was adsorbed onto bentonite particles. This antigen preparation was stable for a few days, but the sensitivity of this procedure was reduced to the level of the bioassay (Johnson and Mulberry, 1966).

Detoxification of Shellfish

Administrative guidelines for PSP levels in clams were established in 1949 and modified in 1951 (Magnusson and Carlson, 1951). These guidelines specify that the average toxicity of fresh, frozen,

or canned whole clams shall not exceed 400 M. U. per 100 g and no individual package shall exceed 2, 000 M. U. With minced or chopped clams, the average toxicity shall not exceed 2, 000 M. U. per 100 g and no individual package shall exceed this figure. Since shellfish exposed to blooms of toxic dinoflagellates generally contain levels of PSP higher than those permitted by the Food and Drug Administration (FDA) standards (Quayle, 1969), various natural and artificial methods of detoxifying toxic shellfish have been attempted.

Natural Detoxification

Natural methods of detoxifying shellfish containing PSP have included detoxification in situ and transplantation to nontoxic areas. A reduction in the PSP content of most toxic bivalve mollusks to acceptible levels usually occurs within a few weeks following a toxic dinoflagellate bloom (Quayle, 1969). With butter clams, however, the decrease in toxicity is very gradual and may require as long as four years before acceptable PSP levels are reached. Older butter clams apparently retain PSP for longer periods than do younger clams.

During a toxic dinoflagellate bloom, there is an immediate increase in the PSP content of butter clams. The body of the clam contains 60% of the PSP and the siphon and gills contain the remaining 40%. During the first six weeks following a bloom, the toxicity

of butter clams decreases at a rate approximating the decrease in toxicity of other mollusk species, eliminating over 90% of the PSP. The bulk of this loss apparently is due to the loss of 88% of the toxin from the clam body. In the butter clam siphon, however, there is an almost 23% increase in toxicity (Quayle, 1969), suggesting a movement of part of the PSP from the body to the siphon. After the six week period 85% of the remaining PSP is found in the siphon of the butter clam (ibid.).

Natural detoxification by transplantation of toxic butter clams has been investigated in Canada and Alaska. Dassow (1966) reported that when toxic butter clams were transplanted from an area of moderately high toxicity to an area of low toxicity there was little, if any, reduction in toxicity after 13 months. Quayle (1969) reported that butter clams transplanted to nontoxic areas gradually detoxified themselves, but also noted that the rate of detoxification was not different from that of clams in their native beds.

Artificial Detoxification

Several procedures for detoxifying butter clams during processing have been proposed. By removing the liver, mantle and siphon of the butter clam it is possible to reduce PSP levels by approximately 40% and still retain 75% of the original weight of the clam. Steaming of the clam meats prior to canning further reduces the

toxicity by 25-35% depending on the length of the steaming period (Magnusson, 1966). A processing procedure recommended by the Bureau of Commercial Fisheries involves the removal of clam siphons, steaming of clam meats for 10 min, and retorting at 250° F for 75 min (Dassow, 1966). This procedure reduces the toxin content of the clams by as much as 93% and results in a canned product which would be acceptable under the established FDA tolerances (Dassow, 1966; Magnusson, 1966). However, no substantial use of this process has been made by the shellfish industry, because it involves the loss of too much clam meat to be economically feasible (Tillion, 1966).

Investigations of chemical detoxification procedures have been limited to the pretreatment of butter clams with mild acid solutions (U. S. Dept. Interior, 1966). No reduction in PSP was observed in butter clams after holding either live clams or chopped clam meat in acid solutions of pH as low as 5.0 for periods up to 17 hr. Acid solutions of pH 2-3 have been shown to be extremely effective in removing PSP from clam siphons during the purification of PSP (Schantz et al., 1957), but boiling toxic butter clams in solutions of pH 1.8-4.9 for periods from 2.5-10 min failed to show any meaningful reduction in toxicity (Quayle, 1969). Severe acid treatment is of doubtful commercial value, since it would render the product inedible (U. S. Dept. Interior, 1966).

Drug-Protein Interactions

The effect of a biologically active compound can often be attributed to or modified by the interaction of the active compound with macromolecules in the organism (Van Os, 1966). Many antimicrobial agents including penicillins, tetracyclines, and sulfonamides (Clausen, 1966; Rolinson and Sutherland, 1965), hormones such as progesterone (De Moor, Heirwegh, and Steeno, 1963) and analgesics such as acetylsalicylic acid (Ali and Routh, 1969) interact reversibly with serum albumin. With certain penicillins, a close correlation has been shown between the diminution of antibacterial activity and the extent to which the drug is bound to serum albumin (Joos and Hall, 1969).

Interactions between proteins and small molecules have been studied by a variety of methods. In most of these investigations, techniques including equilibrium dialysis (Hansch et al., 1965), ultrafiltration (Bird and Marshall, 1967) and gel filtration (Acred et al., 1963) have been used, although other techniques such as nuclear magnetic relaxation (Fischer and Jardetzky, 1965) and pulse radiolysis (Phillips et al., 1970) have also been employed.

Equilibrium Dialysis

Equilibrium dialysis is the most common technique used to

study the reversible binding of small molecules to macromolecules, since it is simple to perform and multiple dialyses can be carried out simultaneously with portions of the same sample (Rose, 1969). One of the problems associated with equilibrium dialysis concerns the rate of equilibrium across the dialysis membrane. If the ligand is added to a solution containing a macromolecule and then dialyzed out, a pseudo-equilibrium may result with an artificially high percentage of the ligand apparently bound to the macromolecule (ibid.). This pseudo-equilibrium may result if the rate of dissociation is very slow compared to the rate of equilibrium, and can be prevented by dialyzing the ligand into the compartment containing the macromolecule (ibid.). Other problems associated with equilibrium dialysis include a possible interaction of the small molecule with the dialysis membrane, artificially high or low apparent binding due to the establishment of a Donnan equilibrium (Keen, 1965), and the long periods of time generally required for equilibration across the dialysis membrane (Rose, 1969). The amount of interaction between ligand and macromolecule is determined after equilibration by measuring the amount of free ligand present on both sides of the dialysis membrane (Keen, 1966).

Ultrafiltration

Ultrafiltration has been used by Keen (1965), Rolinson and

Sutherland (1965) and Bird and Marshall (1967) to study the binding of antibiotics to blood serum. The principal advantage of ultrafiltration over equilibrium dialysis is the short period of time required to complete experiments (Keen, 1965). In these experiments protein-penicillin solutions of 5-30 ml were filtered under pressure for up to 15 min and yielded from 0.5 to 2 ml of ultrafiltrate. The amount of penicillin in the filtrate was subtracted from the original amount added in order to determine the amount of penicillin bound to the protein. Corrections for the Donnan equilibrium were made by measuring the concentration of sodium and chloride in the ultrafiltrate and plasma (ibid.).

Gel Filtration

The use of cross-linked dextran gels to separate molecules of different sizes was first reported by Porath and Flodin (1959). Since that time, gel filtration has been used to study a great many reversible interactions between small molecules and macromolecules including the protein binding of drugs (Acred et al., 1963), peptide-protein interactions (Fairclough and Fruton, 1966), nucleotide-protein interactions (Weissbach, Redfield, and Kaback, 1969), and progesterone-protein interactions (De Moor et al., 1963). Several different procedures utilizing gel filtration have been used in these investigations and they include the following: application of a protein

sample to the gel filtration column and elution with a solution containing the small molecule being tested (Clausen, 1966); application of a sample containing the small molecule and elution with a protein solution (Barlow, Firemark, and Roth, 1962); and application of a sample containing protein and the small molecule, and elution with a buffer solution (De Moor et al., 1963). Gel filtration is preferrable to equilibrium dialysis and ultrafiltration in the study of protein binding for several reasons. Gel filtration is much more rapid than dialysis, an advantage when dealing with labile materials (Acred et al., 1963); gel filtration avoids the use of an interposed potentially adsorbing membrane (Barlow et al., 1962); and gel filtration can be used to indicate both the amount of interaction and the strength of the interaction (ibid.). Although superior to dialysis and ultrafiltration in some ways, there are also disadvantages to gel filtration that include a dilution effect as molecules pass through the column (Acred et al., 1963) and an interaction of the dextran gel with some compounds (Brook and Munday, 1970).

Melanin

The pigment of a great variety of dark tissues is generally known as melanin. Melanins are mainly heterogeneous polymers of dihydroxyphenyl compounds with complex and irregular structures (Bruenger, Stover, and Atherton, 1967) and are considered to be

catabolic products of tyrosine (Fox, 1966). Melanin isolated from rat melanomas has been shown to be similar to melanin synthesized from L-β-3, 4-dihydroxyphenylalanine (L-DOPA) and oxygen in the presence of tyrosinase (Nicholaus, Piattelli, and Fattorusso, 1964).

In vivo, melanin is believed to exist as particles 0.1 to 1.0 μ in diameter (White, 1958). Crude preparations of melanin granuals are completely insoluble in water and in all common organic solvents but are soluble in hot aqueous sodium hydroxide, in which melanin probably depolymerizes (ibid.), and in ethylene chlorhydrin (Lea, 1945).

Melanins are widely distributed in nature (Fox, 1966), and occur in mollusks as visceral pigments in the mantle and hepato-pancreas, and in areas exposed to light such as the siphon tips of bivalve mollusks (Comfort, 1951).

The ability of melanin to interact with low molecular weight molecules was first reported by White (1958), who indicated that melanin granules from both mammalian and invertebrate sources have the capacity to act as cation exchangers of considerable activity. The cation-exchanging ability of melanin was further examined by Bruenger et al. (1967) in an attempt to explain the presence of metals in naturally occuring melanins. They found that both mammalian melanin and synthetic melanin interacted with metal cations, and that the reactions were similar to ion exchange reactions.

Although the significance of the cation-exchange activity of melanin is unknown, White (1958) has suggested that melanin may absorb electrons and thus have a protective function against ionizing radiation.

The methods used to study interactions between melanin and small molecules and ions are much simpler than those used in protein binding experiments. Because of the granular nature of melanin (Fox and Vevers, 1960), binding experiments require only a short incubation of melanin in a solution containing the compound being tested, and then removal of the melanin from the suspension by centrifugation. Adsorption of molecules onto melanin is rapid and an equilibrium is usually established within 1 hr in binding experiments (Bruenger et al., 1967). The amount of melanin binding can easily be determined either directly by using radioactive compounds and measuring the activity in the melanin pellet after centrifugation (ibid.), or indirectly by measuring the concentration of the test compound remaining in the supernatant (White, 1958).

PSP Binding Studies

Attempts to determine the mechanism by which PSP is bound in the hepatopancreas of mussels have been unsuccessful. Schantz (1960) reported that ground mussel hepatopancreas tissue, when mixed with an equal volume of water containing PSP to form a mixture that contained 100 M. U./ml of PSP, was able to bind less than

5% of the PSP present in the solution. The results were the same with sliced or ground mouse liver, skeletal muscle, or kidney. Although these experiments were unsuccessful in demonstrating in vitro binding of PSP by mussel hepatopancreas tissue, Schantz (1969b) suggested that the binding of PSP in the hepatopancreas should be similar to the binding on a weak cation exchange resin.

EXPERIMENTAL

Sources of PSP

Purified PSP

Purified PSP was obtained from Dr. E. J. Schantz, Fort Detrick, Md. The powdered PSP was dissolved in 0.001 N HCl and stored at 4° C. A solution containing 100 μg of PSP per ml was also provided by Dr. E. J. Schantz and was used as a standard.

Crude PSP

A crude preparation of PSP was obtained from cultures of Gonyaulax catenella. A culture of G. catenella was provided by Mr. W. E. Esaias, Department of Oceanography, Oregon State University, Corvallis, Oregon. Stock cultures were maintained in an enriched sea water medium (Esaias, 1970) at 15° C, illuminated by cool-white fluorescent lights supplying 400 foot candles at the surface of the medium, and transferred every 14-17 days. Mass cultures were incubated in one-gallon jugs containing one liter of medium and incubated as described above. Cells were collected in a continuous centrifuge (Sorvall) according to the method of Burke et al. (1960). The packed cells were homogenized in a Waring blendor and adjusted to pH 2.5 with 5 N HCl. Repeated chromatography of the extract on Amberlite 1RF-97 cation exchange resin, formerly XE-64 (Simpson,

1970), according to the method of Schantz et al. (1966), yielded a crude preparation of PSP. In order to remove low molecular weight contaminants, the crude PSP solution of pH 3.0 to 3.5 was concentrated under vacuum at 30 to 35° C, passed onto a 2.5 x 34 cm column of Sephadex G-15 (Pharmacia Fine Chemicals, Inc.), and eluted with 0.02 N HCl. Fractions containing the bulk of the PSP were pooled and stored at 4° C.

PSP Assays

Mouse Bioassay

A standard mouse bioassay (Horwitz, 1960) was used to determine PSP concentrations in biological samples. Four female white mice, weighing from 16 to 20 g, were inoculated intraperitoneally with 1-ml portions of each sample. Death times were determined and the samples diluted with 0.001 N HCl to obtain death times of 5-7 min. Concentrations of PSP were recorded as mouse units, defined as the amount of PSP necessary to kill a 20 g mouse in 15 min. Control mice were inoculated with 1-ml portions of 0.001 M HCl. Conversion from mouse units to μg of PSP was not made since relative toxin levels rather than absolute values were required.

Modified Jaffe Test

A modification of the Jaffe test (McFarren et al., 1958) was used. Four-milliliter portions of PSP solutions were mixed with 1.0 ml of 1.2% picric acid and 1.0 ml of 3.6% NaOH. The mixtures were incubated at 38° C for 20 min, and the absorbance at 510 nm measured against a distilled water blank on a Bausch and Lomb Spectronic 20 spectrophotometer. Concentrations of PSP as low as 2 μg/ml could be accurately determined by this method.

Folin-Ciocalteau Test

The procedure used was a modification of the method employed by Hitchings (1941) to quantitate guanine and xanthine, and was used for routine PSP assays in binding experiments. In each assay, 2.0-ml samples containing PSP were mixed with 0.5 ml of distilled water, 0.25 ml of Folin-Ciocalteau reagent (Fisher Scientific Co.), and 0.5 ml of a saturated aqueous sodium carbonate solution. The mixture was incubated at 25° C for 1 hr, and the absorbance at 660 nm was measured by a spectrophotometer (Spectronic 20, Bausch and Lomb). Purified PSP standards, and distilled water or buffer controls were assayed in the same manner.

PSP-Protein Interactions

Gel Filtration

Sephadex G-15 dextran gel (Pharmacia Fine Chemical Inc.) and bio gel P-4 polyacrylamide gel (Bio-Rad Laboratories) were used in preliminary binding experiments. The gels were allowed to swell in distilled water for 3 hr and then poured in 1.5 x 30 cm Sephadex gel filtration columns yielding a final bed height of 27 cm. Filter paper discs were placed on the columns, and the columns were washed with 1 to 2 liters of distilled water at a rate of 10 ml/hr. Solutions containing 10-15 μg of PSP, 1-10 mg of bovine serum albumin (BSA, Calibiochem), or a PSP-BSA mixture were incubated for 1 hr at 25°C, placed on the gel, and eluted with distilled water or buffer at a flow rate of 10 ml/hr. Fractions (1.5-ml) were collected and assayed with the Folin-Ciocalteau reagent.

Ultrafiltration

Ultrafiltration experiments were carried out in 25 mm ultrafiltration cells (Millipore Corp.) connected to a cylinder of compressed nitrogen gas. The ultrafiltration cells had a capacity of 17 ml and could be operated at pressures up to 100 psig. Pellicon PSAC ultrafiltration membranes (Millipore Corp.) and Diaflow UM-2

ultrafilters (Amicon Corp.) were used in these experiments. Both of these membranes have molecular weight cutoffs of approximately 1,000. Solutions of BSA were dialyzed against distilled water for 18 hr at 4° C prior to ultrafiltration experiments.

Before each binding experiment, the ultrafiltration membranes were rinsed by filtering 50-ml portions of a 1/10 dilution of McIlvaines standard buffer (Lange, 1967) at pH 3.0, 6.0 and 8.0 through the Pellicon membranes, or 50 ml of veronal buffer (Keen, 1966) at pH 7.0 and 8.0 or 0.15 M citrate buffer at pH 3.0-6.0 through the Diaflow membranes. Protein solutions containing 10 mg of dialyzed BSA or 20 mg of bovine fibrinogen (Calbiochem) in 10 ml of distilled water were then added to the ultrafiltration cells. The protein was rinsed with buffer by filtering the solution under 25-50 psig of pressure, collecting 5 ml of filtrate, adding another 5 ml of buffer to the cell, and filtering the solution again. This procedure was repeated until the filtrate gave a negative Folin-Ciocalteau reaction.

PSP solutions containing approximately 20 μg of crude toxin were added to the cells and mixed with the protein. After incubation for 15 min at 25° C, the solutions were filtered under 50 psig of pressure and 1-2 ml of filtrate collected. The filtrates were assayed for PSP with the Folin-Ciocalteau reagent, and the amount of PSP bound to protein determined by differences between the

amount of PSP added and the amount recovered in the filtrate. Protein and PSP controls were processed in the same manner.

Equilibrium Dialysis

Dialysis experiments were conducted in the multicavity equilibrium dialysis cell shown in Figure 2. The dialysis cell was constructed from two 12" x 3" x 3/4" pieces of transparent acrylic plastic. Seven cavities, 1" in diameter and 3/8" deep, were drilled into each half-cell; each cavity had a volume of approximately 2.5 ml. Holes, 3/1.6" in diameter, were drilled from the top of the cell into each cavity providing an opening for introducing or withdrawing solutions. Metal bolts (1/4" x 2") with wing nuts were used to clamp the two half-cells together. Dialysis tubing (1-1/8" round diameter, Union Carbide Corp.) was soaked in distilled water, split open and used as a dialysis membrane between the two half-cells.

The rate at which PSP diffused across the membrane was determined by dialyzing 40 μg of purified PSP in 0.01 M phosphate buffer at pH 7.0 against the same buffer at 4° C. Samples were removed at 12, 24, 36, and 48 hr and assayed with the Folin-Ciocalteau reagent. In control cells, the dialysis membrane was removed.

Small molecular weight components of BSA solutions were removed prior to binding experiments by dialyzing 2-ml solutions

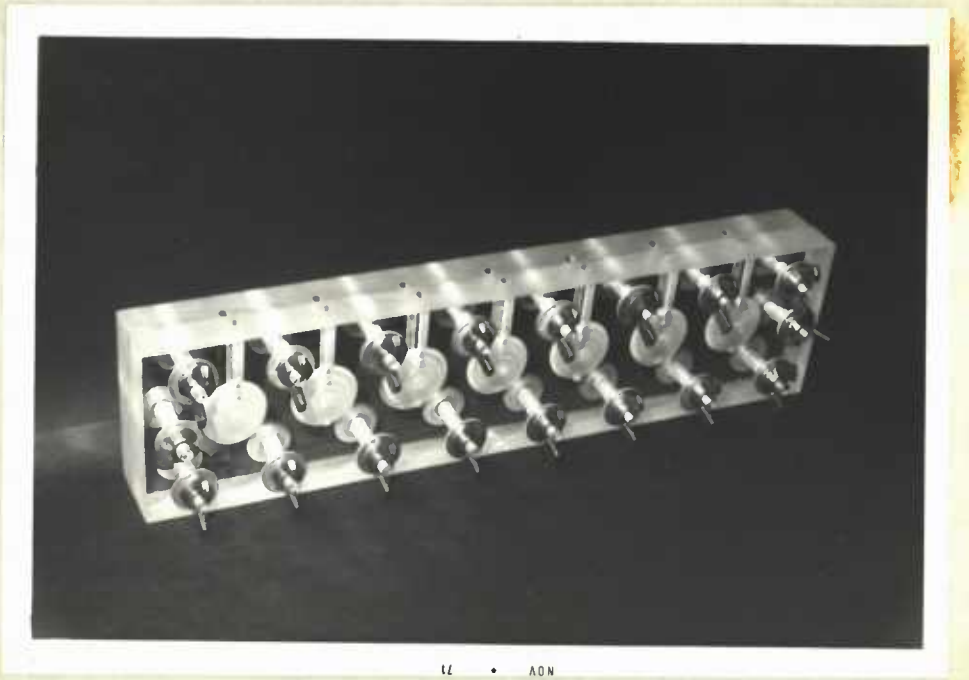

Figure 2. Seven chambered equilibrium dialysis cell.

of BSA in buffer against the same buffer for three 8-hour periods at 4° C. Buffers used in the equilibrium dialysis experiments included 0.02 M disodium phosphate-0.01 M citric acid buffer at 3.0, 5.0, and 7.0; and 0.1 to 0.20 M citric acid-sodium citrate buffer at pH 6.0.

After preliminary dialysis of the protein solutions, toxin solutions containing from 35-40 μg of crude or purified PSP were added to the cavities across from the protein solutions. The openings in the cavities were then sealed and the dialysis cell incubated on a shaker platform at 4° C for 24 hr. At the end of this period, the concentration of PSP in the diffusate was determined with the Folin-Ciocalteau reagent. Both protein and PSP control cells were used in all experiments. The amount of PSP bound to the protein was determined by subtracting the amount of free PSP in the binding cells from that of PSP in the control cells.

PSP-Clam Siphon Homogenate Interactions

Preparation of Clam Siphon Homogenate

Sixty-four grams of butter clam siphons were homogenized with an equal volume of distilled water. Ten-milliliter portions of the homogenate were then mixed with 70 ml of distilled water and dialyzed against tap water for 48 hrs at 4° C. After dialysis, the

homogenate was decanted into a 200 ml bottle, 5 mg of Thimerosal (K & K) were added, and the volume was adjusted to 100 ml with distilled water. Oxygen was removed from the solution by bubbling N_2 through the solution for 5 min. Dialyzed homogenates were then frozen at -20° C until used.

Binding Procedures

Prior to binding experiments, the dialyzed homogenate was heated to 75° C for 10 min to inactivate enzymes. One-milliliter portions of this heat treated homogenate were added to equilibrium dialysis cells and dialyzed against buffer for six 4-hr periods at 4° C.

Binding experiments using equilibrium dialysis were performed as described previously. Buffers used in these experiments included 0.001-0.1 M phosphate buffer at pH 6.0-8.0, and 0.01 M acetate buffer at pH 3.5-5.5.

PSP-Melanin Interactions

Synthetic Melanin

Synthetic melanin was prepared from L-β-3, 4-dihydroxyphenylalanine (L-DOPA, Sigma Chemical Company) with mushroom tyrosinase (o-Diphenol:O_2 oxidoreductase, E. C. 1.10.2.1, Sigma

Chemical Company). Five milligrams of L-DOPA and 500 units of tyrosinase in 20 ml of a buffer prepared from 0.01 M citric acid and 0.02 M Na_2HPO_4 at pH 6.0 were incubated at 25° C with shaking for 24 hr. Under these conditions, 5 mg of L-DOPA yielded 2.8 to 3.0 mg of pure melanin.

Butter Clam Melanin

Melanin from butter clam siphons was isolated as follows: Approximately 57 g of pooled siphons were placed in 150 ml of 1/10 dilution of McIlvaines standard buffer (Lange, 1967) at pH 6.0. The siphon suspension was subjected to sonication for 3 min using a Biosonic sonic oscillator (Brownwill BP 111 with standard probe) and then filtered through a layer of cheese cloth to remove the remaining siphons. Cellular debris was removed from the suspension by centrifugation at 200 g for 20 min. Melanin granules were collected by centrifugation at 20,000 g for 20 min, washed twice by centrifugation at 20,000 g for 20 min with buffer at pH 6.0, and suspended in 300 ml of 0.05 M phosphate buffer at pH 7.0. Protein was removed from the suspension by treatment with papain (E.C. 3.4.4.10) at 25° C for 48 hr, according to the method of Arnon (1970). The granular suspension of melanin was washed by centrifugation with three 100-ml portions of distilled water and then with 0.1 N HCl until the supernatant gave a negative ninhydrin reaction. The

melanin preparation was suspended in 100 ml of 0.1 N HCl and stored at 4° C. The preparation contained approximately 1.5 mg melanin by dry weight per ml.

Buffers

Unless otherwise noted, the buffers used throughout the melanin-PSP studies were prepared as follows. Buffers from pH 3.0 to 8.0 were a 1/10 dilution of McIlvaines standard buffer (Lange, 1967) prepared from 0.01 M citric acid and 0.02 M disodium phosphate. Buffers at pH 2.0 and pH 2.5 were prepared from 0.01 M citric acid and adjusted with 1 N HCl.

Filtration Binding Procedure

Approximately 3 mg of synthetic melanin was washed by centrifugation with two 10-ml portions of buffer, and resuspended in 1.0 ml of buffer. Twenty-seven micrograms of purified PSP in 0.4 of 0.001 N HCl and 1.6 ml of buffer were added, and the mixture was incubated with shaking at 25° C. After 1 hr incubation, the PSP-melanin mixture was filtered through a MF-Millipore HA filter (Millipore Corp.) under 50 psig of pressure. The amount of PSP in the filtrate was determined with the Folin-Ciocalteau reagent. Controls containing melanin or PSP were processed in the same manner.

Centrifugation Binding Procedure

Clam melanin suspensions in 0.1 N HCl were washed by centrifugation at 20,000 g for 20 min three times with buffer prior to binding experiments. The binding of PSP to clam melanin was measured by adding 27 μg of PSP in 0.001 N HCl to melanin suspended in 2.6 ml of buffer and incubating at 25° C in a shaking water bath. After 1 hr incubation, the melanin was separated by centrifugation at 20,000 g for 20 min, and the supernatant fluid assayed for PSP.

In experiments showing the influence of pH or salts on binding, buffers from pH 2.0 to 8.0 or buffered 0.01 M salt solutions at pH 6.0 were used both for washing melanin and for binding experiments.

Desorption of Bound PSP

The release of bound PSP was measured by washing melanin-PSP complexes once with 3 ml of buffer at pH 6.0 and then suspending the PSP-melanin complexes in 3-ml portions of buffer from pH 3.0 to 6.0 or in 3-ml portions of buffered 0.01 M salt solutions at pH 6.0. The mixtures were incubated for 15 min, the melanin separated by centrifugation, and the supernatants assayed for PSP. In pH studies, this procedure was repeated 4 times.

Melanin Assay

Purification of Melanin From Fresh Clam Siphons

Melanin was separated from clam siphons and treated with papain as described previously. After being washed with 0.1 N HCl, the melanin granules were washed by centrifugation twice with distilled water, once with 50% aqueous ethanol, twice with 95% ethanol, and twice with acetone. The dehydrated melanin was then dried under N_2 at room temperature.

Purification of Melanin From Frozen Toxic Butter Clam Siphons

Frozen toxic butter clam siphons were thawed for 6 hr at 25° C. The thawed siphons were drained for 15 min, and the drip and the siphons were divided into equal portions. One portion of each was homogenized with an equal volume of distilled water, adjusted to pH 3.0 with 5 N HCl, and frozen until bioassayed for PSP. The remaining siphons were mixed with an equal volume of distilled water, adjusted to pH 3.0 with 5 N HCl, and sonicated as described previously. After sonication, the siphon residue was separated from the melanin suspension by filtration through cheese cloth. The siphon residue, the melanin suspension, and the drip collected after thawing were homogenized separately, adjusted to pH 3.0 with 5 N

HCl, separated into equal portions and frozen.

Melanin was purified from one portion of each of the fractions according to the procedure previously described.

Colorimetric Assay

The colorimetric assay for melanin was essentially that of Ruban et al. (1969). Dried melanin was dissolved in 0.5 N NaOH by incubating the mixture at 50° C for 2 hr in a shaking water bath. After cooling the solution to room temperature, the melanin was precipitated by adjusting the pH to 2.0 with 5 N HCl. The precipitate was washed twice by centrifugation with 0.01 N HCl and dissolved in 90 ml of 0.5 N NaOH at 50° C. After 1 hr incubation, the volume was adjusted to 100 ml with 0.5 N NaOH and the solution filtered through a sintered-glass filter (UF, Corning Glass Works). Absorbance at 420 nm was measured by a spectrophotometer (Spectronic 20, Bausch and Lomb). All solutions were adjusted with 0.5 N NaOH to give absorbance values between 0.2 and 0.4. Conversion to absolute amounts of melanin were not made, since relative rather than absolute values were required.

RESULTS AND DISCUSSION

PSP Assays

Mouse Bioassay

The standardized mouse bioassay for PSP (Horwitz, 1960), although used to determine PSP levels in biological samples, was not suitable for PSP in binding experiments due to its poor accuracy on samples of low toxicity and because interferences from buffer salts could also decrease its accuracy (McFarren et al., 1958).

Modified Jaffe Test

Preliminary experiments using the modified Jaffe test for PSP developed by McFarren et al. (1959) indicated that this procedure could accurately measure concentrations of PSP as low as 2.0 μg/ml in buffer solutions. Salt solutions containing 0.01 M NaCl, NaBr, NaI, KCl, LiCl, and $NaNO_3$, however, interfered with the Jaffe test and caused irregular and abnormally high absorbance readings, thus reducing the accuracy. Because of this decreased accuracy in the presence of salts and the fairly complex nature of the assay procedure, the Jaffe test was judged not suitable for determining PSP levels during binding experiments.

Folin-Ciocalteau Test

A simple and accurate test was developed for determining the concentration of PSP in buffer solutions by using the Folin-Ciocalteau phenol reagent (Fisher Scientific Company). The use of this reagent was suggested by the similarity of the structure of PSP (Figure 1) proposed by Rapoport et al. (1964, as cited by Russell, 1967) with a derivative of guanine, and on the observation by Funk and Macallum (1913) that guanine and xanthine will react with the phenol reagent.

The procedure used for PSP assays was essentially that of Hitchings (1941) with a final volume reduced to 3.25 ml to minimize dilution of the sample and to increase the sensitivity of the procedure. The volumes of reagents used in the assay were selected to minimize precipitation during incubation.

Color development with this reagent was rapid as shown in Figure 3. The color stabilized after 50 min and did not change during 50 to 120 min incubation. The Folin-Ciocalteau reagent could accurately measure concentrations of PSP in buffer or dilute salt solutions as low as 1.0 μg/ml as shown in Figure 4.

The Folin-Ciocalteau test used in this study has several advantages over the modified Jaffe test: the Folin-Ciocalteau test is less affected by the presence of buffers or salts in the sample; the procedure is simple and does not require extractions prior to absorbance measurements; and the reaction yields a stable color for which

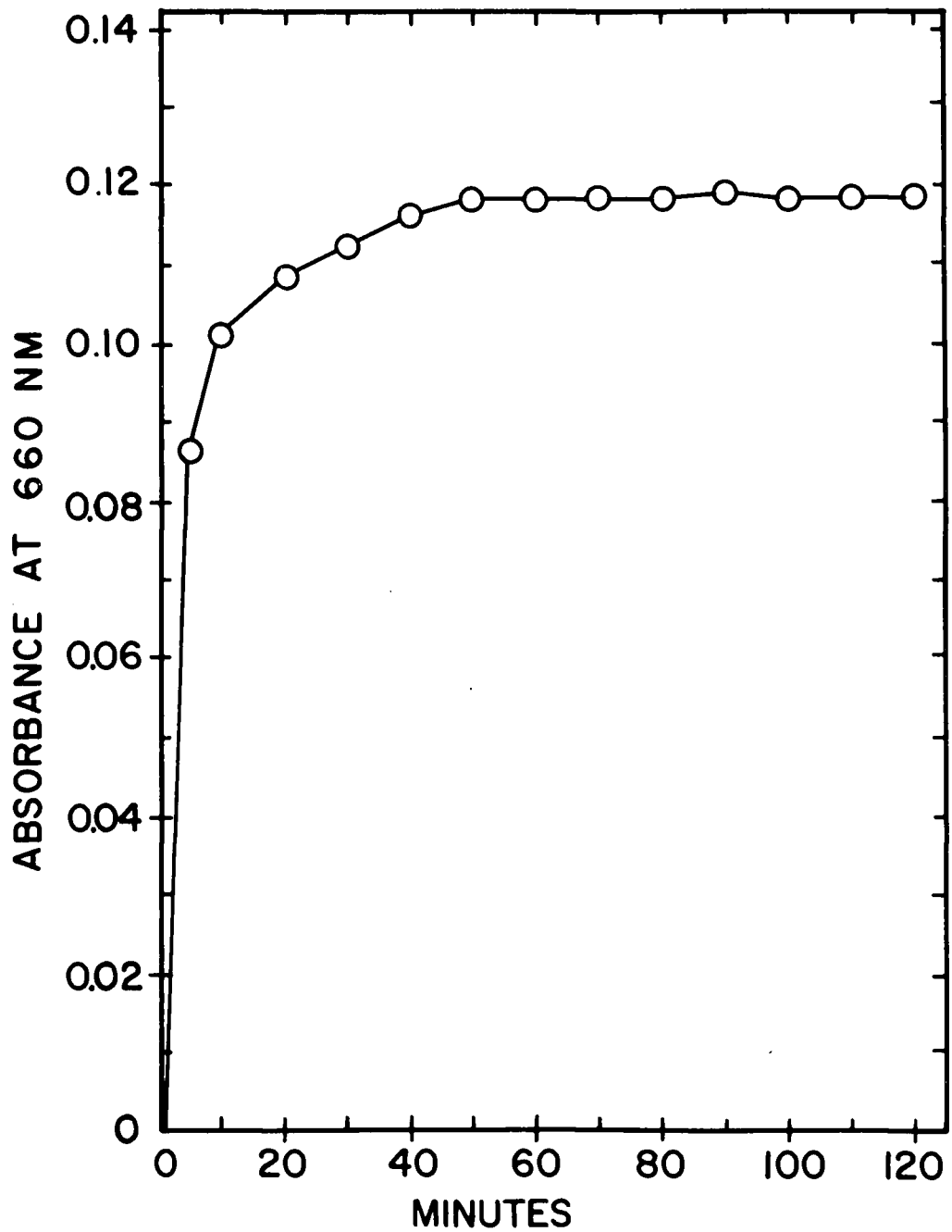

Figure 3. Rate of color development with Folin-Ciocalteau reagent and PSP (10 μg).

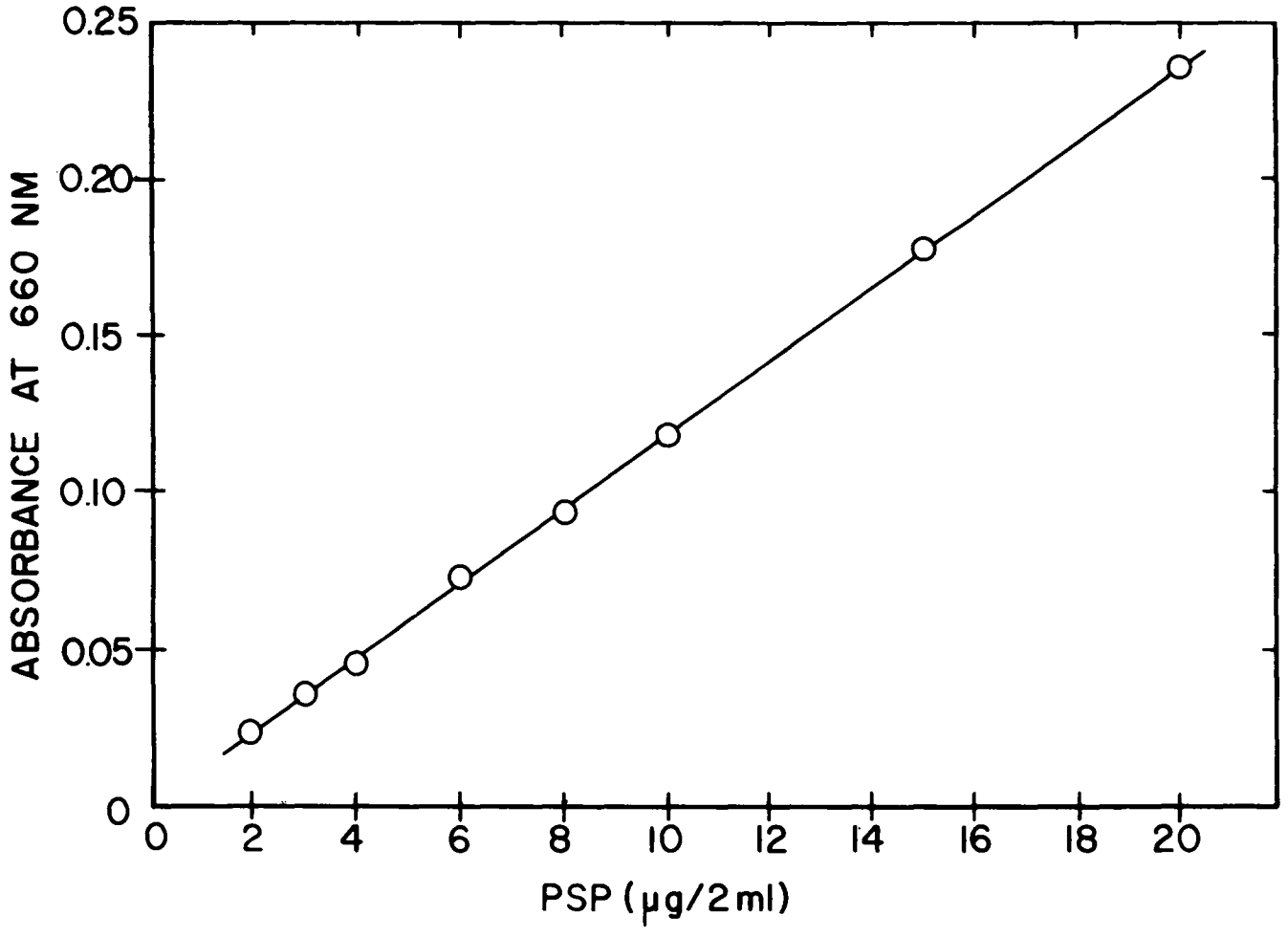

Figure 4. Linearity of absorbance vs. PSP concentration by Folin-Ciocalteau test.

the absorbance remains constant for at least one hour.

A limitation of the Folin-Ciocalteau reaction, its lack of specificity for PSP, is shared by the Jaffe test which is primarily a creatinine test (Jaffe, as cited by Bonsnes and Taussky, 1945). The Folin-Ciocalteau reaction has been used to quantitate guanine and xanthine (Hitchings, 1941), phenols (Diehl, 1970), and proteins (Snell and Snell, 1954). Other substances which interfere with the Folin-Ciocalteau reaction include aromatic amines, unsaturated hydrocarbons, glucose, urea, hydrogen peroxide, and hydrogen sulfide (Snell and Snell, 1953). Although the Folin-Ciocalteau reaction is nonspecific, it could be used to determine PSP concentrations in binding experiments where the protein and melanin preparations were freed from interfering compounds by repeated buffer washes, and the PSP solutions were essentially free of protein and melanin.

PSP-Protein Interactions

Proteins can interact with small biologically active molecules *in vitro* and by doing so alter the activity of the small molecule (Joos and Hall, 1969); it has also been suggested that similar interactions may take place *in vivo* (Rolinson and Sutherland, 1965). Many of these interactions are reversible and may be electrostatic or hydrophobic in nature (Keen, 1966; Fischer and Jardetzky, 1965). Because of this ability of proteins to bind small molecules,

interactions between PSP and proteins were investigated to determine if PSP-protein interactions could be responsible for the retention of PSP in butter clams.

Gel Filtration

Preliminary gel filtration experiments with purified PSP and Sephadex G-15 gel indicated that PSP interacted with the Sephadex gel in distilled water and remained bound to the gel until eluted from the column with 0.001 N HCl. Because this interaction could possibly interfere with an interaction between PSP and protein, or could prevent an accurate interpretation of experimental results, the use of Sephadex gel was discontinued. Further experiments with Biogel P-4 indicated that this gel would not interact with PSP, and Biogel P-4 was used in filtration binding experiments.

The results of interaction experiments with 50 μg of PSP and 10 mg of BSA in 0.1 M phosphate-citrate buffer at pH 3.0 and 7.0 were inconclusive. A single peak apparently containing both PSP and protein was eluted from the columns. The amount of PSP in the fractions or the location of the PSP within the peak could not be accurately determined since the Jaffe and Folin-Ciocalteau reagents reacted with both protein and PSP. Attempts to improve the separation of PSP and protein fractions by decreasing the amount of BSA to 1 mg, slowing the elution rate, and decreasing the fraction size

were equally not successful.

Ultrafiltration

An advantage of ultrafiltration over gel filtration in PSP-protein interaction studies is the physical separation of the protein from the ultrafiltrate minimizing interference from protein during PSP assays.

Preliminary experiments using 10 mg of BSA in 3 ml of 0.1 M phosphate buffer at pH 7.0 under 50 psig of N_2 indicated an apparent leakage of BSA across the Pellicon PSAC membrane filters. Attempts to eliminate this leakage by predialysis or repeated ultrafiltration of the protein solution, and by reducing the pressure applied to the ultrafiltration cell were unsuccessful. Since the retention of BSA varied from membrane to membrane and ranged from 13 to 81% under identical conditions, the use of these membranes was discontinued.

Further experiments with Diaflo ultrafilters (UM-2) indicated that these filters effectively retained BSA and fibrinogen. Binding experiments using 20 mg of fibrinogen and 20 μg of crude PSP in 0.15 M citrate buffer at pH 3.0 and 6.0, and 0.15 M veronal buffer at pH 8.0 indicated that no interaction had taken place between PSP and fibrinogen under these conditions. Similar experiments with BSA instead of fibrinogen indicated an apparent interaction between these compounds, but the results could not be repeated regularly.

Variations in the time required to obtain an amount of filtrate sufficient for PSP assays were probably responsible for the poor reproducibility of ultrafiltration experiments. Since these variations could not be controlled without varying the experimental procedure, a series of equilibrium dialysis experiments were conducted to determine if the observed interactions between PSP and BSA were artifacts or true interactions.

Equilibrium Dialysis

By using a seven chambered equilibrium dialysis cell, several binding experiments and controls could be conducted concurrently under controlled conditions, thus minimizing errors due to differences in experimental conditions. The advantage of physical separation of the protein solution from the PSP solution in ultrafiltration applies equally to equilibrium dialysis. An additional advantage is the ease with which the protein solutions can be dialyzed against buffer prior to binding experiments, in the same chambers that are used for the experiments.

A preliminary experiment dialyzing PSP against 0.01 M phosphate buffer at pH 7.0 indicated that an equilibrium across the membrane was attained in less than 12 hr at 4° C and remained unchanged for at least 48 hr. There was no apparent degradation of the PSP during the 48 hr period.

Initial binding experiments conducted to clarify the apparent interaction observed in ultrafiltration experiments with crude PSP indicated that 20 mg of BSA apparently bound 9 μg of PSP in 0.10 M citrate buffer and 30 μg of PSP in 0.06 M citrate buffer at pH 6.0. Attempts to obtain similar results using purified PSP were unsuccessful indicating that the crude PSP preparation could have contained a compound other than PSP which interacted with BSA and also reacted with the Folin-Ciocalteau reagent. No effort was made to identify the impurity and the use of crude PSP was discontinued.

The results of binding experiments between 20 mg BSA and 35 μg PSP in 0.01 to 0.2 M citrate buffer at pH 6.0 are shown in Table 2. From the results of previous studies on the properties and purification of PSP (Schantz et al., 1957; McFarren et al., 1958), an electrostatic interaction between PSP and protein should be at a maximum at or near pH 6.0. However, there was no appreciable binding of PSP by BSA at pH 6.0 throughout the range of ionic strength employed. The small amounts of PSP bound by BSA in these experiments are not significant, since the values are below the accuracy of the Folin-Ciocalteau assay.

Since the PSP molecule is positively charged at pH 6.0 and BSA carries a negative net charge at this pH (Keen, 1966), there is an unequal distribution of PSP across the membrane due to the Donnan effect (ibid.). Although this effect can be overcome by

adding an excess of a neutral salt (Rose, 1969), this method could not be employed since the binding of PSP in butter clams is believed to be electrostatic in nature (Schantz, 1969b). In any event, a correction for the Donnan effect would further reduce the observed amounts of PSP bound to BSA.

Table 2. Effect of ionic strength on the binding of PSP to BSA.[a/]

Citrate buffer (M)	PSP bound (μg)
0.010	1.5
0.025	0.7
0.050	0
0.075	0
0.10	1.5
0.15	0
0.20	0

[a/] 20 mg BSA, 35 μg PSP; 4° C; pH 6.0.

Additional experiments with 40 μg of PSP and 20 mg of BSA in 0.01 M phosphate-citrate buffer showed further the inability of PSP to bind significantly to BSA. These experiments have shown that only 1.8, 2.6, and 1.8 μg of PSP could be bound to the BSA at pH 3.0, 5.0, and 7.0 respectively.

The results of these experiments suggest that protein does not

significantly interact with PSP, and that protein probably does not play an important role in the retention of PSP by butter clams. In addition, an interaction between PSP and protein would not satisfactorilyexplain the large variation in PSP retention among different clam species (Quayle, 1969). These results also suggested the presence of a unique substance in butter clam siphons that preferentially binds with PSP.

PSP-Clam Siphon Homogenate Interactions

In butter clams, PSP is bound and retained primarily in the siphon, gill, and mantle (Quayle, 1969). Since protein-PSP interactions do not appear to be responsible for this binding, the interaction between PSP and a butter clam siphon homogenate was investigated to determine if PSP would bind to clam siphon material *in vitro*.

Preparation of Siphon Homogenate

Bacterial growth in clam siphon homogenates at 4° C was rapid enough to cause interference in binding experiments. Adding 1 mg of thimerosal per 20 ml of homogenate effectively inhibited bacterial growth during the binding experiments. Interference due to the diffusion of other Folin-Ciocalteau reacting material across the dialysis membrane was minimized by heating the homogenate to 75° C

for 10 min to inactivate enzymes present in the homogenate.

Equilibrium Dialysis

Because of the large amount of low molecular weight material present in the siphon homogenates, the homogenates were dialyzed against buffer for six 4-hr periods prior to binding experiments. This dialysis after heat treatment effectively removed dialyzable components of the homogenate that would react with the Folin-Ciocalteau reagent. Phosphate buffers instead of citrate buffers were used in binding experiments with siphon homogenates to minimize bacterial growth during the experiments.

The effect of pH on the binding of PSP by 1.0 ml of the butter clam siphon homogenate is shown in Figure 5. The maximum amount of PSP bound to the homogenate was 12.1 μg in 0.01 M phosphate buffer of pH 7.5. The binding of PSP to the homogenate was influenced by pH; the amount of PSP bound increased as the pH increased from 3.5 to 7.5, and then remained essentially constant from pH 7.0 to 8.0. The discontinuity in the curve at pH 5.5 to 6.0 is due to the different buffers used; similar buffer effects have been reported previously in binding experiments (Keen, 1966).

The effect of buffer concentration on the interaction between PSP and 1.0 ml of clam siphon homogenate is shown in Table 3. The increased binding of PSP by the homogenate with decreasing ionic

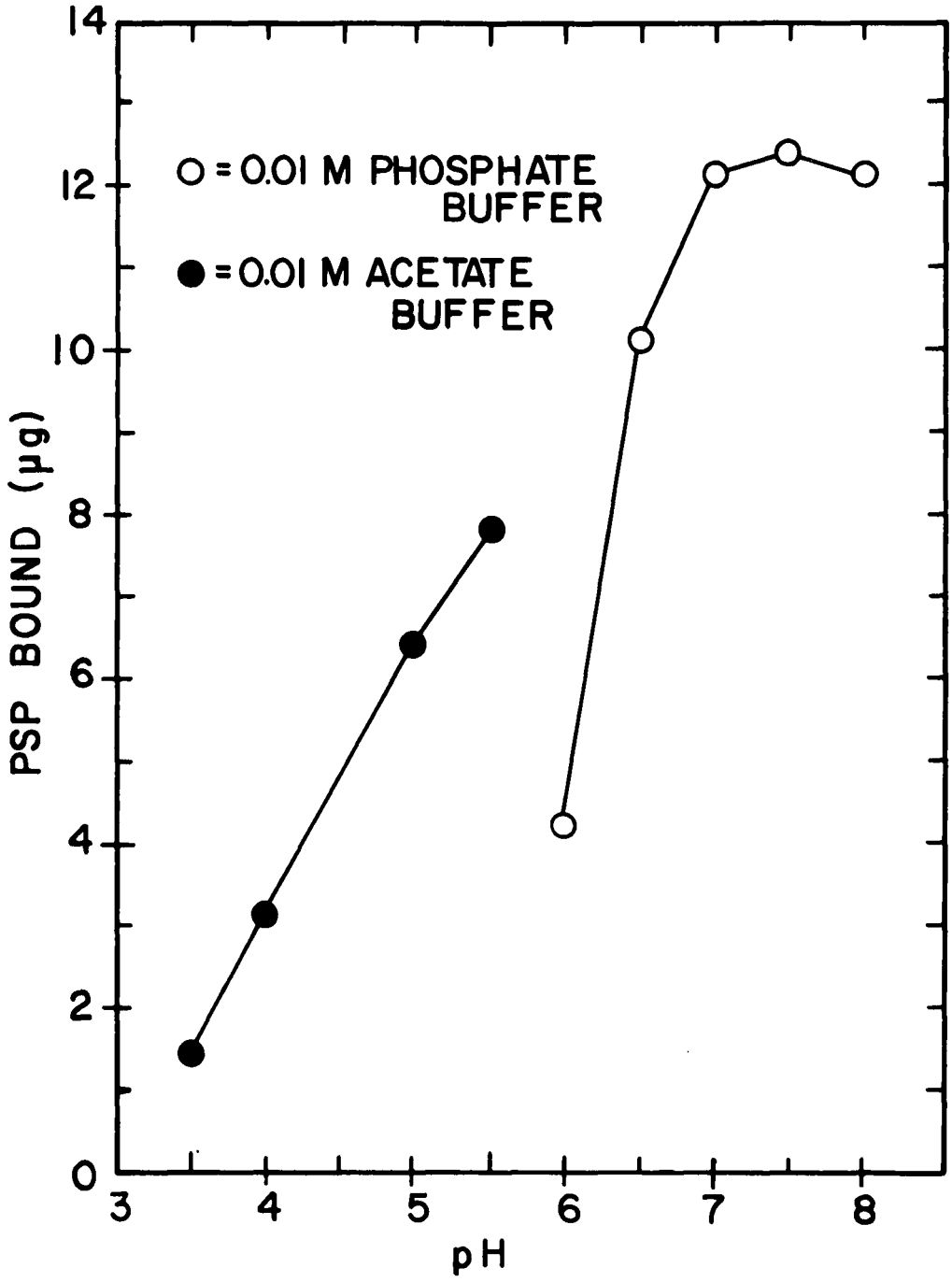

Figure 5. Effect of pH on the binding of PSP (35 µg) to clam siphon homogenate (1.0 ml) at 4° C.

strength suggests an electrostatic type of interaction between PSP and some component of the siphon homogenate.

Table 3. Effect of buffer concentration on the binding of PSP to clam siphon homogenate.[a/]

Phosphate buffer (M)	PSP bound (μg)
0.001	15.7
0.01	12.1
0.10	1.0

[a/] PSP (35 μg), clam siphon homogenate (1.0 ml), pH 7.0.

Although the results of these equilibrium dialysis binding experiments appear to indicate an electrostatic interaction between PSP and some component of the butter clam siphon homogenate, the results may be misleading due to the Donnan effect. Since PSP is present as a charged molecule in the pH range studied, and macromolecules in the clam siphon homogenate probably also carry a net charge, an unequal distribution of dialyzable ions across the dialysis membrane can occur and may be significant enough to invalidate the data shown in Figure 5 and Table 3.

The Donnan effect can either be supressed by adding a high concentration of salt to the solution, or a correction for it should be made (Tanford, 1961). Addition of salt had to be ruled out. As shown in Table 3, the interaction between PSP and the clam siphon

homogenate was influenced by ionic strength, and increasing the ionic strength of the solutions by increasing the salt concentration would probably eliminate or minimize any interaction taking place. The other alternative, correcting for the Donnan effect, requires that the molar concentration and the net charge of the macromolecule be known (ibid.). Since the butter clam siphon homogenate probably contains a great number of macromolecules, and it is practically impossible to determine the molarity of the macromolecules in the homogenate, a correction for the Donnan effect could not be attempted.

The apparent electrostatic interaction found in these studies together with the apparent lack of interaction between PSP and protein noted previously, suggest that some nonprotein component of the clam siphon homogenate may be interacting with PSP and may be responsible for the retention of PSP in butter clam siphons.

PSP-Melanin Interactions

Melanin is present in the mantle, hepatopancreas, and siphon tips of bivalve mollusks (Comfort, 1951), and these areas of melanin pigmentation correspond to the areas where PSP is concentrated in butter clams (Table 4). In butter clam siphons, PSP is concentrated in the black siphon tips and the concentration decreases from the distal to the proximal portion of the siphon (Table 4), corresponding to the apparent distribution of melanin in butter clam siphons shown

Table 4. Distribution of PSP in toxic butter clams.[a/]

Clam tissue	Toxicity (μg/100 g)
Siphon (tip)	17,920
Siphon (middle)	13,020
Siphon (inner)	2,560
Gill and Mantle	2,400
Digestive gland	1,920
Gonad	448
Body wall	432
Pallial muscle	368
Foot	192
Adductor muscle	94
Whole Clam	2,400

[a/] From data presented by Quayle (1969).

in Figures 6 and 7.

Because of this parallel relationship between the distribution of PSP and of melanin in butter clam siphons, and the reported cation exchanging activity of melanin (White, 1958), the interaction between PSP and melanin was investigated to assess the role of melanin as a possible factor in the retention of PSP in butter clams.

Purification of Clam Melanin

Microscopic examination of thin sections of butter clam siphons revealed that melanin granules were concentrated in the epithelial layers of the tissue (Figure 8). Sonication of a split butter clam siphon for 1-min intervals in four 10-ml portions of buffer at pH 6.0 demonstrated that the melanin granules could be removed readily by sonication. The effect of sonication on the butter clam siphon shown in Figure 6 is illustrated in Figures 9 through 13. Absorbance measurements on the buffer solutions after sonication are shown in Figure 14. From these results, it appears that melanin is effectively removed from the epithelial layers of the butter clam siphons by sonication.

PSP-Synthetic Melanin Interactions

Synthetic melanin prepared from L-DOPA with mushroom tyrosinase was used in preliminary PSP-melanin interaction studies.

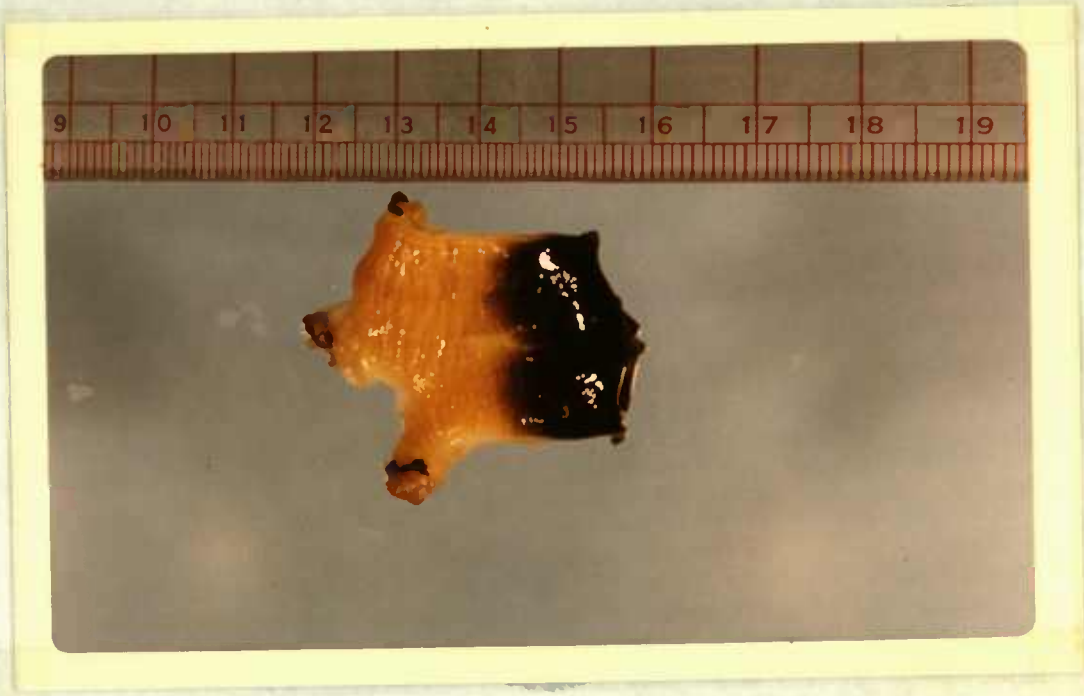

Figure 6. Split butter clam siphon showing exterior distribution of melanin.

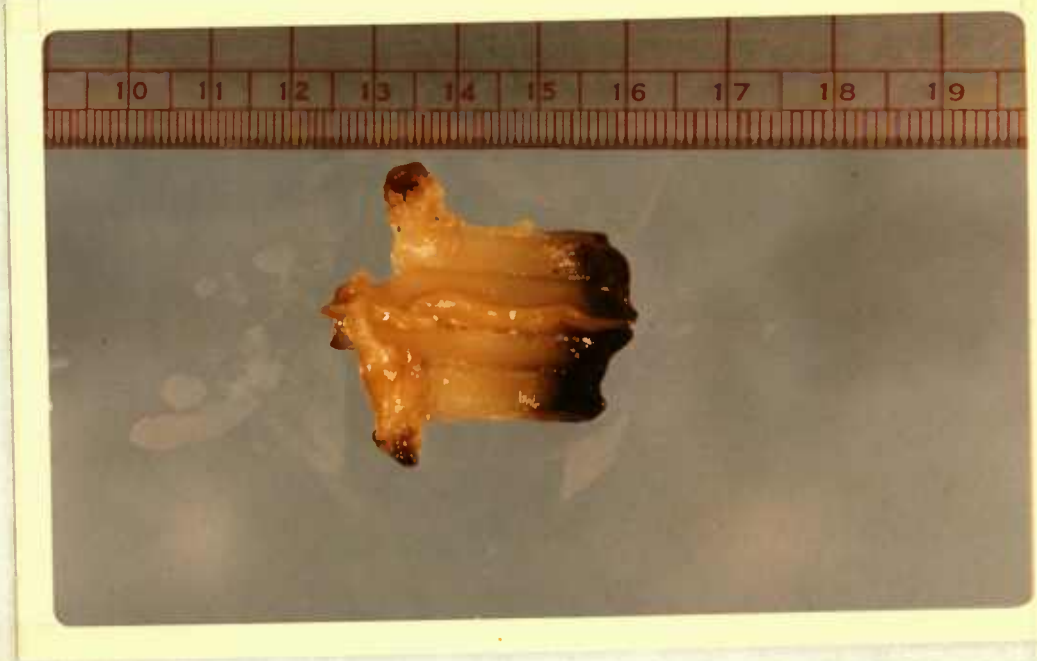

Figure 7. Split butter clam siphon showing interior distribution of melanin.

Figure 8. Inner wall of butter clam siphon showing melanin granules in epithelial cells. Hematoxylin and eosin. x 320.

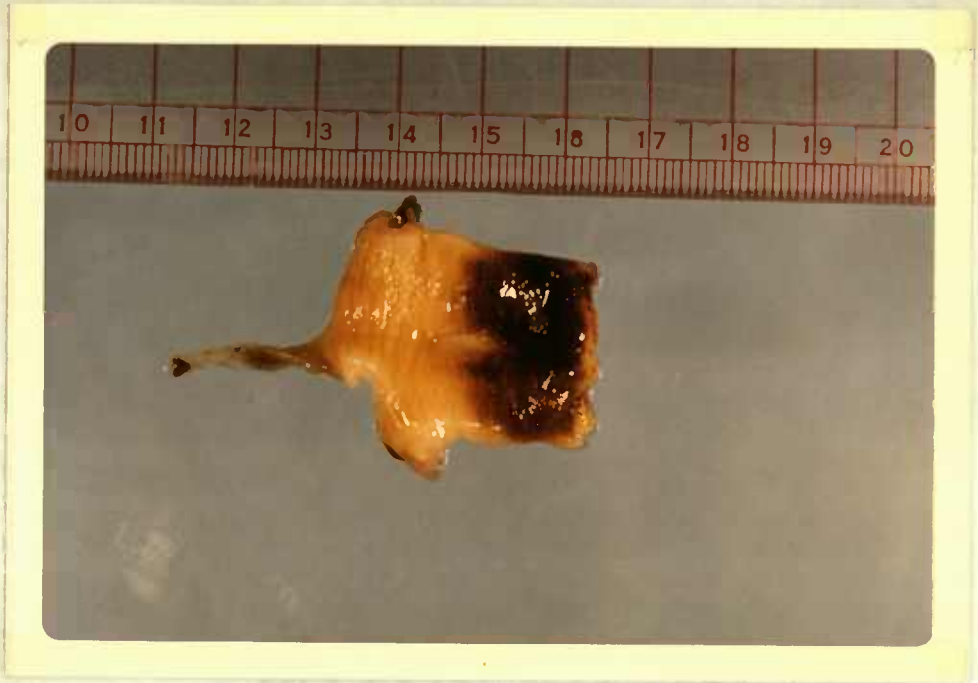

Figure 9. Butter clam siphon after 1 min sonication.

Figure 10. Butter clam siphon after 2 min sonication.

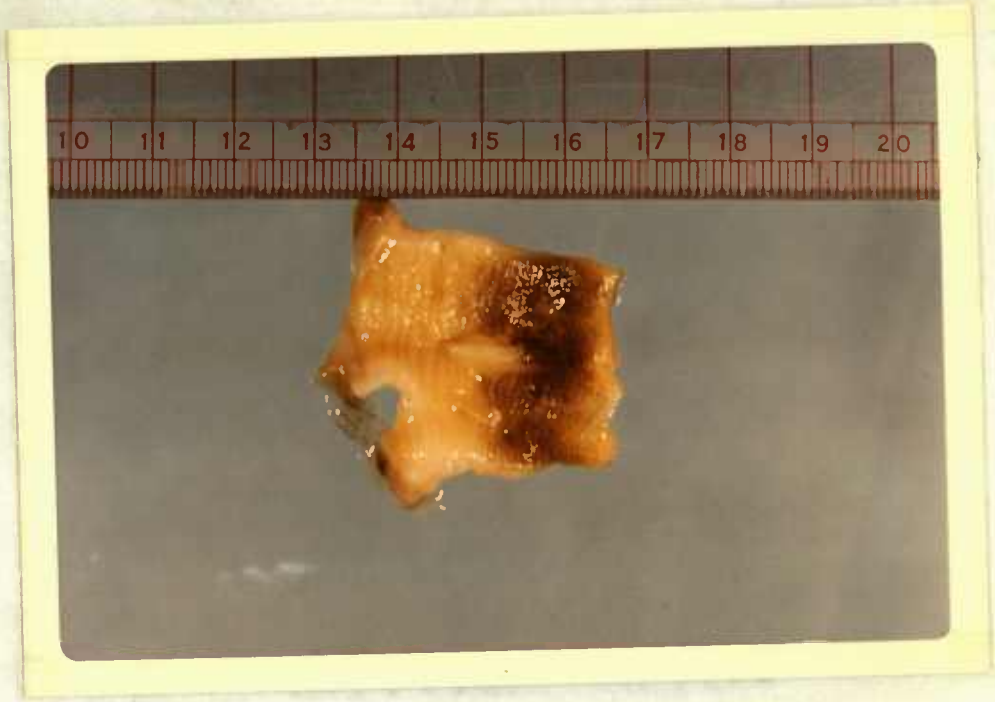

Figure 11. Butter clam siphon after 3 min sonication.

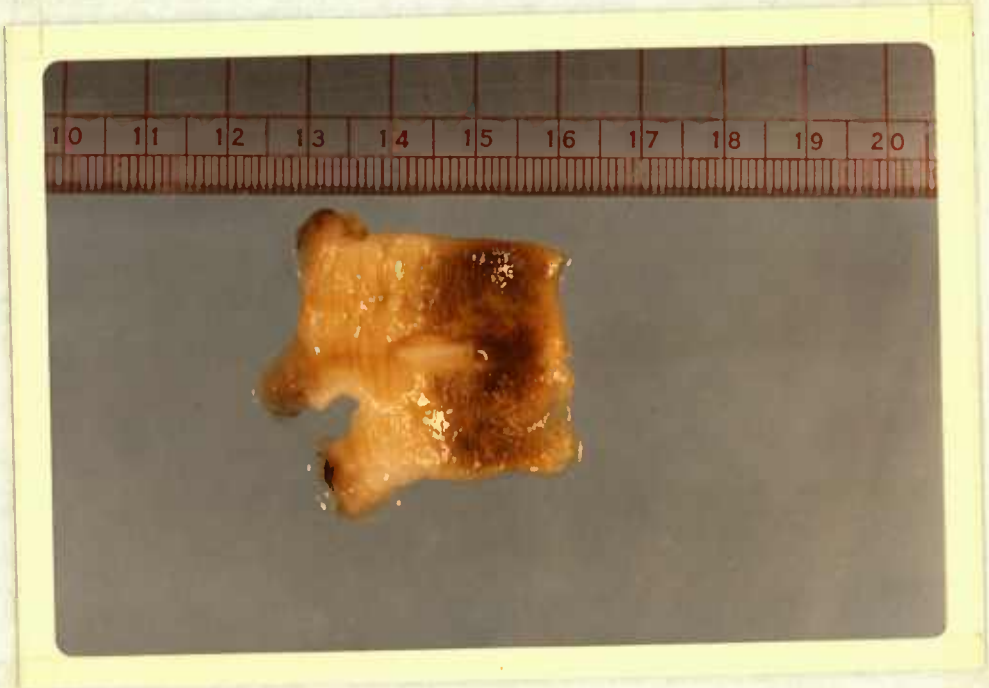

Figure 12. Butter clam siphon after 4 min sonication.

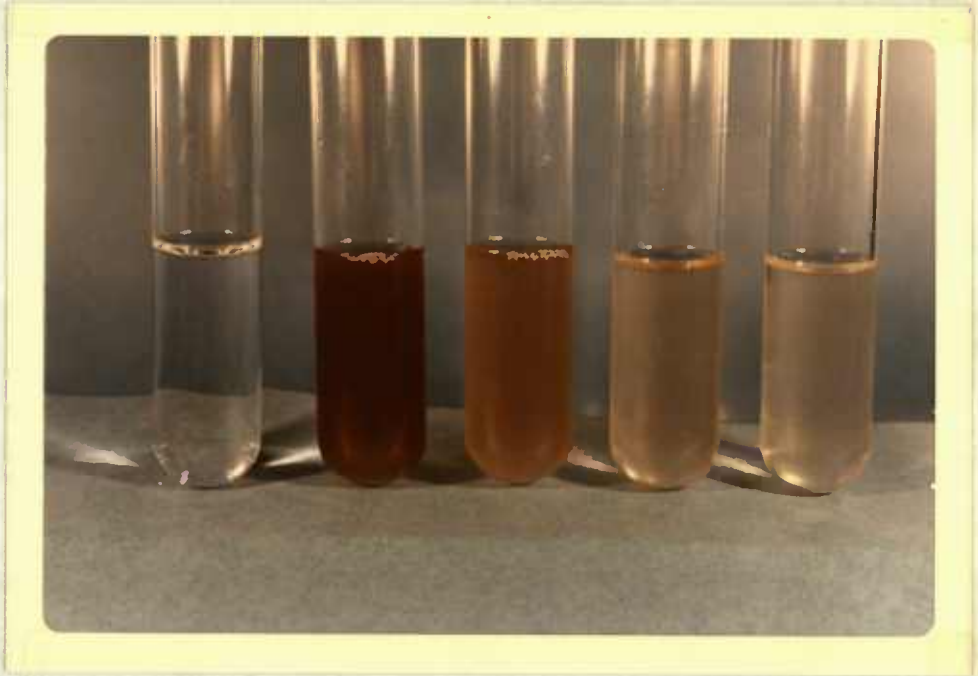

Figure 13. Buffer solutions after sonication of a butter clam siphon for four 1-min intervals. From left to right: buffer control; buffer after 1st min of sonication; buffer after 2nd min of sonication; buffer after 3rd min of sonication; buffer after 4th min of sonication.

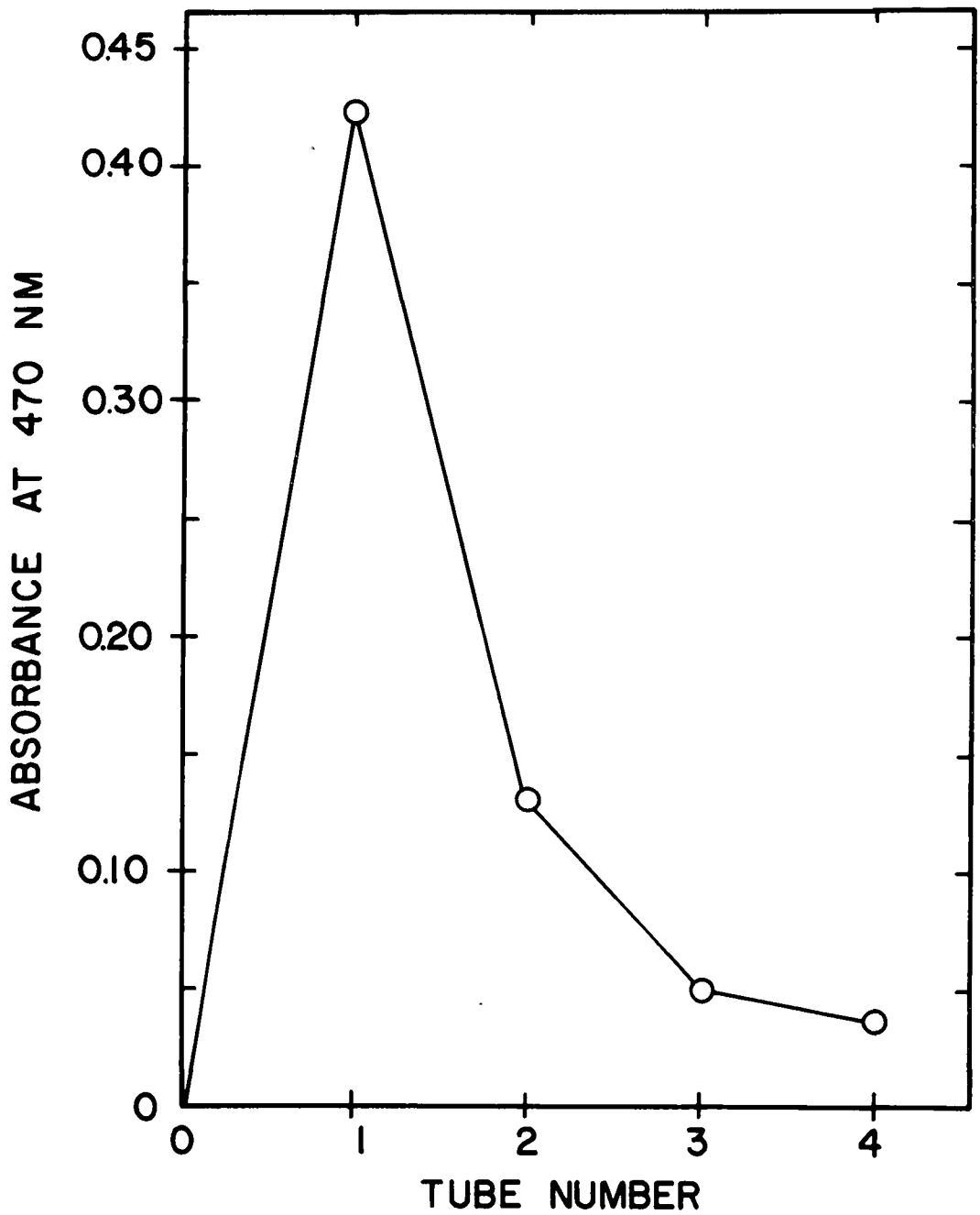

Figure 14. Release of melanin from a butter clam siphon sonicated for 1-min periods in four 10-ml portions of buffer at pH 6.0.

Synthetic melanin prepared in this manner has been shown to be very similar to natural indol melanin, except for the absence of protein generally associated with natural melanin (Bruenger et al., 1967).

The time required for the interaction of PSP and melanin to reach equilibrium was determined by incubating 2.8-mg samples of synthetic melanin with 27 μg of PSP, and assaying for the unbound PSP after 1, 2, 4, 8, 12, and 24 hr. Equilibrium was attained by or before 1 hr and remained constant for at least 24 hr. The precise time for equilibrium could not be determined due to the time required for separation of the melanin from the suspension.

The amount of PSP bound to synthetic melanin in buffer of pH 2.0 to 8.0 is shown in Figure 15. The binding between PSP and synthetic melanin was strongly influenced by pH. The amount of binding increased with increasing pH from pH 2.0 to 5.0, and from pH 4.0 to 8.0 the synthetic melanin bound approximately 27 μg of PSP.

PSP-Clam Melanin Interactions

Melanin synthesized from L-DOPA appeared to be highly polymerized and could easily be separated from the buffer solution by filtration. Clam melanin, however, tended to plug the filters, thus reducing the flow rate and prolonging the experiments. Since the time required to filter the clam melanin solutions varied greatly and possibly affected the results of these experiments, a

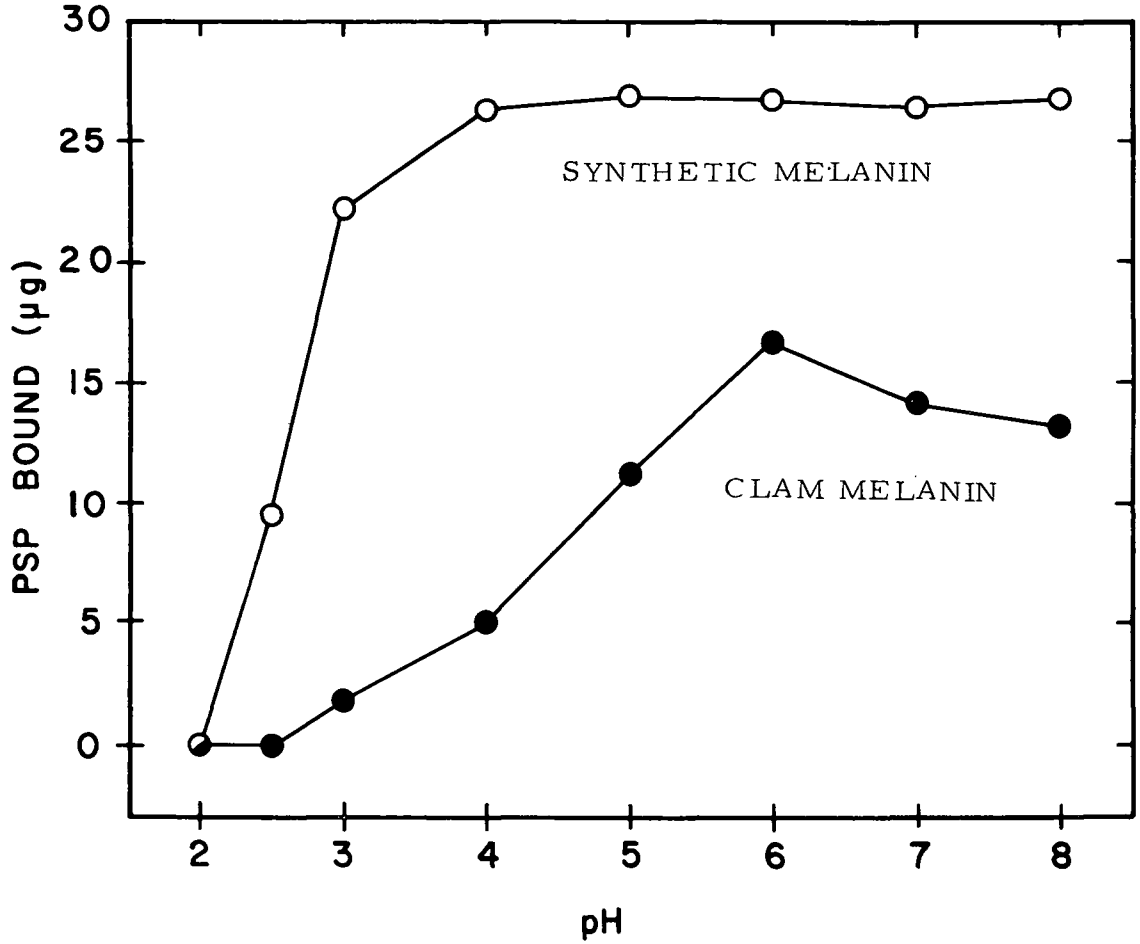

Figure 15. Effect of pH on the binding of PSP to synthetic and clam melanin.

centrifugation procedure was used to separate the melanin granules from the buffer solution after binding experiments. Centrifugation of the melanin solutions at 20,000 g for 20 min effectively removed the clam melanin granules from the PSP-buffer solutions.

Effect of Melanin Concentration on PSP-Melanin Binding

The quantitative relationship between melanin concentration and the degree of PSP-melanin binding is shown in Figure 16. Three milliliters of the stock clam melanin solution, containing approximately 4.5 mg of clam melanin, were used in all binding experiments. This amount of clam melanin bound from 16 to 17 μg of PSP at pH 6.0.

Effect of pH on PSP-Clam Melanin Binding

The percent of PSP bound to clam melanin in buffer at pH 2.0 to 8.0 is shown in Figure 15. The binding of PSP to clam melanin was similar to that of PSP and synthetic melanin in that is was strongly affected by pH. At pH 6.0, 4.5 mg of clam melanin bound approximately 17 μg of PSP.

In these experiments, 2.8 mg of synthetic melanin bound more PSP than 4.5 mg of clam melanin. This difference between synthetic and clam melanin may be due to a difference in the number of reactive sites available for binding. The synthetic melanin appeared to

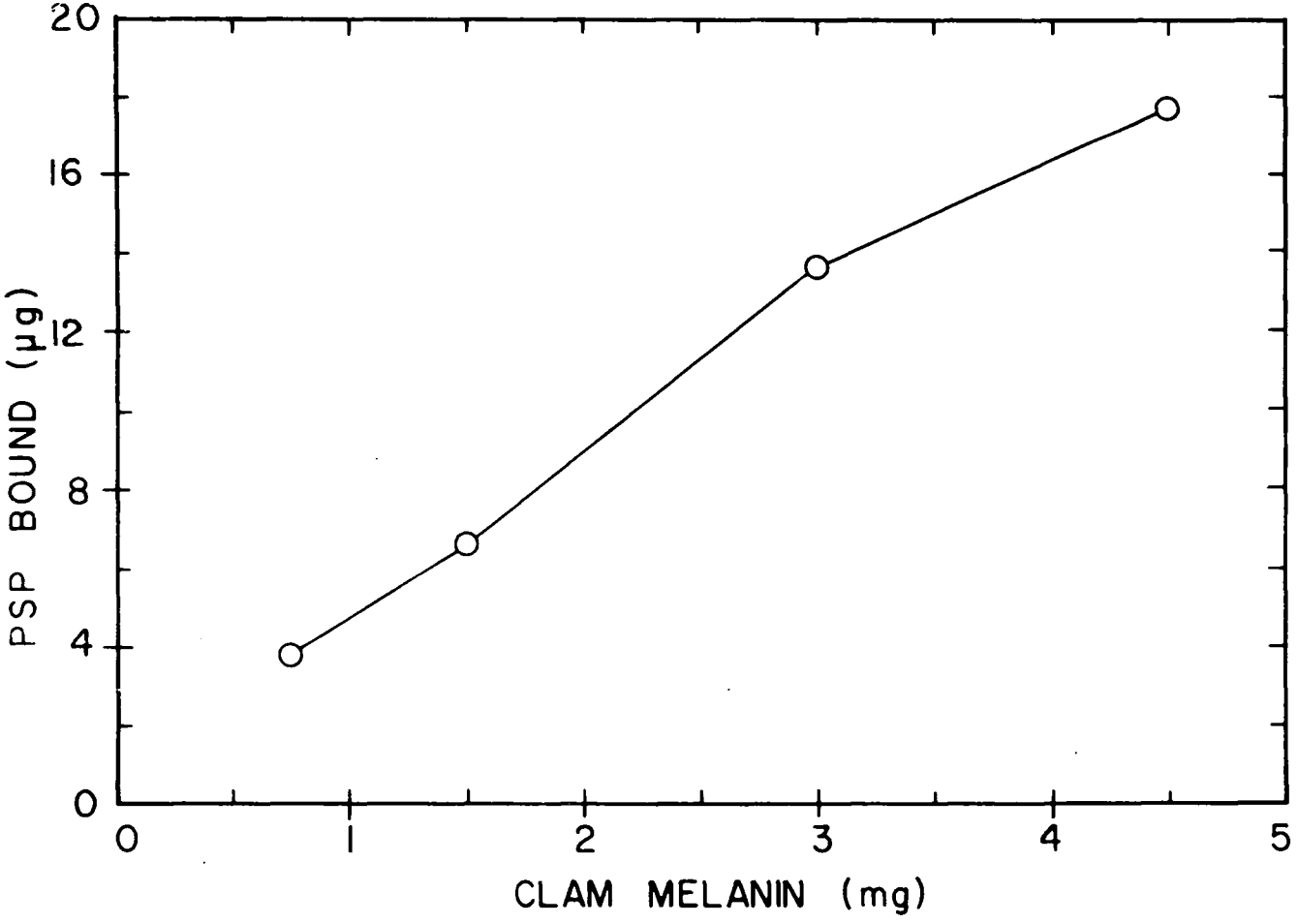

Figure 16. Effect of melanin concentration on PSP-melanin binding at pH 6.0.

have a less organized structure, probably permitting greater exposure of reactive sites to PSP, while natural melanin is found in granular forms and the organized structure of these granules could have reduced the number of available binding sites.

The data in Figure 15 indicates that less PSP was bound to clam melanin at pH 7.0 to 8.0 than at pH 6.0. This may be due to partial solubilization of the melanin at these pH levels. Bruenger et al. (1967) indicated that natural melanin loses some of its granular structure above pH 7.0 and they showed a decrease in the binding of $^{224}Ra^{++}$ to melanin at pH 7.0 and pH 7.5 when compared to the binding at pH 6.0. In our experiments, clam melanin appears to solubilize slightly at pH 7-8 as indicated by a slight coloration in buffer washes. Although PSP was shown to decompose slowly in alkaline solution (Schantz et al., 1957), no decomposition was noted in our experiments.

The reversible nature of the PSP-melanin interaction is shown in Figure 17. The rate of desorption of PSP from the PSP-melanin complex increases with increasing acidity, suggesting a competition between hydrogen ions and PSP for binding sites on the melanin. These results also suggest that the interaction between PSP and clam melanin is fairly weak, since some of the PSP is desorbed at pH 6.0, where the adsorption should be maximum.

Natural melanin contains free carboxyl groups and phenolic

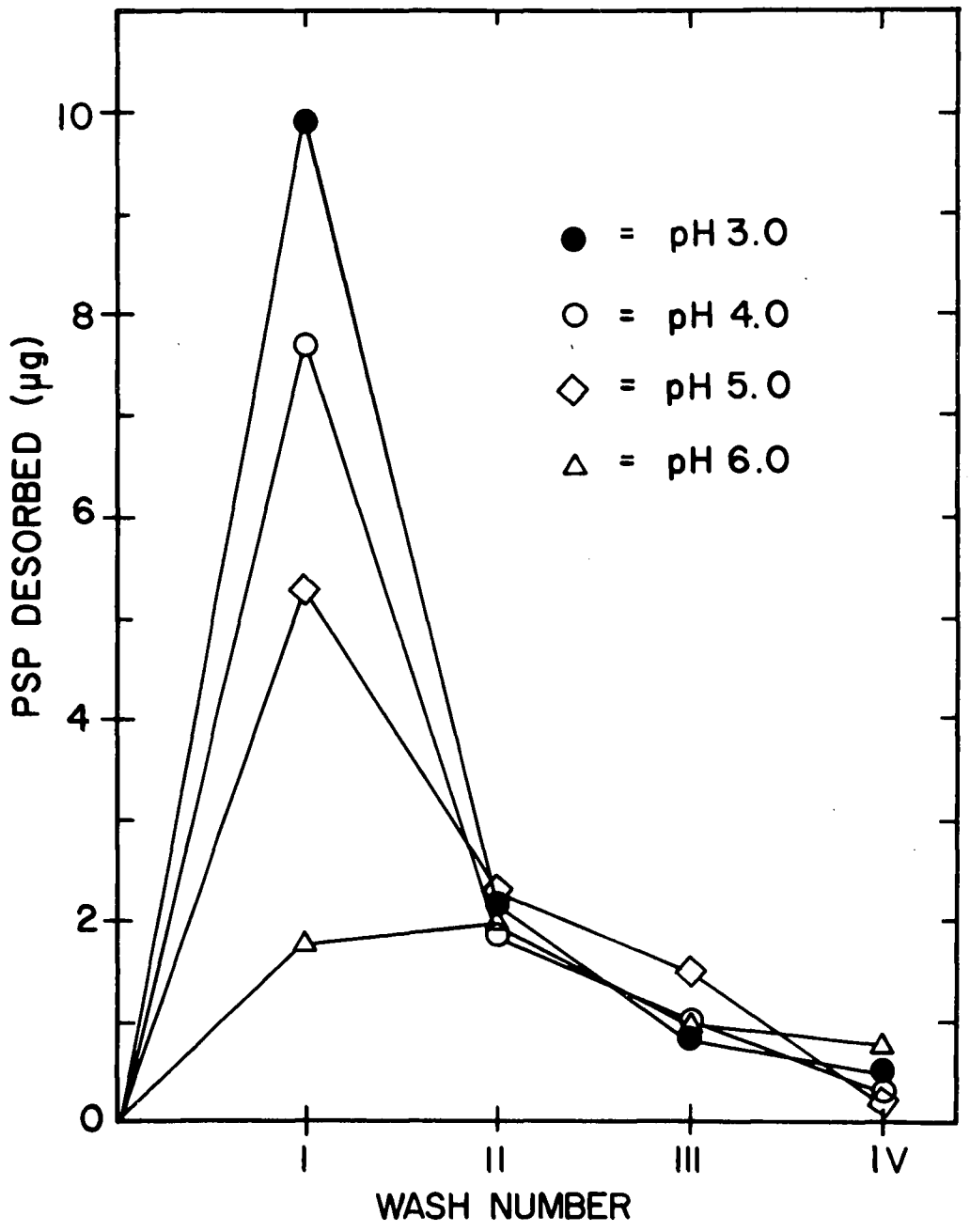

Figure 17. Effect of pH on the desorption of PSP from clam melanin containing 16.5 μ of bound PSP. Melanin was washed with 3-ml portions of buffer at 25° C.

hydroxyl groups and can function as a cation exchanger (White, 1958; Bruenger et al., 1967). The binding of PSP to melanin appears to be such a cation exchange reaction. This explanation is supported by the reversibility of the binding and the increased desorption of PSP from melanin with increasing acidity (Figure 17), and by the decreased PSP uptake at pH 2.0-3.0 (Figure 15). At low pH the weak carboxylic acid groups on melanin are in the hydrogen form (Bruenger et al., 1967), thus the uptake of PSP is probably blocked.

Effect of Cations on PSP-Clam Melanin Binding

The effect of cations on PSP-melanin binding is shown in Tables 5 and 6. The salts used in these experiments were added as nitrates to both binding experiments and controls at final concentrations of 0.01 M.

The data in Table 5 show that cations interferred with the binding of PSP to clam melanin, and that the degree of interference was directly related to the valence of the cation. The data in Table 6 show that salt solutions caused desorption of PSP from PSP-melanin complexes, and that the amount of PSP desorbed was directly related to the cation valence. In both experiments, the order of decreasing cation activity is trivalent, divalent, and monovalent.

The results of these experiments further support the nature of the PSP-melanin interaction suggested previously. The data in

Table 5. Effect of cations on the binding of PSP to clam melanin.[a/]

Cation (0.01 M)	μg PSP Bound	% Decrease in Binding
-	19.4	0
K^+	16.5	15
Na^+	16.2	17
Ba^{++}	8.4	57
Ca^{++}	7.3	62
Al^{+++}	6.2	68

[a/] PSP (27 μg) and clam melanin (4.5 mg), pH 6.0.

Table 6. Effect of cations on the desorption of PSP from clam melanin containing bound PSP.[a/]

Cation	μg PSP desorbed	Net desorption	
		μg	%
-	1.5	0	0
K^+	2.0	0.5	4
Na^+	2.8	1.3	11
Ba^{++}	3.6	2.1	19
Ca^{++}	4.9	3.3	28
Mg^{++}	5.1	3.6	34
Al^{+++}	8.2	6.7	54

[a/] 4.5 mg of clam melanin contained 12 μg of bound PSP. Melanin was washed with 3-ml portions of 0.01 M nitrate solutions in citrate-phosphate buffer at 25° C.

Table 6 supports the reversible nature of the binding, since as much as 54% of the PSP can be desorbed by washing the PSP-melanin complex once with 3 ml of 0.01 M aluminum nitrate. The weak nature of the binding is shown by the slight desorption which takes place when the PSP-melanin complex is washed with the same buffer used in the adsorption of PSP onto the melanin.

The exact mechanism by which butter clams bind and retain PSP is not known. Schantz (1969b), however, has suggested that the binding is similar to the binding of PSP on a weak cation exchange resin. The present observation on PSP-melanin interaction closely resembles that noted between PSP and whole butter clams, and between PSP and cation exchange resins. McFarren et al. (1958) found that PSP adsorbs optimally to Amberlite XE-64 cation exchange resin at pH 5.2, and could be adsorbed at a pH as low as 4.5. A slight increase in acidity below pH 4.5, however, caused desorption of the PSP from the column. Extraction of PSP from butter clams is accomplished with dilute acid solutions at pH 2-3 (Schantz et al., 1957). This correlates well with the rapid desorption of PSP from melanin at pH 3.0 (Figure 17) and the minimal uptake of PSP by clam melanin at this pH (Figure 15). The results shown in Tables 5 and 6 are also consistent with a cation exchange reaction mechanism and with the proposed weak cation exchanging nature of melanin. The affinity of a cation for the cation exchanger increases with

increasing valence, and this effect is greater in the case of weak acid cation exchangers than in strong acid ones (Bruenger et al., 1967). Mono-, di-, and trivalent cations interfere with PSP-melanin binding and cause desorption of bound PSP from melanin, and the effects increase with increasing valence, suggesting a competition for the binding sites on melanin. The increased competition observed with increased valence is consistent with the preference of weak acid ion exchangers for cations of higher valence (ibid.).

Further support for the cation exchange nature of the PSP-melanin reaction can be found in the order of cation interference. There is an apparent relationship between the ability of cations to compete with PSP for binding sites on melanin (Tables 5 and 6), and the hydrated ionic radii of the cations. Although the differences in the amounts of PSP bound or desorbed in the presence of cations of the same valence are small, and may not be significant, there is apparently an increased competition with decreasing hydrated ionic radius. This characteristic is typical of cation exchange reactions (Bruenger et al., 1967).

PSP and Melanin in Clams

Distribution of Melanin in Clam Species

Since melanin may be an important factor in the retention of

PSP by butter clams, an attempt was made to determine if variations in the melanin content of clams could be responsible for the variations in the toxin retention that have been reported among mollusks (Quayle, 1969).

Variation in the Melanin Content of Butter Clam Siphons

A variation in PSP retention has been reported not only among different species of shellfish, but also among individual butter clams (Quayle, 1969). A possible explanation for this variation could be differences in the amount of melanin present in these clams. Figure 18 shows the range of melanin pigmentation among butter clam siphons from clams of approximately the same size. A variation in pigmentation is apparent, but not as dramatic as the variation that was observed among butter clams of different sizes. There appears to be a correlation between size and siphon pigmentation, with smaller and younger butter clams apparently containing less melanin than larger and older clams. This variation in pigmentation may explain the variations in toxin retention reported among butter clams from the same locations (ibid.), since the amount of PSP bound by melanin in binding experiments is directly related to the amount of melanin present (Figure 16).

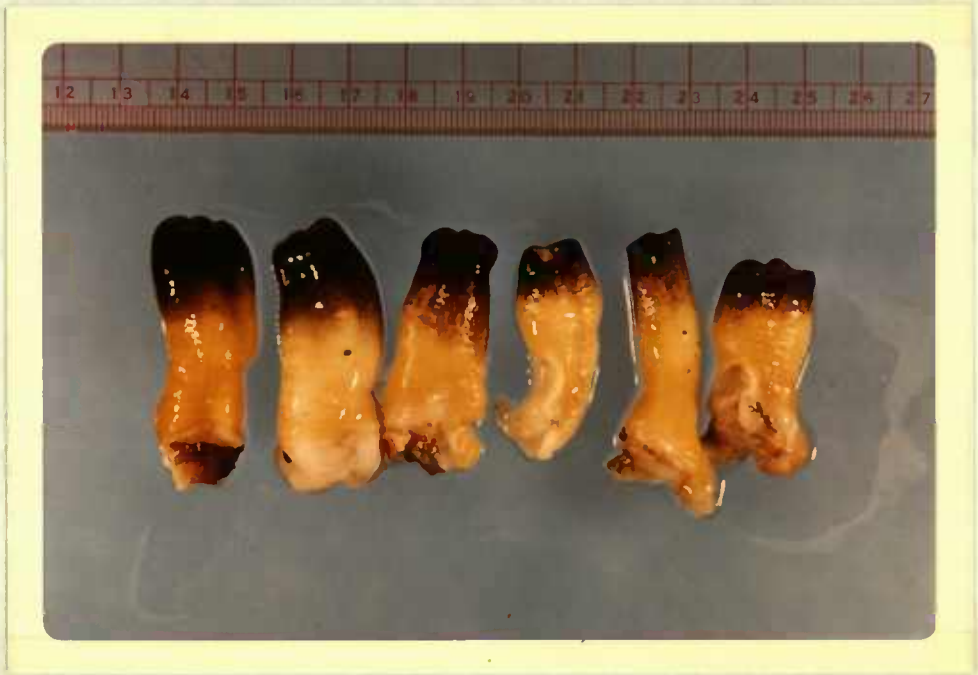

Figure 18. Variation in melanin pigmentation among butter clam siphons.

Variations in the Melanin Content of Mollusk Siphons

In an attempt to determine if differences in melanin pigmentation could explain the large differences in PSP retention among bivalve mollusks, melanin assays were conducted on siphons from four common clam species. For these experiments, the following amounts of siphons were pooled: 18 butter clam siphons with an average weight of 5.7 g; 21 horse clam siphons with an average weight of 6.6 g; 43 littleneck clam siphons with an average weight of 1.0 g; and 46 Manila clam siphons with an average weight of 0.63 g.

Of the species examined, only the butter clam was observed to retain PSP in its siphon for any appreciable length of time (Quayle, 1969). The relative concentration of melanin per siphon is shown in Table 7. These results indicate that butter clams have about five times as much melanin per siphon as the Manila and littleneck clams. Although the horse clam siphons contained almost three times as much melanin as the butter clam siphons, the pigmentation on horse clam siphons is restricted to the exterior of the siphon (Figures 19 to 21).and not exposed to water being siphoned.

The results of these experiments suggest that the melanin in the clam siphon may be a factor in the retention of PSP in clam siphons, and may partially explain why PSP is retained much longer

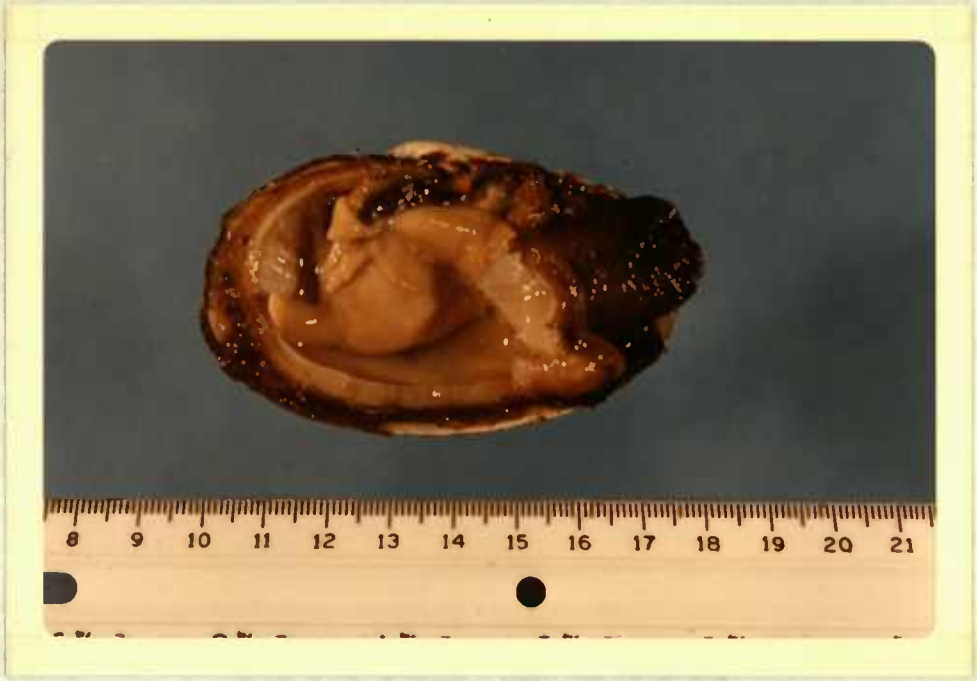

Figure 19. Horse clam showing distribution of melanin on exterior of siphon.

Figure 20. Split horse clam siphon showing lack of melanin on interior of siphon. The horse clam is now classified as Tresus capax (Gould) (Quayle, 1969).

Figure 21. Inner wall of horse clam siphon showing lack of melanin granules in epithelial cells. Hematoxylin and eosin. x 320.

in butter clam siphons than in the siphons of other mollusks.

Table 7. Relative melanin content of various clam siphons.

Species	Relative melanin content per siphon[a/]
Horse clam (Tresus capax)	2.90
Butter clam (Saxidomus giganteus)	1.00
Manila clam (Venerupis japonica)	0.21
Native littleneck clam (Protothaca staminea)	0.19

[a/] Absorbance at 420 nm per 100 siphons divided by the absorbance at 420 nm per 100 butter clam siphons.

Distribution of PSP and Melanin in Toxic Butter Clam Siphons

The distribution of PSP and melanin in fractions obtained from frozen toxic butter clam siphons is shown in Figure 22. The results are derived from melanin assays and bioassays on fractions from 2,233 g of frozen siphons, and indicate a close association between PSP and melanin in toxic butter clam siphons. The filtrate after sonication contained over 50% of the melanin found in the siphon, and also contained almost 50% of the PSP present in the siphon.

The results of these experiments together with the reversible electrostatic interaction between PSP and melanin described previously, suggest a possible reason for the ability of butter clams to bind and retain PSP for extended periods. The PSP in butter clam

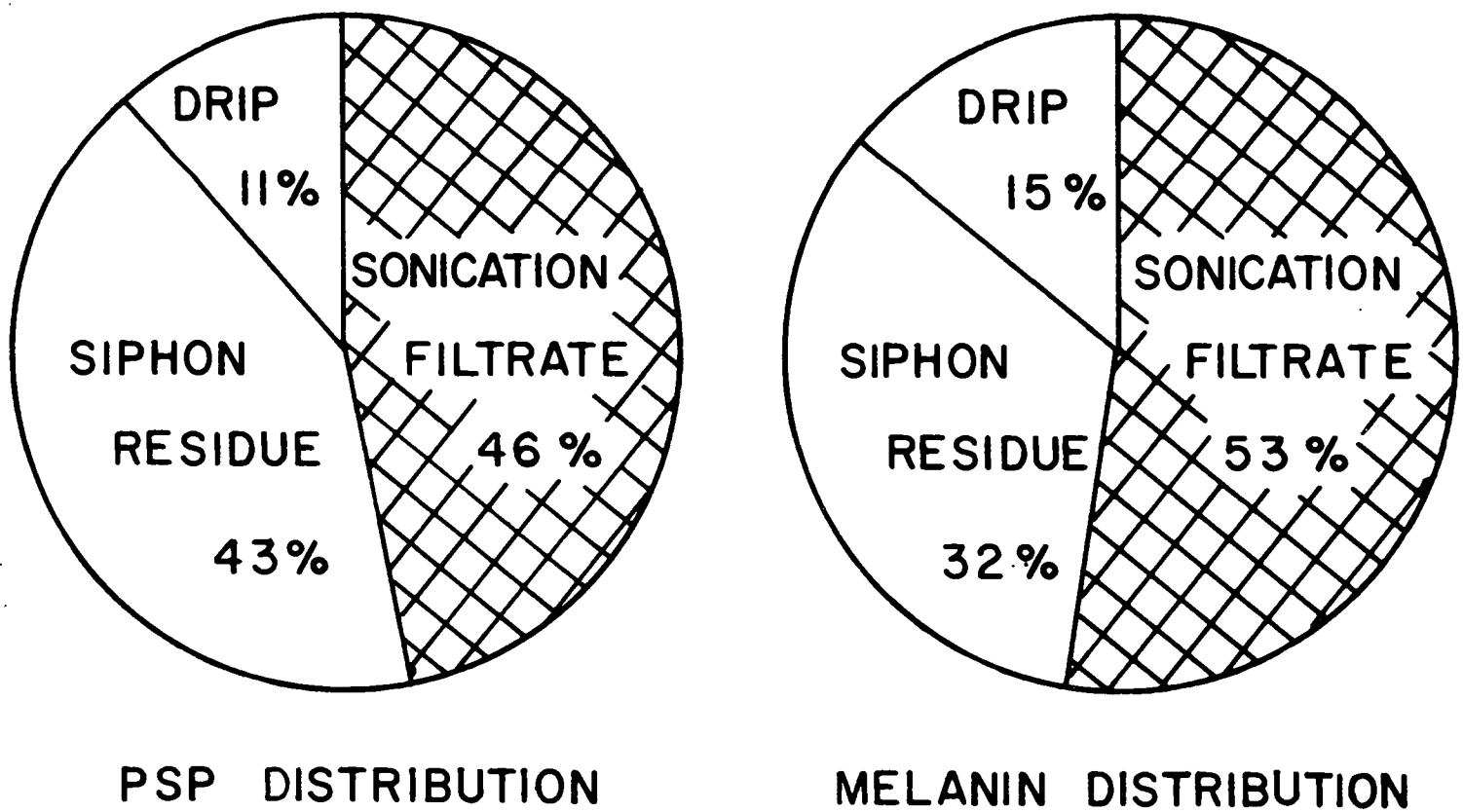

Figure 22. Distribution of PSP and melanin in toxic butter clam siphons.

siphons is closely associated with melanin (Figure 22). It may be bound by the melanin in a manner similar to that described for the interaction of PSP and melanin in vitro. An electrostatic interaction of this nature would explain not only the retention of PSP in butter clam siphons, but also the ability to remove PSP from toxic clams by acid treatment since the interaction is greatly influenced by pH. The weak nature of the PSP-melanin interaction would account for the gradual reduction in toxicity of butter clams in the absence of PSP.

The reversibility of the PSP-melanin interaction in the presence of hydrogen ions or cations suggests that it may be possible to detoxify butter clams during processing of the clams. Although soaking of minced or whole toxic clams in seawater at a pH as low as 5.0 has been shown to be ineffective in reducing PSP levels in clam meats (U. S. Dept. Interior, 1966), no attempts have been made to detoxify butter clams with salt solutions. Since PSP interacts electrostatically with melanin in vitro and may bind similarly in vivo, it may be possible to reduce the PSP levels in toxic butter clams by incubating live clams or soaking minced clams in solutions containing polyvalent cations which would compete with the PSP for binding sites in the clam. A process of this type may prove more economical and feasible than a process involving low pH, removal of butter clam siphons, and extended heat processing to reduce PSP levels.

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