(yeast extract powder, 0.2 percent; peptone, 0.18 percent; ammonium sulfate, 0.15 percent; monopotassium acid phosphate, 0.1 percent; magnesium sulfate, 0.05 percent) supplemented with the dye to be tested. Fifty milliliters of the medium in 100-ml erlenmeyer flasks were inoculated with about 1000 cells of the original culture per milliliter and incubated at 30°C for 48 hours. A few drops of these cultures were diluted in sterilized water, spread on a normal nutrient agar medium, and scored for *petite* mutants after 48 hours of incubation at 30°C.

Table 1 summarizes the results of the experiments with various dyes in varied concentrations. The effects of acriflavine and of caffeine in the collateral tests with the same basal medium are shown for comparison. Most of the inducers are toxic with respect to the yeast and suppress growth at fairly low concentrations. The increase in the frequency of occurrence of the *petite* mutants is, however, greater in the fully grown cultures (up to $3 \times 10^7$ cells per milliliter) than in the inhibited cultures. In other words, maximum induction in terms of increased frequency of the *petite* mutants in the final population appears at concentrations far below the lethal level. Comparison based upon molar concentrations of the dyes instead of on milligrams per liter reveals greater differences in efficacy in both the induction of *petite* mutants and the suppression of growth.

With the exception of acriflavine, the dyes fall into two major groups—namely, triphenylmethane dyes and xanthene dyes. The general trends in the relationship between chemical nature and efficacy may be summarized as follows: (i) all the effective dyes are basic dyes; (ii) efficacy tends to be greater when the number of methyl groups in the molecule is greater; (iii) the aminotriphenylmethane derivatives which have methyl groups introduced into their amino groups are more effective than those which have methyl groups introduced into the benzene nuclei; (iv) the dyes which have ethyl groups instead of methyl groups are as effective as, or sometimes more effective than, the dyes which have methyl groups in the corresponding positions. The Victoria blue dyes B and 4R show rather low efficacy as compared with other triphenylmethane dyes in spite of high affinity (absorption) to the yeast cells. Such characteristics are perhaps due to the presence of a phenyl-α-naphthylamine moiety in the molecule.

An improved modification of the triphenyltetrazolium chloride (TTC) overlay technique was employed for scoring the *petite* mutants in the cultures. The molten agar (1 percent) containing 0.05 percent TTC and 0.5 percent glucose was gently poured onto colonies (200 to 300 per standard petri dish) of the sample cultures growing on nutrient agar of the same composition as the basal medium. The cultures, thus covered 3 to 4 mm deep, were incubated again at 30°C. Color differentiation between normal (red) and mutant (white) colonies appeared more clear-cut and occurred more quickly than color differentiation produced by the procedures hitherto followed (*3*).

The dyes were examined by paper chromatography. The ascending development with acetone, water (4:6) and *n*-butanol, acetic acid, water (4:1:5), respectively, gave fairly good results. No serious admixture or decomposition product has been detected so far (*4*).

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## Acid Mucopolysaccharide of the Crustacean Cuticle

*Abstract.* The acid mucopolysaccharide found in the crab *Hemigrapsus nudus* and containing glucose, galactose, and fucose residues is found both in the cuticle and in the digestive gland of the crab. The concentration of mucopolysaccharide is somewhat higher in the cuticle, where it it the only soluble polysaccharide, than in the digestive gland, where it makes up 10 to 25 percent of the total polysaccharide content.

The occurrence of acid mucopolysaccharides in integumentary and skeletal tissues has been reported frequently in recent years. The present report provides direct chemical evidence of the occurrence of such a substance in the integument and digestive gland of the crab *Hemigrapsus nudus* (Dana). Trim (*1*) reported the occurrence of a nonchitin carbohydrate in the arthropod cuticle. Travis (*2*), using histochemical methods, demonstrated the presence of a mucopolysaccharide in the integument and digestive gland of lobsters. Hu (*3*), in his recent study, observed that a polysaccharide other than glycogen is present in *H. nudus*. The polysaccharide fraction separated by Hu yielded, on hydrolysis, fucose, galactose, and glucose. Nothing was known as to whether this polysaccharide is similar in distribution to that observed by Trim or Travis. Hence, it was thought worth while to study the distribution of this polysaccharide in the crab, with special reference to the cuticle.

The animals were collected from Coos Bay in the vicinity of Charleston, Ore. All the animals used in the experiment were in the $C_4$ stage of the intermoult cycle (*4*).

The cuticle was prepared according to the method used by Frankel and Rudall (*5*) for insects. The polysaccharides were precipitated with alcohol from alkali extracts of the powdered cuticle and of the fresh digestive gland. The polysaccharides were separated by paper electrophoresis at *p*H 5, as suggested by Bera *et al.* (*6*). The Karler-Kirk curtain-electrophoresis apparatus was used (*7*). The polysaccharide was stained on the paper by the method of Köiw and Gronwall (*8*) and of Hammerman (*9*). Quantitative estimation of the polysaccharides after electrophoretic separation was made by the anthrone method of Roe (*10*). For the hydrolysis of the polysaccharide and analysis of the sugars, the methods adopted by Hu (*3*) were followed.

The digestive gland contains, besides glycogen, an acid mucopolysaccharide which gives a positive test with toluidine blue staining on the paper. The acid mucopolysaccharide moves to the cathode and is readily separated from glycogen on paper during electrophoresis. Hydrolysis of the polysaccharide yielded fucose, galactose, and glucose, glucose being more abundant than the other two sugars, as observed by Hu (*3*). The cuticle also showed the presence of acid mucopolysaccharide which

Table 1. Polysaccharide content of digestive gland and cuticle of *Hemigrapsus nudus*.

| Tissue | Polysaccharide | | | |
|---|---|---|---|---|
| | Glycogen (mg/100 gm of dry tissues) | | Acid* (mg/100 gm of dry tissues) | |
| | Mean | Extremes | Mean | Extremes |
| Digestive gland | 5073 | 2506–7392 | 671 | 285–2460 |
| Cuticle | | | 939 | 842–1085 |

* Expressed in terms of glucose equivalents by the anthrone method.

yielded fucose, galactose, and glucose on hydrolysis and which appeared to be identical with that of the digestive gland. Glycogen was not demonstrable in the cuticle; if it was present at all, the amount was negligible as compared with the acid mucopolysaccharide.

Quantitative distribution of the acid mucopolysaccharide in the cuticle and digestive gland is summarized in Table 1.

The cuticle contains an average of 937 mg of acid mucopolysaccharide per 100 gm (dry weight). In the digestive gland, about 10 to 25 percent of the total polysaccharide is in the form of acid mucopolysaccharide.

Recently many interesting observations on the correlation between calcification and distribution of acid mucopolysaccharide have been reported. Rubin and Howard (*11*) observed acid mucopolysaccharide in association with calcification in bones and calcified cartilages and suggested that calcium-binding capacity was due to the presence of the acid mucopolysaccharide. Studies on various types of human tissues have led to the conclusion that a mucopolysaccharide with specific properties is involved in almost all biological calcification. In the study of the organic matrix of the shell of the hen's egg, Simkin and Tyler (*12*) suggest that both acid mucopolysaccharide and protein components of the egg shell are vitally important to the calcification mechanism. Travis (*2*) has observed that the mucopolysaccharide shows some cyclic changes in the digestive gland and the integument in relation to the intermoult stages. On the basis of these observations it is envisaged that the presence of acid mucopolysaccharide in the cuticle of the crab may have an important role in the calcification of the cuticle (*13*).

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## Titration of the Protein Paramyosin

*Abstract.* The titration curve of an aqueous solution of crystalline paramyosin shows a unique buffering zone which can be attributed to a shift in the *pK* of its glutamic acid residues. A possible explanation of this phenomenon on the basis of the presence of polyanionic segments in the molecule is discussed.

In the course of studies on the solubility characteristics of the protein paramyosin and the physiological behavior of muscles containing this protein (*1*), it became apparent that its sharp crystallization point around *p*H 6.4 may be of prime importance to its physiological role. The amino acid composition of paramyosin, as determined by Kominz *et al.* (*2*), shows no amino acid residues which should have a *pK* in this range. The protein contains less than 1 percent histidine but about 158 residues of glutamic acid, 120 of aspartic acid, 73 each of lysine and arginine, and 13 of tyrosine, out of 800 amino acid residues (100,000 molecular weight); of the carboxylic groups, 112 are present as amides. No significant amount of phosphate could be detected.

We have titrated solutions of paramyosin over the *p*H range from 2 to 12, in the hope of determining whether or not crystallization is associated with pecularities in the titration curve.

The procedure was as follows. Paramyosin, isolated as previously described (*1*), was dissolved in 0.3*M* KCl and dialyzed against water. The precipitated paramyosin was isolated by centrifugation and dissolved in 0.3*M* KCl to give a final concentration of 1 percent protein. Thirty milliliters of this solution was placed in a beaker above a magnetic stirrer, under a nitrogen atmosphere, and adjusted to *p*H 12 with KOH. The solution was then titrated with 0.1*N* HCl, with a microburette, and the *p*H was read continuously with a Beckman model G *p*H meter with external electrodes. A blank, consisting of 30 ml of 0.3*M* KCl, was titrated over the same *p*H range, and its curve was subtracted from that of the protein sample. The results, designated as the net titration curve, are shown in Fig. 1. The protein precipitated at a *p*H value near 6.5, as had previously been found to be the case (*1*). The precipitation did not in itself alter the behavior of the protein toward added acid, since it was possible to start on the acid side of the curve with precipitated protein and titrate in the opposite direction with no change in the shape of the curve.

The titration curve shows a buffering zone around *p*H 11.5, corresponding to the known arginine content of the protein. The large buffering zone around *p*H 10 corresponds to the lysine, tyrosine, and terminal amino groups. A large buffering zone is found near the center of the curve, with a *pK* of about 6.3, corresponding to the titration of 130 groups per 800 amino acid residues. No buffering zone for carboxyl groups is found around *p*H 4.3, although such a zone would have been expected on the basis of the known content of glutamic acid. The buffering zone at the low *p*H end of the curve could correspond to free aspartic acid residues as well as terminal carboxyl groups.

The complete absence of a buffering zone around *p*H 4.3 and the presence of a large zone around *p*H 6.3 suggest that the *pK′* of the glutamic acid residues present in the protein has been shifted by approximately 2 *p*H units. Such large shifts in *pK′* are not common in proteins but are common in synthetic polymers. In the case of synthetic polyanions, the shift has been accounted for on the basis of the strong electric field which appears when charges are concentrated along the polymer chain (*3*). Others have sought to explain this effect in proteins on the basis of hydrogen bonding (*4*) or masking of groups by bound water (*5*). However, in view of the solubility behavior of paramyosin, the possibility that polyanionic segments are present in this protein sounds attractive to us.

This possibility has prompted us to calculate the number of charges per unit length of protein molecule necessary to generate an electric field large enough to shift the *pK* of the glutamic acid residues by approximately 2 *p*H units. The following formula, derived by Hartley and Roe (*6*) and modified by Katchalsky (*7*), for polyanions in solution, was used:

$$pH = pK' - 1n\frac{1-\alpha}{\alpha} + \frac{0.4343\,\varepsilon\,\psi}{kT} \quad (1)$$

where *pK′* is the *pK′* of the monomeric group (in this case 4.3), *T* is the abso-

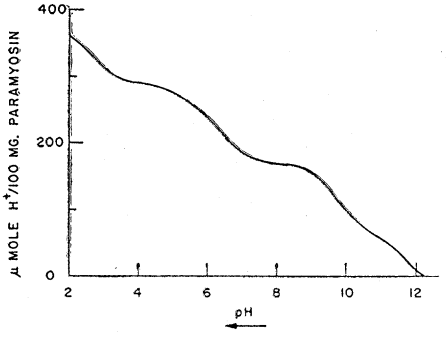


Fig. 1. Net titration curve of paramyosin dissolved in 0.3*M* KCl. The solution was adjusted to *p*H 12 and titrated to *p*H 2 with 0.1*N* HCl.