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Regulation of Betacyanin Efflux from Beet Root by Poly-L-Lysine, Ca-Ion and Other Substances

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Summary. Poly-L-lysine, poly-α, γ-diaminobutyric acid and basic proteins cause efflux of betacyanin from beet root tissues to varying degrees. Membrane activities fall in the order: polylysine > poly-α, γ-diaminobutyric acid > polyarginine (protamine), suggesting the importance of steric factors in side-chain to backbone relations. It was also observed that homopolymer activity > heteropolymer activity, using ribonuclease and lysozyme as examples of the latter. Among polylysines, there appears to be an optimal chain length at a molecular weight equal to 50,000. Lowered activity of larger polymers is interpreted in terms of a diffusion barrier, the cell wall.

Polylysine and Ca^{++} exhibit competitive kinetics, and Ca^{++} otherwise is far more active than other cations. It is assumed that polylysine displaces Ca^{++} from anionic centers on the membrane, but cannot confer equivalent dimensional stability, rendering the membrane leaky. The possible role of cationic shielding in ionic stabilization of the membrane was also considered. The order of divalent ion activity against polylysine was $Ca^{++} > Sr^{++} > Mg^{++}$, suggesting again a specific size-fit relationship.

Washed red beet root sections can be maintained under simple experimental conditions for many days with little or no leakage of the vacuolar pigment, betacyanin. This highly stable system has proven useful in the study of regulators of permeability. Monohydric alcohols have a classical effect, pigment efflux increasing with lengthening carbon chain in the normal series (16). Isomers, particularly the secondary and tertiary alcohols affect permeability far less than do primary straight chain alcohols.

Other experiments have provided evidence for oxidizable membrane sites. At temperatures of 50° or more, the rate of efflux is a function of the equilibrium O_2 level of the ambient solution (18). Comparisons among peroxides made in other experiments suggest that both hydrophobic (organic peroxide-sensitive) and hydrophilic (H_2O_2-sensitive) sites exist (17).

Although the effects of alcohols are reversed by Ca^{++}, that of oxidants is not. Organic peroxides are antagonized by relatively hydrophobic substances such as α-tocopherol and IAA, whereas H_2O_2 is antagonized by water-soluble substances such as CoA and cysteine.

Although Ca^{++} failed to protect beet root tissue against oxidants, the distinctive role of divalent cations in the maintenance of membrane organization and function (4, 6, 10, 13) suggested that the evaluation of organic cations might be of interest. Conceivably a suitable organic cation could compete with calcium for anionic binding sites and, if sufficiently different in spatial configuration, lead to changes in membrane organization that would allow pigment efflux.

It is assumed that the membrane carries a net negative charge (14) — forming a polyanionic sheet, in a sense — hence will interact strongly with polycations. The antibacterial activity of basic polyamino acids and of cationic polypeptide antibiotics such as tryocidin and the polymixins is attributed to interactions with the strongly electronegative bacterial surface leading to changes in permeability, metabolic disturbances, and eventually, death (1, 2, 7, 8, 9). Therefore, such substances, particularly the readily available homopolymers of lysine, seemed well suited as experimental polycations.

Preliminary experiments showed that cationic polyamino acids are in fact highly membrane-active (5); a poly-L-lysine of mol wt ca 8000 at 10 μmoles/liter lysed *Paramecium* in 10 to 12 seconds.

Although partly exploratory, experimentation was directed toward certain specific objectives: A) Reversibility of polycation effect (if any) by Ca-ion; B) site-specificity of reversal; C) uniqueness of calcium relative to other common inorganic cations.

Material and Methods

The procedure used in cutting, washing, and general handling of beet root tissues in earlier experiments (17) was followed precisely except for the use of 6.7 mM phosphate buffer in some experiments with high Ca^{++} levels instead of 67 mM buffer.

Efflux of pigment was followed using the Klett colorimeter with a No. 54 filter. Betacyanin concentration is expressed in Klett units. One Klett unit is equal to an optical density of 0.002. Experiments were run at 23°. No difficulty in maintaining pH 6.6 was encountered, and the necessary change in ionic strength of the buffer caused no substantial change in results. Each experimental vessel was replicated 2 to 4 times, and critical experiments were repeated.

Polylysine-HBr and poly-sodium- L-glutamate were obtained as purified commercial preparations (Pilot Chemical Co.). Poly-α,γ-diamino butyric acid (mol wt 8000) was synthesized in this laboratory (5). Other biochemicals from Calbiochem were the highest purity grades available.

Of the polylysines used, mol wt 84,000 was most readily available in quantity, hence was chosen as a standard for most experiments.

Results

Survey...In poly-L-lysine HBr (mol wt 84,000) at 0.1 %, or 12 μmoles/liter, the course of betacyanin efflux is approximately linear for several hours (fig 1).

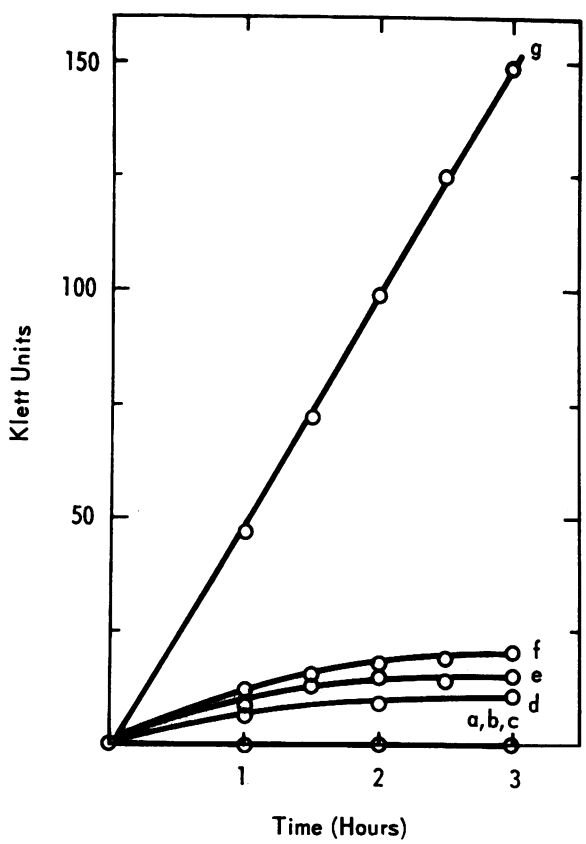


FIG. 1. Course of betacyanin efflux from beet root as affected by 0.1 % solutions of a) buffer only; b) poly-L-glutamate, mol wt 132,000; c) L-lysine HBr; d) lysozyme; e) calf thymus histone; f) poly-α, γ-diamino-butyric acid, mol wt 8000; g) poly-L-lysine, mol wt 84,000. One Klett unit = 0.002 OD.

By adjusting polylysine, L-lysine and other substances to the same weight percentage, it is possible to compare polymers and monomers on an equivalent residue basis. This is of particular importance to establish that the membrane activity of lysyl cations in polymeric form cannot be replaced by an equal number of monomeric lysine cations. The significance of polycationic character is shown by the inactivity of poly-Na-L-glutamate, a polyanion.

Small basic proteins such as lysozyme (mol wt ca 14,000) (12), and histone (mol wt order of 10^4) (3) have a low level of activity, as does ribonuclease (mol wt ca 14,000, table I). These proteins contain varying but substantial proportions of basic amino acids, which determine their basic character. They do not contain a majority of these amino acids however. Their activity is low when compared with an L-lysine homopolymer of approximately the same size (polylysine mol wt 20,000, table I). Protamine (mol wt ca 10^4) (3), approaches the homopolymeric condition with respect to arginine or arginine plus lysine, but exhibited low activity nevertheless. Even poly-α,γ-diamino-butyric acid, is lower in activity than a similar lysine polymer, although more active than most basic proteins. Simpler bases such as aliphatic amines and ammonia were devoid of membrane activity, even at 10-fold higher levels.

Effects of Concentration and Polymer Size. Within experimental time periods the efflux of betacyanin was approximately linear with increasing concentrations of polylysine from ca 1 to 10

Table I. *The Activity of Various Bases in the Beet Root System*

Beet root tissues were incubated at 23° in 6.7 mM phosphate buffer. One Klett unit is equal to an optical density of 0.002.

Substance	Concentration (μ moles/l)	Klett units*
poly-α, γ-Diamino-butyric acid**	ca 100	45 ± 5
Protamine sulfate	ca 100	24 ± 2
Histone (calf thymus)	ca 100	38 ± 6
Lysozyme (egg)	100	24 ± 3
Ribonuclease (pancreatic)	100	20 ± 5
Streptomycin sulfate	100	24 ± 2
1,4-Diaminobutane	1000	0
1-Aminohexane	1000	0
NH_4OH	1000	0
poly-L-lysine***	100	186 ± 16
poly-L-lysine†	100	65 ± 8
L-lysine·HBr	100	0
	1000	0

* Based upon 3 hour readings using the Klett-Summerson photocolorimeter with no. 54 filter.
** Molecular weight ca 8000.
*** Molecular weight 84,000.
† Molecular weight 20,000.

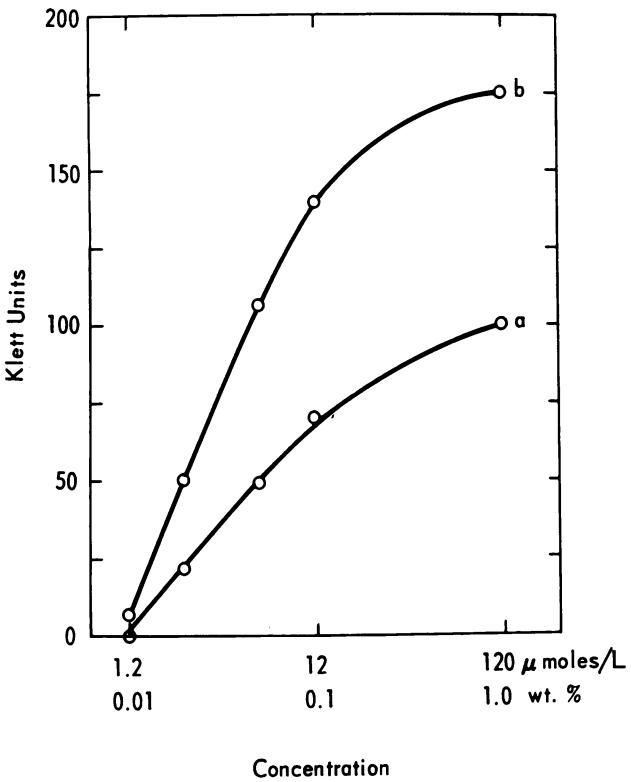


FIG. 2. Efflux of betacyanin from beet root as a function of concentration of poly-L-lysine, mol wt 84,000. a) At 1.5 hours; b) at 3 hours. One Klett unit = 0.002 OD.

μmoles/liter, and with time (fig 2). In the experimental system, activity could not be detected below ca 1 μmole/liter, even after 24 hours.

Using a constant weight-concentration of 0.84 % (0.84 % of mol wt 84,000 = 100 μmoles/l, the efflux of pigment was greatest in solutions of mol wt 50,000, falling about 25 % at mol wt 20,000 and ca 50 % at 110,000 (fig 3). Under these conditions, the molar concentration varies while the lysyl concentration remains constant.

When the molar concentration of polylysine is held constant at 120 μmoles/liter, the total lysyl content varies with mol wt. Here membrane activity rises to a maximum, then falls, but the peak corresponds to a mol wt of 84,000.

Polylysine-Ca^{++} Antagonism. The membrane activity of polylysine was antagonized by a variety of cations at concentrations equivalent to the concentration of lysyl cation residues (33 mM equivalent to 100 μmoles/l of polymer (fig 4). The anti-polylysine activities of Group I chlorides followed the order

$$K^+ > Na^+ \geqslant Li^+$$

and were markedly greater initially than after 3 hours, whereas the Group II series was

$$Ca^{++} > Sr^{++} > Mg^{++}.$$

It was also noted that K^+ and Mg^{++} differed little. The latter was somewhat more active initially, but the distinction disappeared with time, but Ca^{++}-antagonism was largely independent of time.

A few additional experiments showed that Ca^{++} at 0.017 M or 0.5 Ca-ions per lysyl cation was as effective as 33 mM (1 Ca^{++}/lysyl residue). The anti-polylysine activity of Sr^{++} was somewhat more than half at 17 mM as at 33 mM. An equimolar combination of Ca^{++} and Sr^{++}, each 0.017 M, was approximately equal in activity to 17 mM Ca^{++}. When ionic concentrations were reduced to ca 1 mM, only Ca^{++} showed any anti-polylysine activity at all.

The unique character of Ca^{++}-polylysine antagonism was confirmed, and shown to be competitive in nature (fig 5). Values for pigment efflux at 3 hours were used, with 20 to 200 μmoles polylysine and Ca^{++} at 0.5 and 2.0 mmoles/liter.

Discussion

The enhancement of pigment release in the beet root may be direct or indirect. That is, the experimental treatment may involve direct action on cell membranes, or may operate through intermediaries (enzymes, for example) released within the cell. We have observed that several lipases and proteinases, especially phospholipase C, can in fact increase permeability, but for the present, it is

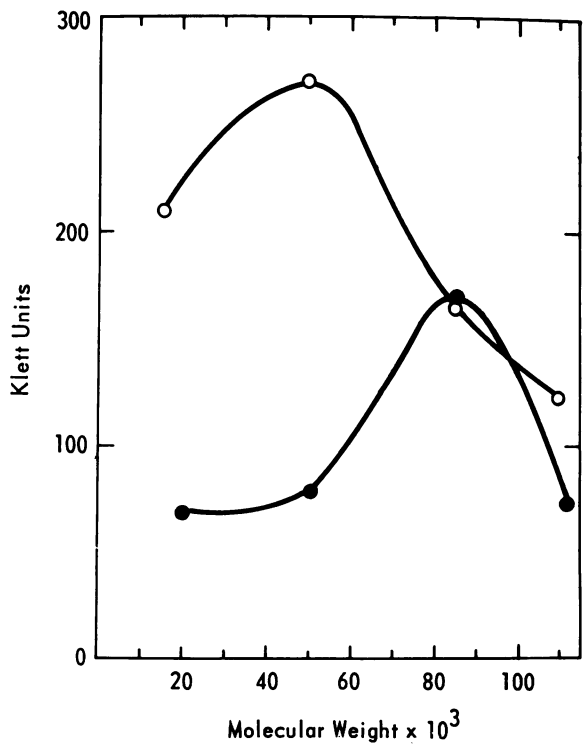


FIG. 3. Efflux of betacyanin as a function of molecular size of poly-L-lysines. ○: At constant weight concentration, 0.84 %; ●: At constant μmolar concentration, 100 μmoles/liter.

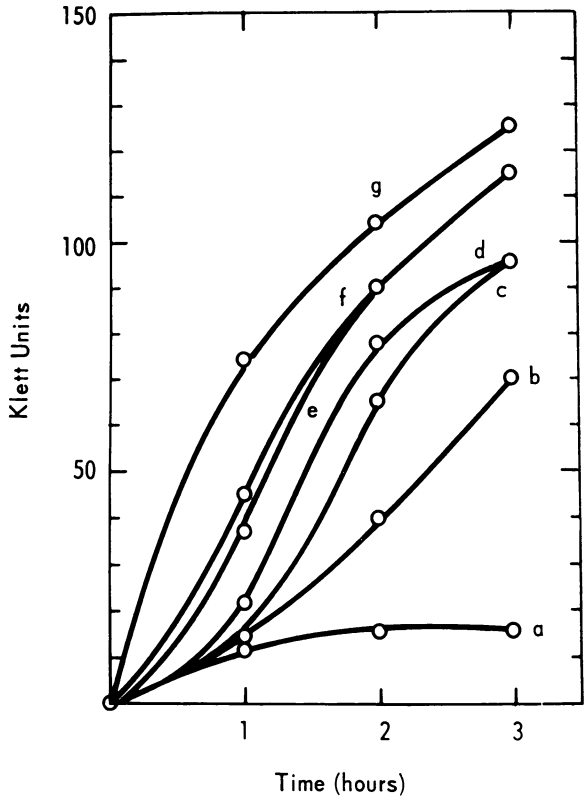


FIG. 4. Effects of mono- and divalent ions on the course of polylysine-induced betacyanin efflux from beet root. Ionic concentrations are all 33 mM, or one ion per lysyl cation in the polymer. Polylysine, 100 μmoles/liter, mol wt 84,000. a) $CaCl_2$; b) $SrCl_2$; c) $MgCl_2$; d) KCl; e) NaCl; f) LiCl; g) polylysine alone. One Klett unit = 0.002 OD.

assumed that the activities of alcohols, oxidants and polycations may be referred directly to the membrane.

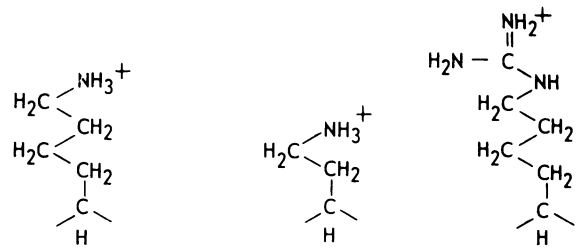
The membrane activity of alcohols was related to their structure and Ca^{++}-reversible, hence may have involved anionic sites associated with lipoidal materials or fatty acids. Structure was also important in the performance of peroxides, but without the involvement of anionic centers. The experiments with polycations reveal another kind of specificity, and also involve Ca^{++}-sensitive sites.

Polycationic character is obviously required for activity, but is not sufficient in itself to give more than a minimal effect. Thus an assortment of polybasic substances have similar low degrees of membrane activity and include molecules such as protamine which is virtually an arginine homopolymer and ribonuclease (RNAse) which is a basic heteropolymer. The direct activity of RNAse on *Avena* membranes was reported by Ruesink and Thimann (15). They, however, interpreted their results to mean that RNA was involved in membrane integrity. In view of the present data, this conclusion may require reinterpretation. Their system of isolated protoplasts may be more sensitive than beet root however. In experiments with *Paramecium* we have also noted that lysozyme and ribonuclease are nearly as active as polylysines.

When polylysine (poly-Ly), protamine (poly-arginine, poly-Ar), and poly-α,γ-diaminobutyric (poly-DAB) acid of more or less comparable mol wt, ca 10^4 are compared, the relative order of membrane activity is:

poly-Ly > poly-DAB > poly-Ar

As bases the ε- and γ-amino groups of poly-Ly and poly-DAB are similar, and the guanidine group of poly-Ar even stronger. However, since all 3 are almost fully protonated at pH 6.6, the important difference is more likely to be stereochemical, as the polypeptide side chain structures show:



The distance from the asymmetric carbon or peptide bond to the cationic group, and the length of polymethylene chain attached to the $-NH_3^+$, or both, may be important, but our current data provide no evidence bearing on these possibilities. It is clear, however, that membrane activity requires more than homopolycationic character.

Specificity is also evident among the polylysines (fig 3). Comparing the 2 modes of expression as polymer weight increases we have:

	Constant wt	*Constant Molarity*
Total no. of lysyl cations	Constant	Increases
No. lysyl cations/chain	Increases	Increases
Total no. of molecules	Decreases	Constant

At constant weight concentration, membrane activity passes through a maximum at mol wt 50,000, then falls. Thus at lower molecular weights, polymer size, or the no. of lysyl cation/chain must determine activity, but with continuing increase in mol wt the fall in concentration (no. of molecules) may offset the effect of increasing chain length. At constant molarity, membrane activity increases to a maximum at 84,000, but this could be a function either of the number of molecules or number of residues.

In this case, however, the drop in membrane activity with increasing polymer size occurs in spite of the increase in lysyl cation residues. Such an effect must be related to chain length. It seems unlikely that a polymer would be too large to inter-

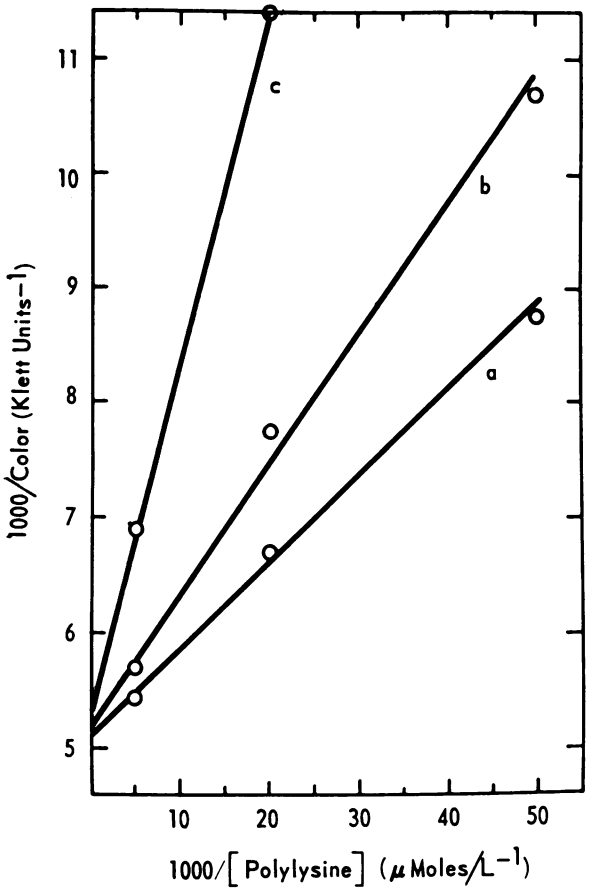


FIG. 5. Lineweaver-Burke double reciprocal plot of betacyanin efflux versus polylysine concentration at 3 $CaCl_2$ concentrations. Polylysine, mol wt 84,000; time, 3 hr. Ca^{++}: a) 0; b) 0.5 moles/liter; c) 2.0 mmoles/liter. One Klett unit = 0.002 OD.

act with the cell surface, hence size must influence access to the surface instead. The cell wall is not ordinarily regarded as a diffusion barrier on the basis of the movement of small electrolytes, but it is the only diffusion barrier possible in the present case, and a reasonable one for a large polyelectrolyte. Additional evidence for such a diffusion barrier comes from the high activity of polylysine on *Paramecium* at a concentration of 1 μmole/liter (5), the threshold concentration for its activity in the beet root.

The membrane activity of the polycations has been examined thus far on a comparative basis without regard to their mode of action.

The evidence gained from ion-antagonism experiments indicates that polylysine displaces cations, especially Ca^{++} from electronegative sites on the membrane. These negative centers presumably depend upon coordination with cations for dimensional stability, and become sufficiently disordered or disorganized to allow pigment leakage when some fraction of the Ca^{++} is removed. In part, Ca^{++} may serve as a counter-ion, reducing the electrostatic repulsion between anionic centers. Such an effect may have accounted for the stabilizing influence of other cations, especially the monovalent ions. These ions were active only at concentrations on the order of 10 mM, whereas Ca^{++} was still active at ca 1 mM. A 1:10 relationship between mono- and divalent species is consistent with their respective shielding strengths (Hofmeister or lyotropic effect) in electrokinetic processes (19). On the other hand, if stabilizing effects of divalent ions were based upon shielding, polylysine itself should be a stabilizing polycation, not a membranolytic agent. If, then, the divalent ions antagonize polylysine in a distinct manner, it is most likely to involve formation of bridging complexes or compounds. Participation in this manner immediately suggests that the observed order of activity, $Ca^{++} > Sr^{++} > Mg^{++}$, is determined by fit. A comparison of ionic radii (11) gives:

Sr^{++} (1.13Å) > Ca^{++} (0.99 Å) > Mg^{++} (0.78 Å),

or a ratio of 1.49:1.27:1.00. As was the case in their activities against polylysine, Sr^{++} and Ca^{++} are closer to one another than are Ca^{++} and Mg^{++}. To some extent this order of cation selectivity is reflected in the complexes formed by certain di- and tribasic acids (12).

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