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THE EFFECTS OF SELECTED NITROGEN COMPOUNDS ON THE GROWTH OF PLANT TISSUE CULTURES

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INTRODUCTION

Plant tissue cultures and various ancillary techniques find an increasing use in the study of growth and metabolism. In the Cornell Laboratory, tissue-culture techniques have been designed primarily to achieve control over the variables which stimulate growth by cell division and which maintain it at maximum, but quantitatively reproducible, rates. In this technique[1] tissue explants (2.5 mg) freshly removed, aseptically, from a specific region of the intact carrot root (1–2 mm from the cambium and in the secondary phloem) are stimulated to grow rapidly by as yet incompletely characterized growth factors which are to be found in the liquid endosperm of the coconut (*i.e.* coconut milk). Other similar natural fluids such as those present in immature corn (*Zea*) grains and immature *Aesculus* fruits may also be used. These growth factors are used as supplements to a basal nutrient medium which contains salts, trace elements, vitamins, and sucrose as prescribed by WHITE[2].

In the absence of the growth factors present in the coconut milk, attempts may be made to detect growth-promoting qualities, equivalent to those that are now familiar in the coconut milk. Alternatively, by the use of the complete nutrient system, in which the tissue will normally grow rapidly, the ability of a test substance to inhibit, or to retard, growth may be investigated. In addition, the same general techniques may be used to measure the interacting effects of both stimulatory and inhibitory substances when these are supplied together.

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The alcohol-insoluble nitrogen, here regarded as the total protein, of the carrot cultures grown on media containing coconut milk was found to be unexpectedly rich in the amino-acid hydroxyproline[3,4]. Hydroxy-L-proline is normally present in plant proteins only in small amounts, and it rarely occurs in plants in the free state. Subsequent analyses of other rapidly proliferating tissue also showed an enhanced hydroxyproline content in their protein. Such tissues were obtained from tissue cultures derived from the potato tuber by the combined action of coconut milk and 2,4-D*, the tissue stimulated to grow by the crown-gall organism acting on *Kalanchöe*, and certain tumors in hybrid strains of tobacco, in which the stimulus originates from the genetic constitution of the plant in question. Thus the occurrence of a protein relatively rich in hydroxyproline seems to be a characteristic feature of such rapidly proliferating plant cells[4].

The presence of a hydroxyproline-containing protein moiety in cultured plant tissues enables attention to be focused upon a specific protein which is synthesized in such growing cells. A parallel study[5] in which various ^{14}C-labeled substrates were supplied to the tissue, showed quite clearly that ^{14}C-proline, endogenously produced from ^{14}C-labeled substrates, was incorporated in the cells directly into this particular hydroxyproline-containing fraction. Once synthesized, this hydroxyproline-containing moiety did not subsequently "turn over". By contrast, the cells also synthesized another kind of protein, the carbon of which was rapidly reincorporated into the metabolism of the cells, and "turned over" more rapidly than the net synthesis of protein alone could explain. All these observations suggested the analogy of the inert hydroxyproline-containing substance with collagen, a metabolically inert[6] protein in the animal body, in contrast to the more metabolically active proteins of the growing cell. Collagen biosynthesis and hydroxyproline metabolism may be of importance in arthritis, in the process of wound healing, and in the formation of neoplastic tissue in the animal body[7]. The availability of various analogs of hydroxyproline[8] for testing as possible chemotherapeutic antimetabolites of hydroxyproline prompted a study of their effects in plant tissue cultures.

As the investigation proceeded, its scope was extended and, in the final outcome, the tests to be described have embraced a variety of other biologically interesting compounds that may be conceived to act in different ways.

METHODS

A basal medium was prepared after the procedure prescribed by WHITE [2], and this was supplemented by the addition of 2% by volume of whole coconut milk. The growth of standard explants of carrot tissue in 10 ml of this medium under the controlled conditions prescribed earlier[1] was used as the standard of comparison against which the effects of additional substances were measured. This growth is measured by the difference in final fresh weight of explants grown in the medium as supplemented by coconut milk and in the basal, unsupplemented medium.

The test substances were added to cultures which contained the complete medium; experience showed that 10 p.p.m. was usually a suitable concentration at which differences in the activity of the substances tested became apparent. All the test substances were added to the medium before it was sterilized by autoclaving and the possibility exists that in some cases they may have been modified by the heat treatment. Each culture tube, containing 10 ml of sterilized medium, received three standard carrot-tissue explants. The effect of each treatment was assessed from the mean behavior of nine explants in three replicate culture tubes; all the explants in a given experiment came from the same carrot root. Substances were usually tested on carrot explants derived from different roots.

* 2,4-dichlorophenoxyacetic acid.

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RESULTS

All the substances tested, by the methods outlined above, are classified in Table I according to their effects on the growth of carrot explants. Since different classes of

TABLE I

CLASSIFICATION OF SOME NITROGENOUS COMPOUNDS BY THEIR EFFECT AS GROWTH INHIBITORS FOR CARROT EXPLANTS IN MEDIA CONTAINING COCONUT MILK

Substances that are inhibitory at 1 p.p.m. (causing more than 50% inhibition)

Gramicidin-S-sulphate

Substances that are inhibitory at 10 p.p.m. (causing more than 50% inhibition)

Hydroxy-L-proline
Allohydroxy-L-proline
O-Acetylhydroxy-L-proline
Allohydroxy-L-prolylglycine
Hydroxy-L-prolylglycine
Azetidine-2-carboxylic acid
Diazoacetylserine (Azaserine)
δ-Hydroxy-L-lysine
5-Hydroxytryptophan
L-Canavanine
Synthetic δ-hydroxyleucine

Substances that are partially inhibitory at 10 p.p.m. (causing 25 to 50% inhibition)

Hydroxy-D-proline
Allohydroxy-D-proline
L-Leucine
α-Hydrazinophenylpropionic acid
α-Hydrazino-*n*-butyric acid
5-Hydroxyindoleacetic acid

Substances that are not inhibitory at 10 p.p.m. (causing 25% or less inhibition)

Allohydroxy-D-proline anilide
4-Keto-L-proline
Poly (hydroxy-L-proline)
Hydroxy-L-proline anilide
1-Guanido-L-proline
4-Thiomethyl, allo-L-proline
1-Guanido, hydroxy-L-proline
N-Acetyl, hydroxy-L-proline lactone
O-Acetyl, allohydroxy-D-proline
Allohydroxy-D-prolylglycine
Allohydroxy-D-proline anilide
N-Nitroso, hydroxy-L-proline
N-Acetyl, hydroxy-L-proline
Stachydrine
Betonicine
Turicine
Acetylbetonicine·HCl
Acetylturicine·HCl
γ-Hydroxyornithine
L-Cysteine
S-Methyl-L-cysteine
γ-Hydroxyglutamic acid
DL-Valine
DL-α-Aminobutyric acid
DL-Alanine
DL-Isoleucine
L-Asparagine
L-Glutamine
L-Pyrrolidonecarboxylic acid
β-Alanine
γ-Aminobutyric acid
L-Arginine
L-Lysine
L-Histidine
L-Serine
L-Threonine
L-Glutamic acid
L-Pipecolic acid
5-Hydroxy-L-pipecolic acid
5-Allohydroxy-L-pipecolic acid
1-Amino-1-carboxycyclopentane
2-Carboxypyrrole
Formaminoglycine
L-Tryptophan
DL, O-Phenylserine
γ-Hydroxyvaline (natural)
γ-Hydroxy, γ-Methylglutamic (synthetic)
DL, β-Hydroxyglutamic (Merck)
DL, Allo, β-Hydroxyglutamic acid (Merck)
DL, β-Hydroxyvaline (Merck)
α-Hydrazinoisovaleric acid
α-Hydrazinoisocaproic acid
α-Hydrazino, β-methyl-*n*-valeric acid
Diazoacetylnorleucine (DON)
δ-Ketolysine
Urocanic acid
Urea
Ammonium chloride
Gramicidin-G-sulphate (1/100 satd. soln.)
Barium inosinate

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compounds were tested, they are now considered with due regard to the nature of the results and the reasons which prompted their selection for use.

Substances related to hydroxy-L-proline

Table II summarizes the effects of a number of compounds which are related structurally, or metabolically, to hydroxy-L-proline. It is apparent that hydroxyproline itself is, quite unexpectedly, a powerful *inhibitor* of the growth of carrot explants in the presence of 2 % coconut milk, rather than a stimulant, as might have been expected from its presence in a protein peculiar to cells which are grown in this manner.

Tests on the effect of the four possible hydroxyprolines show that the L forms are more inhibitory than the D forms, and that the configuration with respect to the hydroxyl group, *i.e.* whether normal or allo, has no effect on the growth-inhibitory properties. The tests made on derivatives of hydroxyproline indicate that all modifications of the hydroxyproline molecule, such as substitution in the hydroxyl group, on the nitrogen atom, at the carboxyl group, or oxidation of the hydroxyl to a keto group, all reduce the growth inhibition by the molecule so altered. In fact, all substitutions on the nitrogen of hydroxyproline remove its growth-inhibiting properties.

Table II also shows that similar substances with a 6-membered piperidine, rather

TABLE II

THE EFFECT AT 10 P.P.M. OF THE VARIOUS HYDROXYPROLINES AND SOME RELATED SUBSTANCES ON THE GROWTH OF CARROT EXPLANTS AS STIMULATED BY COCONUT MILK

(Growth in medium + coconut milk + test substance as % of growth in medium + coconut milk)

Substance	*Per Cent*
Hydroxyprolines	
Hydroxy-L-proline	8.9
Allohydroxy-L-proline	4.7
Hydroxy-D-proline	54
Allohydroxy-D-proline	68
Hydroxy-L-proline derivatives	
N-Acetyl-hydroxy-L-proline	118
O-Acetyl-hydroxy-L-proline	34
N-Nitroso-hydroxy-L-proline	125
N-Dimethyl-hydroxy-L-proline Betaine (Betonicine)	97
N-Dimethyl-allohydroxy-L-proline Betaine (Turicine)	106
Anilide of hydroxy-L-proline	103
Hydroxy-L-prolylglycine	31
4-Keto-L-proline	113
Poly (Hydroxy-L-proline)	98
Guanidino, hydroxy-L-proline	107
Related compounds	
L-Proline	107
Hydroxy-L-pipecolic acid	108
Allohydroxy-L-pipecolic acid	97
L-Pipecolic acid	100
1-Amino, 1-carboxycyclopentane	118
δ-Hydroxylysine	0
γ-Hydroxyornithine·dihydrochloride	119
γ-Hydroxyglutamic acid	123
Azetidine-2-carboxylic acid	3

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than a pyrrolidine, ring, are not inhibitory, while azetidine-2-carboxylic acid, with a 4-membered ring, is strongly inhibitory. γ-Hydroxyornithine and γ-hydroxyglutamic acid, both of which are possible metabolic precursors, or products, of hydroxyproline, are without effect on the growth. Yet, strangely, a possible precursor of hydroxypipecolic acid, namely δ-hydroxylysine, is a powerful inhibitor of growth in this tissue system. This is curious since both hydroxyproline and δ-hydroxylysine are known to occur in animal tissue proteins as constituents of collagen. It is, therefore, a problem to know how these two hydroxyamino acids affect growth in the carrot tissue-culture system. Hydroxylysine, unlike hydroxyproline, is not known to occur in the proteins of the tissue cultures, and it has not been critically established that this substance occurs in higher plants.

The effect of concentration of hydroxy-L-proline and its interaction with casein hydrolysate

Table III shows the inhibitory effect of hydroxy-L-proline at varying concentrations and for explants derived from two carrot roots (A and B). Explants from carrot B were obviously more sensitive to hydroxyproline at 10 p.p.m. than those from carrot A, though at higher concentrations the growth was completely inhibited. The experiments of Table III also indicate that the presence of 200 p.p.m. of casein hydrolysate (enzymic) antagonizes the hydroxy-L-proline inhibition of growth of the carrot explants.

The nature of the interaction between casein hydrolysate and the hydroxyproline growth-inhibition was determined. To do this, some of the individual amino acids of casein hydrolysate were tested, along with ammonia, for their ability to antagonize, or reverse, the growth-inhibitory properties of hydroxy-L-proline in the external medium. The results are given in Table IV, from which it will be apparent that *only* L-proline, of the various amino acids tried, is able to reverse the growth inhibition due to hydroxy-L-proline. In this respect the results parallel a case in fungi, in which proline antagonizes a growth inhibition of *Trichophyton* (a dermatophytic fungus) by hydroxyproline[9].

With Table IV in mind, L-proline was tested for its ability to reverse the inhibition which is due to 9 of the known growth inhibitors in this system, with results which are given in Table V. L-Proline obviously reverses the inhibition due to those six compounds which are structurally related to hydroxyproline, *i.e.* the first six compounds listed in Table V. However, L-proline has no detectable effect on the inhibitory properties of the last three compounds in Table V, none of which bear any obvious structural relationship to hydroxyproline*. This suggests that the growth-inhibitory actions of the first six compounds are all effective at a site where L-proline is synthesized and incorporated into protein, while the inhibitions due to the other three compounds are only effective at another site, or sites, in the cell.

Causes of growth inhibition

Six of the growth inhibitors in Table I do not operate as competitive proline antagonists. Of these, diazoacetylserine is known as an inhibitor of purine metabolism, in particular of cytosine synthesis via inosinic acid, and involving glutamine[10];

* Mr. H. BARRALES, working in the Cornell Laboratory with excised embryos of *Zea* in aseptic culture, has recently demonstrated a similar growth inhibition by hydroxy-L-proline and its reversal by proline. This extends the effect to organized growth.

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TABLE III

THE EFFECT OF HYDROXY-L-PROLINE AND CASEIN HYDROLYSATE ON THE GROWTH OF CARROT EXPLANTS IN MEDIA CONTAINING 2 % COCONUT MILK

(Growth in absence of hydroxyproline = 100)

Hydroxyproline concentration (p.p.m.)	*Carrot A*		*Carrot B*	
	Relative growth		*Relative growth*	
	No casein	*Casein 200 p.p.m.*	*No casein*	*Casein 200 p.p.m.*
0	100	100	100	100
0.35	117	100	94	73
1.00	106	97	96	84
3.50	91	105	69	73
10.0	58	91	11	67
35.0	— 1	77	3	72
100.00	—10	50	0	64

TABLE IV

THE EFFECT OF CERTAIN AMINO ACIDS AND OF AMMONIUM CHLORIDE ON THE GROWTH OF CARROT EXPLANTS IN THE PRESENCE OF 100 P.P.M. OF HYDROXY-L-PROLINE

(Growth expressed as per cent of growth in 2 % coconut milk without added hydroxyproline)

Basal media supplement by:	*Concn. of added substance*	*Growth as a per cent of growth in media containing 2 % coconut milk*	
		Carrot A	*Carrot B*
2 % Coconut milk		100	100
2 % Coconut milk + 100 p.p.m. Hydroxy-L-pyroline		3	4
2 % Coconut milk + 100 p.p.m. Hydroxy-L-proline plus:			
L-Proline	100	104	111
	10	59	53
L-Leucine	100	3	1
	10	3	4
L-Lysine hydrochloride	100	4	12
	10	3	6
L-Arginine hydrochloride	100	0	30*
	10	4	13
L-Histidine hydrochloride	100	5	7
	10	3	6
L-Serine	100	5	17
	10	2	6
L-Threonine	100	4	14
	10	2	5
L-Glutamic acid	100	4	27*
	10	3	4
Ammonium chloride	100	2	9
	10	3	6

* Carrot B seemed able to utilize both arginine and glutamic acid, both of which are closely related metabolically to proline; and which may be acting as endogenous sources of proline and so antagonizing the effect of hydroxy-L-proline. Carrot A on the other hand, seemed unable to use these amino acids.

canavanine inhibits growth of microorganisms[11] and higher plants[12], and in both cases the inhibition is reversed by arginine; but, at the time of these experiments, nothing was known which could suggest the metabolic site at which the other four compounds might act. However, it was a possible extension of the results obtained with proline and hydroxyproline that lysine, leucine and tryptophan might be antagonized by their hydroxy derivatives. No clues to possible antagonists for the action of gramicidin-S were, or are, available. Some of the possibilities indicated above were experimentally tested, with the results shown in Table VI.

L-Tryptophan clearly reverses the inhibition of growth of carrot explants due to 5-hydroxytryptophan, and L-arginine partially antagonizes the inhibition due to canavanine.

Apparently L-glutamine will *not* reverse the inhibition of growth of carrot explants due to azaserine. According to LEVENBERG *et al.*[10], azaserine inhibits the utilization of glutamine during inosinic acid synthesis, but in a manner which would be very hard to demonstrate by competitive experiments of the type reported in Table VI.

Lysine on the other hand *will* reverse the inhibition of δ-hydroxylysine, although the data are less striking because L-lysine itself, at a concentration of 100 p.p.m. is somewhat inhibitory. More data are needed to describe the exact interaction of these two substances in the growing cell, but the present data suggest that the lysine and hydroxylysine competitively interact in their effect on growing carrot cells. In chicken embryos, δ-hydroxy-L-lysine is about twice as toxic as hydroxy-L-proline: the former is deadly at 8–10 mg per chick embryo, the latter at a dose of 20 mg per chick embryo*. An inhibitory action on protein synthesis or growth in certain tumor cells has also been reported for δ-hydroxylysine. This particular inhibition was relieved by the administration of small doses of glutamine[13].

Referring again to Table I it may be stated in summary that 12 substances have been observed to inhibit the growth of carrot explants which is induced by coconut milk; six partially inhibitory substances have been observed and sixty non-inhibitory substances, listed in Table I, have been tested. The effect of five hydroxyproline compounds as competitive inhibitors for proline has been demonstrated, and the role of azetidine-2-carboxylic acid as a competitive inhibitor of proline, by virtue of its similar structure, has also been demonstrated. Other hydroxyamino acids (hydroxytryptophan, hydroxylysine) seem also to affect the growth of the carrot tissue by interfering with the use of their corresponding amino acids. Attempts to reverse the effects of hydroxyleucine have not been made. Two other substances (canavanine and azaserine) which affect the growth of the carrot explants, appear to do so by intervening in the utilization of specific amino acids (arginine and glutamine respectively).

The strong inhibitory action of gramicidin-S may be related to the mechanism of its antibiotic activity. When gramicidin-S or copolymers of leucine and ornithine[14] inhibit bacterial growth, it is believed that these polypeptides affect the cells in ways which allow inorganic phosphate to leak through the membranes**. Whether such a mechanism obtains in carrot tissue cultures will have to be investigated.

* Personal communication from Dr. C. MITOMA, National Heart Institute.
** Personal communication from Prof. E. KATCHALSKI.

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TABLE V

THE EFFECT OF 100 P.P.M. OF L-PROLINE ON THE INHIBITION OF GROWTH OF CARROT EXPLANTS BY CERTAIN KNOWN INHIBITORS

Substance added to medium containing 2% coconut milk:	*Concn.*	*Growth as % of growth in medium + 2% coconut milk*	
		Without proline	*100 p.p.m. L-proline added*
Hydroxy-L-proline	100	0	92
	10	13	105
Allohydroxy-L-proline	50	0	57
	5	4	93
Hydroxy-L-prolylglycine	100	14	129
	10	83	105
Allohydroxy-L-prolylglycine	10	65	111
O-Acetylhydroxy-L-proline	100	1	72
	10	33	99
Azetidine-2-carboxylic acid	10	3	85
Diazoacetylserine	100	0	0
	10	0	0
	1	86	99
Gramicidin-S-sulphate	Sat./10*	0	0
	Sat./100	0	0
	Sat./1000	0	2
δ-Hydroxy-L-lysine	100	0	0
	10	0	0
	1	87	91

* Of all the compounds studied so far, Gramicidin-S-sulphate, a cyclic decapeptide of known structure, is the most powerful inhibitor, being strongly inhibitory at a concentration of one thousandth saturation or at an approximate concentration of 0.3 to 0.5 p.p.m.

TABLE VI

THE EFFECT OF CERTAIN INHIBITORS AND POSSIBLE ANTAGONISTS OF THESE INHIBITORS ON THE GROWTH OF CARROT EXPLANTS

Substance added to medium containing 2% coconut milk	*Growth as % of growth in media + 2% coconut milk*	
	Carrot A	*Carrot B*
10 p.p.m. Azaserine	0.0	0.0
10 p.p.m. Azaserine + 100 p.p.m. of L-glutamine	0.0	0.0
10 p.p.m. Azaserine + 10 p.p.m. of L-glutamine	0.0	0.0
10 p.p.m. synthetic δ-hydroxylysine**	0.0	0.0
10 p.p.m. of synthetic δ-hydroxylysine plus 100 p.p.m. of L-lysine·HCl	61	10
10 p.p.m. of synthetic δ-hydroxylysine plus 10 p.p.m. of L-lysine·HCl	0.0	1.0
10 p.p.m. Canavanine	0.0	3.9
10 p.p.m. Canavanine + 25 p.p.m. L-arginine	19	26
10 p.p.m. Canavanine + 50 p.p.m. L-arginine	44	26
10 p.p.m. 5-Hydroxytryptophan	32	28
10 p.p.m. 5-Hydroxytryptophan + 10 p.p.m. tryptophan	135	170
10 p.p.m. 5-Hydroxytryptophan + 100 p.p.m. tryptophan	161	270
10 p.p.m. L-tryptophan	131	188

** The synthetic δ-hydroxylysine used was a mixture of 60% DL-allo, δ-hydroxylysine and 40% DL-δ-hydroxylysine.

DISCUSSION

These experiments demonstrate the use that may be made of plant tissue-culture systems in the investigation of the antimetabolic action of compounds upon the growth of cells; in particular, the inhibition of the synthesis of a hydroxyproline-rich but metabolically inert, alcohol-insoluble, protein that seems to be characteristic of the rapidly proliferating carrot cells which grow in response to coconut milk.

Insofar as the compounds act by virtue of their similarity to proline and hydroxyproline, all the data are consistent with the following interpretation. Free proline, synthesized as such in the tissue, is apparently incorporated directly into a particular protein moiety and thereafter oxidized to hydroxyproline. (In other experiments, still to be published, this has been confirmed by the use of ^{14}C-proline.) Hydroxy-L-proline does not normally exist free in the cells, and this substance acts at the site of protein synthesis as a competitive inhibitor for proline synthesis and incorporation in the protein, presumably because it occupies the same site but cannot itself be directly used in protein synthesis. The specificity of this effect is shown by the fact that only proline reverses both the effect of hydroxyproline and of all the less inhibitory nitrogen compounds which bear a close structural relationship to hydroxyproline. Furthermore, the L-hydroxyprolines are more inhibitory than the D-forms, and chemical substitution in the hydroxyproline molecule—especially on the nitrogen atom—reduces the ability of the molecule to interfere with proline.

Along lines parallel to those above, C. MITOMA, National Institutes of Health, in experiments made with chick embryos has also observed that free hydroxyproline inhibits growth and proline incorporation into protein.

It is interesting that azetidine-2-carboxylic acid, an analogue of proline, seems also to inhibit growth by competing with proline, because it occurs naturally in quite a few liliaceous plants[15].

Although hydroxylysine is not known to occur either free or combined in the carrot tissue cultures, like hydroxyproline, it strongly inhibits growth. Hydroxylysine, however, acts at a different metabolic site from hydroxyproline, since its effect is reversed by lysine but not by proline. Presumably hydroxylysine exerts its effect by interfering with lysine metabolism during protein synthesis. In the animal body hydroxylysine, like hydroxyproline, occurs in collagen, and here again it has been suggested by PIEZ AND LIKENS[16] as well as by SINEX AND VAN SLYKE[17] that the hydroxy acid cannot be directly used in synthesis, in contrast to lysine, and that the conversion of lysine to hydroxylysine occurs in a collagen precursor.

In a somewhat similar manner 5-hydroxytryptophan has been shown to be inhibitory to the growth of carrot explants, and its effect is antagonized by tryptophan.

Although the explanations are of a different kind, there are a few substances which also seem to have growth-inhibitory properties to the carrot tissue. Of these azaserine may well be implicated in glutamine metabolism and canavanine in arginine metabolism. The action of gramicidin-S, the most powerful of all these inhibitors, is as yet, unexplained.

With these more general observations, the results and conclusions are summarized below.

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SUMMARY

Carrot phloem explants, growing and dividing rapidly in a nutrient medium containing coconut milk, synthesize a protein which, like collagen in animals, is rich in hydroxyproline and quite inert metabolically. Hydroxyproline strongly inhibits the growth of such tissues, the L forms of both allohydroxyproline and hydroxyproline being more inhibitory than the D forms. Chemical alteration of the hydroxyproline molecule markedly reduces this inhibitory effect, *i.e.* the inhibition is quite specific. The inhibition by hydroxy-L-proline is reversed by enzymic casein hydrolysate and only by L-proline of the eight L-amino acids tried. Among twelve inhibitors demonstrated, six are structurally related to proline, and like hydroxy-L-proline, are much less inhibitory in the presence of proline. Of the other six inhibitors, azaserine is not antagonized by glutamine, hydroxy-L-lysine is antagonized by L-lysine, 5-hydroxytryptophan is antagonized by tryptophan and canavanine by arginine. No antagonist is yet known for the most powerful inhibitor of all, gramicidin-S, sulphate. In addition to the twelve inhibitors, six partial inhibitors were found and sixty substances which were tested showed no growth inhibition. These results are interpreted in terms of the effects of the substances in question on protein metabolism.

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