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Direct Incorporation of Hydroxyproline into *Avena* Coleoptile Proteins*

Robert Cleland and Alfred C. Olson

ABSTRACT: The *cis* isomer of L-hydroxyproline (allohydroxyproline), when present at a growth-inhibitory level (0.5 mM), is directly incorporated into *Avena* coleoptile proteins at a rate that is about three times greater than the normal rate of formation of *trans*-hydroxyproline. This incorporation is predominately into cytoplasmic proteins, is linear with time, and is only slightly affected by 0.15 mM α,α-dipyridyl. The ability of proline to prevent this incorporation suggests that the *cis*-hydroxyproline is being incorporated into protein in place of proline. Label from [^{3}H]*trans*-hydroxyproline also appears in protein-bound hydroxyproline, but the bulk of this incorporation appears to be indirect (*i.e.*, through proline) since it can be severely inhibited by dipyridyl. Both isomers of hydroxyproline can be converted to proline by *Avena* coleoptile tissues, but the *trans* isomer is more efficiently utilized in this reaction. The lack of correlation between the incorporation of hydroxyproline isomers into protein and their ability to inhibit cell elongation indicates that while the direct incorporation of hydroxyproline into protein may contribute to the inhibition of auxin-induced growth, it is unlikely to be its principal cause.

The widespread occurrence of protein-bound hydroxyproline in plants is well established (Vanetten *et al.*, 1963; Lamport, 1965). As in animal systems (Stetten, 1949), proline rather than hydroxyproline is the normal precursor of the bound hydroxyproline (Steward and Pollard, 1958; Olson, 1964; Lamport, 1965). The possibility that some direct incorporation of hydroxyproline can occur in plants was suggested, however, by the finding that free hydroxyproline is an effective inhibitor of plant growth (Steward *et al.*, 1958; Cleland, 1963).

Studies on the direct incorporation of hydroxyproline have been hampered by the indirect incorporation of hydroxyproline, *i.e.*, incorporation into protein only after prior conversion into proline. Thus label in protein-bound hydroxyproline can arise from labeled free hydroxyproline either by direct incorporation or indirectly by conversion into proline followed by reconversion into hydroxyproline. For instance, Cleland and Olson (1967) showed with *Avena* coleoptiles that when free hydroxyproline is at a noninhibitory level, the transfer of label from free to protein-bound hydroxyproline is due to the indirect incorporation pathway. The best evidence, to date, for direct incorporation of hydroxyproline into plant proteins is the finding of Holleman (1967) that α,α-dipyridyl interferes to a greater extent with the conversion of proline into hydroxyproline (Hurych and Chvapil, 1965; Hutton *et al.*, 1967) than with the transfer of label from free to protein-bound hydroxyproline.

Advantage has been taken in this study of the fact that the *cis* isomer of L-hydroxy-4-proline (*cis*-Hypro[1]), which is more effective than the normal *trans* isomer (*trans*-Hypro) as a growth inhibitor (Cleland, 1967a), is not normally found in *Avena* coleoptile proteins and is not formed from proline. The appearance of any *cis*-Hypro in protein following incubation with free *cis*-Hypro can only be due to direct incorporation of this isomer. It is shown here that direct incorporation of *cis*-Hypro into *Avena* coleoptile proteins does occur.

Experimental Section

Materials

The plant material consisted of 14-mm sections cut from 25–32-mm coleoptiles of *Avena sativa*, var. Vic-

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[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: *cis*-Hypro, *cis*-hydroxy-4-proline (allohydroxyproline); *trans*-Hypro, *trans*-hydroxy-4-proline; IAA, indoleacetic acid; CHA, cycloheximide; DiP, α,α-dipyridyl.

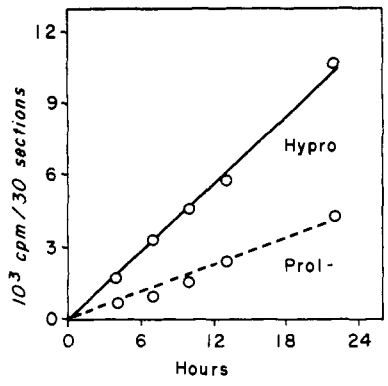

FIGURE 1: Time course of incorporation of label from [^{14}C]*cis*-hydroxyproline into protein-bound proline and hydroxyproline. Sections incubated in 5 ml of basal medium containing [^{14}C]*cis*-Hypro (1.7 μCi, 0.5 mM).

tory. Seedlings were grown and sections were prepared as detailed in Cleland (1960). Leaves were removed from all sections. Each section weighed about 10 mg and contained about 65 μg of protein.

L-[5-^{3}H]*trans*-Hydroxyproline (187 mCi/mmole) and L-[U-^{14}C]proline were obtained from New England Nuclear Corp.[2] L-*trans*-Hydroxyproline and L-*cis*-hydroxyproline were from California Corp. for Biochemical Research, while D-*cis*-hydroxyproline was from Sigma Chemical Co. The DL-[2-^{14}C]*cis*-hydroxyproline which was used in the initial experiments was obtained by separating [2-^{14}C]hydroxyproline mixed isomers (California Corp. for Biochemical Research, lot 35739, 18.5 mCi/mmole) into its *cis* and *trans* isomers by descending chromatography on Whatman No. 3MM paper in a system of *n*-butyl alcohol saturated with 10% aqueous diethylamine (Hardy and Holland, 1952). Identical results were obtained when in subsequent experiments DL-[3,4-^{14}C]*cis*-hydroxyproline (8.9 mCi/mmole) from Schwarz BioResearch, Inc., was utilized. The purity of each of the hydroxyproline isomers was checked before use by chromatography in isopropyl alcohol–formic acid–water (15:2:2, downflow) or in the diethylamine–butyl alcohol system. No significant cross-contamination was detected for any of the isomers.

Methods

Incubations. Unless otherwise stated, sections were incubated for 22 hr in a basal medium containing potassium maleate buffer (2.5 mM, pH 4.7), sucrose (2% w/v), IAA (5 μg/ml), penicillin G (0.1 mM), and with other additions as indicated. When required, α,α-dipyridyl was used at 0.15 mM, a concentration which in *Avena* coleoptiles causes a maximal inhibition of hydroxyproline formation with a minimal inhibition of proline incorporation (unpublished results). Groups of 30 sections were incubated in 5 ml of medium in test tubes which were rotated at 1 rpm on a Rollardrum. Groups of 120 and 200 sections were incubated in 20 or 40 ml of medium in beakers which were rotated at 30 rpm on a gyrotory shaker.

Preparation of Materials. After incubation, sections were washed in distilled water, extracted three times for 5 min with 10 ml of boiling 80% ethanol, and washed with the trichloroacetic acid series of Peterson and Greenberg (1952). Protein was then hydrolyzed in 6 N HCl at 110° for 16 hr. After this, humin was removed by filtration, and the filtrate was reduced to near dryness with heat and a nitrogen stream and was streaked across the end of a 1.25-cm wide strip of Whatman No. 3MM paper. In experiments where no label was present during the incubation period, 5 μl of a solution containing [^{14}C]proline and [^{14}C]hydroxyproline mixed isomers (less than 0.1 μg of each) was added to each strip to act as a marker. In certain experiments the first two ethanol extracts, which contain the soluble amino acids, were combined, concentrated to near dryness, and also placed on paper strips.

When a separation of tissues into cytoplasmic and wall protein fractions was desired, the sections were ground in 2 ml of Tris buffer (0.05 M, pH 7.5) in an all-glass homogenizer, and then subjected to further homogenization in a Virtis "45" homogenizer with 200-μ glass beads and Tris buffer (Kivilaan *et al.*, 1959). The walls were separated by filtration through glass beads and the protein of the filtrate was precipitated with 5% trichloroacetic acid at 4°. Both walls and cytoplasmic protein fractions were then washed with the trichloroacetic acid series and treated as indicated above.

Chromatography. The chromatograms were developed for 16 hr in isopropyl alcohol–formic acid–water (15:2:2, downflow). This system gives essentially complete separation of proline and hydroxyproline, the only two amino acids which are labeled to a significant extent following incubation of *Avena* coleoptiles with labeled hydroxyproline or proline (*e.g.*, see Figure 1 of Cleland and Olson, 1967). Proline and hydroxyproline areas were then located with a radiochromatogram scanner.

When a separation of hydroxyproline into its *cis* and *trans* isomers was desired, the hydroxyproline region was eluted, concentrated, and applied to a second strip of Whatman No. 3MM paper. This was then developed for 7 days with *n*-butyl alcohol saturated with aqueous 10% diethylamine (downflow). The location of the two isomers was determined with a radiochromatogram scanner. The identity of the suspected *cis*-Hypro was confirmed by cochromatography with authentic *cis*-Hypro in high-voltage electrophoresis at pH 1.9 (Sheehan and Whitney, 1963).

Analyses. Proline was assayed by the method of Troll and Lindsley (1955) and hydroxyproline was determined by method 1 of Prockop and Udenfriend (1960). To determine ^{14}C, 0.05–0.3 ml of eluate was dried on aluminum planchets and counted in a gas-flow counter. Counts were corrected to infinite thinness. Tritium was measured by combining 0.05–0.2 ml of eluate with 12 ml of the dioxane counting mixture of Butler (1961), and counting it in a liquid scintillation spectrometer.

[2] Reference to a company or product name does not imply approval or recommendation of the product by the U. S. Department of Agriculture to the exclusion of others that may be suitable.

Results

Avena coleoptiles which are freshly harvested or have been incubated with proline or *trans*-Hypro have, at best, only traces of *cis*-Hypro in their proteins (Table I). Significant amounts of bound *cis*-Hypro are found, however, after incubation with growth-inhibitory levels of free L-*cis*-Hypro (Table I and IV). The ability of the protein synthesis inhibitor cycloheximide (CHA) to prevent this incorporation without blocking the uptake of this imino acid indicates that the *cis*-Hypro has been incorporated into protein. This is further indicated by the inability of the D isomer of *cis*-Hypro to be utilized (Table I). Finally, the incorporation can be prevented by the inclusion in the medium of sufficient L-proline (0.5 mM) to reverse the hydroxyproline-induced growth inhibition (Table II).

The possibility that the incorporation of *cis*-Hypro may be due to bacterial contamination has been considered and discounted for two reasons. First, the addition of 50 μg/ml of streptomycin and 5×10^{-6} M gramicidin to the medium had no effect on the amount of incorporation from a medium that contained [^{14}C]*cis*-Hypro (data not shown). Secondly, the rate of incorporation of [^{14}C]*cis*-Hypro is constant over a 22-hr period (Figure 1); this would not be expected if the incorporation was due to bacteria.

The rate at which *cis*-Hypro is incorporated into *Avena* coleoptile proteins is impressive when compared with the normal rate of increase of protein-bound *trans*-Hypro (because of the lack of turnover in protein-bound hydroxyproline (Cleland, 1968) this is equivalent to the rate of synthesis of *trans*-Hypro). For instance, the amount of *cis*-Hypro which is incorporated during a 22-hr incubation is three times as great as the amount of *trans*-Hypro that is synthesized during the same period (Table I). As a result, at the end of the 22-hr period the cells contain 60–70% as much protein-bound *cis*-Hypro as they do *trans*-Hypro. The amount of *cis*-Hypro that is incorporated is only slightly affected by the presence or absence of auxin (data not shown).

The majority of the *trans*-Hypro in *Avena* coleoptiles is found in the cell wall fraction and about half of the *trans*-Hypro which is formed during a 22-hr incubation appears in this fraction (Cleland, 1968). In contrast, the *cis*-Hypro is localized in the cytoplasmic protein fraction with only a minor amount appearing in cell wall protein (Table III).

In addition to the direct incorporation of *cis*-Hypro, some indirect incorporation also occurs as indicated by the appearance of protein-bound [^{14}C]proline after an incubation with labeled *cis*-Hypro (Table IV). One way in which the amount of this indirect incorporation can be estimated is by comparing the ratios of radioactive hydroxyproline to proline in protein (H/P ratio) after incubation with radioactive proline and *cis*-Hypro. If the incorporation is entirely indirect (*i.e.*, through proline), the ratio should be the same regardless of the source of the label. The fact that the ratio is 30–50 times higher when *cis*-Hypro is the source

TABLE I: Incorporation of *cis*-Hydroxyproline into Protein of the *Avena* Coleoptile.[a]

Added to Medium (mM)	2 μg in Protein[b]	
	trans-Hypro	*cis*-Hypro
None	13.6	0.8[c]
L-*trans*-Hypro (1)	12.8	0.3
L-*cis*-Hypro (0.5)	12.4	8.6
L-*cis*-Hypro (0.5) + CHA (4 μg/ml)	10.9	0.8
D-*cis*-Hypro (0.5)	13.0	0.7
Before incubation	10.7	0.3

[a] Sections (120, 14 mm) were incubated 22 hr in 20 ml of basal medium, minus IAA, and containing additions as indicated. [b] Results expressed as μg/120 sections. [c] Values below 1 μg are not significant and represent incomplete separation of the two isomers.

TABLE II: Prevention by Proline of *cis*-Hydroxyproline Incorporation into Protein.[a]

Medium (mM)		μg in Protein[b]		Growth (%/22 hr)
cis-Hypro	Proline	*trans*-Hypro	*cis*-Hypro	
0	0.5	11.1	<0.5	65
0.5	0	8.7	5.8	23
0.5	0.5	12.5	<0.5	55

[a] Sections (120, 14 mm) incubated 22 hr in 20 ml of basal medium with *cis*-Hypro and proline as indicated. [b] Results expressed as μg/120 sections.

TABLE III: Localization of Incorporated *cis*-Hydroxyproline in Cytoplasmic Proteins.[a]

Protein Fraction	*cis*-Hypro in Medium	μg in Protein[b]	
		trans-Hypro	*cis*-Hypro
Cytoplasmic	−	4.5	0.3
	+	4.3	6.9
Cell Wall	−	10.9	0.3
	+	9.4	1.1

[a] Sections (200, 14 mm) incubated 26 hr in 40 ml of basal medium with or without 0.5 mM *cis*-Hypro. [b] Results expressed as μg/200 sections.

TABLE IV: Effect of Hydroxyproline Concentration on Incorporation of Hydroxyproline Isomers into Protein.[a]

Hypro (mM)	Tracer	mμmoles of Tracer Incorp into[b]		
		Proline	Hypro[c]	H/P
trans-Hypro (0.02)	[^{14}C]Proline	1.75	0.12	0.07
trans-Hypro (1)	[^{14}C]Proline	1.80	0.08	0.04
trans-Hypro (0.02)	[^{3}H]*trans*-Hypro	7.4	0.5	0.07
trans-Hypro (1)	[^{3}H]*trans*-Hypro	5.6	13.8	2.5
cis-Hypro (0.02)	[^{14}C]*cis*-Hypro	0.19	0.40	2.1
cis-Hypro (0.5)	[^{14}C]*cis*-Hypro	10.0	34	3.4

[a] Sections (30, 14 mm) incubated 22 hr in 5 ml of basal medium with additions of Hypro isomer and tracer as indicated. [b] Calculated from cpm/30 sections on the basis of specific activity of tracer. [c] Hydroxyproline isomers in protein not separated.

TABLE V: Effect of α,α-Dipyridyl on Incorporation of Hydroxyproline Isomers into Protein.[a]

Hypro (mM)	Tracer (μCi)	DiP	10^2 cpm Incorp into		
			Proline	Hypro[b]	H/P
trans-Hypro (1)	[^{14}C]Proline (1)	−	2250	118	0.048
		+	1850	13	0.007
trans-Hypro (1)	[^{3}H]*trans*-Hypro (200)	−	314	1490	4.7
		+	167	235	1.4
cis-Hypro (0.5)	[^{14}C]*cis*-Hypro (1)	−	0.96	3.8	4.0
		+	0.38	2.5	6.6

[a] Sections (30, 14 mm) incubated 22 hr in 5 ml of basal medium with or without 0.15 mM dipyridyl and with Hypro isomer and tracer as indicated. [b] Protein-bound hydroxyproline not resolved into isomers.

of the label (Table IV) indicates that only a minor amount of indirect incorporation of *cis*-Hypro takes place. Confirmation of this has been obtained by a separation of the protein-bound hydroxyproline into its two isomers after incubation with *cis*-Hypro; as expected, only a small amount (less than 10%) of the label was in *trans*-Hypro.

We have already shown (Cleland and Olson, 1967) that when *Avena* coleoptiles are incubated with non-inhibitory levels of [^{3}H]*trans*-Hypro, label is incorporated into both proline and hydroxyproline. The fact that the H/P ratio is the same as when the source of the label is [^{14}C]proline indicates that under these conditions the free *trans*-Hypro is incorporated only after conversion into free proline.

An increase in *trans*-Hypro to a growth-inhibiting level (1 mM) has little effect on the amount of free *trans*-Hypro which is incorporated into proline, but it does cause a 25-fold increase in its incorporation into protein-bound hydroxyproline (Table IV). This is not due to a nonspecific absorption of *trans*-Hypro onto proteins since over 95% of the incorporation can be prevented by 4 μg/ml of cycloheximide. It may reflect direct incorporation of *trans*-Hypro under these conditions or it may be due to a compartmentalization in proline incorporation; *i.e.*, the proline which is formed from free *trans*-Hypro may not enter the free proline pool but may be preferentially incorporated into those proteins in which hydroxyproline formation will subsequently take place.

α,α-Dipyridyl, which is an effective inhibitor of the oxidation of proline to hydroxyproline in animal (Hurych and Chvapil, 1965) and plant systems (Holleman, 1967; Hague, 1967), has been used to distinguish between these two possibilities. If the label in protein-bound hydroxyproline arises *via* free proline, dipyridyl should cause a marked reduction in the H/P ratio, while if the incorporation is direct, the H/P ratio should not be affected by the dipyridyl. As expected, 0.15 mM dipyridyl caused a severe decrease in the H/P ratio when the source of the label was [^{14}C]proline while it produced no reduction in the ratio when the tissues were incubated with [^{14}C]*cis*-Hypro (Table V). When used in the presence of 1 mM [^{3}H]*trans*-Hypro, dipyridyl caused an almost fourfold decrease in the H/P ratio. This indicates that the bulk of the free *trans*-Hypro was incorporated into protein-bound hydroxyproline only after prior conversion into free proline and that the

effect of the *trans*-Hypro concentration on the H/P ratio is most likely to be due to compartmentalization of the newly formed proline within the cells.

Both isomers of hydroxyproline are converted into proline by *Avena* coleoptile tissues, but they differ in ability to act as a substrate for this conversion (Table VI). For instance, 30 times as much proline is formed from *trans*-Hypro when both are at 0.015 mM. More equal amounts of proline are formed when the concentration of the hydroxyproline isomers is raised to growth-inhibiting levels, but even under these conditions conversion from *trans*-Hypro predominates.

TABLE VI: Comparison of Ability of Hydroxyproline Isomers to be Converted into Proline.[a]

Hypro Isomer (mM)	μg of Proline Formed in[b] Protein	Soluble
trans-Hypro (0.015)	1.2	0.14
trans-Hypro (1)	1.7	0.85
cis-Hypro (0.15)	0.04	0.01
cis-Hypro (0.5)	0.76	0.01

[a] Sections (30, 14 mm) incubated 22 hr in 5 ml of basal medium containing [^{14}C]*trans*-Hypro (1.5 or 5 μCi) or [^{14}C]*cis*-Hypro (1.5 or 3 μCi). [b] Microcuries of proline calculated from specific activity of initial Hypro isomer and counts per minute in proline.

Discussion

Although the normal pathway of hydroxyproline synthesis from proline has been extensively studied (Stetten, 1949; Udenfriend, 1966), little attention has been focused on the possibility that some direct incorporation of free hydroxyproline into protein may also occur. The observation that label from free hydroxyproline can find its way in small amounts into protein-bound amino acids including proline and hydroxyproline was originally made by Stetten (1949) and has since been confirmed for animal (Gianetto and Bouthillier, 1954; Wolf *et al.*, 1956; Mitoma *et al.*, 1958), plant (Pollard and Steward, 1959; Cleland and Olson, 1967; Holleman, 1967), and bacterial systems (Katz *et al.*, 1962). However, the appearance of label in protein does not, in itself, indicate that direct incorporation of hydroxyproline has taken place since the hydroxyproline may have been metabolized to some other free amino acid prior to incorporation into protein. For instance, in certain animal (Adams and Goldstone, 1960) and bacterial systems (Adams, 1959), free hydroxyproline can be converted *via* glutamate into free proline. In *Avena* coleoptiles the conversion into proline occurs by a pathway which apparently does not involve glutamate (Cleland and Olson, 1967). This proline can, in turn, be incorporated and reconverted into hydroxyproline.

One way to assess the amount of direct hydroxyproline incorporation is to compare the ratios of radioactivity in protein-bound hydroxyproline and proline (H/P ratio) after incubation of tissues with either labeled proline or hydroxyproline. In the absence of direct incorporation of hydroxyproline the H/P ratio should be the same regardless of the source of the label, but if direct incorporation of hydroxyproline has taken place, the H/P ratio should be higher than when the source of label is proline. Mitoma *et al.* (1958) used this method to show that a small amount of direct incorporation of hydroxyproline does occur into chick embryo proteins while the same method has been used by Pollard and Steward (1959) and Cleland and Olson (1967) to show that incorporation into protein from noninhibitory levels of hydroxyproline is entirely indirect in carrot callus and *Avena* coleoptile tissues.

The most convincing evidence for direct incorporation of hydroxyproline into plant proteins has come from the work of Holleman (1967) on sycamore cambium callus suspensions. He has shown that α,α-dipyridyl almost completely blocks the conversion of proline to hydroxyproline but causes only a small reduction in the H/P ratio of cells which are incubated with labeled *trans*-hydroxyproline. This suggests that the label in protein-bound hydroxyproline must have arisen by direct incorporation since production of hydroxyproline from proline should have been blocked by the dipyridyl.

Caution must be used in interpreting the results obtained by this ratio method, as is illustrated by the data obtained in this study with *trans*-Hypro. The 30-fold greater H/P ratio which occurs when *Avena* coleoptiles are treated with growth-inhibitory levels of labeled hydroxyproline rather than with labeled proline would seem to indicate that direct incorporation of *trans*-Hypro is occurring in this tissue. However, this conclusion is not consistent with the marked decrease in the H/P ratio which is produced by dipyridyl. Dipyridyl may be preferentially blocking the direct incorporation of *trans*-Hypro into protein in this system, but this seems unlikely since it does not have a similar effect on the incorporation of *cis*-Hypro. A more likely possibility is that the enhancement of the H/P ratio by free hydroxyproline is due to a compartmentalization of the proline which is formed from free hydroxyproline, *i.e.*, that this proline is preferentially incorporated into proteins in which hydroxyproline will be formed and is not equilibrated with the general pool of free proline in the cells. Further experiments will be necessary in order to decide between these two possibilities.

In the present study, the deficiencies of the ratio method are overcome by the use of the *cis* isomer of hydroxyproline. Since *cis*-Hypro is not normally found in *Avena* coleoptiles and is not produced from proline, *trans*-Hyp, or D-*cis*-Hypro, any *cis*-Hypro which appears in protein after incubation with free L-*cis*-

Hypro can only be due to direct incorporation. It is shown here that a sizable amount of direct incorporation of *cis*-Hypro does occur in *Avena* coleoptile tissues.

The ability of cycloheximide and puromycin to prevent this incorporation indicates that the *cis*-Hypro is being incorporated by the normal protein synthesis pathway. In order for *cis*-Hypro to be incorporated, then, it must be activated by an amino acid–RNA synthetase. The possibility that this activation is accomplished by prolyl-tRNA synthetase is suggested by the ability of free proline to prevent *cis*-Hypro incorporation. It should be noted that a low level of hydroxyproline activation by this enzyme has been reported for rat liver (Fraser and Klaus, 1963) and *Escherichia coli* (Norton, 1964), although no activation of hydroxyproline could be detected with the prolyl-tRNA synthetase from two plant tissues (Peterson and Fowden, 1965).

If hydroxyproline is replacing proline in protein, it should cause a decrease in the rate of proline incorporation. A calculation can be made of the amount of this decrease, based on the fact that *cis*-Hypro is incorporated at two to three times the normal rate of hydroxyproline formation and that the rate of proline incorporation is about 20 times greater than the rate of hydroxyproline synthesis. This calculation gives an inhibition of proline incorporation of 10–15%, which agrees well with the experimental results which have already been obtained (Cleland, 1967b).

Proline which is incorporated into protein can have two fates: it can remain as proline or it can be converted into hydroxyproline. It has been shown earlier (Cleland, 1967b) that in auxin-treated tissues, proline incorporation into hydroxyproline is inhibited to a much greater extent by free *cis*-Hypro than is total proline incorporation. This raises the question as to whether *cis*-Hypro replaces proline at random or whether it specifically replaces those prolines which would normally be converted to hydroxyproline. In the former case a defective protein would be formed which will have hydroxyprolines in unaccustomed positions while in the latter case normal protein will be formed. Although a definitive answer cannot yet be given, it seems likely that *cis*-Hypro replaces proline at random, since the amount of *cis*-Hypro which is incorporated far exceeds the normal amount of hydroxyproline which would be synthesized. Unless the production of hydroxyproline-containing proteins is stimulated by *cis*-Hypro, the excess *cis*-Hypro must be replacing some amino acids other than the hydroxyproline-destined proline. Furthermore, in the absence of auxin free *cis*-Hypro has no effect on proline incorporation into hydroxyproline (Cleland, 1967b) even though the incorporation of *cis*-Hypro into protein still occurs under these conditions.

What are the biological effects of the direct incorporation of *cis*-Hypro? The fact that *cis*-Hypro is a potent inhibitor of auxin-induced cell elongation in *Avena* celeoptiles (Cleland, 1967a) might suggest that the direct incorporation of *cis*-Hypro with the subsequent production of defective proteins is the cause of the growth inhibition. Holleman (1967) has concluded that the inhibition of sycamore cambium callus growth by hydroxyproline is due to such a mechanism. There are two reasons to question this conclusion for *Avena* coleoptiles, however. First, growth is also inhibited by *trans*-Hypro, even though direct incorporation of *trans*-Hypro into protein, if it occurs at all, is certainly considerably less than that of *cis*-Hypro. Secondly, studies on the growth inhibition have indicated that *cis*-Hypro has no effect on the growth capacity of the tissues unless it is given in the presence of auxin, even though it is incorporated into protein in both the presence and absence of auxin. A more likely cause of the growth inhibition in *Avena* coleoptiles is that free hydroxyproline inhibits the formation or utilization of a specific hydroxyproline-containing protein which is required for auxin-induced growth. Evidence to support this possibility has been published earlier (Cleland, 1967b).

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Participation of Redundant Transfer Ribonucleic Acids from Yeast in Protein Synthesis*

P. L. Bergquist, D. J. W. Burns,† and Carol A. Plinston

ABSTRACT: Four glycine tRNAs have been isolated from brewers yeast. They appear to recognize the same code word but are known to be structurally different. In the present experiments, each glycine tRNA has been precharged with radioactive glycine and has been used in the *in vitro* synthesis of protein directed by bacteriophage R17 RNA in an *Escherichia coli* S-30 system that was dependent on added transfer ribonucleic acid (tRNA) for maximal activity. Preliminary experiments established that the yeast glycyl-tRNAs transferred their amino acid into the same proteins that were synthesized when *E. coli* tRNA was used. Large-scale preparations fractionated on sucrose gradients allowed the isolation of the coat protein subunits synthesized *in vitro*.

Thin-layer mapping of tryptic digests of protein synthesized *in vitro* has shown that for the most part, the glycine code words are translated with considerable fidelity by the yeast glycyl-tRNAs. The yeast tRNAs show a characteristic pattern of competition between themselves, and also when compared to *E. coli* $tRNA^{Gly}$ when used in pairs for the synthesis of coat protein. One of the peptides in the R17 coat has more glycine incorporated into it from yeast $tRNA^{Gly}$ than would be expected from the known composition of the peptide. It appears likely that a code word for another amino acid in the R17 RNA is being translated as glycine by the yeast, but not the *E. coli*, tRNAs. These observations have formal similarities to reports of suppression of missense being brought about by tRNA.

Results from several laboratories have shown that the genetic code is highly degenerate (Nirenberg *et al.*, 1965; Söll *et al.*, 1965, 1966). Fractionation of tRNA has demonstrated the existence of more than one acceptor RNA for many amino acids (for review, see Miura, 1967). A number of workers have reported that degenerate tRNAs recognize distinct code words (Weisblum *et al.*, 1962, 1965; von Ehrenstein and Dais, 1963; Bennett *et al.*, 1965; Gonano, 1967).

Recognition of synonym code words by separable amino acid specific RNAs also has been demonstrated (Kellog *et al.*, 1966; Söll *et al.*, 1967; Söll and RajBhandary, 1967). The basis of synonym code word recognition almost certainly residues in the codon–anticodon relationship of m- and tRNAs. It is not known how a single tRNA is able to recognize more than one triplet, although a theoretical basis for the phenomenon has been suggested by Crick (1966).

If many tRNAs show multiple codon recognition it would be anticipated that a large number of species would not be required for recognition of all the sense code words. In fact, evidence from fractionation studies shows that the number of separable tRNA species may be large (Bergquist *et al.*, 1965; Söll *et al.*, 1966; Gillam *et al.*, 1967). The multiplicity of tRNAs specific for a single amino acid appears to arise from two causes. Degenerate species are able to recognize the synonym triplets in the genetic code and differ minimally in the sequence of their anticodon triplets. It is reasonable to assume that no more than 64 degenerate tRNA species could exist.

Acceptor RNA species that respond to the same code word but are structurally different have been termed redundant (Söll *et al.*, 1966; Bergquist, 1966) and, theoretically, their number may be limited only by the pos-

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† Imperial Chemical Industries (N. Z.) research fellow.