that the molecular structure of an auxin may control its activity in growth by influencing the reaction reported here.

On the basis of these experiments it is proposed that auxin may act in plant growth and metabolism at least in part by reacting with coenzyme A. It may be assumed that the reaction product is a high-energy ester like the thiol-esters of other acids with coenzyme A. From its position as an ester with coenzyme A, it is further suggested that auxin may act to control the wide variety of synthetic and metabolic reactions in plants which are known to require coenzyme A, and that in this manner the growth hormone may regulate plant growth.

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[1] Bonner, J., and Bandurski, R. S., *Ann. Rev. Pl. Phys.*, **3**, 59–86 (1952).
[2] Bentley, J. A., and Bickle, A. S., *J. Exptl. Bot.*, **3**, 406–423 (1952).
[3] Commoner, B., and Thimann, K. V., *J. Gen. Phys.*, **24**, 279–296 (1941).
[4] Galston, A. W., PROC. NATL. ACAD. SCI., **35**, 10–17 (1949).
[5] Gordon, S. A., and Wildman, S. G., *J. Biol. Chem.*, **147**, 389–398 (1943).
[6] Hansch, C., Muir, R. M., and Metzenberg, R. L., *Plant Phys.*, **26**, 812–821 (1951).
[7] Jones, H. W., and Wade, R., *Science*, **118**, 103–107 (1953).
[8] Lynen, F., *Liebigs Ann. Chem.*, **574**, 33–37 (1951).
[9] Lynen, F., Wessely, L., Wieland, O., and Rueff, L., *Angew. Chem.*, **64**, 687 (1952).
[10] McRae, D. H., and Bonner, J., *Pl. Phys.*, **27**, 834–838 (1952).
[11] Overbeek, J. van, Blondeau, R., and Horne, V., *Pl. Phys.* **26**, 687–696 (1951).
[12] Plaut, G. W. E., and Plaut, K. A., *J. Biol. Chem.*, **190**, 141–151 (1942).
[13] Price, W. H., Cori, C. F., and Colowick, S. P., *Ibid.*, **160**, 633–634 (1945).
[14] Rhodes, A., and Ashworth, R. B., *Nature*, **169**, 76 (1952).
[15] Stern, J. R., and Ochoa, S., *J. Biol. Chem.*, **198**, 313–321 (1952).
[16] Thimann, K. V., In Skoog (Ed.), *Plant Growth Substances*, Univ. of Wis. Press (1951).
[17] Thimann, K. V., *Arch. Biochem. Biophys.*, **44**, 242–243 (1953).
[18] Thimann, K. V., and Schneider, C. L., *Am. J. Bot.*, **26**, 328–333 (1939).

EXPERIMENTAL COUPLING OF INDOLEACETIC ACID TO PEA ROOT PROTEIN IN VIVO AND IN VITRO

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In attempting to understand the great biological effectiveness of low concentrations of the plant growth hormone, indoleacetic acid (IAA), plant physiologists have frequently invoked the concept that IAA, like many of the vitamins, constitutes the prosthetic group of an enzyme. According to this concept, IAA to be physiologically effective must first combine with a specific protein to form an enzyme which then catalyzes a reaction essential

to the growth process. In support of this concept, it has been shown that free auxin is released from plant tissues and plant proteins by proteolytic agents;[5, 6, 7, 10] that labeled synthetic auxins quickly find their way into substances sedimented by typical protein precipitants[2] and that the kinetics of auxin action in Avena coleoptiles are best understood in terms of a two-point attachment of auxin to some acceptor, presumably protein.[1] Despite the suggestive nature of these data, the "auxin-protein" has remained a hypothetical entity rather than a tangible reality.

In the course of work on the adaptive formation of IAA oxidase in pea roots, it was observed that proteins deposited by trichloroacetic acid (TCA) treatment of breis from roots previously incubated in 10^{-3} *M* IAA were pink, while those from control roots were white. Since TCA could be shown to yield a pink color with as little as 5 μg. of IAA on filter paper, it was reasoned that the pink color indicated protein-bound IAA. This conclusion was reinforced by treatment of the washed TCA precipitates with the Salkowski colorimetric reagent for IAA,[9] which caused the appearance of a stable deep pink color only in those proteins derived from roots previously fed IAA.

In extending this original observation, we have found that IAA is incorporated into pea root proteins in vivo by an energy-requiring process. Further, the reaction coupling IAA to protein has been successfully consummated in vitro by the use of adenosine triphosphate (ATP).

Methods.—The terminal 5 mm. of root from 2- or 3-day Alaska pea seedlings germinated at 26°C. on moist paper towels were excised and employed in experimental work within one hour after removal from the seedling. Tissue samples of 200 or 250 mg. fresh weight (*ca.* 40–50 root tips) were incubated in 50-ml. Erlenmeyer flasks in 10 ml. of *M*/60 pH 6.1 phosphate buffer, with or without added IAA. The flasks were incubated in an Aminco Dubnoff metabolic shaking incubator thermostated at 30°C. After a suitable interval, roots were recovered from the reaction vessels, rinsed with buffer, and ground in cold buffer with the aid of washed sea sand. Breis thus prepared were centrifuged at *ca.* 2000× G for 10 minutes to remove cellular debris, and the supernatant made up to a standard volume of 10 ml.

In quantitative studies of the proteins, 2 ml. aliquots of brei were treated with an equal volume of 1 *M* TCA, refrigerated one hour, then centrifuged and the supernatant decanted. The precipitates were resuspended in fresh 0.5 *M* TCA, and after standing for several minutes, recentrifuged. The precipitates were then redissolved in 1 or 2 ml. of 0.1 *N* NaOH (final pH *ca.* 11), and the Salkowski colorimetric determination of IAA carried out in the usual manner with the aid of a Klett-Summerson photoelectric colorimeter equipped with a No. 54 green filter. The proteins were analyzed for nitrogen by digestion and direct Nesslerization.[3]

Properties of the IAA-Protein.—Whether precipitated by 0.5 *M* TCA, 70% saturated ammonium sulfate or 80% aqueous acetone, the IAA-protein contained the same amount of IAA. That the IAA was firmly bound to the protein was shown by its failure to be removed by prolonged boiling, and by its persistence over the pH range 2.5 to 11.0. The IAA-protein is stable for at least 72 hours when stored at 2°C. Some of the bound IAA is released after storage for 144 hours at 2°C. or after 20 hours at 30°C.

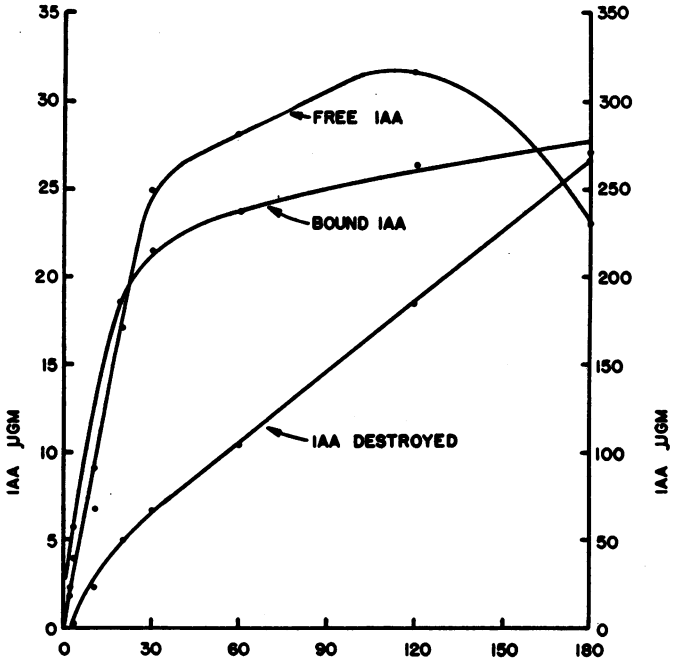


FIGURE 1

Kinetics of IAA assimilation by excised pea root tips. Left ordinate refers to curves labeled "free" and "bound IAA"; right ordinate refers to the curve labeled "IAA destroyed."

The Salkowski color of the IAA-protein is stable for at least 48–72 hours when left in contact with the reagent (which contains 50% H_2SO_4), whereas the pink color characteristic of free IAA fades in several hours to a pale orange. This is taken as further evidence of firm attachment of the IAA to the protein.

The IAA-protein was localized in the non-particulate fraction of the cell by usual centrifugation techniques.[4] Calculations of the molar ratio of IAA:protein were made for several precipitates, assuming a mean molec-

ular weight of protein of 10^5. This ratio varies from 0.1 when 10^{-4} *M* IAA is supplied to more than 1 when 10^{-3} *M* IAA is supplied.

Characteristics of the in Vivo Coupling Reaction.—TCA-precipitable IAA could be detected after one minute of incubation of the root tips with 5×10^{-4} *M* IAA. The binding of IAA to protein proceeded linearly with time up to 20 minutes of incubation, then continued at a decreased rate up to 120 minutes, approaching an asymptote at about 180 minutes. Figure 1 presents graphically the kinetics of absorption, binding and destruction of IAA fed to pea roots.

Protein-bound IAA could be detected when the external concentration of IAA was as low as 10^{-6} *M;* both the amounts bound and the ease of detection increased with increasing external IAA concentration up to 10^{-3} *M*, the highest concentration tested.

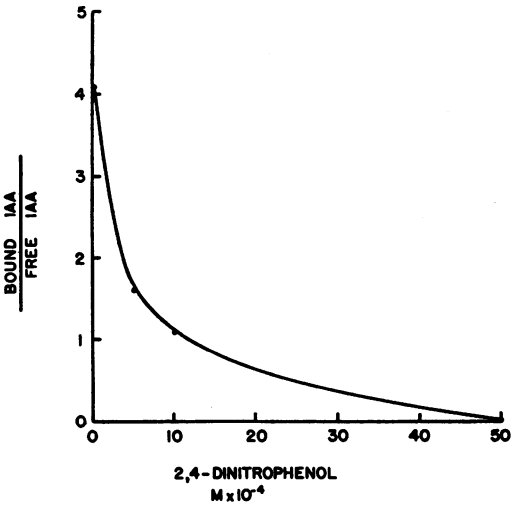


FIGURE 2

Inhibition of the in vivo coupling of IAA to pea root protein by 2,4-dinitrophenol.

The reaction coupling IAA to protein proceeds at a negligible rate at a temperature of 2°C., but rapidly at 25° or 30°C. The reaction is inhibited by 2,4-dinitrophenol (DNP), iodoacetic acid, potassium cyanide and sodium azide. These compounds at high concentrations inhibit both uptake of IAA and the binding of auxin to protein. However at lower concentrations, uptake is unaffected, but the ratio of bound:free IAA is sharply reduced, indicating that the coupling reaction itself is being interfered with. Typical inhibitional data, obtained with DNP, are shown in figure 2. Coupling of IAA to protein is also completely inhibited by 10^{-3} *M* KCN, 10^{-3} *M* NaN_3, and 5×10^{-4} *M* iodoacetic acid. Synthetic IAA analogs are also able to block the reaction, 5×10^{-4} *M* α-naphthaleneacetic acid producing a 50% inhibition.

In Vitro Studies.—Preliminary attempts to couple IAA to pea root protein by mixing protein and IAA in vitro yielded products in which IAA was much less firmly attached than in the auxin protein made in vivo. For instance, most of the IAA bound to protein in vitro was readily removed by washing of the precipitate with acetone, or by treatment with Salkowski reagent. Since the in vivo synthesis had been inhibited by various substances which interfere with the generation of energy-rich phosphate bonds, it was reasoned that the in vitro reaction might be driven by the addition of ATP.

The addition of 40 micromoles of ATP to 2 ml. of reaction mixture containing Coenzyme A, Mg^{++}, reduced glutathione, and KF was found to result in a reduction of the already low-level of IAA coupling to protein. Since Speck[8] had found a similar inhibition of glutamine synthesis to be due to excessively high levels of ATP, we reasoned that an effect of ATP might be shown if the brei were first depleted of endogenous ATP. This reduction of endogenous ATP was accomplished by aging of the brei for 16 hours at 2°C., and by preparation of an acetone powder of the pea roots.

TABLE 1

THE EFFECT OF ADDED ATP ON THE IN VITRO COUPLING OF IAA TO PEA ROOT PROTEIN

Each tube contained 1.0 ml. of the acetone powder extract, 250 μg. IAA and 40 micromoles of ATP. There were no other addenda. Autoclaved brei was treated for 15 minutes at 15 lbs. pressure.

CONDITIONS	SALKOWSKI COLOR OF REDISSOLVED TCA PRECIPITATE	PROTEIN-BOUND IAA, μG.	BOUND IAA CORRECTED, μG.
+ATP	107	13.3	8.7
−ATP	69	9.0	4.6
Autoclaved brei + ATP	30	4.6	...
Autoclaved brei − ATP	28	4.4	...

When added to aged brei, 20 micromoles of ATP stimulated the binding of IAA to protein by more than 30%. Still greater stimulations were obtained in experiments with acetone powders. Sixteen grams of freshly excised root tips from 2-day old peas were frozen on a block of solid CO_2, and were then ground in a mortar with washed sea sand and chips of solid CO_2. The frozen slurry was then exhaustively extracted with acetone previously chilled to −15°C., and the resulting material dried to a fine powder. The powder was then dissolved in $M/15$ K_2HPO_4, centrifuged free of undissolved material, made up to 25 ml. and used as a source of protein. With material prepared in this fashion, the stimulatory effect of ATP on the binding of IAA to protein was demonstrable (table 1). If correction is made for the IAA non-specifically bound to the protein in the autoclaved controls, then ATP is seen to stimulate the binding of IAA to protein by 85%.

Contrary to expectation, it was found that the coupling reaction proceeds less well in the presence of CoA than in its absence. An explanation of this anomalous situation was later supplied by the finding that the IAA-protein is rapidly cleaved into protein and TCA-soluble IAA by the addition of CoA (table 2). This cleavage appears to be specific for CoA, since other sulfhydryl-containing substances such as reduced glutathione and cysteine are, respectively, slightly active and completely inactive.

The fact that CoA removes IAA from the protein helps to explain the apparent stimulation of the in vitro coupling reaction by iodoacetate. Since iodoacetate is known to combine with sulfhydryl groups, its apparent stimulation of the coupling reaction may most probably be attributed to its combination with CoA, and thus a prevention of the reaction through which CoA removes auxin from protein. In support of this hypothesis, it was

TABLE 2

THE EFFECT OF COENZYME A AND OF OTHER SULFHYDRYL-CONTAINING REAGENTS ON THE DISSOCIATION OF IAA FROM THE IAA-PROTEIN

The IAA protein was first made by an ATP-driven in vitro reaction (60 minutes, 30°C.). Replicate tubes were then supplemented by 0.36 micromole of the sulfhydryl reagents and incubated for an additional 40 minutes. The TCA precipitates were then prepared and assayed for IAA in the usual manner.

CONDITIONS	TCA-SOLUBLE IAA, μG.	PROTEIN-BOUND IAA, μG.	TOTAL IAA, μG.
Initial	42.4	4.0	46.4
After 40 minutes			
$+H_2O$	26.0	3.5	29.5
+CoA	34.6	0	34.6
+Glutathione	30.4	2.5	32.9
+Cysteine	26.8	3.6	31.4

shown that the removal of IAA from protein in vitro by CoA is inhibited by added iodoacetate, 50% inhibition of the reaction being conferred by 5×10^{-3} M iodoacetate.

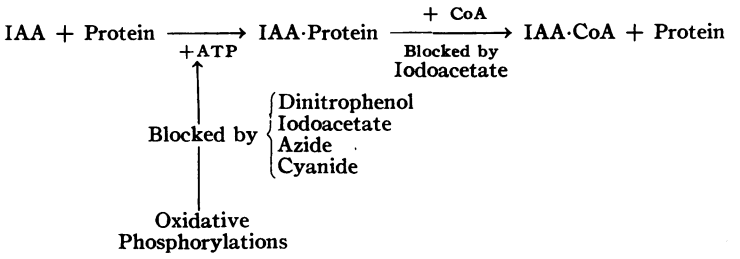
The IAA released from protein as a result of incubation with CoA was found to be largely ether insoluble at pH 3.0. Since free IAA is known to be readily soluble in ether under acid conditions, most of the IAA released from protein is not free IAA itself, but rather some water-soluble derivative. A combination of IAA with CoA or with a peptide fragment derived from the protein would satisfy these prerequisites.

Discussion.—The present work provides unambiguous confirmation of the existence of auxin-proteins in plant cells and furthermore indicates that coupling of auxin to protein is an endergonic process which can be driven by energy-rich sources such as ATP.

Whether or not the auxin-protein described here is the catalytic entity long postulated to be involved in the growth reaction, the fact that free

auxin may be firmly bound to protein must have some implications for growth. Catalytically inactive auxin-proteins may function as sequestering agents for IAA, preventing its destruction by IAA-oxidase. Alternatively, they may constitute reservoirs of IAA readily releasable and available for growth. Whatever their nature, they are of interest to the plant physiologist.

Our conception of the mechanism of the formation and cleavage of the IAA protein in vivo and in vitro may be summarized by the scheme below:



Summary.—Indoleacetic acid fed to excised pea root tips is quickly and firmly bound to protein, approximately 0.1–1.0 mole of IAA being bound per mole of protein of assumed molecular weight of 100,000. The in vivo coupling is inhibited by cyanide, azide, iodoacetate, and dinitrophenol, and is also interfered with by the presence of another auxin such as α-naphthaleneacetic acid. Auxin is not removed from the protein by dilute acid or alkali, acetone or boiling, and the auxin protein persists unchanged in vitro for at least 72 hours at 2°C.

In vitro, the coupling of auxin to protein may be accomplished with the aid of ATP. This coupling reaction is apparently inhibited by the addition of coenzyme A, which also can be shown to remove IAA in ether-insoluble form from the protein. This reaction between CoA and IAA-protein presumably involves thioether formation, since it can be prevented by the addition of iodoacetate.

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[1] Foster, R. J., McRae, D. H., and Bonner, J., these PROCEEDINGS, **38**, 1014–1022 (1952).

[2] Liverman, J. L., and Bonner, J.; unpublished experiments.

[3] Miller, G. L., and Miller, E., *Anal. Chem.*, **20**, 481–488 (1948).

[4] Millerd, A., Bonner, J., Axelrod, B., and Bandurski, R. S., these PROCEEDINGS, **37**, 855–862 (1951).

[5] Moewus, F., *Planta*, **37**, 413–430 (1950).

[6] Muir, R. M., these PROCEEDINGS, **33**, 303–312 (1947).

[7] Skoog, F., and Thimann, K. V., *Science*, **93**, 64 (1940).
[8] Speck, J. F., *J. Biol. Chem.*, **179**, 1387–1403 (1949).
[9] Tang, Y. W., and Bonner, J., *Arch. Biochem.*, **13**, 11–25 (1947).
[10] Wildman, S. G., and Bonner, J., *Ibid.*, **14**, 381–413 (1947).

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*THE VISCOSITY AND THE CONDUCTANCE-VISCOSITY PRODUCT OF ELECTROLYTE SOLUTIONS IN BROMINE**

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The conductance measurements reported by Darby[1] and by Moessen and Kraus[2] for solutions of trimethylammonium chloride and tetrabutylammonium bromide in bromine indicate that these salts are highly dissociated into their ions in this solvent at higher concentrations. However, in the absence of viscosity data and in view of the apparently high viscosity of these solutions, it is not possible to draw any valid conclusion as to the extent to which ionization takes place. According to Walden's rule, the product of limiting equivalent conductance and viscosity, $\Lambda_0\eta$, has the same value for a given electrolyte in different solvents. This rule does not hold very closely for salts of small ions, particularly in water. For salts of large ions the departure from Walden's rule is not great. In solutions at higher concentrations, the conductance-viscosity product provides us with a means of approximating the extent to which a salt is ionized at different concentrations if the limiting value of the product may be evaluated for the fused salt.

We have measured the viscosity of solutions of trimethylammonium chloride and tetrabutylammonium bromide up to the highest concentration at which conductance data are available and we have extended conductance measurements for the chloride to concentrations higher than heretofore reported.

Viscosities were measured by means of viscometers of the Ostwald type, modified to meet the requirements of the material employed. They were fitted with stopcocks and ground glass caps to exclude the atmosphere and to provide for transfer of the solution from one leg of the viscometer to the other. They were calibrated with respect to water at 25° and the efflux time was obtained as a fraction of the volume of water in the viscometer so that in measuring the viscosity of different solutions it was not necessary to adjust the volume of liquid to a definite value. The amount of bromine in the viscometer was determined by weighing the viscometer and its contents.