Phytochemistry, 1969, Vol. 8, pp. 1109 to 1120. Pergamon Press. Printed in England.

COMPARATIVE NET BIOSYNTHESIS OF INDOLEACETIC ACID FROM TRYPTOPHAN IN CELL-FREE EXTRACTS OF DIFFERENT PARTS OF *PISUM SATIVUM* PLANTS

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(*Received* 21 *January* 1969)

Abstract—Some optimum conditions for assaying the enzymic conversion of tryptophan to indoleacetic acid (IAA) in crude cell-free extracts of pea (*Pisum sativum* L.) seedlings are described. The assay is based upon the use of tryptophan-^{14}C and the isolation of IAA-^{14}C by TLC. Degradation of the enzymic product, measured by the rate of decarboxylation of IAA-1-^{14}C which was added to enzyme extracts, accounted for the absence of absolute direct proportionality between yield of IAA and either enzyme concentration or reaction time. A comparison was made of the capacities for net biosynthesis of IAA from tryptophan in extracts of various parts of light-grown pea seedlings and immature pea seeds. The greatest net auxin production occurred in extracts of terminal buds, young stems and young leaves of seedlings. Lesser amounts of net auxin production occurred in extracts from older stems and leaves and from root tips. Extracts from developing pea seeds exhibited an auxin-synthesizing capacity comparable to that of terminal buds of seedlings. Several experiments employing various antibiotics, sterile filtration and aseptic culture of seedlings *in vitro* indicated that microbial contamination was not a significant problem in the assay procedure.

INTRODUCTION

RECENT reports from the author's laboratory[1,2] have presented evidence that L-tryptophan is converted to indoleacetic acid (IAA) in cell-free extracts of shoot tips of pea (*Pisum sativum* L.) seedlings. Addition of α-ketoglutaric acid, pyridoxal phosphate and thiamine pyrophosphate to cell-free enzyme extracts enhanced conversion of tryptophan to IAA. Indolepyruvic acid, as the 2,4-dinitrophenylhydrazone, and indoleacetaldehyde, after reduction to tryptophol with sodium borohydride, were isolated from reaction mixtures. Thus the collective data which have been reported previously are consistent with a pathway of auxin biosynthesis being operative in *Pisum* which is initiated by a transamination reaction of tryptophan to form indolepyruvic acid, proceeds via decarboxylation of indolepyruvic acid to indoleacetaldehyde, and terminates in the oxidation of indoleacetaldehyde to IAA. These findings agree closely with the report by Wightman and Cohen[3] on an auxin-synthesizing enzyme system in cell-free extracts of mung bean (*Phaseolus aureus* Roxb.), a legume which is closely related to *Pisum sativum*. One purpose of the present report is to characterize the optimum assay conditions for the previously studied enzyme system from *P. sativum*.

There have been numerous investigations of the interrelationship between endogenous auxin content and growth in different parts of seed plants. Representative of such studies are those of Thimann[4] using etiolated *Avena sativa* seedlings, Thimann and Skoog[5] with

[1] T. C. MOORE and C. A. SHANER, *Plant Physiol.* **42**, 1787 (1967).
[2] T. C. MOORE and C. A. SHANER, *Arch. Biochem. Biophys.* **127**, 613 (1968).
[3] F. WIGHTMAN and D. COHEN, in *The Physiology and Biochemistry of Plant Growth Substances* (edited by G. SETTERFIELD and F. WIGHTMAN), in press, Runge Press, Ottawa (1968).
[4] K. V. THIMANN, *J. Gen. Physiol.* **18**, 23 (1934).
[5] K. V. THIMANN and F. SKOOG, *Proc. R. Soc. Lond.* Ser. B, **114**, 317 (1934).

Vicia faba seedlings, Van Overbeek *et al.*[6] using vegetative *Ananas comosus* plants, and Scott and Briggs[7–9] using *P. sativum* seedlings. In general, a strong correlation between "free" auxin content, as determined by diffusion and/or extraction combined with bioassay techniques, and growth has been demonstrated by these investigations.

The factors controlling the amounts of auxin available for growth processes in different parts of a plant are incompletely understood. Among the factors that may be involved are differential capability for auxin transport[7–10], differential binding of auxin to protein,[11] differential conjugation of auxin with aspartic acid[12] or other compounds, and differential capacities for enzymic auxin destruction.[13] Another factor which may be correlated with the variation in endogenous auxin content in the different parts of a plant is differential auxin biosynthesis. It is commonly recognized that the most active sites of biosynthesis in a seed plant are those parts with relatively high metabolic activity, that is, shoot and root apices, developing flowers and fruits, and young leaves.[14] Furthermore, it is widely believed that the enzyme systems required for auxin biosynthesis (at least from tryptophan) are present in all the metabolically highly active loci.[15] However, a detailed comparative study of auxin biosynthesis in the different parts of a single plant species apparently has not been made. This paper presents results of quantitative comparisons of the capacities for net biosynthesis of IAA-^{14}C from tryptophan-^{14}C in cell-free enzyme extracts of various parts of light-grown pea seedlings and immature pea seeds.

A serious potential problem confronting investigations of auxin biosynthesis is microbial contamination, since numerous species of microorganisms are known to convert tryptophan to IAA. The influence of epiphytic bacteria on auxin metabolism in preparations from pea plants has been documented extensively in recent years by Libbert *et al.*[16] and Wichner and Libbert.[17, 18] Hence several experiments were performed to determine the possible influence of microbial metabolism in the system employed by the author.

RESULTS AND DISCUSSION

Characterization of the Assay System Using Enzyme Extracts From Shoot Tips of Green Seedlings

Dialysis of enzyme extracts prepared from shoot tips of green seedlings caused highly significant increases in yields of IAA-^{14}C as compared to yields obtained with non-dialyzed extracts (Fig. 1). The yield of IAA-^{14}C was 7- to 8-fold higher in preparations containing enzyme extract that had been dialyzed for 8–16 hr at 2–5° than in preparations containing

[6] J. VAN OVERBEEK, E. S. VÁZQUEZ and S. A. GORDON, *Am. J. Botany* **34**, 266 (1947).
[7] T. K. SCOTT and W. R. BRIGGS, *Am. J. Botany* **47**, 492 (1960).
[8] T. K. SCOTT and W. R. BRIGGS, *Am. J. Botany* **49**, 1056 (1962).
[9] T. K. SCOTT and W. R. BRIGGS, *Am. J. Botany* **50**, 652 (1963).
[10] W. P. JACOBS, *Am. J. Botany* **37**, 248 (1950).
[11] A. WINTER and K. V. THIMANN, *Plant Physiol.* **41**, 335 (1966).
[12] W. A. ANDREAE and M. W. VAN YSSELSTEIN, *Plant Physiol.* **35**, 225 (1960).
[13] A. W. GALSTON, in *Photoperiodism* (edited by R. B. WITHROW), Am. Assoc. Adv. Sci., Washington, D.C. (1959).
[14] S. A. GORDON, in *Hand. Pflanzenphysiologie* (edited by W. RUHLAND), Vol. XIV, p. 620, Springer, Berlin-Göttingen-Heidelberg (1961).
[15] S. A. GORDON, in *The Chemistry and Mode of Action of Plant Growth Substances* (edited by R. L. WAIN and F. WIGHTMAN), Butterworths Scientific Publications, London (1956).
[16] E. LIBBERT, S. WICHNER, U. SCHIEWER, H. RISCH and W. KAISER, *Planta* **68**, 327 (1966).
[17] S. WICHNER and E. LIBBERT, *Physiol. Plantarum* **21**, 227 (1968).
[18] S. WICHNER and E. LIBBERT, *Physiol. Plantarum* **21**, 500 (1968).

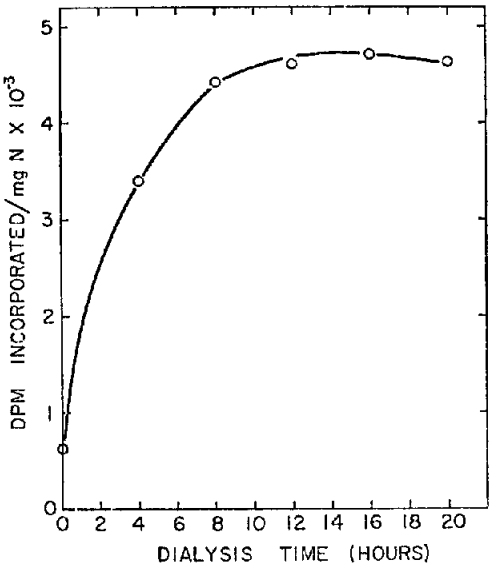

FIG. 1. EFFECT OF DURATION OF DIALYSIS OF ENZYME EXTRACTS AGAINST 0·1 M KH_2PO_4–Na_2HPO_4 BUFFER (pH 7·4) AT 2 TO 5° ON ACTIVITY OF THE TRYPTOPHAN-IAA SYSTEM.

Each reaction mixture consisted of 3·0 ml of enzyme extract and 3·0 ml of phosphate buffer containing 6 μc of DL-tryptophan-^{14}C, 30 μmoles each of non-radioactive DL-tryptophan and α-ketoglutaric acid, and 0·6 μmole each of pyridoxal phosphate and thiamine pyrophosphate. Incubation was for 120 min in darkness at 30°. The data were obtained by liquid scintillation counting, and the values presented are means based on duplicate samples from duplicate reaction mixtures. Mean dpm values obtained for reaction mixtures containing boiled enzyme extract were subtracted from mean gross experimental values for each determination.

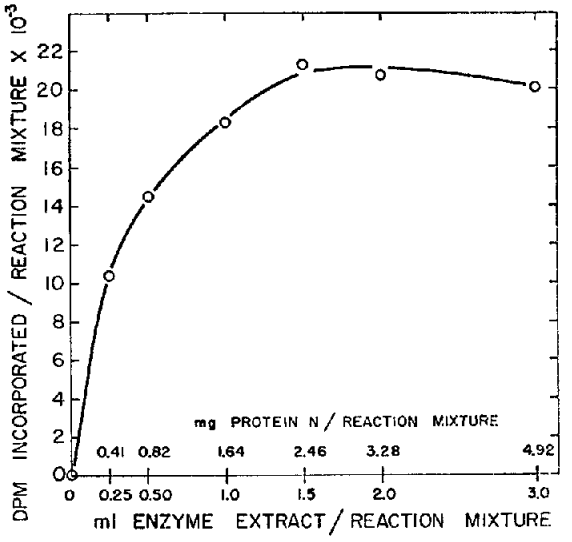

FIG. 2. RELATIONSHIP BETWEEN ENZYME CONCENTRATION AND YIELD OF IAA-^{14}C.

Reaction conditions were as described in legend for Fig. 1, except for enzyme concentration which was varied. The data presented are means based on duplicate samples from duplicate reaction mixtures, and have been corrected for values obtained with reaction mixtures containing boiled enzyme extract.

non-dialyzed enzyme extract. Decline in enzyme activity was observed when dialysis was extended beyond 18–20 hr. Therefore, in all other experiments reported, enzyme extracts were dialyzed for approximately 16 hr.

Activity was not directly proportional to enzyme concentration, even at relatively low enzyme concentrations (Fig. 2), but did increase with concentration from zero to 2·5 mg of protein N per reaction mixture (approximately equivalent to 0·4 mg of protein N, or 2·5 mg of protein/ml of reaction mixture). The deviation from direct proportionality between product yield and enzyme concentration, even at low enzyme levels, is due at least partially to enzymic product degradation, a fact which will be discussed later in connection with the data presented in Fig. 7. Despite the lack of direct proportionality, a concentration of

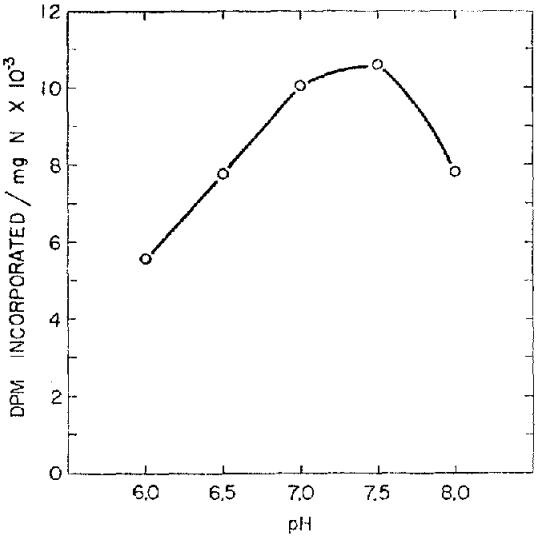

FIG. 3. EFFECT OF HYDROGEN-ION CONCENTRATION ON ACTIVITY OF THE TRYPTOPHAN-IAA ENZYME SYSTEM.

Phosphate–borate buffer (0·1 M, pH 7·4) was used in the preparation of enzyme extract and as the dialyzing solution. The pH of each reaction mixture was adjusted to the desired pH by adding concentrated KOH or H_3PO_4 before and immediately after enzyme extract (1·5 ml) was added. Reaction conditions otherwise were as described in legend for Fig. 1. The mean dpm value obtained from zero-time reaction mixtures was subtracted from the mean gross experimental value for each pH. The data are based on duplicate samples from duplicate reaction mixtures.

approximately 0·4 mg of protein N per ml of reaction mixture was selected as standard for all other experiments, in order to obtain maximum yields of IAA-^{14}C.

The optimum pH for the enzyme system was approximately 7·4 to 7·5 (Fig. 3). Data for pH values greater than 8·0 are not presented; however, an interesting result was noted at pH 8·0 to 9·0. The dpm incorporated into IAA-^{14}C in both experimental and control reaction mixtures increased sharply from pH 8·0 to 8·5 and decreased slightly from 8·5 to 9·0. While no attempt was made to explain this result, it may be speculated that the activity observed at pH of about 8·0–9·0 was the result of polyphenolic oxidation of tryptophan to IAA, as reported by Gordon and Paleg[19] for cell-free preparations of mung bean, oat and sunflower seedlings. Gordon and Paleg reported that phenols, under conditions favoring

[19] S. A. GORDON and L. G. PALEG, *Plant Physiol.* **36**, 838 (1961).

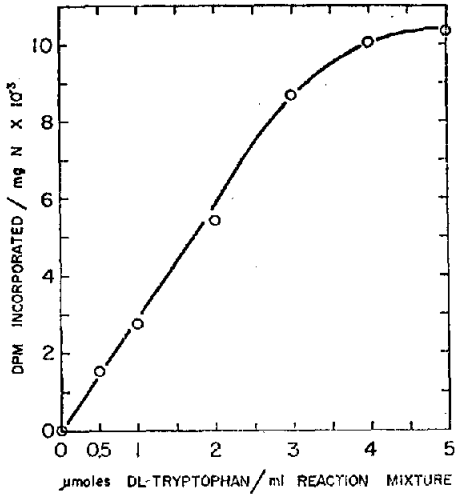

FIG. 4. EFFECT OF SUBSTRATE CONCENTRATION ON YIELD OF IAA-^{14}C.

Each reaction mixture consisted of 1·5 ml of enzyme extract (~2·5 mg of protein N) and 4·5 ml of phosphate buffer (0·1 M, pH 7·4) containing 30 μmoles of α-ketoglutaric acid, and 0·6 μmole each of pyridoxal phosphate and thiamine pyrophosphate. The concentration of DL-tryptophan-^{14}C was reduced proportionately with non-radioactive tryptophan. Incubations were for 120 min in darkness at 30°. The data presented are corrected means based on duplicate samples from duplicate reaction mixtures.

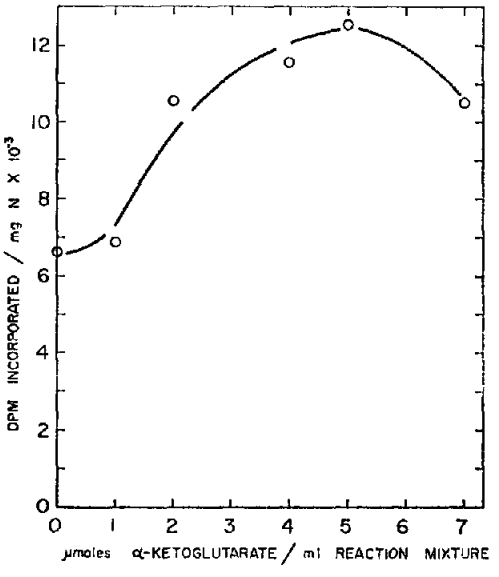

FIG. 5. EFFECT OF CONCENTRATION OF α-KETOGLUTARIC ACID ON YIELD OF IAA-^{14}C.

Reaction conditions were as described in legend for Fig. 4, except that each reaction mixture contained 6 μc of tryptophan-^{14}C and 30 μmoles of non-radioactive tryptophan and the concentration of α-ketoglutaric acid was varied. The mean dpm for reaction mixtures containing boiled enzyme extract and no α-ketoglutaric acid were subtracted from gross experimental values.

their oxidation, react with tryptophan to form IAA. The reaction was catalyzed by polyphenolases and occurred spontaneously at alkaline pH, with maximum activity occurring at pH 8·5. Based on the results presented in Fig. 3, pH 7·4 was adopted for routine use in the experiments. This, coincidentally, is the same pH used by Gordon[20] in his early investigations of auxin biosynthesis in cell-free extracts of mung bean seedlings.

Substrate saturation was achieved with 5 μmoles of DL-tryptophan per ml of reaction mixture, or more specifically with approximately 8·2 μmoles of tryptophan per mg N or approximately 1·3 μmoles per mg protein (Fig. 4). The optimum concentration of α-ketoglutaric acid, which served as the co-substrate for the transamination reaction, also was

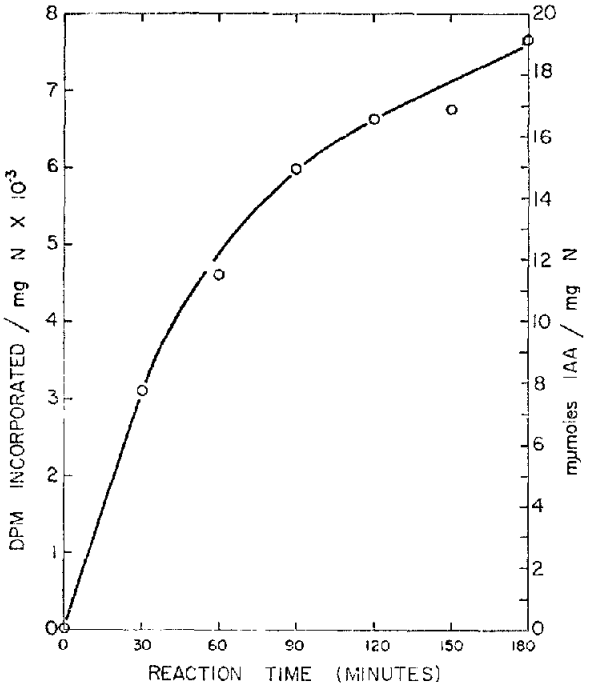

FIG. 6. TIME-COURSE OF CONVERSION OF TRYPTOPHAN-^{14}C TO IAA-^{14}C IN CELL-FREE EXTRACTS. Assays were prepared as described in the legend for Fig. 4, except that each mixture contained 6 μc of tryptophan-^{14}C and 30 μmoles of non-radioactive tryptophan and time was varied. The mean dpm obtained for zero-time control preparations were subtracted from gross experimental values. The data presented are means based on duplicate samples from duplicate reaction mixtures.

5 μmoles per ml of reaction mixture (Fig. 5). Higher concentrations of α-ketoglutaric acid caused reductions in yield of IAA-^{14}C, such that 10 μmoles of the keto-acid per ml reduced the yield of IAA-^{14}C to the level obtained with no added keto-acid.

In separate experiments in which the concentrations of pyridoxal phosphate and thiamine pyrophosphate were varied individually under otherwise optimum conditions, the optimum concentration of both coenzymes was found to be 0·1 μmole per ml of reaction mixture. The concentrations of pyridoxal phosphate and thiamine pyrophosphate were varied from 0 to 0·13 and 0 to 0·2 μmole per ml of reaction mixture, respectively.

The relationship between reaction rate and time was determined using the optimum

[20] S. A. GORDON, *Plant Physiol.* 33, 23 (1958).

conditions described previously. Direct proportionality between yield of IAA-^{14}C and time was not observed, although yield did increase with time up to 180 min (Fig. 6). In an effort to ascertain the reason for the lack of linearity of the rate with a reasonable period of time, assays were conducted for IAA decarboxylating activity in cell-free extracts.

Extrapolation of a straight line from zero time through 90 min on the curve presented in Fig. 6 provided a basis for estimating theoretical gross yields of IAA. These calculations were based on the assumption that increasing product degradation with time might account for the deviation from linearity. Thus, concentrations of IAA-1-^{14}C ranging from 0 to

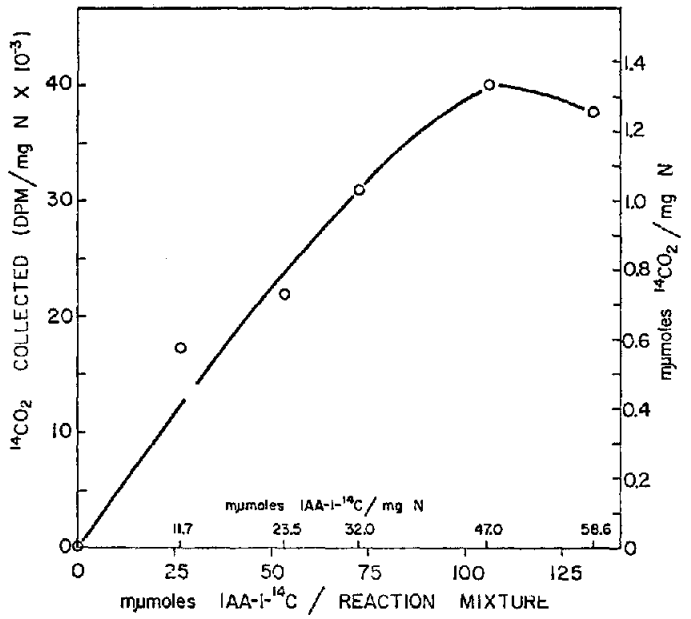

FIG. 7. RATE OF DECARBOXYLATION OF IAA-1-^{14}C IN CELL-FREE ENZYME PREPARATIONS.

Reaction mixtures were prepared as described in the legend for Fig. 4, except that tryptophan and tryptophan-^{14}C were omitted and the indicated amounts of IAA-1-^{14}C were added. Incubations were for 180 min in darkness at 30°. The radioactivity in aliquots of the alkali used to trap $^{14}CO_2$ was measured by liquid scintillation counting. The data presented are means based on duplicate samples from duplicate reaction mixtures and have been corrected by subtracting values obtained with boiled enzyme extract.

133 mμmoles per reaction mixture were used as substrate in reaction mixtures which were complete except for the total omission of tryptophan. IAA decarboxylating activity was observed (Fig. 7), and activity increased with increasing concentrations of IAA-1-^{14}C up to approximately 0·1 μmole per reaction mixture. In another experiment the rate of decarboxylation of IAA-1-^{14}C was found to be proportional to time through 180 min. From these results it seems reasonable to conclude that product degradation is at least partially responsible for the lack of direct proportionality between amount of IAA-^{14}C formed from tryptophan and both reaction time and enzyme concentration.

To the extent that the data are comparable, the results of the present investigations agree well with the data presented by Wightman and Cohen[3] for the enzymic conversion of tryptophan to IAA in cell-free extracts of mung bean seedlings. There is some similarity

also between the data for the *Pisum sativum* system and the data of Phelps and Sequeira[21] for a system from an avirulent strain (B-1) of *Pseudomonas solanacerum*, and, to a lesser extent, the findings of Lantican and Muir[22] for an enzyme system prepared from various seed plants.

Thus in *Pisum sativum*, *Phaseolus aureus*, the B-1 strain of the vascular pathogen *Pseudomonas solanacerum*, and some other organisms, enzymic conversion of tryptophan to IAA via indolepyruvic acid and indoleacetaldehyde seems to be well documented. However, available data certainly do not exclude the probability that other reactions besides those for which evidence has been presented are operative in cell-free enzyme extracts of pea seedlings, or that pathways independent of tryptophan are involved. The lack of absolute dependency of dialyzed enzyme extracts upon added co-substrate and coenzymes, which has been reported previously[1,2] and confirmed in the present investigations, point to this probability. Additional preliminary investigations indicate that there is less labeling of IAA from tryptophan-^{14}C labeled in the 2-position, and perhaps also the 3-position, of the alanine side-chain than from tryptophan labeled in the 2-position of the indole ring. Certainly it is clear that other pathways are operative in other species,[23] and reactions other than those described here may yet be found to be involved in IAA biogenesis in *Pisum*. The enzyme assay and procedure for analysis of IAA which have been utilized in the present investigations should prove useful in further investigations, using partially purified and fractionated enzyme extracts, to elucidate more completely pathways of auxin biosynthesis and other features of auxin metabolism.

Comparative Net Auxin Biosynthesis in Cell-free Extracts of Different Parts of Pea Plants

Tryptophan was converted to IAA in cell-free enzyme extracts of all parts of "Telephone" pea seedlings and in extracts of immature "Telephone" and "Alaska" pea seeds (Table 1). Auxin yields are expressed as the amount of radioactivity from DL-tryptophan-2-^{14}C incorporated into IAA-^{14}C per g fresh weight of tissue, a physiologically more meaningful

TABLE 1. COMPARATIVE NET BIOSYNTHESIS OF IAA-^{14}C FROM TRYPTOPHAN-^{14}C IN CELL-FREE EXTRACTS OF DIFFERENT PARTS OF PEA PLANTS*

Plant part(s)	Dpm incorporated into IAA-^{14}C/g fr. wt./2 hr	Mean fr. wt. of parts/plant (mg)	mg N/g fr. wt.
Terminal bud	61,166	24	6·31
Stem above 5th node	40,181	98	1·59
Leaves above 5th node†	51,776	77	4·96
Shoot tip (all above 5th node)	38,933	305	3·55
Stem below 5th node	19,200	478	0·68
Leaves below 5th node†	10,240	545	1·62
Apical 2 cm of primary root	7,786	34	0·98
Developing seeds‡	56,000	171	2·22
Developing seeds§	89,266	—	—

* Collective data from three separate experiments.
† Including stipules, rachis, leaflets and tendrils.
‡ Approximately half-grown "Alaska" pea seeds.
§ Approximately half-grown "Telephone" pea seeds.

[21] R. H. PHELPS and L. SEQUEIRA, *Phytopath.* **57**, 1182 (1967).
[22] B. P. LANTICAN and R. M. MUIR, *Plant Physiol.* **42**, 1158 (1967).
[23] R. H. PHELPS and L. SEQUEIRA, *Plant Physiol.* **42**, 1161 (1967).

basis than per unit protein or nitrogen for comparing the auxin-synthesizing capacities of the different parts of seedlings, because of the marked differences in protein and nitrogen concentration of the plant materials. On this basis the regions of most active auxin production apparently were the terminal bud, young stem below the terminal bud and young leaves. Lesser amounts of net auxin biosynthesis occurred in enzyme extracts prepared from older stems and leaves and from root tips. Thus, in general, the data agree well, to the extent that they are comparable, with the amounts of "free" auxin obtained by diffusion and extraction from young light-grown pea seedlings.[7–9] Developing pea seeds exhibited an apparent auxin-synthesizing capacity which was comparable to or higher than that of terminal buds of seedlings.

Two primary considerations must be taken into account in interpreting the data presented, however. In the first place, it is not known to what extent the auxin-synthesizing capacities in cell-free enzyme extracts reflect the actual *in vivo* capacities of the various plant parts to produce IAA from tryptophan. Secondly, in the experiments here described net IAA production was measured, and there is no accounting for concomitant auxin degradation by the IAA oxidase system in the preparations. Previously it was shown (Fig. 7) that IAA-1-^{14}C (carboxyl-labeled), when added to cell-free enzyme extracts of shoot tips in estimated substrate quantities, was enzymically decarboxylated. Hence it is reasonable to assume that IAA oxidase activity was present, and at variable levels, in the enzyme extracts of the various plant parts. Based on Galston's[13] investigations of the distribution of IAA oxidase activity and of inhibitor of the enzyme in green pea epicotyls, it appears probable that the relative IAA oxidase activity in the cell-free enzyme extracts varied inversely with net IAA production. Galston[13] found that the IAA oxidase inhibitor was most abundant in young tissues of all kinds (apical bud, stem and leaf) and that it decreased in concentration in progressively older tissues. Relatively high IAA oxidase activity in roots has also been well documented (see, for example, Pilet and Gaspar[24]).

Thus there appears to be a good correlation among capacity for net auxin biosynthesis from tryptophan, distribution of IAA oxidase inhibitor, and endogenous "free" auxin content of the various parts of young green pea seedlings. There still remains the question of whether there are differences in the capacities of the various parts of the seedling plant for gross auxin production, or whether the differences in apparent auxin-producing capacity noted in the present investigation are correlated primarily with differential IAA oxidase activity. Scott and Briggs,[9] while considering also other possible contributing factors, felt that auxin destruction seemed to be the best alternative to account for the variations in "free" auxin content in different parts of green pea epicotyls. However, these investigators stated in a previous paper[7] that the apical bud appeared to be the only source of "free" auxin in the epicotyl, whereas the present study demonstrates that the *potential* for some auxin production is present in virtually all parts of the young pea seedling. Based on all available data it appears probable that the different parts of the pea seedling do differ in potential auxin-synthesizing capacity as well as in IAA oxidase activity, and that differential auxin biosynthesis *in situ* is one factor among many which accounts for variations in auxin content in the various parts of pea seedlings.

Investigations of Possible Influence of Microbial Contamination in Cell-free Assays

Experiments employing the use of antibiotics, sterile filtration of enzyme extracts and other solutions, and aseptic culture of seedlings *in vitro* indicated that microbial contamina-

[24] P. E. Pilet and Th. Gaspar, *Physiol. Plantarum* **17**, 324 (1964).

tion was not a serious, if at all significant, factor in the assays (Table 2). The results of these experiments may not be directly compared with one another because of variations in procedures; for example, the concentrations of protein N in the four experiments varied from 1·13 to 1·75 mg N per 6-ml reaction mixture. In the previous investigations by the

TABLE 2. INVESTIGATIONS OF POSSIBLE CONTRIBUTION BY MICROBIAL METABOLISM TO AUXIN BIOSYNTHESIS FROM TRYPTOPHAN IN CELL-FREE ENZYME EXTRACTS OF PEA PLANTS

Exp. No.	Conditions	IAA-^{14}C yield (dpm/mg protein N/2 hr)
1	Different antibiotics used in preparation of enzyme extracts from shoot tips of young seedlings grown in a growth chamber; extracts centrifuged at 48,000 × *g* for 20 minutes; 200 μg/ml of antibiotic in enzyme extracts, 100 μg/ml final concentration of antibiotic in reaction mixtures	
	(a) No antibiotic	15,677*
	(b) Penicillin G	18,600*
	(c) Streptomycin sulfate	15,231*
	(d) Chloramphenicol	14,342*
	(e) Actinomycin D	17,527*
2	(a) Enzymes extracted in present of 200 μg/ml of chloramphenicol and dialyzed against buffer containing 200 μg/ml of chloramphenicol; 200 μg/ml final concentration of chloramphenicol in reaction mixtures	13,696†
	(b) Same as "a", except no chloramphenicol used anywhere in procedure	13,423†
3	Enzyme extracts prepared from whole epicotyls of 10-day-old seedlings cultured aseptically on 1·5% "Difco Bacto-Agar"; 200 μg/ml of chloramphenicol in enzyme extract, 100 μg/ml final concentration in reaction mixtures	17,770‡
4	(a) Enzyme extracts prepared from shoot tips of seedlings which were grown in a growth chamber and which were surface-disinfected with 1% NaClO (10 min) and rinsed five times with autoclaved distilled water before being homogenized; 200 μg/ml of chloramphenicol in all solutions; all solutions, including enzyme extracts both before and after dialysis, were "Millipore"-filtered (mean pore size 0·22 μ); all glassware and utensils were autoclaved	18,939§
	(b) Same as "4a," except that shoot tips were not surface-disinfected	19,738§
	(c) Same as "4a," except that shoot tips were not surface-disinfected and no solutions were "Millipore"-filtered	20,012§

* Mean based on duplicate samples from duplicate reaction mixtures; corrected by subtracting values for reaction mixtures containing boiled enzyme extract.

† Corrected mean based on duplicate samples from three reaction mixtures.

‡ Corrected mean based on duplicate samples from five reaction mixtures.

§ Corrected mean based on duplicate samples from duplicate reaction mixtures.

author and his associates[1,2] routine precautions against influences of microbial metabolism included surface-sterilization of seeds prior to planting and hydroponic culture in a growth chamber, preparation of enzyme extracts in the presence of 200 μg/ml each of penicillin G and streptomycin sulfate, and centrifugation of enzyme extracts at 10,000 × *g*, or more recently 48,000 × *g*, for 20 min prior to dialysis. Experiment No. 1 showed that the yield of IAA was

not appreciably different when no antibiotic or any one of four antibiotics was used in concentrations in excess of those commonly employed in such investigations. Similar results were witnessed in Experiment No. 2 which compared auxin yields from preparations containing 200 μg/ml and no chloramphenicol throughout. Experiment No. 3 revealed that enzyme extracts prepared from seedlings which were cultured aseptically *in vitro* also catalyzed conversion of tryptophan to IAA in appreciable yield, even if, as in a similar experiment, the extracts were also sterile-filtered both before and after dialysis, and all glassware and utensils were sterilized.

There remained the possibility that, while whole bacteria apparently were not confounding the assays, tryptophan-converting enzymes from epiphytic microorganisms were being solubilized in the process of homogenizing shoot tips of seedlings. Experiment No. 4 (Table 2) was designed to test this possibility. The results show that perhaps solubilized microbial enzymes might indeed have been responsible for some of the observed IAA production, but the effect, if indeed significant, was relatively slight. This conclusion is supported by the recent report by Henry *et al.*,[25] who examined with an electron microscope tissues from young internodes of "Alaska" pea seedlings which were handled and grown under conditions similar to those used in the present investigations. They reported that no bacteria were seen in electron micrographs of tissues which were fixed directly from intact seedlings.

EXPERIMENTAL

Preparation of Enzyme Extracts and Reaction Conditions

Seedlings of the "Tall Telephone" (or "Alderman") variety of *Pisum sativum* (W. Atlee Burpee Company, Riverside, California), cultured as described previously[1] under a 16-hr photoperiod at $20 \pm 1°$, an incident light intensity of 9800–10,800 lux and an 8-hr dark period at $16 \pm 1°$, were utilized in all but a few experiments in which "Alaska" peas were substituted. Shoot tips were commonly excised immediately above the fifth node of 12-day-old seedlings, and samples were homogenized in 4 volumes (4 ml: 1 g fr. wt.) of KH_2PO_4–Na_2HPO_4 buffer (0·1 M, pH 7·4) containing 0·3 M sucrose and 200 μg/ml each of penicillin G and streptomycin sulfate. Homogenization was done with a high-speed Thomas teflon-to-glass homogenizer in a cold room ($\sim 5°$), and the homogenates were kept in an ice bath throughout the preparative procedures. Crude homogenates were centrifuged at $10{,}000 \times g$ or $48{,}000 \times g$ for 20 min at 4°, and the resulting supernatant was used as the enzyme extract. Enzyme extracts were dialyzed against an approximate 200-fold volume excess of 0·1 M phosphate buffer for 16–18 hr, except as noted otherwise, at 2–5° with three changes of external buffer. A fresh enzyme extract was prepared for each experiment reported, although limited evidence has indicated that dialyzed enzyme extracts are stable at $-20°$ for at least 2 weeks.

In experiments using different plant parts, the plants were harvested when 12 days of age. Tissues were harvested immediately before use, and each sample consisted of the parts of at least 25, and more commonly 80 to 150, plants. Roots were surface-disinfected with 0·5% NaOCl and rinsed repeatedly with sterile distilled water prior to being homogenized. Seeds were excised from pods when the seeds were approximately half mature size; seeds were homogenized in 5 volumes of phosphate buffer.

Each complete reaction mixture consisted of, except where described as being otherwise: 1·5 ml of enzyme extract (equivalent to approximately 2·5 mg of protein N or 15·6 mg of protein); and 4·5 ml of 0·1 M KH_2PO_4–Na_2HPO_4 buffer (pH 7·4) containing 6 μc of DL-tryptophan-2-^{14}C (ring labeled; sp. act. $\simeq$ 22 mc/mmole), 30 μmoles each of non-radioactive DL-tryptophan and α-ketoglutaric acid, and 0·6 μmole each of pyridoxal phosphate and thiamine pyrophosphate. Incubations routinely were for 120 min at 30° in darkness in glass-stoppered 12-ml tubes. Reactions were stopped by lowering the pH to 3·0 with concentrated H_3PO_4. Complete reaction mixtures containing boiled enzyme extract were routinely used in duplicate as controls, and all values reported are corrected by subtraction of the dpm observed in the appropriate control. Reaction mixtures were prepared in duplicate, and duplicate samples of each acidic extract were analyzed for IAA-^{14}C. Determinations of protein N concentration were made on duplicate 1·0- or 1·5-ml aliquots of each enzyme extract by a micro-Kjeldahl procedure.[26]

Extraction and chromatographic isolation of IAA-^{14}C was by a procedure described previously,[1] except

[25] E. W. HENRY, J. G. VALDOVINOS and T. E. JENSEN, *Plant Physiol.* **43**, 1730 (1968).

[26] *Official Methods of the Association of Agricultural Chemists* (edited by W. HORWITZ), 10th edition, p. 744 (1965).

that ethyl acetate was used as the extractant instead of CH_2Cl_2. Radioactivity in samples of isolated IAA-^{14}C was measured by liquid scintillation counting on a Packard Model 3375 Liquid Scintillation Spectrometer. Each sample of IAA-^{14}C was added to 10 ml of Bray's[27] solution; counting efficiency varied between 71 and 76 per cent. Counts per minute have been converted to disintegrations per minute (dpm).

Determination of pH Optimum

Phosphate–borate buffers were prepared using a modification of the procedure described by Clarke and Mann,[28] in order that the same buffer constituents could be utilized over the pH range 6·0–9·0. Phosphate–borate buffer was prepared by mixing equal volumes of 0·1 M KH_2PO_4 and 0·1 M H_3BO_3 and adjusting the pH to 7·4 using concentrated KOH. Enzyme extract was prepared in and dialyzed against a 200-fold volume excess of this buffer for 16–18 hr. After dialysis, aliquots of the stock buffer were adjusted to desired pH by adding concentrated H_3PO_4 or KOH. Addenda for each reaction mixture were then prepared in buffer at the desired pH. After adding enzyme extract to each reaction mixture, the pH was again adjusted to the desired pH. Reaction mixtures were incubated at 30° for 120 min. Zero-time controls were prepared for each pH. Each control mixture was prepared as described previously, except that the reaction was stopped and the mixture extracted immediately after addition of enzyme extract.

*Measurement of Rate of Decarboxylation of IAA-*1-^{14}C

Rates of decarboxylation of IAA were determined by adding substrate amounts of IAA-1-^{14}C (sp. act. $\simeq$13·5 mc/mmole) to cell-free reaction mixtures, which were prepared as described in the first part of this section except for the omission of tryptophan, and measuring the rate of $^{14}CO_2$ release. The procedure used was a modification of the method described by Kerstetter and Keitt.[29] Reaction mixtures were contained in 9-ml vials suspended with nickel chromium wire inside 35-ml shell vials fitted with serum caps. In the bottom of each vial was 0·5 ml of 0·5 N NaOH. After 180 min incubation in darkness at 30°, a predetermined amount of concentrated H_3PO_4, adequate to lower the pH to 3·0, was dispensed with a syringe into each reaction mixture; then the vials were allowed to stand for 60 min in darkness at 30°. Next, 15 ml of Bray's solution were added to each sample of ^{14}C-carbonate; duplicate samples of this solution then were removed, each was added to 10 ml of Bray's solution and the radioactivity was measured. Reaction mixtures were prepared in duplicate and duplicate aliquots of each ^{14}C-carbonate sample were counted.

Reagents

The critical chemicals used and the sources from which they were obtained were: DL-tryptophan and penicillin G (Sigma Chemical Company, St. Louis, Missouri); α-ketoglutaric acid, pyridoxal phosphate hydrochloride, chloramphenicol, and thiamine pyrophosphate chloride (CalBiochem, Los Angeles, California); DL-tryptophan-2-^{14}C (ring labeled; sp. act.$\simeq$22 mc/mmole; Schwarz BioResearch, Orangeburg, New York); indoleacetic acid-1-^{14}C (sp. act.$\simeq$13·5 mc/mmole; New England Nuclear, Boston, Massachusetts); streptomycin sulfate (Nutritional Biochemicals, Cleveland, Ohio); indoleacetic acid (Mann Research Laboratories, New York, New York); 1,4-*bis*-2′-(5′-phenyloxazolyl)-benzene (POPOP) and 2,5-diphenyloxazole (PPO) (Packard Instrument Co., La Grange, Illinois); *para*-dioxane (Matheson Coleman & Bell, Cincinnati, Ohio); actinomycin D (Merck Sharp & Dohme, Rahway, New Jersey); naphthalene (Eastman Organic Chemicals, Rochester, New York); and "Bacto-Agar" (Difco Laboratories, Detroit, Michigan). The sterile filtration apparatus was obtained from Millipore Corporation, Bedford, Massachusetts. All organic solvents used in extraction and chromatographic procedures were redistilled.

Acknowledgements—These investigations were supported by Grant GB-7386 from the National Science Foundation. The author also gratefully acknowledges the conscientious technical assistance of Mrs. Coralie A. Shaner, Mrs. Lynda Tyrl and Miss Lourminia A. Carino. The author expresses special gratitude to Dr. Donald J. Reed for his continued advice and counsel and for his critical reading of the manuscript.

[27] G. A. BRAY, *Anal. Biochem.* **1**, 279 (1960).
[28] A. J. CLARKE and P. J. G. MANN, *Biochem. J.* **65**, 763 (1957).
[29] R. E. KERSTETTER and G. W. KEITT, JR., *Plant Physiol.* **41**, 903 (1966).