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TUBULIN SYNTHESIS AND AUXIN-INDUCED ROOT INITIATION IN *PHASEOLUS*

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Key Word Index—*Phaseolus vulgaris*; Leguminosae; french bean; hypocotyls; auxin; roots and tubulin.

Abstract—The auxin-induced formation of roots in the hypocotyls of *Phaseolus vulgaris* can be prevented by treatment with actinomycin D, colchicine or cytochalasin B if applied within 40 hr of initiation. Shortly after auxin pretreatment, there is an increase in translatable messenger RNA activity. Analysis of the labelled cell-free products indicate, among other changes, a striking increase in a protein co-migrating with tubulin, in the case of RNA isolated from indolebutyric acid (IBA) pretreated hypocotyls. An increase in tubulin content *in vivo* can also be demonstrated on the basis of SDS-polyacrylamide gel analysis of membrane proteins and functional assays for tubulin polymerization. An increase in the synthesis of tubulin *in vivo* can also be demonstrated after IBA pretreatment. In addition, the auxin is also able to promote tubulin polymerization when added *in vitro*. It is suggested that tubulin synthesis and microtubule assembly are early events in auxin-mediated root differentiation.

INTRODUCTION

Among the factors which regulate adventitious root formation, auxin is of primary importance [1]. Excised or intact hypocotyls and epicotyl segments undergo redifferentiation in response to auxin treatment, leading to the formation of root primordia. The morphological and biochemical events underlying this process have been analysed. In auxin-treated pea epicotyls or soybean hypocotyls, the parenchymatous cells in the cortex appear swollen and exhibit few cell divisions 24 hr later. After 48 hr, the number of cell divisions increases and disintegration of many cortical cells sets in, leaving lacunae. Root primordia originate from cambial cells near vascular tissues and become recognizable after 3 days. The primordial cells continue to divide and occupy the lacunae. In 4–5 days, the new roots break through the epidermal layers of the auxin-treated segments [2–4].

Several biochemical changes have been recorded during the process of auxin-induced new root formation. These include an increase in RNA, DNA and protein contents, a shift in polysomes, and an increase in the labelling of rRNA, tRNA and DNA-like RNA and ribosomes [4–7]. After 3 days of auxin treatment, a significant increase in the activities of cellulase, pectin esterase and β $(1 \rightarrow 3)$ gluconase have been reported [3]. The increase in cellulase activity is totally abolished if the segments are treated with actinomycin D or azaguanine or puromycin together with auxin [4].

The present study is an attempt to identify specific auxin-induced, early macromolecular changes in the hypocotyls of *Phaseolus vulgaris* during new root formation induced by auxin treatment.

RESULTS

In our laboratory, it was shown that auxin pretreatment of hypocotyls obtained from *Phaseolus vulgaris* seedlings leads to an increase in actinomycin D insensitive protein synthesis within 30 min. Pretreatment of hypocotyl segments with cycloheximide together with auxin greatly delays and diminishes the formation of new roots. In *ca* 2 hr after auxin pretreatment, there is an increase in the synthesis of RNA and actinomycin D sensitive protein synthesis [8].

In the present study, the effects of actinomycin D, cytochalasin B and colchicine on root production by auxin-pretreated hypocotyls were tested. All three drugs completely inhibited the emergence of root primordia when added at any time during the first 36–40 hr after IBA pretreatment, but permitted almost normal root formation when added after this time period (data not presented). In view of the earlier observations on the IBA-mediated increase in total RNA synthesis and RNA-dependent protein synthesis [1], and the fact that actinomycin D and the other two drugs would interfere with transcription and microtubule integrity respectively, the effects of auxin pretreatment on messenger RNA activity and the nature of the protein products synthesized were studied.

The total and poly (A)-containing RNAs were isolated from untreated and IBA-pretreated hypocotyl segments at different periods of time. The messenger activity of total RNA was tested in the wheat germ cell-free system. The messenger RNA content as assayed in terms of translatable activity increases by 2- to 4-fold over a period of 6–48 hr after IBA pretreatment (Fig. 1). It is also seen that at equivalent concentrations, poly (A)-containing RNA preparations from IBA-pretreated hypocotyls at different periods of time uniformly exhibit 1.5-fold higher translational efficiency than the RNA preparations from control segments at the corresponding time periods (Table 1). It thus appears that auxin pretreatment enhances messenger RNA content as well as its translational efficiency.

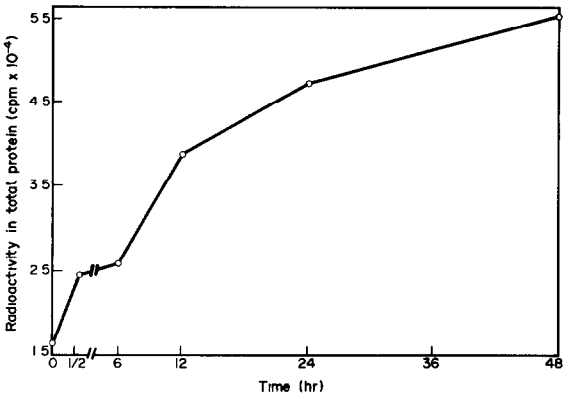

Fig. 1. Effect of IBA pretreatment on the translatable activity of total RNA from *Phaseolus vulgaris*. Total RNA was isolated from the hypocotyls at various stages of development after IBA pretreatment, and 30 μg of RNA from each sample was translated using the wheat germ cell-free system in a total volume of 100 μl. Other experimental details are given in the text.

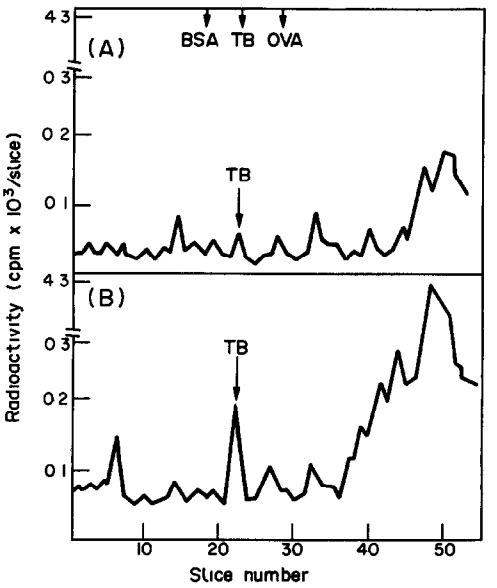

Fig. 2. Radioactivity profile of the cell-free translation products programmed with poly (A)-containing RNA isolated from control and IBA-pretreated hypocotyls of *Phaseolus vulgaris*. Poly (A)-containing RNA from control and IBA-pretreated hypocotyls was isolated at 12 hr. The labelled cell-free products programmed with 5 μg of RNA were precipitated with trichloroacetic acid and analysed on 8% SDS-polyacrylamide slab gels. The gels were sliced into 1.5 mm pieces, digested with H_2O_2, and the radioactivity was measured as given in the text. (A) Control RNA; (B) RNA from IBA-pretreated hypocotyls. Arrows indicate the positions of tubulins.

Table 1. Effect of IBA pretreatment on the translatable activity of poly (A)-containing RNA from *Phaseolus vulgaris*

RNA source	Time after IBA pretreatment	[^{3}H]Leucine incorporated into TCA-precipitable proteins (cpm/5 μg of poly (A)-RNA) I	II	III
Control*	All the time intervals	47 200	39 400	46 000
IBA	30 min	62 000	61 500	60 500
IBA	6 hr	64 800	64 300	63 500
IBA	12 hr	—	70 000	68 000

Poly (A)-containing RNA was isolated from the hypocotyls at various stages of development after IBA pretreatment and 5 μg of RNA from each sample was translated using the wheat germ cell-free system in a total volume of 100 μl.

*Control RNA samples used in the different experiments were isolated from hypocotyls without IBA pretreatment, but kept in Hoagland's medium for different periods of time.

The radioactivity profiles, after SDS-polyacrylamide gel electrophoresis (SDS-PAGE), of the translated products obtained in the wheat germ cell-free system programmed with poly (A)-containing RNA from control and auxin-pretreated hypocotyls are depicted in Fig. 2. There are some qualitative and quantitative differences in the radioactivity profiles obtained in the two cases. In particular, a striking increase in the labelling of the M_r 55 000–58 000 protein among the translation products after IBA pretreatment is evident.

It was suspected that the M_r 55 000–58 000 product could be tubulin on the basis of its mobility in SDS-polyacrylamide gels and in view of the inhibitory effects of cytochalasin B and colchicine treatment on auxin-induced root formation, implicating microtubule involvement. Therefore, attempts were made to determine whether the concentration of the M_r 55 000–58 000 protein showed any change *in vivo* after auxin pretreatment of the hypocotyl segments. From the SDS-polyacrylamide gel analysis of the microsomal protein fraction of the hypocotyl tissues (Fig. 3), it can be seen that there is a striking increase in the content of the M_r 55 000 protein as early as 18 hr after IBA pretreatment. It is known that tubulin has two distinct subunits, α and β, and the change in the β-subunit (M_r 58 000) concentration is perhaps masked by another protein with the same mobility.

To substantiate further that tubulins were synthesized in response to IBA pretreatment, functional assays were carried out. Tubulin polymerization in a polymerization buffer can be measured using the turbidometric assay procedure and the initiation of polymerization is accompanied by GTP breakdown [9, 10]. Tubulin polymerization was thus measured in the presence of microsomal membranes from control and IBA-pretreated hypocotyls, exogenously added tubulin, GTP and other components of the polymerization buffer. Several interesting features are apparent from the results presented in Table 2. The endogenous GTPase activity as well as the tubulin polymerization measured turbidometrically of IBA-pretreated hypocotyl membrane in the tubulin polymerization buffer are significantly higher than those of the control membranes. Exogenous addition of tubulin enhances polymerization strikingly in the case of control membranes. Addition of IBA *in vitro* stimulates tubulin polymerization significantly. The maximum level of polymerization observed in the presence of exogenous tu-

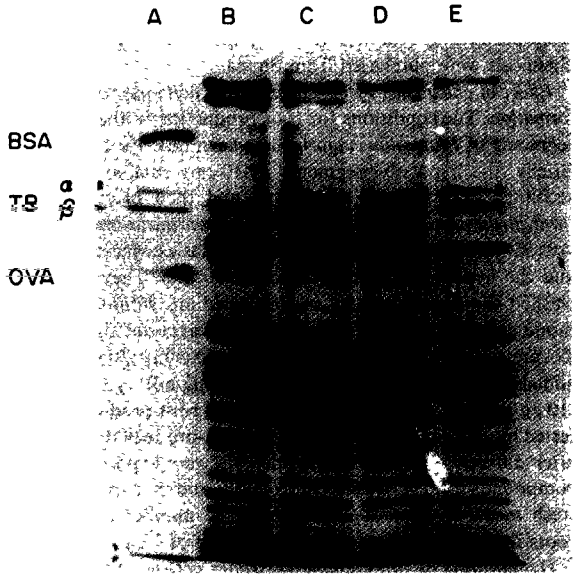

Fig. 3. SDS-Polyacrylamide slab gel electrophoresis of microsomal proteins from control and IBA-pretreated hypocotyls of *Phaseolus vulgaris*. 100 μg of microsomal protein was analysed on 8% SDS-polyacrylamide slab gels and stained with Coomassie blue. (A) Marker proteins; (B) control (hypocotyls incubated for 12 hr but without IBA pretreatment); (C) IBA-pretreated, 12 hr; (D) IBA-pretreated, 24 hr; (E) IBA pretreatment, 36 hr. BSA, bovine serum albumin; TB, tubulin; OVA, ovalbumin.

bulin is the same in control and IBA-pretreated hypocotyl membranes, with the endogenous tubulin polymerization activity of the latter being significantly higher than that of the control membranes.

Finally, tubulin synthesis was measured *in vivo* by labelling the hypocotyl segments for 6 hr (at the 18 to 24th hr) with ^{14}C-labelled *Chlorella* protein hydrolysate. The microsomal membrane fraction was brought into solution and subjected to immunoprecipitation with tubulin antibody as described in the Experimental. The labelled immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and the gel was dried and analysed by autoradiography. Figure 4 clearly indicates that the labelling of the tubulin subunits in the IBA-pretreated hypocotyls is significantly higher than that of the corresponding control segments.

DISCUSSION

The present studies clearly identify tubulin synthesis as one of the early events in IBA-induced root redifferentiation in French bean hypocotyls. Tubulin was identified on the basis of its mobility in SDS-PAGE as well as by functional assays using GTPase activity and turbidometric measurements under conditions favouring tubulin polymerization. The striking increase in tubulin content and enhanced synthesis as demonstrated by radiolabelling and immunoprecipitation, as well as the higher endogenous tubulin polymerization activity of microsomal membranes isolated from IBA-pretreated hypocotyls at 24 hr, implicate tubulin synthesis as an early event in root redifferentiation. It is evident that at least part of the newly synthesized tubulin is still present in an unassembled form (as a monomer pool) at 24 hr after IBA pretreatment, and perhaps microtubule assembly and reorientation leading to new root formation takes place over a period of 36–40 hr after IBA pretreatment. This suggestion is made on the basis that addition of colchicine or cytochalasin B fails to inhibit root production when added after this time.

It can be seen that in the case of IBA-pretreated hypocotyls, radiolabelling followed by immunoprecipitation and SDS-PAGE (Fig. 4) reveals certain higher M_r and lower M_r proteins in addition to tubulins, being labelled more than those of the corresponding control tissue, although the total amount of radioactivity taken for immunoprecipitation is the same in both the cases. While there could be non-specific precipitation during the direct immunoprecipitation procedure employed, it is likely that these proteins are tubulin-associated proteins

Table 2. Effect of IBA on tubulin polymerization

Treatment	GTP hydrolysed (μmol phosphorus/mg protein per 60 min)	Tubulin polymerized *A* at 350 nm
Control		
Microsomes	0.66	0.02
Microsomes + plant tubulin	1.30	0.72
Microsomes + IBA (*in vitro*)	0.72	0.04
Microsomes + plant tubulin + IBA (*in vitro*)	3.30	1.56
IBA-pretreated		
Microsomes	1.12	0.13
Microsomes + tubulin	2.01	0.83
Microsomes + IBA (*in vitro*)	1.86	0.26
Microsomes + plant tubulin + IBA (*in vitro*)	3.12	1.54

Microsomal membranes were isolated from control and IBA-pretreated (24 hr) hypocotyls. Tubulin polymerization was followed by measuring the attendant GTP hydrolysis as well as the change in *A* at 350 nm during the polymerization reaction. When added *in vitro*, IBA was used at a concentration of 10 ng/500 μl. The above experimental details are given in the text.

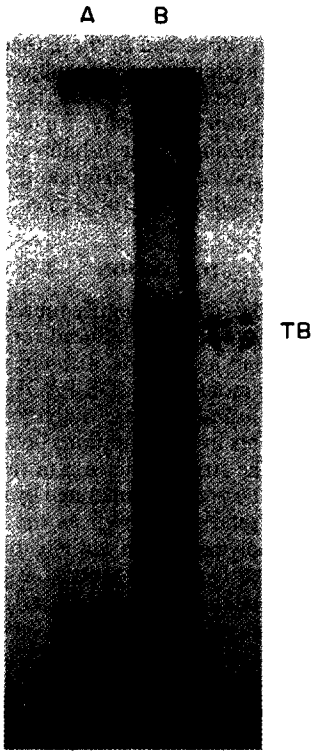

Fig. 4. IBA-induced synthesis of tubulin *in vivo*. The control and IBA-pretreated hypocotyls were labelled for 6 hr (18–24 hr) with 25 μCi of ^{14}C-labelled *Chlorella* protein hydrolysate. The microsomal fraction providing 5×10^{-6} cpm of trichloroacetic acid precipitable radioactivity was taken for immunoprecipitation in both cases. (A) Control; (B) IBA-pretreated. TB, Tubulins (α and β units). The tubulin bands accounted for 0.02 and 0.14% of total microsomal proteins labelled in the case of control and IBA pretreatment, respectively.

such as 'maps' and other proteins, which are involved in the polymerization process [11].

Another significant observation is the effect of IBA in promoting tubulin polymerization when added *in vitro* (Table 2). It appears that the hormone enhances tubulin synthesis as well as having a direct effect on the polymerization process. The effect of IBA on tubulin synthesis is perhaps a transcriptional effect, as indicated by the enhanced amount of M_r 55 000–58 000 protein formed in the wheat germ cell-free system programmed with RNA isolated from IBA-pretreated hypocotyls. In addition, the hormone treatment enhances the total messenger RNA activity significantly and at least some of the proteins coded could be tubulin-associated proteins governing the polymerization and orientation of the microtubule assembly, leading to redifferentiation and adventitious root formation.

EXPERIMENTAL

Seeds of *Phaseolus vulgaris* L. were purchased from the Lalbagh Seed Nursery, Bangalore, India. ^{14}C-Labelled *Chlorella* protein hydrolysate (27 Ci/m atom C) was purchased from the Bhaba Atomic Research Centre, Bombay. [^{3}H]Leucine (105 Ci/mmol) was purchased from the Radiochemical Centre, Amersham, U.K. Oligo dT-cellulose was purchased from Colloborative Research Inc., Waltham, U.S.A. All other biochemicals were purchased from Sigma.

Effect of colchicine, cytochalasin B and actinomycin D on root formation. The conditions for root production in the hypocotyl segments of *Phaseolus vulgaris* have been described in ref. [8]. Briefly, the hypocotyl segments (8–10 cm) obtained from the seedlings were washed thoroughly and then placed in half-strength Hoagland's nutrient medium. Hormone treatment involved the addition of IBA to the medium at a concn of 10 μg/ml and the hypocotyls were exposed for 30 min. They were then washed thoroughly, placed in fresh medium without the hormone, and the number of emerging roots was counted after 72 hr. To prevent bacterial contamination, ampicillin (30 μg/ml) was added. The effects of actinomycin D (10 μg/ml), cytochalasin B (10 μg/ml), and colchicine (2.5 mg/ml) on root production were tested by adding these components to IBA-pretreated hypocotyls after different periods. The hypocotyls were exposed to these compounds for 6 hr, washed thoroughly, and then transferred to fresh media without the inhibitors. The number of emerging roots in each case was counted at the end of 72 hr.

Quantitation of translatable messenger activity. For this purpose, total and poly (A)-containing RNAs were isolated from control and IBA-pretreated hypocotyl segments at different periods of time. Total RNA was isolated from the hypocotyl segments using phenol–$CHCl_3$ extraction procedure [12]. Poly (A)-containing RNA was isolated from total RNA as described in ref. [13].

The messenger activity of the RNA preparations was assayed in the wheat germ cell-free system [14]. The assay mixture in a vol. of 100 μl contained Hepes, 20 μM; GTP, 20 μM; ATP 1 mM; phosphocreatine, 8 mM; $Mg(OAc)_2$, 3 mM; KCl, 100 mM; DTT, 2 mM; amino acid mixture without leucine, 25 μM each; [^{3}H]leucine (105 Ci/mmol), 20 μCi; and S-30 fraction, 1 A_{260} unit of RNA (an optimum concn in the range giving a linear response). The mixture was incubated at 25° for 60 min and 5 μl portions were transferred to Whatman filter paper discs, washed with hot and cold CCl_3COOH (TCA) and then with Et_2O twice, and used for measuring radioactivity incorporated into total proteins. The radioactivity incorporated into the TCA-precipitable protein product at identical concentrations of different RNA preparations is taken as the measure of messenger activity.

Radioactivity profile of the cell-free products. The labelled cell-free products were precipitated with cold TCA (10% w/v in final concn) and washed with Et_2O. The pellet was dissociated with 2% (w/v) SDS containing 10 mM Tris–HCl (pH 6.8), 5% β-mercaptoethanol and 10% (v/v) glycerol at 100° for 2 min. The protein products were analysed using 8% SDS-polyacrylamide slab gels [15]. The gels were stained with Coomassie blue, destained, and then each slot was cut into 1.5 mm slices. The slices were digested with 0.5 ml H_2O_2 (30% soln) at 60° overnight and the radioactivity was measured using 0.5% (w/v) 2,5-diphenyloxazole in Triton–toluene (1:2, v/v) as solvent.

Analysis of membrane proteins. For this purpose, IBA-pretreated and control hypocotyl segments were homogenized in 0.25 M sucrose containing 20 mM KPi buffer, pH 7.2 and 1 mM phenylmethylsulphonyl fluoride. The homogenate was filtered through 2 layers of cheesecloth and the filtrate was centrifuged at 10 000 *g* for 15 min. The supernatant was spun at 105 000 *g* for 90 min and the pellet was used as the microsomal fraction. The proteins were analysed using 8% SDS-polyacrylamide slab gels.

Tubulin polymerization was followed by assaying two parameters. In one case, the attendant GTPase activity was measured in the presence of microtubule reassembly buffer [9]. The reassembly buffer in a total vol. of 500 μl contained 5 mM

morpholine ethane sulphonate (MES) buffer (pH 6.8), 1 mM, EGTA, 0.5 mM $MgCl_2$, 50 mM KCl, 100 μg of microsomal protein fraction, and 20 μg of tubulin obtained from either rat brain or hypocotyl segments. After the reaction mixture was equilibrated in a shaking water bath at 27° for 10 min, the reaction was initiated by the addition of GTP (1 mM in final concn). The reaction was terminated at different time intervals by adding 50% TCA to a final concn of 10%. The tubes were kept at 0° for 30 min, centrifuged, and the supernatant was used for the estimation of Pi content by the method of ref. [16].

In the other method, tubulin polymerization was followed using the turbidometric assay procedure [17]. Phosphocellulose-purified plant tubulin (1 mg/ml) was incubated in MES buffer (100 mM MES, 0.5 mM $MgCl_2$, 1 mM EGTA and 50 mM KCl, pH 6.8) in the presence of microsomal membranes from control and IBA-pretreated hypocotyls at 37°. The reaction was initiated by the addition of GTP and A was measured at 350 nm as a function of time.

Synthesis of tubulin in vivo. After 18 hr of incubation in half-strength Hoagland's medium, both control and IBA-pretreated hypocotyl segments (2–4 cm length) were placed in 5 ml of fresh medium containing 25 μCi of ^{14}C-labelled *Chlorella* protein hydrolysate. After another 6 hr, the segments were thoroughly washed and the microsomal fraction was isolated. The membrane proteins were rendered soluble in 20 mM Tris–HCl (pH 7.4) containing 0.15 M NaCl, 1% Na deoxycholate and 1% Triton X-100. Immunoprecipitation of tubulin was carried out by the addition of a 2-fold excess of anti-tubulin IgG based on the equivalence point. The incubation was carried out at 37° for 30 min and then at 4° overnight. The ppts. were washed with the immunoprecipitation buffer and finally with Tris–NaCl buffer. The immunoprecipitate was then analysed on SDS-polyacrylamide slab gels (8%) as described earlier and subjected to fluorography [18]. The tubulin bands were also sliced, digested with H_2O_2, and the radioactivity was measured.

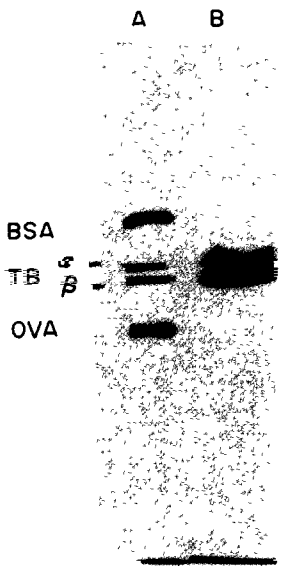

Fig. 5. Purification of tubulin from the hypocotyls of *Phaseolus vulgaris*. Experimental details are given in the text. (A) Marker protein: BSA, bovine serum albumin; TB, tubulins (α and β units); OVA, ovalbumin. (B) Purified plant tubulin (30 μg protein).

Other procedures. Antibodies were raised in rabbits for rat brain ubulin prepared according to the method of ref. [19], with certain minor modifications. The IgG fraction was prepared from the antisera and its cross-reaction with purified plant tubulin and the plant microsomal membrane fraction was ascertained by the Ouchterlony technique. Plant tubulin was isolated from IBA-pretreated hypocotyl segments by the procedure employed for the isolation of rat brain tubulin. An SDS-polyacrylamide slab gel electrophoretic profile of the final preparation is given in Fig. 5. The anti-tubulin IgG was titrated against the plant tubulin. IgG was also titrated against the plant microsomal membrane proteins rendered soluble by procedures described above, and at the equivalence point, 500 μg IgG protein was found to immunoprecipitate 3.2 mg of the microsomal proteins quantitatively. Protein content was measured by the method of ref. [20].

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