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Side Chain Degradation of Epimeric 20-Hydroxy-4-pregnene-3-ones by *Penicillium lilacinum* NRRL 895

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Penicillium lilacinum NRRL 895, capable of cleaving the side chain of progesterone, was shown to carry out the same transformation with 20α- and 20β-hydroxy-4-pregnene-3-one. The 20β-epimer was transformed into 4-androstene-3,17-dione with progesterone as an intermediate. When the 20α-epimer was transformed into 4-androstene-3,17-dione no intermediate could be detected. The two substrates showed different fermentation kinetics. The results thus indicate the possibility of different mechanisms for the pregnane side chain cleavage by *P. lilacinum*. The progesterone C_{17-20}-lyase of *P. lilacinum* was shown to be of a substrate-induced type.

Cleavage of the pregnane side chain is a well-known microbial steroid transformation. Hitherto the only clearly elucidated mechanism for this transformation is the so called biochemical Baeyer-Villiger oxidation of 20-ketones into 17β-acetates, *e.g.* progesterone into testosterone acetate.[1] Since 1960 this pathway has been demonstrated in some fungi [1–7] and very thorough studies of this mechanism have been carried out by Rahim and Sih [2] and by Tamaoki *et al.*[8,9]

One of the first microorganisms which was demonstrated to carry out the side chain cleavage of C_{21}-steroids was the mold fungus *Penicillium lilacinum*.[1] The exact nature of the pregnane side chain cleavage by this microbe is still not quite clear. Sebek and co-workers have proposed a pathway including 20β-hydroxy-4-pregnene-3-one as an intermediate.[10] In a previous study carried out at this laboratory [4] *P. lilacinum* and *Aspergillus flavus* were incubated with progesterone in the presence of the powerful esterase inhibitor diisopropyl-fluoro phosphate (DFP). Under these conditions *A. flavus* gave rise to sizeable amounts of testosterone acetate as the sole product, thereby demonstrating a pathway of the Baeyer-Villiger type. However, *P. lilacinum* produced great amounts of 4-androstene-3,17-dione and traces of testosterone but no testosterone acetate. On account of this one might exclude the Baeyer-Villiger pathway for the latter fungus. The pathway including 17α-hydroxy-progesterone, proposed in that paper, also seems to be ruled out for several

reasons. First, when *P. lilacinum* was incubated with 21-^{14}C-progesterone it was not possible to detect any 17α-hydroxyprogesterone (Carlström, K., unpublished observation). Secondly, 17α-hydroxyprogesterone seemed to be a rather poor substrate for the formation of 4-androstene-3,17-dione compared with progesterone. Thirdly, very few microorganisms have been shown to 17α-hydroxylate progesterone, contrary to mammalian steroidogenic tissues.[1,11]

In order to get more detailed information about the C_{17-20}-lyase activity of *P. lilacinum*, fermentations have been carried out with the epimeric 20-hydroxy-4-pregnene-3-ones and with progesterone. From the results of these experiments the possibility of alternative mechanisms and the role of the 20-hydroxy-4-pregnene-3-ones in the pregnane side chain cleavage by *P. lilacinum* will be discussed.

MATERIALS AND METHODS

Reagents. Progesterone, 4-androstene-3,17-dione, testosterone, and testosterone acetate were obtained from Sigma Chemical Company, St. Louis, Mo., U.S.A. The two epimeric 20-hydroxy-4-pregnene-3-ones were obtained from Ikapharm, Ramat-Gan, Israel.

Beer wort was obtained from Pripp-Bryggerierna AB., Stockholm, Sweden. After dilution with one part of water it had a concentration of 10—11 % dry substance and 150—200 mg α-amino nitrogen per litre.

All other reagents came from commercial sources and were of A.R. grade.

Fermentation technique, kinetic experiment. Penicillium lilacinum NRRL 895 (CSB) was grown on 300 ml of beer wort-distilled water 1:1 at 25°C on a shaking table. After one week of fermentation the cells were harvested, washed twice with 300 ml of 0.01 M sodium phosphate buffer pH 6.9 and resuspended to original density in the same kind of buffer. The cell concentration was 27.0 mg dry weight per ml. The suspension was distributed in 20 ml portions into 100 ml cotton-plugged Erlenmeyer flasks.

Steroid substrates were added in following amounts: Progesterone 42.3 μmoles in 0.5 ml of dimethyl formamide (DMFA), 20α-hydroxy-4-pregnene-3-one 37.5 μmoles in 0.5 ml of DMFA and 20β-hydroxy-4-pregnene-3-one 40.2 μmoles in 0.5 ml of DMFA. After addition of the steroids the cultures were incubated on a shaking table at 25°C. All incubations took place at the same occasion and duplicate flasks were run for each steroid tested. At definite intervals 2.0 ml samples were withdrawn, thoroughly mixed with 6.0 ml of methanol and left at room temperature for 3 h. Then the samples were centrifugated and the supernatants were collected. The cells were washed with 2.0 ml of 75 % aqueous methanol and the washing liquids were combined with the supernatants.

For quantitative gas chromatographic (GLC) analysis, 4.0 ml aliquots were withdrawn from the methanolic solutions. Estrone 3-methyl ether was added as internal standard and the samples were evaporated to dryness under reduced nitrogen pressure. 3 ml of water and 6 ml of ethyl acetate were added to the dry residues and the mixtures were shaken vigorously. After separation of the phases the water layer was removed by suction. The washed samples were evaporated to dryness and acetylated by heating with 0.1 ml of pyridine and 0.5 ml of acetic anhydride at 60°C for 1 h. After evaporation of the acetylating agents the samples were dissolved in ethyl acetate and subjected to GLC analysis. To ensure that no endogenous substance from the cells interfered with the steroids during the analysis, a "mycelium blank" without any steroid was processed exactly as the fermentation samples, but with no internal standard added. No interfering substance could be detected.

The substrate steroid concentration at time 0 was determined using 20 ml portions of phosphate buffer with appropriate amounts of steroid added. These buffer solutions were treated as the fermentation samples. This was done in order to study the binding effect between the substrate steroids and the cells (see discussion, first part).

For qualitative thin layer chromatography (TLC) analysis, 2 ml aliquots of the methanolic solutions were treated exactly as the GLC samples but without addition of the internal standard.

Fermentation technique, induction experiment. The fungus was grown on 300 ml of beer wort-distilled water as described above. After one week of fermentation a 25 ml sample was withdrawn. The sample was washed twice with 25 ml of 0.01 M sodium phosphate buffer pH 6.9 and resuspended to original density in the same kind of buffer solution. From this sample two 10 ml aliquots were transferred into 100 ml cotton-plugged Erlenmeyer flasks and immediately frozen at −22°C.

211 μmoles of progesterone in 2.0 ml of DMFA were added to the remaining culture. The incubation continued on the shaking table. After 4 h a 45 ml sample was withdrawn, washed, resuspended and frozen in four 10 ml portions as described above. After 8 and 12 h 25 ml samples were treated in the same way.

After 48 h all frozen samples were thawed. From two of the 4-h samples 5.0 ml aliquots were withdrawn and treated with 15 and 5 ml of methanol as described for the kinetic experiment. From the 75 % methanolic solutions 2.0 ml aliquots were purified and analyzed by GLC as described for the kinetic experiment.

To all remaining samples 21.1 μmoles of progesterone in 0.2 ml of DMFA were added and the samples were incubated on the shaking table for 5 h. After that the samples were analyzed as described above.

Analytical technique, chromatographic systems. For the GLC a Perkin-Elmer F-11 Gas Chromatograph with a flame ionization detector was used. Quantitative analysis was performed on a 3 m × 3 mm i.d. glass column, packed with 2.5 % OV-1 on AW-DMCS Chromosorb G, 80—100 mesh. Typical conditions: Column temperature 235°C, injection temperature 250°C, and nitrogen carrier gas flow 35 ml/min. Typical retention times are given in Table 1.

For identification purposes additional qualitative GLC was done, using a column of similar dimensions but packed with 2.5 % OV-17 on the same support. Column temperature was 250°C, injection temperature 260°C and nitrogen carrier gas flow 50 ml/min. Some typical retention times are given in Table 1. Also, for identification purposes, TLC was done in two systems: Silica gel G plates 200 × 100 mm with benzene-hexane-ethyl acetate 1:1:1 (vol.) as solvent and Al_2O_3 G plates of the same dimensions with 0.4 % (vol.) ethanol in benzene as solvent. Typical R_F-values are given in Table 1. TLC R_F-

Table 1. TLC R_F-values and GLC retention times. Conditions are given in the text.

Steroid	R_F-value		Relative retention time	(cholestane = 1.00)
	Silica G	Al_2O_3G	OV-1	OV-17
Progesterone	0.38	0.27	1.70	2.10
20α-Hydroxy-4-pregnene-3-one	0.19		1.71	
20α-Acetoxy-4-pregnene-3-one	0.40		2.17	
20β-Hydroxy-4-pregnene-3-one	0.22		1.70	
20β-Acetoxy-4-pregnene-3-one	0.44		2.17	
Testosterone	0.20	0.09	1.10	
Testosterone acetate	0.47	0.30	1.38	
4-Androstene-3,17-dione	0.29	0.22	1.09	1.44
Estrone 3-methyl ether	0.70		0.76	

values are hardly reproducible without rigorous and time-consuming standardization of the procedure. Therefore simultaneous running of standard substances was done beside the fermentation extracts for each plate.

Identification technique, isolation of progesterone and 4-androstene-3,17-dione. From each fermentation the remaining methanolic solutions and the remaining samples from the GLC, containing the greatest amounts of the steroid to be studied, were pooled. After evaporation the samples were purified by ethyl acetate-water partition as described

previously. The samples containing 4-androstene-3,17-dione as main component were acetylated with acetic anhydride and pyridine. After that the samples were subjected to TLC on Silica gel G. The standard spot was visualized with 10 % $SbCl_5$ in chloroform. The corresponding zone in the extract area was scraped off and eluted with ethanol. It was rechromatographed on Al_2O_3 G. The substance isolated from the Al_2O_3 G plate was subjected to qualitative GLC on OV-1 and OV-17 and to gas chromatographic-mass spectrometric (GLC-MS) investigation. The 4-androstene-3,17-dione samples were also tested with the Zimmerman colour reaction. The progesterone, formed from 20β-hydroxy-4-pregnene-3-one, was isolated from the 6 and 9 h samples and the 4-androstene-3,17-dione formed from the same substrate was isolated from the 12.5 and 15 h samples. The 4-androstene-3,17-dione, formed from 20α-hydroxy-4-pregnene-3-one was isolated from the 15 and 21.5 h samples.

Identification technique, GLC-MS and Zimmerman colour reaction. The GLC-MS was performed in an LKB 9000 Gas Chromatograph-Mass Spectrometer. The column used was a 3 m×3 mm i.d. glass tube, packed with 1 % SE-30 on Chromosorb G. Column temperature was 220°C and carrier gas flow 30 ml/min. Retention times were 12 min for 4-androstene-3,17-dione and 16.5 min for progesterone. The mass spectra were recorded on the tops of the peaks.

The Zimmerman colour reaction for the 17-keto group of 4-androstene-3,17-dione was performed according to Vestergaard [12] and the Zimmerman spectra were recorded in a Beckman B spectrophotometer, using 10 mm glass cuvettes.

RESULTS

Kinetic experiment. The time-courses of the three fermentations are given in Fig. 1. All substrates gave 4-androstene-3,17-dione as main product. From progesterone and 20β-hydroxy-4-pregnene-3-one minor amounts of testosterone were formed during the first phase of the fermentation. Only traces of testosterone could be detected when 20α-hydroxy-4-pregnene-3-one was the substrate. Sizeable amounts of progesterone were formed from 20β-hydroxy-4-pregnene-3-one before the appearance of any other steroid metabolite. At the end of the fermentations all three substrates gave rise to a compound with a rather polar behaviour in the TLC ($R_F = 0.05$ on Silica gel G and 0.07 on Al_2O_3 G). It could not be clearly detected by GLC. Formation of testololactone from 4-androstene-3,17-dione by this fungus is well known and it may be possible that this polar metabolite will be testololactone. However, because of its late appearance it was not further investigated.

The formation of testosterone and 4-androstene-3,17-dione from progesterone was confirmed for this particular strain in a previous study at this laboratory.[4] The testosterone formed from the two epimeric 20-hydroxy-4-pregnene-3-ones was tentatively identified by its chromatographic behaviour in the TLC and GLC, as free alcohol as well as 17β-acetate.

The identity of the 4-androstene-3,17-dione formed from the two 20-hydroxy-4-pregnene-3-ones was established by the following criteria: Chromatographic behaviour in the two TLC systems and on the three GLC phases was identical to that of the authentic reference compound. The Zimmerman colour reaction showed an absorption maximum at 520 nm, indicating a 17-keto group. The mass spectra of the two samples from the fermentations and of the authentic reference substance were nearly identical and showed only minor differences in the relative peak intensities. All three spectra had the molecular ion *m/e* 286 as their base peak and strong peaks at *m/e* 244 (M − 42), *m/e* 201 (M − 85) and *m/e* 124 appeared as typical for the Δ^4-3-keto group.[13]

The progesterone formed from the 20β-hydroxy-4-pregnene-3-one was identified by similar chromatographic and mass spectrometric studies. The mass spectra of the progesterone sample from the fermentation and of the progesterone standard were also nearly identical and they had their base peak *m/e* 124 which is typical for progesterone. [14] Other strong peaks were *m/e* 314 (molecular ion), *m/e* 272 (M − 42), *m/e* 244, *m/e* 229 (M − 85), and *m/e* 147.

Induction experiment. The effect of the substrate induction time on the utilization of progesterone by the cells is shown in Table 2. The amount of

Table 2. Effect of induction time on the progesterone C_{17-20}-lyase activity of *Penicillium lilacinum* NRRL 895. Initially added progesterone (after induction)21.1 μmoles.

Induction time, h	Utilized progesterone, μmoles	Formed 4-androstene-3,17-dione, μmoles
0	4.2	Traces
4	7.1	0.9
8	10.0	1.6
12	12.4	2.0

consumed progesterone as well as the amount of formed 4-androstene-3,17-dione increases with increasing induction time, clearly showing the adaptive nature of the progesterone C_{17-20}-lyase of *P. lilacinum*. The samples from the two flasks not receiving the second addition of progesterone showed no steroid peaks in the GLC analysis and therefore the washing procedure for the cells seemed to be sufficient for the removal of the remaining extractable inducer progesterone.

DISCUSSION

The results of the kinetic experiment clearly show that *P. lilacinum* was capable of cleaving the side chain of the three substrates tested. When carrying out a comparative study of this kind it will be necessary to ensure that secondary factors, especially adsorption phenomena, do not influence the results. Therefore the binding effect between the steroid substrates and the cells was studied as described under "materials and methods". After 0.25 h of incubation about 25 % of the substrate was bound to the cells and this bound steroid could not be recovered by repeated washings with 75 % aqueous methanol. This is in accordance with the findings of Hartman and Holmlund.[15] Using a culture of *Penicillium canescens* they found that several steroids were bound to the cells at a varying degree and that complete recovery of the added steroid could be acquired only after lyophilization of the culture. The three substrate steroids used in this study were bound to the same degree. The main product was identical for the three substrates. Thus the influence of binding on the fermentation kinetic experiment might be neglected.

Sebek and co-workers have proposed 20β-hydroxy-4-pregnene-3-one to be an intermediate in the side chain cleavage of progesterone by *P. lilacinum*.[10] However, the results in diagrams I and II on Fig. 1 indicate that progesterone

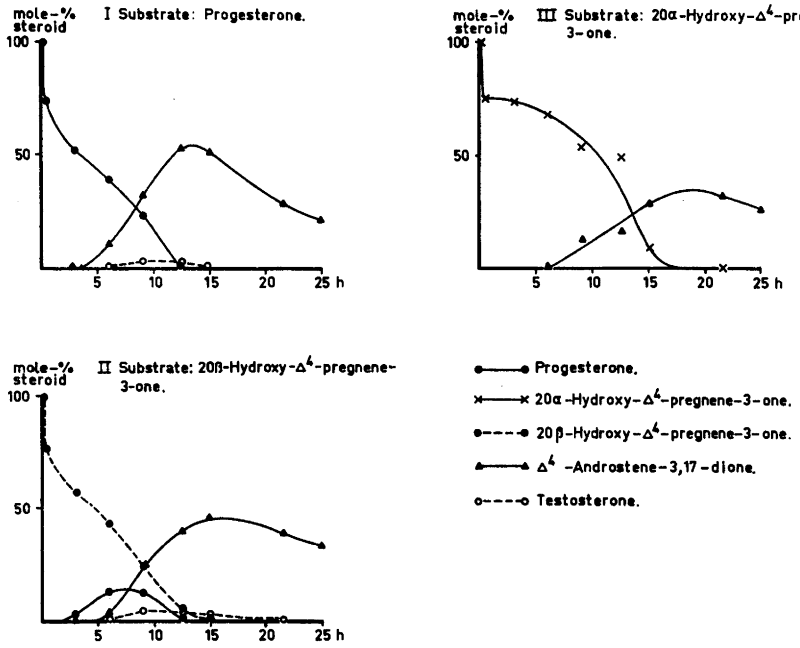


Fig. 1. Time-course of fermentations of progesterone (I), 20β-hydroxy-4-pregnene-3-one (II) and 20α-hydroxy-4-pregnene-3-one (III) with *P. lilacinum*.

is in fact an intermediate in the side chain scission of 20β-hydroxy-4-pregnene-3-one. The best substrate for the formation of 4-androstene-3,17-dione is progesterone. No 20β-hydroxy-4-pregnene-3-one could be clearly detected during this progesterone fermentation but sizeable amounts of progesterone appeared before any other steroid metabolite when 20β-hydroxy-4-pregnene-3-one was the substrate. Furthermore the product 4-androstene-3,17-dione was formed somewhat faster from progesterone than from 20β-hydroxy-4-pregnene-3-one.

The result of the induction experiment shows the adaptive character of the progesterone C_{17-20}-lyase of *P. lilacinum*. Therefore the fermentation patterns for progesterone and for 20β-hydroxy-4-pregnene-3-one can be outlined as I and II in Fig. 2. Of course overlapping between the different steps will occur. It is possible that 20β-hydroxy-4-pregnene-3-one *per se* will induce the lyase activity in the second step of II, but an induction experiment with this steroid will be difficult to interprete because of its rapid transformation into progesterone. Recent experiments with 17α-hydroxy-progesterone and 4-androstene-3,17-dione indicate that these steroids possess induction capabili-

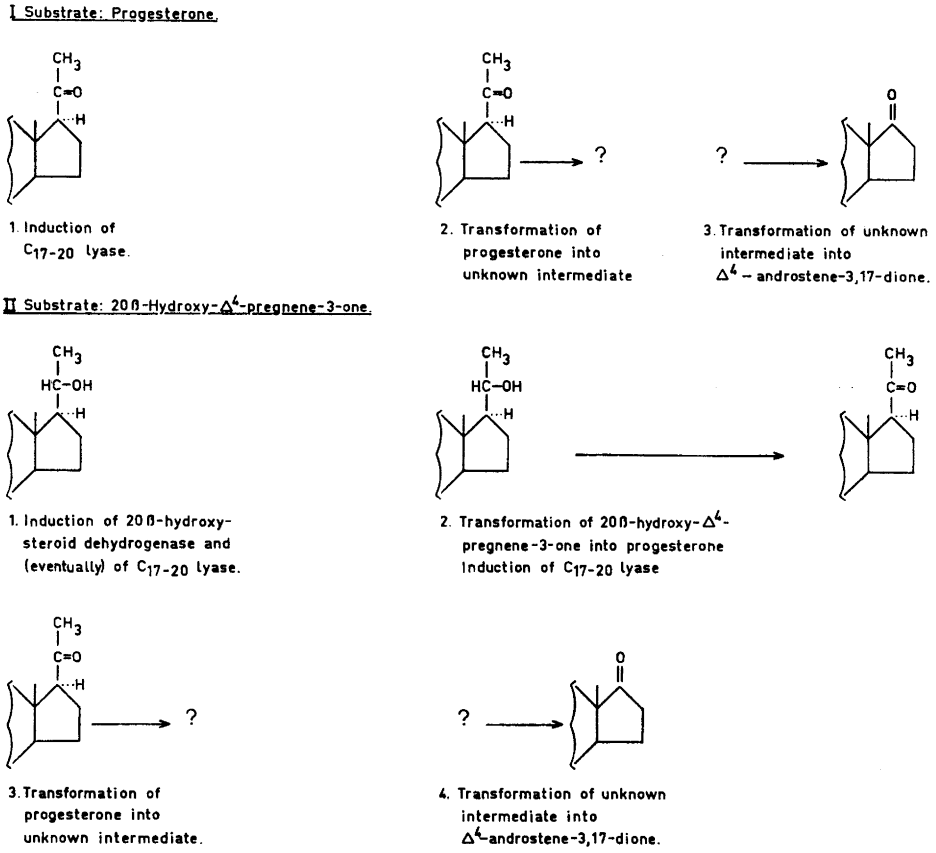


Fig. 2. Pathways for the side chain cleavage of progesterone (I) and 20β-hydroxy-4-pregnene-3-one (II) by *P. lilacinum*.

ties comparable with that of progesterone (Carlström, K., to be published). The adaptive nature of some other microbial steroid lyases has been demonstrated previously by other workers.[2,9,16]

The 20β-hydroxy-steroid dehydrogenase activity of *P. lilacinum* has been demonstrated by Murray and Peterson [17] and by Sebek and co-workers.[10] The latter workers found 20β-hydroxy-4-pregnene-3-one in the early stages of a progesterone fermentation with *P. lilacinum*. From the results presented in this paper this might be considered as a product of this dehydrogenase activity only. When the substrate progesterone concentration decreases this metabolite will probably be dehydrogenated back into progesterone and further metabolized into C_{19} steroids. A recent work by a Soviet group [18] has shown that beside such known factors as aeration and pH the age and the growth phase of the culture also influences the alcohol/ketone ratio in the 20β-hydroxy steroid dehydrogenase reaction of *Mycobacterium globiforme*.

It may therefore be possible that this dehydrogenase activity of *P. lilacinum* also will be dependant of these factors.

The fermentation of 20α-hydroxy-4-pregnene-3-one seems to follow a pattern, quite different from that of the two other substrates. The substrate disappearance curve is considerably less steep and the product 4-androstene-3,17-dione is formed at a later time and in a noticeably lower yield. No intermediate between the substrate and the product could be detected neither by TLC nor by GLC.

These findings indicate the possibility of a pathway different from that of 20β-hydroxy-4-pregnene-3-one/progesterone. It may therefore be assumed that at least two different pathways will exist for the pregnane side chain cleavage by *P. lilacinum*.

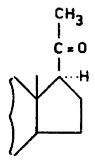
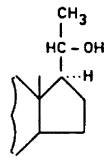
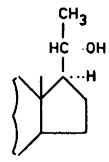
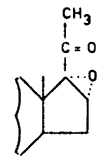
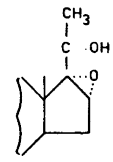
Organism.	Reference.					
Septomyxa affinis.	6	Degradation (BV)	No degradation.	Degradation.		
Cylindrocarpon radicicola.	3	Degradation (BV).	No degradation		Degradation	No degradation
Aspergillus ochracaeus.	7	Degradation (BV)	No degration.	No degradation.		
Penicillium lilacinum.	This paper.	Degradation	Degradation.	Degradation.		

Fig. 3. Summary of the results from different studies of the microbial side chain degradation of 20-hydroxy-4-pregnene steroids. (BV) means Baeyer-Villiger oxidation of 20-ketones into 17β-acetates.

Recent studies of the role of 20-hydroxy-4-pregnene steroids as substrates for microbial C_{17-20}-lyases have been undertaken by Rahim and Sih,[2] Singh and Rakhit,[5] and Tan and Smith.[6] Their results together with those of the present study are summarized in Fig. 3. Besides *P. lilacinum*, *S. affinis* was capable of cleaving the side chain of 20α-hydroxy-4-pregnene-3-one and appearantly following a pathway different from that of progesterone. *S. affinis* did not metabolize the 20β-epimer. *C. radicicola* and *A. ochracaeus* did not metabolize any of the 20-hydroxy-4-pregnene steroids. While the mechanism for the side chain degradation of progesterone by *A. ochracaeus*, *C. radicicola*, and *S. affinis* was shown to follow the Baeyer-Villiger pathway, the corresponding reaction of *P. lilacinum* is rather obscure. On account of the results presented in this paper one may exclude the 20-hydroxy-4-pregnene-3-ones as intermediates in this reaction. The missing link between progesterone and the C_{19}-steroids still remains to be demonstrated.

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