Effects of cytochrome P450 inhibitors and of steroid hormones on the formation of 7-hydroxylated metabolites of pregnenolone in mouse brain microsomes

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Abstract

Hydroxylations of pregnenolone (PREG) at the 7α- and 7β-positions have been reported in numerous murine tissues and organs and responsible cytochrome P450 (CYP) species await identification. Using thin layer chromatography and gas chromatography–mass spectrometry, we report identification of 7α-hydroxy-PREG and 7β-hydroxy-PREG metabolites produced in mouse brain microsome digests and kinetic studies of their production with apparent K_M values of 0·5 ± 0·1 µM and 5·1 ± 0·6 µM for 7α- and 7β-hydroxylation respectively. Investigation of CYP inhibitors and of steroid hormone effects on both 7α- and 7β-hydroxylations of PREG showed that: (i) different CYP were involved in 7α- and 7β-hydroxylation of PREG because solely 7α-hydroxylation was extensively inhibited by metyrapone, α-naphthoflavone, ketoconazole and 3β-hydroxysteroids, (ii) CYP 1A2, 2D6, 2B1 and 2B11 were not responsible for 7α- and 7β-hydroxylation of PREG because respective specific inhibitors furafylline, quinidine and chloramphenicol triggered no inhibition, (iii) CYP 1A1 was responsible for only part of the 7β-hydroxylation of PREG because use of α-naphthoflavone, which inhibits specifically CYP 1A1, did not suppress entirely 7β-hydroxylation, while ketoconazole, metyrapone and antipyrine, which do not inhibit CYP 1A1, decreased part of the 7β-hydroxylation, (iv) 7α-hydroxylation of PREG may be shared with other 3β-hydroxysteroids such as isandrosterone and 5-androstene-3β,17β-diol which were strong inhibitors, but not with dehydroepiandrosterone which was a non-competitive inhibitor as weak as 3-oxosteroids, and (v) 7β-hydroxylation of PREG was not markedly changed by other steroids. Taken together, these findings will be of use for identification of the CYP species responsible for 7α- and 7β-hydroxylation of PREG and for studies of their activities in brain.


Introduction

For a long time pregnenolone (3β-hydroxy-5-pregnen-20-one, PREG) was considered as a physiologically inactive steroid and solely as a precursor for dehydroepiandrosterone (DHEA), progesterone and glucocorticoid hormones. Cholesterol is converted to PREG by the cytochrome P450 scc in mitochondrial membranes of rat brain oligodendrocytes and PREG accumulates in rat brain independently of gonads and adrenals (Corpechot et al. 1983, Hu et al. 1987, Jung-Testas et al. 1989a). Cytochrome P450 scc was detected in brain by immunocytochemistry (Le Goascogne et al. 1987), and the enzymatic reaction has been demonstrated to take place in brain mitochondria (Walther et al. 1987), and in mitochondria from cultured oligodendrocytes (Hu et al. 1987). It was also reported that brain can convert PREG into progesterone in vitro (Jung-Testas et al. 1989b) and that memory-enhancing effects were obtained with PREG and its metabolites (Flood et al. 1992). In addition, both PREG and DHEA are irreversibly hydroxylated at the 7α and 7β position by mouse and rat brain preparations (Akwa et al. 1992, Morfin & Courchay 1994, Doostzadeh & Morfin 1996). 7α-Hydroxylated and 7β-hydroxylated derivatives of PREG and DHEA were shown to be more efficient than their parent precursors at increasing the immune response in mice (Morfin & Courchay 1994). It was suggested that their action was derived from anti-glucocorticoid potencies (Morfin et al. 1994, Padgett & Loria 1994). These findings implied that the cytochromes P450 (CYP) responsible for 7α- and 7β-hydroxylation of PREG were key enzymes for the regulation of glucocorticoid action and of immune processes.

Specificity of brain and pituitary CYP species for 7α-hydroxylation of 3β-hydroxysteroids was reported (Guiraud et al. 1982, Warner et al. 1989, Akwa et al. 1992). None of the other 3-ketosteroids and 3α-hydroxysteroids tested were hydroxylated at the 7α-position. Few studies were concerned with 7β-hydroxylation of PREG and we demonstrated recently that yeast-expressed mouse and
human CYP 1A1 transformed PREG into 7β-OH-PREG (Doostzadeh et al. 1996, 1997). None of the other yeast-expressed CYPs was responsible for 7-hydroxylation of PREG.

Since many molecules have been identified as inhibitors of several CYP species and since physiologically active steroid hormones may interfere with 7α- and 7β-hydroxylation of PREG, effects of several CYP inhibitors and of steroid hormones were tested on PREG transformations by mouse brain microsomes. Investigations were aimed at three purposes: (i) to relate inhibitory effects with specifically inhibited CYPs, (ii) to identify inhibitors which should be used in studies of PREG 7α- and 7β-hydroxylating CYPs of brain microsomes, and (iii) to identify possible specific structures which are required for 7-hydroxylation of steroid hormones.

Materials and Methods

Animals

Six-week-old male mice of the C57BL/6 strain (19–21 g) were purchased from Ifa–Credo (L’Arbresle, 69, France). Animals were caged for 1 week in the laboratory’s animal room with food and water freely available prior to killing. Animals were kept with a ratio of 12 h light:12 h darkness, with lights on 0800 h to 2000 h, and the temperature was set at 20–21 °C.

Preparation of tissue microsomes

Mice were killed by decapitation. Brains were collected in ice-cold PBS containing 20% glycerol, 1 mM EDTA, 0·1 mM pepstatin, and 0·1 mM phenylmethylsulfonyl fluoride. Brains were placed in a dish on ice and were cut into small pieces with scissors. Homogenization was carried out at 4 °C in three volumes of the collection medium with 10 up and down strokes in a glass–teflon homogenizer set at 20–21°C. The microsomal pellet was finally suspended in 67 mM Na2HPO4/KH2PO4 (pH 7·4) containing 0·25 mM sucrose, 20% glycerol, 1 mM EDTA, 0·1 mM pepstatin, 0·1 mM phenylmethylsulfonyl fluoride, and 2 µM leupeptin. Protein concentrations were measured according to Lowry et al. (1951) with the Folin–Ciocalteau reagent at 650 nm. The prepared microsomal fractions were stored at −80 °C prior to the extraction procedure.

Steroids

[4-14C]DHEA (51 mCi/mmol) was purchased from NEN (Du Pont de Nemours, Les Ulis, France). [20-14C]PREG (55 mCi/mmol) was produced (custom synthesis CFQ 6416) by Amersham International (Cardiff, UK). The radiochemical purity of both 14C-labeled steroids was periodically controlled by crystallization to constant specific radioactivity of a carrier-diluted portion. Non-radioactive steroids were purchased either from Sigma–Aldrich (St Louis, MO, USA) or from Steraloids (Wilton, NH, USA). 7α-Hydroxy-PREG, and 7β-hydroxy-PREG were prepared as previously reported (Akwa et al. 1992).

Reagents

Solvents, salts of analytical grade and chromatographic supplies were purchased from Merck (Darmstadt, Germany). PBS, sucrose, EDTA, NADPH, pepstatin, leupeptin, phenylmethylsulfonyl fluoride, bis-(trimethylsilyl) trifluoroacetamide, α-naphthoflavone, chloramphenicol and metyrapone were obtained from Sigma–Aldrich. Ketoconazole, quinidine, antipyrine and furafylline were from RBI (Natick, MA, USA).

Incubations

Solutions in ethanol of the radiolabeled steroid substrate (0·5 nmol) and those of the different molecules tested (from 0 to 150 µM) were dried under vacuum (Speed-vac concentrator, Savant Instrument Corp., Hicksville, NJ, USA) at the bottom of 10 ml glass tubes. Incubations were carried out in a total volume of 1 ml. The buffer was 67 mM Na2HPO4/KH2PO4 (pH 7·4) containing 1 mM EDTA. Buffer was first added and followed by 0·5 mg of microsomal protein. The tubes were vortex-mixed at room temperature and warmed at 37 °C. Incubations in a shaking water bath were started by addition of NADPH for a final concentration of 0·5 mM. The tubes were left open during the incubation period. Unless otherwise stated, a 30 min incubation time was used because preliminary tests with mouse brain microsomes showed a linear increase of the produced metabolites during a 0–30 min period (zero order kinetics). Incubations were stopped by addition of 2 ml acetone followed by 2 ml ethyl acetate. The tubes were stored at −80 °C prior to the extraction procedure.

Extraction and separation of radiosteroids

Acetone–ethyl acetate supernatants were collected from the frozen incubation mixtures and the thawed water phase was extracted twice with 2 ml ethyl acetate. Recoveries in the organic phase were in the 92–99% range. The extracts were dried under vacuum, taken up in 0·1 ml ethyl acetate, and applied to silica-gel F254 thin-layer plates (Merck) for thin-layer chromatography (TLC). Reference steroids were chromatographed on separate lanes. The plates were developed once in ethyl acetate. In
this system, the reference fronts ($R_f$) of 7α-hydroxy-PREG, 7β-hydroxy-PREG and PREG were 0.28, 0.53 and 0.82 respectively.

Quantification of radioactive metabolites
The relative amounts of [14C]steroids were measured by scanning the TLC plates with a Multitrace-master model LB-2832 instrument (Berthold Analytical Instruments, Nashua, IL, USA). Other radioactivity measurements were carried out in 5 ml of Aquasafe 300 scintillation fluid (Zinsser Analytic, Maidenhead, UK) with an Intertech-nique SL-4000 liquid scintillation spectrometer (Kontron, St Quentin en Yvelines, France).

Formation of derivatives and gas chromatography–mass spectrometry (GC-MS)
The trimethylsilyl ether (TMS) derivatives were prepared before GC-MS by reacting the dried steroids with 0.1 ml bis-(trimethylsilyl) trifluoroacetamide at 60 °C for 30 min. Appropriate dilutions were made with n-hexane. GC-MS analysis used a Hewlett-Packard HP 5890 series II gas chromatograph coupled with an HP 5989A mass spectrometer. GC was carried out on a fused silica capillary column (12 m, 0.2 mm internal diameter) coated with bonded HP-1 stationary phase. Splitless injection of TMS derivatives of steroid references and of samples used helium (5 ml/min) as a mobile gas phase. Oven temperature was programmed from 100 to 230 °C with a 10 °C/min increment. The column was directly coupled to the quadrupole mass spectrometer ionization chamber where the source was set at 300 °C and the energy of bombarding electrons at 70 eV. Ion detection and mass spectra were recorded and processed by model B1500A # AA0 Hewlett-Packard software on a HP Apollo series 400 computer.

Statistical analysis
Linear regression analysis and computation of standard errors used the least square method with the ‘Biostal’ program. An analysis of variance procedure was used for statistical comparisons.

Results
Identification of 7-hydroxylated metabolites of PREG
Brain microsomes were incubated in ten tubes with carrier-diluted (10:1) [20-14C]PREG for 60 min and extracts were pooled. After TLC separation, radioactively labeled steroids were recovered at the $R_f$ of reference 7α-hydroxy-PREG and 7β-hydroxy-PREG were transformed into diTMS derivatives and analyzed by GC-MS.

At the retention time of authentic 7α-hydroxy-PREG diTMS and of authentic 7β-hydroxy-PREG diTMS, characteristic ion fragment patterns included absence of the molecular ion at $m/z$ 476 ($M^+$), intense $m/z$ 386 ($M^--90$), small amounts of $m/z$ 296 ($M^-290$) and $m/z$ 143 ($C_7H_15OSi^+$) and both $m/z$ 129 and $m/z$ 73 ions currently found in mass spectra with TMS derivatives of authentic 3β-hydroxy-C$_{21}$-steroids. At the same retention times the same ion fragment patterns were obtained for 7α-hydroxy-PREG and 7β-hydroxy-PREG metabolites isolated from digests of mouse brain microsomes (Table 1).

Kinetic parameters of the 7α- and 7β-hydroxylation of PREG
Mouse brain microsomes were incubated with increasing concentrations of [20-14C]PREG for 30 min. Quantities and production rates of 7α- and 7β-hydroxylated metabolites were deduced from experiments repeated four times. Computations, using both the double reciprocal plot and the Eadie–Scatchard plot, gave apparent $K_M=0.5 \pm 0.1 \mu M$ and $V_{max}=5.17 \pm 0.24$ pmol/min per mg protein for the 7α-hydroxylating system, and gave apparent $K_M=5.1 \pm 0.6 \mu M$ and $V_{max}=2.59 \pm 0.19$ pmol/min per mg protein for the 7β-hydroxylating system.
Effects of cytochrome P450 inhibitors on PREG 7-hydroxylation

Effects of cytochrome P450 inhibitors were tested in triplicate incubations of 0·5 µM [20-14C]PREG with NADPH-fortified brain microsomes. Each experiment was repeated three times. Inhibitor concentration range was 5–150 µM (Fig. 1).

7α- and 7β-hydroxylation of PREG were not significantly changed by chloramphenicol, furafylline or quinidine (P>0·3), but were extensively inhibited by antipyrine (P<0·001). Ketoconazole was a weak inhibitor of the 7β-hydroxylation (P<0·02) and a strong inhibitor of the 7α-hydroxylation reaction (P<0·001). Metyrapone and α-naphthoflavone markedly inhibited 7α-hydroxylation (P<0·001) but were less efficient on 7β-hydroxylation (P<0·01) and only at low concentrations (5–25 µM). With larger concentrations (50–150 µM), 7β-hydroxylation returned to inhibitor-free levels (Fig. 1B).

Effects of steroid hormones on PREG 7-hydroxylation

Effects of steroid hormones were tested in triplicate incubations of 0·5 µM [20-14C]PREG with NADPH-fortified brain microsomes. Each experiment was repeated three or four times. Steroid concentration range was 5–150 µM (Fig. 2).

Inhibition of the 7α-hydroxylation reaction was obtained with steroids which share with PREG a 3β-hydroxylated structure. Thus, extensive inhibition (P<0·001) was obtained with 3α-hydroxy-5α-androstan-17-one; 5-androstenediol = 5-androsten-3β,17β-diol; dehydroepiandrosterone = 3β-hydroxy-5-androsten-17-one; 4-androstenedione = 4-androsten-3,17-dione.
Inhibition of PREG 7α-hydroxylation by DHEA was studied further and several concentrations of [20-14C]PREG (2.5, 5 and 10 μM) were incubated with brain microsomes in the absence or in the presence of 10–50 μM DHEA. It was confirmed that DHEA induced no change in [20-14C]7β-hydroxy-PREG production rates, but production rates of [20-14C]7α-hydroxy-PREG were significantly decreased by DHEA. Use of the double reciprocal plot with data from three experiments showed decreased Vmax and unchanged Km, providing evidence for a non-competitive inhibition of PREG 7α-hydroxylation by DHEA, and use of the Dixon plot confirmed this conclusion and permitted measurement of Ki at 57.6 ± 6.1 μM (Fig. 3).

Complementary to this finding, it was obvious to test whether PREG inhibited 7α-hydroxylation of DHEA. Therefore, several concentrations of [4-14C]DHEA (0.5, 1 and 2 μM) were incubated with brain microsomes in the absence or in the presence of 0.5–8 μM DHEA. No change in production rate of [4-14C]7β-hydroxy-DHEA was obtained, but velocities of [4-14C]7α-hydroxy-DHEA were significantly decreased by PREG. Use of the double reciprocal plot and of the Dixon plot showed decreased Vmax and unchanged Km, which characterized a non-competitive inhibition by PREG of DHEA 7α-hydroxylation and allowed a Ki measurement of 2.0 ± 0.3 μM (data shown elsewhere).

All tested 3-oxosteroids significantly inhibited the 7α-hydroxylation of PREG (P<0.02) but were weaker inhibitors than isoandrosterone and 5-androstene-3β,17β-diol. Increasing inhibitory potencies were found for corticosterone, testosterone, androstenedione and progesterone (Fig. 2A). Test of these steroids on 7β-hydroxylation of PREG showed no marked change due to corticosterone, testosterone or androstenedione but a transient increase of 7β-hydroxy-PREG production when progesterone was used at 25 and 50 μM (P<0.03) (Fig. 2B).

Discussion

The NADPH-dependent 7α- and 7β-hydroxylations of PREG by mouse brain microsomes have been previously reported (Morfin & Courchay 1994, Doostzadeh & Morfin 1996, Doostzadeh et al. 1997). Based on GC-MS identifications, our present findings ascertained that 7α-hydroxy-PREG and 7β-hydroxy-PREG were produced from PREG by brain microsomal enzymes. Since both 7α- and 7β-hydroxylated derivatives of PREG were produced by several enzyme systems contained in brain microsomes, each apparent Km value derives from relative proportions of these microsomal enzymes. We report now for PREG 7α-hydroxylation an apparent Km value 10 times lower with mouse brain microsomes (0.5 ± 0.1 μM) than the apparent Km obtained under identical conditions with rat brain microsomes (4.4 ± 3.2 μM) (Akwa et al. 1992). Km data for 7β-hydroxylation in rat brain microsomes were not available for comparison, but difference in
7α-hydroxylation appears to be related to the change of species, since our measurements of apparent \( K_M \) in mouse brain microsomes are consistent with those reported in other mouse tissue microsomes (Doostzadeh & Morfin 1996).

The first conclusion which may be drawn from previous work and from our present findings with CYP inhibitors and 3β-hydroxysteroids is that different CYPs are responsible for 7α- and 7β-hydroxylation of PREG by mouse brain microsomes. Our previous report of CYP 1A1 detection in brain microsomes and of its ability to transform PREG into 7β-hydroxy-PREG without any production of 7α-hydroxy-PREG (Doostzadeh et al. 1996), and our findings that metyrapone, α-naphthoflavone, ketoconazole and all steroids tested inhibited 7α-hydroxylation to a much larger extent than 7β-hydroxylation support this conclusion and infer that the α-naphthoflavone inhibition of PREG 7α-hydroxylation in brain may be a characteristic of the responsible CYP which is not CYP 1A1.

The specificity of several inhibitors towards CYP species allows identification of CYPs which were obviously not involved into PREG 7α- and 7β-hydroxylation. Thus, furafylline which is known as a specific inhibitor of CYP 1A2 (Kunze & Trager 1993, Tassaneeyakul et al. 1993), quinidine which inhibits specifically CYP 2D6 (Halpert et al. 1994, Newton et al. 1994), and chloramphenicol which inhibits specifically CYP 2B1 and 2B11 (Ciaccio et al. 1987), had no effect on either 7α- or 7β-hydroxylation of PREG. Therefore, our second conclusion is that CYPs 1A2, 2D6, 2B1 and 2B11, which had already been detected in brain (Ravindranath & Boyd 1995), are not involved in the 7α- and 7β-hydroxylation of PREG by mouse brain microsomes. This second conclusion is also supported by our previous report with yeast-expressed human CYPs which showed that CYPs 1A2 and 2D6 did not hydroxylate PREG at the 7-position (Doostzadeh et al. 1997).

Our third conclusion may be drawn from our finding that α-naphthoflavone, a known inhibitor of CYPs 1A1 and 1A2 (Tassaneeyakul et al. 1993, Chang et al. 1994), did not inhibit 7β-hydroxylation to the extent which was to be expected if CYP 1A1 was solely involved in 7β-hydroxylation of PREG (Doostzadeh et al. 1996). This indication that, second to CYP 1A1, another CYP species may be responsible for 7β-hydroxylation of PREG, is also supported by the fact that ketoconazole, metyrapone and antipyrine, which are not specific inhibitors of CYP 1A1, decreased only part of PREG 7β-hydroxylation. Therefore, it may be proposed that ketoconazole, metyrapone and antipyrine inhibited a CYP different from CYP 1A1, and that in such case the remaining production of 7β-hydroxy-PREG was due to CYP 1A1.

If several leads exist now for identification of the CYP species responsible for 7β-hydroxylation of PREG, much less information on 7α-hydroxylation of PREG is presently available. Our studies of steroid hormone effects on 7α-hydroxylation bring out the fourth conclusion, which concerns the specificity of 7α-hydroxylation towards steroid substrates. Our evidence for weak interferences brought about by several 3-oxosteroids may be explained by previous work which showed that, in contrast to PREG, DHEA, 5α-androstan-3β,17β-diol and isoandrosterone, neither testosterone nor androstenedione was 7α-hydroxylated in rat brain microsomes (Akwa et al. 1992). Therefore, competitive inhibition of PREG 7α-hydroxylation by the 3-oxosteroids tested in mouse brain microsomes could be excluded. In marked contrast, use of 5-androsten-3β,17β-diol and of isoandrosterone, which share with PREG a 3β-hydroxylated structure, led to extensive inhibition of PREG 7α-hydroxylation. Specificity of 7α-hydroxylation towards 3β-hydroxysteroids may be deduced from these findings and from previous work which proved 7α-hydroxylation of DHEA, PREG, 5α-androstan-3β,17β-diol, isoandrosterone, 3β-hydroxy-5α-pregnan-20-one-20-one and 27-hydroxycholesterol substrates by murine brain and pituitary microsomes (Guiraud et al. 1982, Warner et al. 1989, Akwa et al. 1992, Strömstedt et al. 1993, Zhang et al. 1995).

Our present findings demonstrated that DHEA was a non-competitive inhibitor of PREG 7α-hydroxylation (\( K_i=57.6 ± 6.1 \mu M \)) and that PREG was a non-competitive inhibitor of DHEA 7α-hydroxylation (\( K_i=240 ± 0.3 \mu M \)). Non-competitive inhibition of 3β-hydroxysteroid 7α-hydroxylation has already been described with estradiol-17β on PREG 7α-hydroxylation in rat brain microsomes (\( K_i=4.5 \mu M \)) (Akwa et al. 1992) and of 5α-androstan-3β,17β-diol 7α-hydroxylation in rat prostate (\( K_i=5 \mu M \)) (Isaacs et al. 1979). Indeed, 7α-hydroxylase from rat prostate microsomes and from rat brain microsomes appeared identical for 5α-androstan-3β,17β-diol (Warner et al. 1989) and for 3β-hydroxy-5α-pregnan-20-one (Strömstedt et al. 1993). Nevertheless, among 3β-hydroxysteroids tested for their inhibiting potencies on 7α-hydroxylation of 3β-hydroxy-5α-pregnan-20-one by a rat prostate CYP reconstituted system, PREG was one of the least efficient ones (Strömstedt et al. 1993). In addition to these facts, it is already known that different CYPs are responsible for 7α-hydroxylation of cholesterol in liver and of 27-hydroxycholesterol in liver and brain (Martin et al. 1993, Zhang et al. 1995), and our findings that 7α-hydroxylation of PREG was much more inhibited by isoandrosterone and 5-androsten-3β,17β-diol than by DHEA support the proposal made by Zhang et al. (1995) that several CYPs may be responsible in brain for the production of 7α-hydroxylated metabolites of 3β-hydroxysteroids. Nevertheless, this suggestion does not exclude formally other possibilities such as two enzymes acting together on PREG and DHEA, but with different affinities for the two substrates, or a two-step reaction involving a carrier protein for the substrate. In order to solve these problems,
further studies will need to be carried out with several 3β-hydroxysteroid substrates and a reconstructed 7α-hydroxylation system containing a responsible CYP species.

At present, it is possible that one CYP responsible for 7α-hydroxylation of 3β-hydroxysteroids has been identified, since recent evidence for a novel (hct-I) CYP expressed primarily in rat and mouse brain provided its full cDNA and amino acid sequences (Stapleton et al. 1995). Interestingly, hct-I amino acid sequence analysis revealed a 39% homology with liver cholesterol 7α-hydroxylase and that it contained the steroidogenic domain of other steroid-metabolizing CYPs.

The hct-I translated protein is now awaited for reconstitution of its monoxygenase activity and studies of steroid substrate specificity and of hydroxylated metabolites produced. In those experiments, data reported in this paper will necessarily be used when the required comparisons with native brain microsomes are performed.

Final identifications may lead to the finding that, including hct-I, several CYP species are devoted to the specific 7α- and 7β-hydroxylations of circulating 3β-hydroxysteroids in brain where their metabolic products may contribute to regulation of neuronal function and immunity.

Acknowledgements

This work is contained in Jaleh Doostzadeh’s DSc thesis of the University of Paris 6 which was defended on November 27 1996. This work was supported by a grant from Pasteur–Mérieux Sérums and Vaccins (Marcy l’Étoile, 69, France) and by a fellowship to J Doostzadeh from the Mérieux Foundation (Lyon, France). The authors are grateful to Professor P Beaune and his group for valuable discussion and to Dr Ph Manchon for his help with the ‘Biostal’ program for statistical analysis of the data. The authors wish to thank Professor F Dray (Vitasterol) and Professor J F Desjeux (Chairman of Biology, CNAM) who made this work possible through financial support and by providing equipment and laboratory space.

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Received 11 April 1997

Accepted 27 May 1997