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Journal of Physiology (1995), **486.2**, *pp.*517–522

Acceleration of puberty onset in female mice by male urinary proteins

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1. Puberty onset in female mice is accelerated by exposure to conspecific adult male urine, which acts through the vomeronasal organ and the accessory olfactory system. A distinctive component of adult male mouse urine is the major urinary protein complex (MUP), which is a lipocalin; it has a hydrophobic pocket that binds small endogenous volatile molecules. The MUP gene family also codes for a hexapeptide, which has four residues in common with the *N*-terminal region of MUP.

2. MUP, the volatiles bound to MUP and the MUP-related hexapeptide have been tested for the induction of puberty acceleration by measuring the increase in uterus weight related to the first pro-oestrus phase. MUP, together with its bound volatiles, induces puberty acceleration. Its activity is retained even when the volatiles have been removed either by organic extraction or competition displacement with a high-affinity ligand.

3. MUP-related hexapeptide also induces puberty acceleration in female mice. In contrast, the odorants bound to MUP do not exert this effect.

4. It is proposed that the vomeronasal organ contains receptors that recognize the short *N*-terminal consensus sequence, *N*-Glu-Glu-Ala-X-Ser (where X is a polar residue), common to both MUP and the hexapeptide.

Puberty onset is a complex phenomenon of neural, hormonal and environmental interactions. In mice (*Mus musculus*), it has been shown that exposure of prepubertal females to conspecifics affects the timing of puberty onset. In particular, these effects can only be triggered by exposure to the conspecific urine, suggesting that they are related to the peculiar chemical composition of the stimuli. Most priming pheromones elicit physiological effects by acting on the vomeronasal organ, since lesions of the vomeronasal organ or the accessory olfactory bulb prevent pheromonal effects, while lesions of the main olfactory system do not impair pheromonal communication (Kaneko, Debski, Wilson & Whitten, 1980; Keverne, 1983). Accessory olfactory system stimulation, in turn, increases both dopaminergic activity in the hypothalamus (Dluzen, Guan & Vandenbergh, 1992), and luteinizing hormone (LH) release from the hypophysis, so that ultimately reproductive behaviour is affected (Bronson & Maruniak, 1976).

Sexual maturation of female mice can be either delayed by volatile substances obtained from the urine of females that are housed together (Novotny, Jemiolo, Harvey, Wiesler & Marchlewska-Koj, 1986), or accelerated by components of adult male urine (Vandenbergh, 1969; Vandenbergh, Whitsett & Lombardi, 1975). However, male pheromones are thought not to be volatile since they act through the accessory olfactory system (Kaneko *et al.* 1980). A partial purification of the puberty-accelerating pheromone from male mouse urine has indicated that it could be bound to a protein or part of a protein (Vandenbergh *et al.* 1975; Vandenbergh, Finlayson, Dobrogosz, Dills & Kost, 1976), but the active substance, probably a peptide, has never been characterized further. The pheromone appears to be androgen dependent, since urine from castrated or subordinate males cannot induce puberty acceleration (Vandenbergh *et al.* 1975; Lombardi & Vandenbergh, 1977).

Several substances that have been identified in the urine from adult male mice are dependent upon high levels of testosterone. Some of these substances are volatiles, such as 2-(*sec*-butyl)thiazoline and 2,3-dehydro-*exo*-brevicomin. On the basis that they can elicit aggression between males, these two compounds have been reported to be releaser pheromones (Novotny, Harvey, Jemiolo & Alberts, 1985).

Among the non-volatiles, proteins of the major urinary protein (MUP) complex are present in adult male mouse urine at very high concentrations, approximately 1–5 mg ml^{-1} (Clissold, Hainey & Bishop, 1984). MUP, which has a molecular mass of about 18 kDa, is synthesized under multiple gene control in the liver, where MUP mRNA

This manuscript was accepted as a Short Paper for rapid publication.

is 4% of the total mRNA. MUP is coded by MUP genes of Group 1 (for definition of the MUP gene groups see Al-Shawi, Ghazal, Clark & Bishop, 1989). The full-length protein has eight β-sheets barrel structure, common to all lipocalins, with a small hydrophobic cavity. MUP can bind highly hydrophobic molecules with low molecular mass, forming a long-lived complex (Cavaggioni, Findlay & Tirindelli, 1990), and it has naturally bound volatile substances (Bacchini, Gaetani & Cavaggioni, 1992). This has also been confirmed by structural studies, which have demonstrated a small ligand bound within the hydrophobic pocket in the core of the protein (Böcskei *et al.* 1992). MUP genes of Group 2 are derived from Group 1 genes by mutation of a glycine codon into a stop codon. Their major expression product is the MUP-related peptide *N*-Glu-Glu-Ala-Arg-Ser-Met, which differs from the *N*-terminal leading sequence of MUP by two residues only. MUP and the peptide were suggested to be the pheromones involved in puberty acceleration (Clark, Ghazal, Bingham, Barrett & Bishop, 1985).

The aim of the present work is to investigate the role of MUP, its volatile ligands and the peptide in the acceleration of the puberty onset of female mice.

METHODS

Animals

Mice were derived from a Swiss strain stock and reared in our laboratory. Parent couples were each housed in 27 × 42 × 15 cm plastic cages with their respective litters. Food (16% protein, 2% fat, mouse diet chow; Mucedola, Milan, Italy) and water were freely available. The light schedule was 12 h light : 12 h dark, with light commencing at 6.00 h. Room temperature was 23 ± 1 °C, and humidity was 40–50%. The cage bedding of wood shavings was changed every 5 days. The litters were weaned at 21 days of age. Half of the females from each litter received experimental treatment, and the other half control treatment. Since all females in the two groups were treated at the same time, seasonal variations were avoided. Four to five mice were housed together in each cage. Each experiment, which consisted of testing an experimental group and a control group from the same litters, was performed separately because there were not enough females in each litter to cover all the experiments at the same time.

Urine collection and purification

Urine was collected from at least eight animals and immediately stored at −20 °C until use. All chemical manipulations were performed with the use of glass vials and glass instruments, in order to avoid contamination of odorants.

Test urine was collected from adult males that were 2–5 months old. Control urine was collected from prepubertal males on the day of weaning (postnatal day 21).

Adult male urine was thawed, centrifuged at 500 *g* for 5 min and the supernatant was filtered through paper. The supernatant was then buffered at pH 4·7, and brought to 50% ammonium sulphate saturation at 0 °C. After centrifuging at 2000 *g* for 20 min, the pellet was discarded and the supernatant brought to 70% ammonium sulphate saturation. After 30 min at 0 °C the preparation was again centrifuged (2000 *g*, 20 min), and the pellet stored at −20 °C. When appropriate, the pellet was dialysed overnight against water with a 8000/15000 molecular mass cutoff membrane. MUP was further purified by molecular sieve chromatography on Sephadex G-50 (Pharmacia, Milan, Italy) and by ion exchange chromatography on DE52 cellulose (Whatman, Maidstone, Kent) (Cavaggioni *et al.* 1990). MUP was always administered to mice at a physiological concentration (2 mg ml^{-1}).

The protein content in urine was determined by a colorimetric test with MUP as a standard (BioRad Protein Assay). Protein concentration in solutions was determined spectrophotometrically, assuming a molar extinction coefficient of 11340 cm^{-1} M^{-1} (λ = 280 nm). Polyacrylamide gel electrophoresis, under denaturing conditions, was performed on all solutions tested.

The peptide *N*-Glu-Glu-Ala-Arg-Ser-Met was synthesized using 9-fluorenylmethoxycarbonyl-amino acids and stored at −20 °C.

Organic extraction of the natural ligands

MUP solution was treated with one volume of dichloromethane by shaking in a centrifuge tube. Following separation of the organic and water phase by centrifugation on a bench-top centrifuge (6000 *g*, 10 min), the phase of interest was collected using a Pasteur pipette with a sharpened tip. The phases were stored at −20 °C in glass vials.

Competition displacement of the natural volatile ligands

Natural volatile ligands bound to MUP were substituted with the high-affinity ligand 2-isobutyl-3-methoxypyrazine (IBMP; Aldrich), as described by Cavaggioni *et al.* (1990). Briefly, 1 μmol MUP in 2 ml of water was equilibrated with 1 mM IBMP at 37 °C for 15 min. The solution was then dialysed against 3 l of water at 8 °C for 20 h with three changes of water to remove free IBMP. Following extraction in dichloromethane, gas chromatograms (GC) were performed on all the tested solutions. One microlitre of the organic phase, separated by centrifugation (1000 *g*, 10 min), was analysed by GC, using an OV17 capillary column (30 m long, 0·32 mm diameter; Dani, Milan, Italy), PTV injector and flame ionization detector in the split–splitless mode. The injector temperature was 225 °C, the temperature program was 1 min at 61 °C, then increasing at 5 °C min^{-1} for 25 min up to 185 °C. The carrier gas was helium. Mass spectroscopy (MS) was performed with an ion-trap mass analyser. The identification of substances in gas chromatography was by retention time, and in mass spectroscopy was by fragmentation, compared with synthetic samples (Bacchini *et al.* 1992).

Bioassay

All experiments conform to the European Community regulations regarding laboratory animals (86/609/EEC). A total of 227 mice were tested in the experiments reported in this paper. The sample sizes, ranging from twelve to twenty-two for each experimental condition, are given in Results.

For three consecutive days, starting from postnatal day 30, female mice, weighing 24 ± 3 g, were given 40 μl of the appropriate solution on the oronasal groove. The Hamilton syringe used for delivering the solution was different for each treatment. On the fourth day (postnatal day 33), mice were killed by cervical dislocation. The uteri were then promptly removed and weighed by an experimenter who was unaware of the treatment received by each mouse.

The data on uterus weight were log transformed by the method of Vandenbergh *et al.* (1975), and submitted to one-way between-subjects ANOVA (experimental *vs.* control). Differences between

body weight data from the experimental and control treatment groups at the beginning of each treatment (postnatal day 30) were assessed using the Mann–Whitney U test, to exclude the possibility that the difference in uterus weight could be due to a difference in body weight.

RESULTS

Effect of male urine protein fraction

Experiment 1 aimed to assess the reliability of the bioassay procedure. Twenty-two females were examined in each group. Urine from adult males was used for the experimental group and water was used for the control group. The results from the Mann–Whitney U test showed that there was no difference in body weight between the two groups ($U = 209$; $P = 0{\cdot}4386$). ANOVA results confirmed that adult whole urine accelerated puberty by increasing uterus weight ($F_{(1,42)} = 7{\cdot}639$, $P < 0{\cdot}01$). Means and S.E.M. are shown in Fig. 1 for this and for the following experiments. These results suggest that the test procedure of administering the solutions over 3 days was reliable.

Experiment 2 aimed to confirm that the protein fraction containing MUP was active (Vandenbergh *et al.* 1976). Twelve mice were treated with MUP, which had been isolated by ammonium sulphate precipitation and extensive dialysis, and which had then been dissolved in prepubertal male mice urine. Twelve control mice were treated with prepubertal male urine. This urine was used as the control carrier solution because no MUP could be detected either by gel electrophoresis or by colorimetric protein assay. In addition, a previous study has demonstrated that prepubertal male urine by itself has no influence on female puberty onset (Drickamer, 1988).

No difference could be observed in body weight prior to the treatments ($U = 60{\cdot}5$, $P = 0{\cdot}5067$). Increase in uterine weight occurred only in the mice treated with MUP, ($F_{(1,22)} = 5{\cdot}044$, $P < 0{\cdot}05$), demonstrating that MUP was related to puberty acceleration. This effect of isolated MUP could be due to the protein itself, or to the natural low molecular mass volatile substances that are tightly bound to MUP, as 2-(*sec*-butyl)thiazoline, 2,3-dehydro-*exo*-brevicomin and 4-(ethyl)phenol.

Odorants extracted from MUP are not active

Experiment 3 aimed to confirm that the volatile ligands separated from MUP are not involved in the anticipation of puberty. Natural tightly bound volatile ligands were extracted in dichloromethane from MUP, which had been purified by ammonium sulphate precipitation and dialysis. Fifteen female mice were treated with 5 μl of the organic

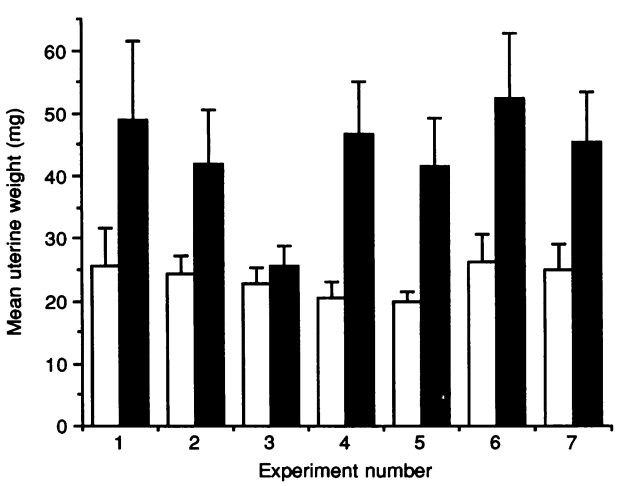

Figure 1. Uterine weight is increased by exposure to MUP

Means and S.E.M. are shown. Open bars refer to control groups and filled bars to experimental groups. Experiment 1: the experimental group was treated with urine from adult males, the control was treated with water. Experiment 2: MUP was purified from adult male urine by ammonium sulphate precipitation and dialysis, and diluted at a physiological concentration in urine from prepubertal males. Experiment 3: natural odorants were extracted from MUP in dichloromethane and added to prepubertal male urine; the control group received the urine with the solvent alone added. Experiment 4: both groups received dichloromethane, but for the experimental group this was suspended in adult male urine, while for the control group it was suspended in prepubertal male urine. Dichloromethane did not inhibit the anticipation of puberty in the experimental group. Experiment 5: MUP was extracted 3 times with dichloromethane to remove all the ligands. It was then lyophylized and dissolved in prepubertal male urine. Experiment 6: the natural ligands were replaced with IBMP in MUP purified by chromatography; the control group received prepubertal male urine, in which free IBMP had been added. Experiment 7: the experimental group received the synthetic peptide dissolved in prepubertal male urine; the control group received only prepubertal male urine.

extract suspended in urine from prepubertal mice. Sixteen females were treated with 5 μl of the solvent suspended in urine from prepubertal mice. There were no significant differences between the two groups for uterine weight ($F_{(1,29)} = 0{\cdot}526$, $P = 0{\cdot}474$) or for body weight ($U = 100{\cdot}5$, $P = 0{\cdot}4408$). This result suggested that the volatiles extracted in the organic phase, without protein, did not affect puberty onset.

A control experiment (Experiment 4) was conducted in order to check that the solvent dichloromethane did not inhibit the effect of pheromones. Sixteen female mice were given 35 μl of adult male urine. Fifteen female mice were given 35 μl of prepubertal male urine. In both cases 5 μl of dichloromethane was added to the urine. No difference in body weight was detected ($U = 102{\cdot}5$, $P = 0{\cdot}4891$). The weight of uteri from the group which had been administered adult male urine was significantly greater than those from the females which had been administered prepubertal male urine ($F_{(1,29)} = 11{\cdot}459$, $P < 0{\cdot}005$). Clearly, dichloromethane did not inhibit puberty acceleration. As such, the lack of effect in Experiment 3 could not be attributed to solvent inhibition.

These experiments demonstrate that, by themselves, the small volatile ligands were not active when extracted in organic phase. However, the possibility that they could have been active when bound to MUP cannot be excluded.

Effect of MUP without natural ligands

In Experiment 5, after isolation from urine by ammonium sulphate precipitation and dialysis, MUP was separated from the tightly bound volatile ligands with three successive extractions in dichloromethane. Gas chromatograms performed on the last organic phase indicated that the extraction was complete. Subsequently, the aqueous phase containing MUP was lyophilized for 24 h at 0·1 mmHg, in order to remove traces of solvent. Fifteen mice received the separated MUP dissolved in urine from prepubertal males. Fifteen female mice received only prepubertal male urine. Body weight was similar for the two groups ($U = 76{\cdot}5$, $P = 0{\cdot}1354$). Acceleration of puberty was present in the former group only ($F_{(1,28)} = 10{\cdot}122$, $P < 0{\cdot}005$). This experiment showed that the ligands were not necessary for puberty acceleration; MUP devoid of volatile ligands was still active.

In Experiment 6 we removed the natural ligands from MUP using a different method, competition displacement with a high-affinity ligand. The displacing ligand was IBMP (2-isobutyl-3-methoxypyrazine), a high-affinity ligand ($K_d = 0{\cdot}34$ μM; Cavaggioni *et al.* 1990) for MUP. The displacement was carried out on MUP that had been purified by ammonium sulphate precipitation, dialysis, molecular sieve and anion exchange chromatography (Cavaggioni *et al.* 1990). Before competition, MUP had

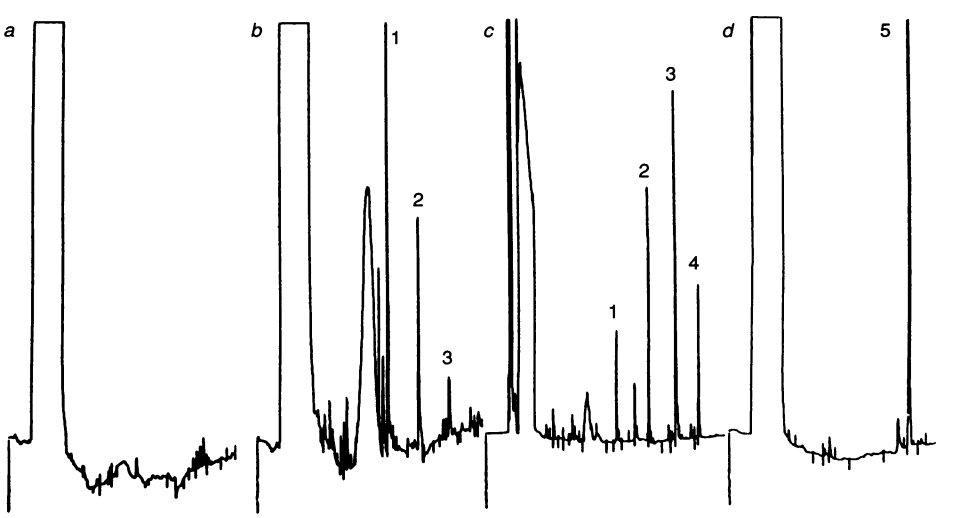

Figure 2. Gas chromatograms of MUP before and after displacement of the natural ligands

a, prepubertal male urine. No GC peaks are detected. *b*, adult male urine. *c*, MUP from adult male urine; the signal was attenuated 10 times with respect to the other traces. *d*, MUP after treatment with 2-isobutyl-3-methoxypyrazine. The GC peak of this compound only is present. Peak 1, 2,3-dehydro-*exo*-brevicomin (retention time, 8 min 17 s); peak 2, 2-(*sec*-butyl)thiazoline (retention time, 10 min 19 s); peak 3, 4-(ethyl)phenol (retention time, 12 min 0 s); peak 4, a terpene (retention time, 13 min 29 s); peak 5, 2-isobutyl-3-methoxypyrazine (retention time, 11 min 33 s).

bound several natural volatiles, including 2-(*sec*-butyl)-thiazoline, 2,3-dehydro-*exo*-brevicomin and 4-(ethyl)phenol, which were identified by GC–MS. However, after competition displacement, the GC showed that MUP had bound only IBMP (Fig. 2). Sixteen female mice received MUP which had bound only IBMP, dissolved in prepubertal male urine. Fifteen female mice received equivalent IBMP, without MUP, dissolved in prepubertal male urine. There was no difference for body weight ($U = 108{\cdot}5$, $P = 0{\cdot}6494$). Puberty acceleration was evident only in the group that had been treated with MUP ($F_{(1,29)} = 5{\cdot}597$, $P < 0{\cdot}05$).

Effect of the MUP-related hexapeptide *N*-Glu-Glu-Ala-Arg-Ser-Met

We also tested the hexapeptide sequence most likely to occur in nature, as it has been deduced by gene sequencing (Clark *et al.* 1985). Eighteen female mice received the synthetic hexapeptide dissolved in prepubertal male urine at a final concentration of 500 μg ml^{-1}, and eighteen mice received prepubertal male urine only. No difference could be detected in body weight ($U = 139$, $P = 0{\cdot}4668$). Mice receiving the peptide had a mean uterine weight greater than controls ($F_{(1,34)} = 4{\cdot}194$, $P < 0{\cdot}05$). This experiment shows that the peptide *N*-Glu-Glu-Ala-Arg-Ser-Met can induce puberty acceleration.

DISCUSSION

As early as 1975, Vandenbergh and colleagues first reported puberty-accelerating activity in the protein fraction of adult male mice urine. Subsequent characterization did not seem to confine this activity to the high molecular mass fraction. Rather, an active peptide with a molecular mass of about 860 Da was suggested (Vandenbergh *et al.* 1975, 1976). The current results demonstrate that purified MUP is a priming pheromone, which is sufficient to accelerate puberty in prepubertal female mice, and that the dialysable agent is the hexapeptide coded for by the Group 2 MUP genes. Our initial focus was upon the small volatile molecules which are so tightly bound to MUP that they copurify with MUP and cannot be disposed of, even with sequential precipitation, dialysis, molecular sieve and ion exchange chromatography (Bacchini *et al.* 1992). It could be argued, in fact, that these tightly bound ligands are the active molecules, MUP being merely a carrier or reservoir for these. Two observations made this possibility unlikely. Firstly, the volatiles, extracted in the organic phase and delivered to the nose of the female mouse, are not active. This was first demonstrated with an ether extract by Vandenbergh *et al.* (1975), and repeated in the current study with a dichloromethane extract in a carrier solution in which no MUP could be detected (prepubertal mouse urine). Secondly, MUP without the natural volatiles, obtained either by organic extraction or by competition displacement with a high-affinity ligand, retains its activity. The absence of natural volatiles, at least in any measurable quantity, was tested using gas chromatography (GC). While traces below the GC detection limit cannot be excluded, to our knowledge MUP has not previously been purified of volatiles to such an extent. The finding that the activity of MUP was irrespective of the method for isolating it from its ligands, makes it possible to state that MUP is not merely a carrier of, or reservoir for, pheromones, but is in itself a pheromone. The activity of the MUP-related synthetic hexapeptide, which carries no volatiles, further points to the existence of a receptor protein specific for MUP. As mentioned previously, lesions of the vomeronasal organ or accessory olfactory bulb prevent puberty acceleration, whereas lesions of the main olfactory system do not prevent acceleration (Kaneko *et al.* 1980; Keverne, 1983). This is in agreement with the notion that the accessory or vomeronasal olfactory system is also stimulated by proteins. For example, the vomeronasal organ of the garter snake can perceive a polypeptide excreted by earthworms (Jiang, Inouchi, Wang & Halpern, 1990). More relevant to this study, male hamsters are attracted by female vaginal discharge, which contains a protein named aphrodisin. This protein is categorized within the same lipocalin superfamily as MUP, and it, too, acts through the vomeronasal system (Singer, Macrides, Clancy & Agosta, 1986). It is not known, however, whether aphrodisin binds some ligands and their possible effects, or which part of the protein is active. The demonstration that MUP (and the related *N*-terminal hexapeptide) is a pheromone which stimulates the vomeronasal organ does not exclude a role for the volatile ligands. For example, ligand odour may well help a mouse search in the field, it may convey some ancillary information about the diet, metabolism and social status of the releaser, or it may modulate the activity of MUP.

It is proposed that MUP and MUP-related peptides should be considered as a new family of bioactive proteins/peptides, which is characterized by a similar *N*-terminal region. The sequence of the *N*-terminal hexapeptide of MUP is *N*-Glu-Glu-Ala-Ser-Ser-Thr and this suggests a consensus sequence *N*-Glu-Glu-Ala-X-Ser, where X is a polar residue, which is recognized by a vomeronasal receptor. Despite large variability in peptide chain length, members of the endogenous opioid family also share a short (four residues) *N*-terminal consensus (Schwyzer, 1986). In MUP the consensus sequence is far away from the opening of the β-sheets barrel structure, but close to the bottom pole, a region that has been proposed for lipocalin–receptor interaction (Flower, North & Attwood, 1993).

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Acknowledgements

We thank Drs K. M. B. Bennett and Roberto Tirindelli for helpful suggestions, and Mr Fabrizio Orzi for animal care. This work was supported by the National Research Council of Italy (special project RAISA, subproject 4).

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Received 24 March 1995; accepted 11 May 1995.