On the existence of receptors to the pheromonal steroid, 5α-androst-16-en-3-one, in porcine nasal epithelium

M.R. Hancock+, J.N. Gennings° and D.B. Gower*

Department of Biochemistry, Guy's Hospital Medical School, London SE1 9RT, England

Received 16 November 1984; revised version received 28 November 1984

The binding of the odorant, 5α-androst-16-en-3-one, to porcine nasal tissues, has been investigated using methods normally employed for studying both cytosolic and membrane-bound receptors. 5α-Androst-16-en-3-one was generally taken up more avidly by homogenates of olfactory (nervous) tissue than by respiratory tissue, but binding to the former was only partially prevented by prior heating or by excess ligand, suggesting some degree of specific binding. At low protein concentration, saturable binding was noted but these data were not reproducible. The binding of a non-odorant, DHA, was only 2% that of 5α-androst-16-en-3-one. Using agarose gel electrophoresis, some evidence was obtained for binding protein(s) to the odorous 16-adrostene in porcine respiratory tissues, that were absent from previously heated tissue. Experiments with SDS-treated, or cell-membrane-enriched preparations, of nasal epithelium did not show improved binding of 5α-androst-16-en-3-one. We conclude that the extreme hydrophobicity of 5α-androst-16-en-3-one is probably responsible for the high degree of non-specific binding noted and for variability in results. This is discussed in relation to other known odorous ligand/receptors in olfactory tissue, particularly that of 5α-androstan-3-one [10].

Olfaction *5α-Androst-16-en-3-one* *Porcine* *Pheromone* *Receptor* *Steroidal odorant*

1. INTRODUCTION

Many attempts have been made to illustrate the presence of high affinity, low capacity binding of odorants to olfactory mucosal fractions [1], using techniques employed for insulin receptor studies [2] and for cytosolic receptors to steroid hormones. Amino acids, known to act as olfactory stimuli in the rainbow trout (*Salmo gairdneri*) [3], are known to bind to its olfactory tissue [4] and in rats, camphor binds to the olfactory epithelium with high affinity (K_{diss}, 1.5×10^{-9} M) [5]. This assay was complicated by less specific binding which exhibited a maximum binding capacity 110 times higher than that of the high-affinity site. Similarly, saturable binding of the odorant, 2-isobutyl-3-methoxypyrazine was demonstrated specifically in the olfactory mucosa of cow, rabbit and dog, with the K_{diss} value in the cow being 8×10^{-5} M [6]. Receptor proteins to anisole (methoxybenzene) also occur in dog olfactory epithelium [7–9].

Using the urinous odorant, 5α-androstanone, binding studies with crude homogenates of sheep olfactory tissue proved to be inconclusive and irreproducible, due largely to the extreme

+ Present address: Pathology Laboratory, High Royds Hospital, Menston, Ilkley LS29 6AQ, England
° Present address: Department of Medical Oncology, Charing Cross Hospital, Fulham Palace Road, London W6 8RF, England
* To whom correspondence should be addressed

Abbreviations: 5α-androstenone, 5α-androst-16-en-3-one; DHA (dehydroepiandrosterone), 3β-hydroxy-5-androsten-17-one; 5α-androstanone, 5α-androstan-3-one; pregnenolone, 3β-hydroxy-5-pregnen-20-one; testosterone, 17β-hydroxy-4-androsten-3-one; 5α-dihydrotestosterone (5α-DHT), 17β-hydroxy-5α-androstan-3-one; oestradiol-17β, 1,3,5(10)-oestratrien-3,17β-diol; RIA, radioimmunoassay; BSA, bovine serum albumin

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/85/$3.30

hydrophobicity of the steroid. Evidence was presented [10], however, for the presence of specific, saturable binding using 12000 × *g* supernatant fractions. The amount of non-specific binding was large so that estimations of an affinity constant (7.0×10^8 M^{-1}) were subject to considerable error.

Because of the pheromonal importance [11,12] of the urinous 5α-androstenone, the present work was concerned with attempts to detect specific binding of this steroid to sow olfactory epithelium.

2. MATERIALS AND METHODS

Materials used were essentially as described earlier [13]. Collagenase from *Clostridium histolyticum* (EC 3.4.24.3) was supplied by Boehringer, Lewes, Sussex and hydroxyapatite (Biogel HTP) by BioRad Laboratories, Richmond, CA. Ethmoturbinate and septal epithelium ('sensory' tissue) and respiratory epithelium ('non-sensory' tissue) were dissected from sow and gilt nasal chambers [13]. Nasal mucus was collected as before [13]. [7α-^{3}H]DHA (specific radioactivity 23 Ci/mmol) was obtained from Amersham International, Bucks.

2.1. *Separation of bound and free steroid*

Homogenates (10%, w/v) of heated (60°C for 10 min) respiratory tissue were prepared in TEMP buffer (Tris, 10 mM; EDTA, 1 M; 2-mercaptoethanol, 0.1% (v/v); 1,3-propanediol, 1% (v/v), pH 7.4). Portions (0.2 ml) were incubated with 5α-[5,6-^{3}H]androstenone (10 nmol/l) at 4°C in a final volume of 0.4 ml at a protein concentration of 1.7 mg/ml [14]. Charcoal suspensions (200 μl), containing Dextran T70 and BSA, each 0.4% (w/v), were added to the incubation mixture, with a 3 s agitation time and 10 min standing time, before centrifugation at 2000 × *g* for 10 min. Radioactivity in the supernatant was assessed; this treatment removed 91% steroid. In the absence of any tissue fraction, this 'charcoal efficiency' was 98.3% and, in subsequent studies, this figure was assessed at each concentration of 5α-androstenone and was corrected for in the calculation of bound steroid. It was found essential to prepare the charcoal fresh in order to keep the figure above 95%.

2.2. *Binding of 5α-[^{3}H]androstenone in the presence and absence of excess non-radioactive steroid*

Homogenates (10%, w/v) of sensory and respiratory tissue in TEMP buffer were subjected to a preliminary digestion by collagenase (0.1%, w/v) before incubation (1 ml, 1.7 mg protein), in duplicate, with an equal volume of buffer containing 5α-[^{3}H]androstenone (0.25 nmol/l) for 30 min at 4°C. Parallel incubations were carried out containing radioactive steroid plus cold 5α-androstenone (63 nmol/l). The incubated samples were centrifuged at 4000 × *g* for 10 min to remove cell debris, and portions (0.4 ml) of the supernatant subjected to charcoal treatment as in section 2.1. Radioactivity in the supernatant was assessed and expressed as the percentage of that in the original incubation. The contribution of specific sites was calculated as the total charcoal-resistant radioactivity recovered from incubations containing 5α-[^{3}H]androstenone only, less that recovered in the presence of excess cold steroid.

2.3. *Preparation of a membrane-enriched fraction from sow olfactory epithelium*

Homogenates of sow olfactory epithelium (section 2.1) were filtered through gauze and then centrifuged at 7300 × *g* for 30 min; the pellet (designated fraction P) [15] is thought to be membrane-enriched. Separation of bound and free 5α-androstenone here and in section 2.4 was achieved using hydroxyapatite [16].

2.4. *Preparation of an SDS extract of sow olfactory epithelium*

Homogenates of sow olfactory epithelium (section 2.1) were centrifuged (30000 × *g*, 30 min), giving a 'detergent-free' extract. The pellet was rehomogenized in TEMP buffer (pH 7.4) containing SDS (0.1%, w/v) [7] and centrifuged at 30000 × *g* for 30 min. The supernatant is referred to as 'SDS extract'.

2.5. *Separation of bound and free steroid by electrophoresis*

After incubation of porcine nasal tissue preparations and mucus with 5α-[^{3}H]androstenone, electrophoresis on Agarose slab gels was performed [13].

3. RESULTS AND DISCUSSION

3.1. *Binding of 5α-[^{3}H]androstenone to sensory and non-sensory porcine nasal tissues*

Table 1 shows that homogenates of sensory tissue exhibited 38% charcoal-resistant binding at a concentration of 5α-[^{3}H]androstenone of 0.25 nmol/l. When excess unlabelled steroid was present this value was reduced to 18%, indicating that 20% of the binding at the lower ligand concentration was of the specific type. Heating sensory tissue reduced the percentage of specific binding by more than 50% but heated sensory and non-sensory homogenates also exhibited a proportion of 'specific' binding. Since specific steroid receptors show a high degree of thermolability, it is unlikely that any such components were present in the heated preparation. However, there is a possibility that receptors for endogenous steroids, such as 5α-DHT and oestradiol-17β are present [17,18]. The most likely explanation for the finding of 'specific' binding in the control tissues is a methodological one, since the concentration of unlabelled 5α-androstenone used was very high (63 nmol/l) and it has been reported that, at concentrations greater than 100-fold that of the label, radio-inert ligand may partially compete for binding, even to components of relatively low affinity, thus reducing the level of binding of radioactive ligand and causing an overestimation of specific binding [19].

3.2. *Binding of 5α-[^{3}H]androstenone over a wide range of steroid concentrations*

The results in table 1, using sensory tissues, indicated that there might be some specific binding of 5α-[^{3}H]androstenone, 50% of which could be displaced by adding excess unlabelled ligand. Fig.1 shows the effects on binding of a wide range of ligand concentrations. In sensory tissue, 25% of the total steroid appeared in the charcoal-resistant fraction at 25°C and this value was reduced to 10% at 4°C. Charcoal-resistant binding was reduced in heated sensory tissue to a level similar to that seen in non-sensory tissue fractions. However, in no case was the binding shown to be saturable over the concentration range used.

3.3. *Charcoal-resistant binding at 4°C as a function of protein concentration*

The results depicted in fig.1 suggested that the binding was non-saturable because the protein concentration chosen (0.85 mg/ml) was too high. With a wider range of cold steroid concentration and lower protein concentrations (0.43 mg/ml), a 50% drop in the proportion of charcoal-resistant binding was noted (fig.2A,B). Further reduction in the protein concentration had little effect on binding at high total steroid concentrations but, at the lowest protein level (0.21 mg/ml), a slight curve was discernible at low steroid concentrations, suggesting saturable binding. Heating the sensory homogenates reduced the level of binding at the

Table 1

Percentage charcoal-resistant binding of 5α-[^{3}H]androstenone to porcine nasal epithelium in the presence and absence of excess unlabelled steroid

Tissue type	Charcoal-resistant binding (%)		Specific binding (%)
	Plus 5α-[^{3}H]androstenone (0.25 nmol/l)	Plus 5α-[^{3}H]androstenone (0.25 nmol/l) and cold 5α-androstenone (63 nmol/l)	
Sensory	38	18	20
Heated sensory	17	8	9
Non-sensory	35	24	11
Heated non-sensory	21	12	9

Homogenates (10%, w/v) in TEMP buffer of sensory and non-sensory porcine nasal tissues (1 ml, 1.7 mg protein) were incubated for 30 min at 4°C with TEMP buffer (1 ml) containing 5α-[^{3}H]androstenone. Parallel incubations were also carried out in the presence of excess unlabelled 5α-androstenone. All experiments were done in duplicate. Incubated samples were centrifuged at 4000 × *g* for 10 min and the supernatants subjected to charcoal treatment (see section 2)

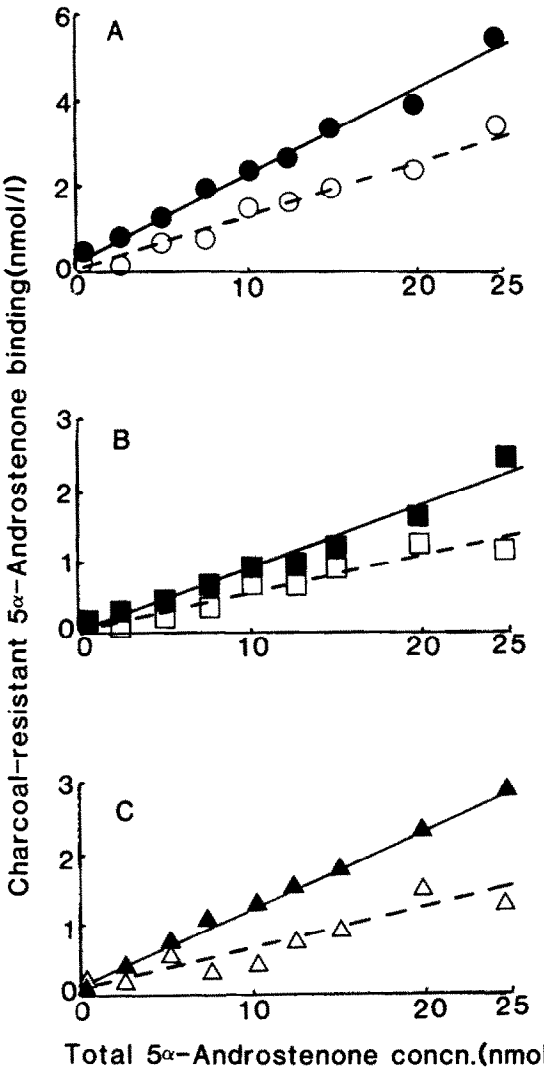


Fig.1. Effect of 5α-androstenone on charcoal-resistant binding of the steroid to sow nasal epithelium. Homogenates (10%, w/v) in TEMP buffer of sow 4 nasal epithelium (final protein concentration 0.85 mg/ml) were incubated at 25°C (closed symbols) and 4°C (open symbols) for 40 min with 5α-[^{3}H]androstenone plus increasing quantities of the unlabelled steroid. Bound and free steroid were separated using charcoal and then centrifuging (see section 2). (A) Sensory nasal epithelium, (B) heated (60°C for 10 min) sensory nasal epithelium, (C) non-sensory nasal epithelium.

highest protein concentration by half but this binding appeared unaffected by lowering of protein concentration. It should be stressed that, in repeat experiments, similar levels of charcoal-resistant binding were obtained but the curve for sensory tissue at a protein concentration of 0.21 mg/ml was not reproducible.

Although the sensory epithelial homogenates of this sow (sow 5) exhibited the same levels of charcoal-resistant binding as those of sow 4 (fig.1), the non-sensory tissue fractions (fig.2C) demonstrated a higher capacity of binding, up to 75% of steroid remaining in the supernatant after charcoal treatment of those incubations containing the highest protein concentration. Such binding was linearly reduced by lowering the protein content of the incubations to 0.43 mg/ml, but a curve was discernible at 0.21 mg/ml.

3.4. *Binding of 5α-androstenone to an SDS-extract of sow olfactory epithelium*

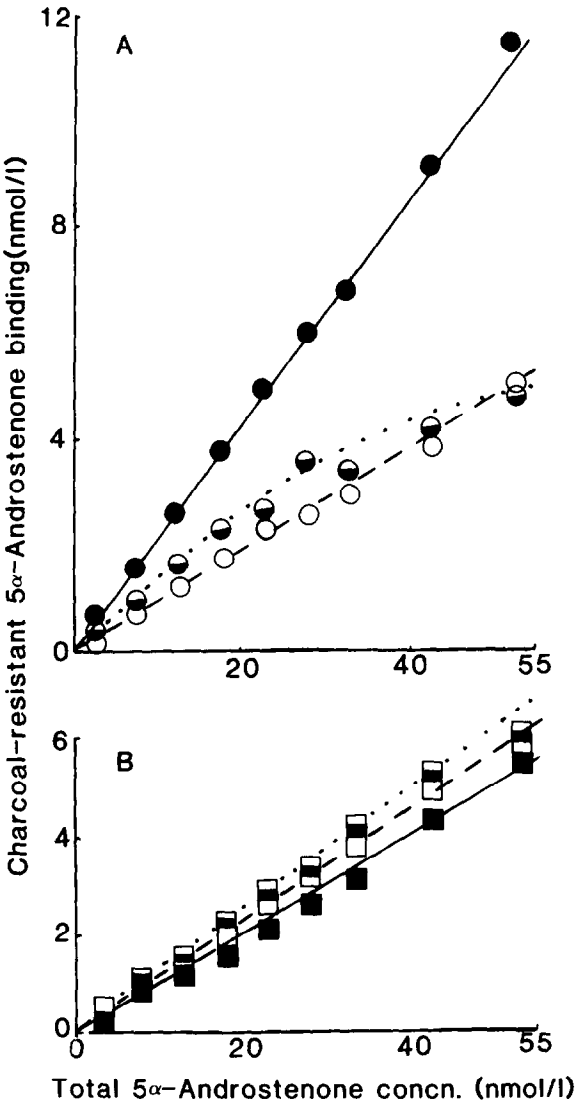
Since there is evidence to indicate that receptor proteins are components of the cell membrane, solubilization using detergents may be required to obtain a sufficiently pure extract and thereby reduce non-specific binding, which is high in our work and in that of others [5,10]. However, the data (not shown) showed a linear increase in bound 5α-[^{3}H]androstenone with increasing ligand (2.2–42.3 nmol/l) in the incubation medium, indicating that specific binding moieties were not associated with the cell membranes.

3.5. *Binding of 5α-androstenone to a cell membrane-enriched fraction of sow olfactory epithelium*

When the membrane-enriched fraction (P) from sow olfactory epithelium (section 2.3) was incubated with 5α-[^{3}H]androstenone (8–116 nmol/l) in the presence or absence of 100 times the concentration of unlabelled 5α-androstenone, no differences were noted in the percentage binding of the ligand compared with the homogenate or fraction S [15]. Neither was there clear evidence for competition for binding sites by unlabelled 5α-androstenone, as would be expected if specific binding were present in the membrane-enriched preparation.

3.6. *Comparison of binding of 5α-androstenone with that of pregnenolone and DHA*

Over the concentration range of 0–50 nmol/l, the binding of pregnenolone to sensory and non-



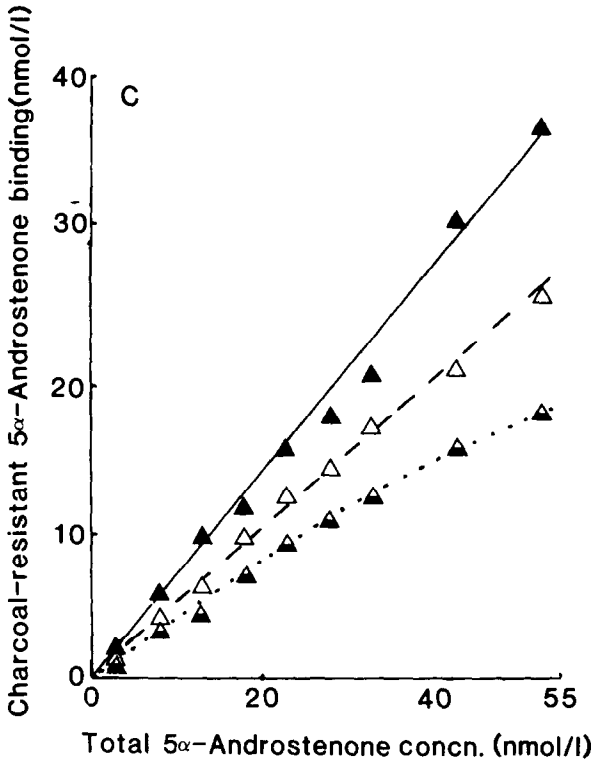


Fig.2. Effect of protein concentration on charcoal-resistant binding of 5α-[^{3}H]androstenone to sow nasal epithelium. Homogenates of nasal epithelium (sow 5), containing protein at concentrations of 0.85 mg/ml (closed symbols), 0.43 mg/ml (open symbols) (A and B) or 0.21 mg/ml (half-closed symbols) (C), were incubated at 4°C for 40 min with 5α-[^{3}H]androstenone. For further details see section 2 and fig.1 legend.

sensory epithelial homogenates was unaffected by heating (unlike that of 5α-androstenone) and was 5–30% that of 5α-androstenone [13]. Here, at steroid concentrations of 100 nmol/l, DHA binding was less than 2% that of 5α-androstenone, when a detergent-free extract of sow olfactory epithelium was examined (section 2.4), indicating a degree of specificity of 5α-androstenone.

3.7. *Electrophoresis of porcine nasal tissues and nasal mucus after 5α-[^{3}H]androstenone incubation*

Agarose gel electrophoresis of sensory tissues or sensory tissue mucus did not reveal any significant differences between non-heated and heated tissues [13]. In the present work, however, a peak of radioactivity was noted at +1.7 to +2.0 cm in preparations of respiratory tissue (fig.3) and of respiratory mucus (not shown). These peaks were not noted in similar extracts from control tissue or mucus that had been heated previously. It is possible that respiratory tissue secretes some component which specifically binds to 5α-androstenone; the flow of mucus would then cause the complex to come into contact with the olfactory epithelium, a receptor with higher affinity possibly competing

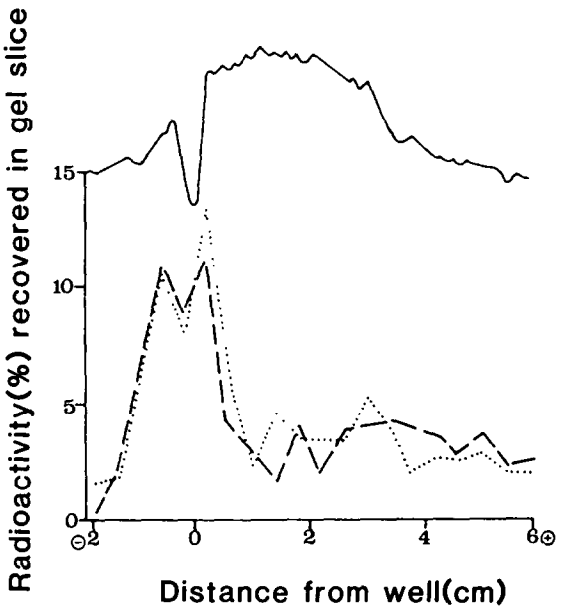


Fig.3. Agarose gel electrophoresis of sow respiratory tissue fractions previously incubated with 5α-[^{3}H]androstenone. A portion of the 12000 × *g* supernatant of sow respiratory epithelium was incubated with 5α-[^{3}H]androstenone (final concentration, 85 nmol/l) for 2 h at 4°C. A 20 μl portion of the incubate (containing 38 μg protein) was electrophoresed on an agarose slab gel [13]. Protein (——) was scanned using a densitometer and radioactivity in gel slices was estimated for both non-heated (– – –) and heated (···) tissue.

there for steroid binding. Such a mechanism would aid the passage of the hydrophobic pheromone through the watery mucus. Interactions have previously been demonstrated between odorant molecules and olfactory mucus; a 15-fold increase in water-solubility of octane was noted after passage through the frog olfactory sac [20]. A similar, though more pronounced, form of 16-androstene binding has been shown to occur in boar submaxillary, but not parotid, saliva [21].

Our present results may be compared with preliminary results of Persaud et al. [22], who used very similar methods and conditions to those described here (section 3.2) and found that the binding of 5α-androstanone was linearly related to the total steroid concentration. It was only at lower concentrations (0.02–1.0 nmol/l) that charcoal-resistant binding exhibited a slight curve as 5α-androstanone concentration increased [10], giving the K_a value of 7×10^8 M^{-1}. Just as in the present work (sections 3.2, 3.3), the authors [10] experienced non-reproducibility of their results, particularly at low ligand concentrations, and also a high degree of non-specific binding. The former could well be due to the extreme hydrophobic character of 5α-androstenone and 5α-androstanone; both adsorb readily to glass surfaces even when these are silanized [13] or to hydroxyapatite [16]. This means that it is not possible to know exactly how much 5α-androstenone is present in the receptor assay studies, and this could well explain non-reproducibility of results. It is significant that non-specific binding of progesterone represented about 8% of total binding in cytosol preparations of guinea-pig uterus [23]. When the extreme hydrophobicity of 5α-androstenone is taken into account, the magnitude of the problem may be understood.

A second explanation of non-specific binding in the present work is that 5α-androstenone may bind to lipids, as found in RIA [24]. Lipid interference also occurs in RIA of cortisol, DHA sulphate, progesterone and testosterone [25] but is more pronounced with 5α-androstenone, presumably because of its very non-polar nature.

It is also possible that 5α-androstenone may bind to 3α- and 3β-hydroxysteroid oxidoreductases (EC 1.1.1.50/51) known to be present in porcine nasal tissues, both sensory and non-sensory [13]. The apparent K_m values for these enzymes are in the range 0.1–1 μM.

Even though we cannot produce compelling evidence for the presence of specific receptors to 5α-androstenone for reasons discussed above, there is the possibility that the steroid binds with a protein in the saliva of the sow [26]. The pheromone-protein complex might then be transferred to the vomeronasal organ of the female to initiate pheromonal activity since non-volatiles are known to have access to this organ [27].

ACKNOWLEDGEMENTS

We thank Drs L.H. Bannister and W.D. Booth and Mr J.E.T. Jones for help with dissection and obtaining porcine tissues and The Agriculture and Food Research Council for financial support (AG 35/21 and 33).

REFERENCES

[1] Hancock, M.R. (1984) PhD Thesis, University of London, pp.62–67.
[2] Cuatrecasas, P. (1971) Proc. Natl. Acad. Sci. USA 68, 1264–1268.
[3] Cagan, R.H. and Zeiger, W.M. (1978) Proc. Natl. Acad. Sci. USA 75, 4679–4693.
[4] Hara, T.J. (1973) Comp. Biochem. Physiol. (A) 44, 407–416.
[5] Fesenko, E.E., Novoselov, V.I., Mjasoedov, N.F. and Sidirov, G.V. (1978) Stud. Biophys. 73, 71–84.
[6] Pelosi, P., Baldaccini, N.E. and Pisanelli, A.M. (1982) Biochem. J. 201, 245–248.
[7] Price, S. (1978) Chem. Sens. Flav. 3, 51–55.
[8] Goldberg, S.J., Turpin, J. and Price, S. (1979) Chem. Sens. Flav. 4, 207–214.
[9] Price, S. and Turpin, J. (1980) in: Olfaction and Taste, vol.VII (Van der Starre, H. ed.) pp.65–68, IRL Press, London.
[10] Dodd, G. and Persaud, K. (1981) in: Biochemistry of Taste and Olfaction (Cagan, R.H. and Kare, M.R. eds) pp.333–357, Academic Press, New York.
[11] Reed, H.C.B., Melrose, D.R. and Patterson, R.L.S. (1974) Br. Vet. J. 130, 61–67.
[12] Kirkwood, R.N., Hughes, P.E. and Booth, W.D. (1983) Anim. Prod. 36, 131–136.
[13] Gower, D.B. and Hancock, M.R. (1982) in: Olfaction and Endocrine Regulation (Breipohl, W. ed.) pp.267–277, IRL Press, London.
[14] Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
[15] Kreuger, J.M. and Cagan, R.H. (1976) J. Biol. Chem. 251, 88–97.
[16] Gennings, J.N. (1982) M. Phil. Thesis, pp.111–112, University of London.
[17] Stumpf, W. and Sar, M. (1982) in: Olfaction and Endocrine Regulation (Breipohl, W. ed.) pp.11–22, IRL Press, London.
[18] Vanelli, G.B. and Balboni, G.C. (1982) in: Olfaction and Endocrine Regulation (Breipohl, W. ed.) pp.279–284, IRL Press, London.
[19] Chamness, G.C. and McGuire, W.L. (1975) Steroids 26, 538–542.
[20] Hornung, D.E. and Mozell, M.M. (1977) Brain Res. 128, 158–163.
[21] Booth, W.D. (1982) in: Olfaction and Endocrine Regulation (Breipohl, W. ed.) pp.353–354, IRL Press, London.
[22] Persaud, K., Pelosi, P. and Dodd, G.H. (1980) in: Olfaction and Taste VII (Van der Starre, H. ed.) p.101, IRL Press, London.
[23] Milgrom, E., Perrot, M., Atger, M. and Baulieu, E.-E. (1972) Endocrinology 90, 1064–1078.
[24] Bird, S. and Gower, D.B. (1981) J. Steroid Biochem. 14, 213–219.
[25] Rash, J.M., Jerkunica, I. and Sgoutas, D. (1979) Clin. Chim. Acta 93, 283–294.
[26] Booth, W.D. (1984) J. Endocr. 100, 195–202.
[27] Wysocki, C.J., Wellington, J.L. and Beauchamp, G.K. (1980) Science 207, 781–783.