CSIRO PUBLISHING

Australian Journal of Agricultural Research

Volume 50, 1999

A journal for the publication of original contributions towards the understanding of an agricultural system

www.publish.csiro.au/journals/ajar

All enquiries and manuscripts should be directed to
Australian Journal of Agricultural Research
CSIRO PUBLISHING
PO Box 1139 (150 Oxford St)
Collingwood Telephone: 61 3 9662 7628
Vic. 3066 Facsimile: 61 3 9662 7611
Australia Email: jenny.fegent@publish.csiro.au

Published by **CSIRO** PUBLISHING
for CSIRO Australia and
the Australian Academy of Science

Aust. J. Agric. Res., 1999, **50**, 441–5

Reducing metabolic rate by immunoneutralisation of catecholamines

C. Gazzola[AC] *and R. A. Hunter*[B]

Tropical Beef Centre, PO Box 5545, Rockhampton, Qld 4702, Australia.
[A]Queensland Beef Industry Institute, Department of Primary Industries, Rockhampton, Qld 4702, Australia.
[B]CSIRO Tropical Agriculture, Rockhampton, Qld 4702, Australia.
[C]Corresponding author; email: gazzolc@dpi.qld.gov.au

Abstract. A conjugate of noradrenaline and ovalbumin was used to produce anti-noradrenaline antibodies in goats and Brahman steers. Three weeks after the primary immunisation, anti-noradrenaline binding was detected in the sera of 2 of 5 immunised steers but none was detected in any steers 6 weeks after immunisation. Three weeks after immunisation, immunised steers, including the ones with no detectable anti-noradrenaline antibodies, had a larger change ($P < 0.02$) in fasting metabolic rate from pretreatment values (–14.5 W) than did paired controls (+3.3 W). After 6 weeks, the changes in fasting metabolic rate for the treated and control groups were –17.8 W and –36.6 W ($P < 0.08$), respectively. Anti-noradrenaline polyclonal antibodies purified from goat serum had an approximate dissociation constant for binding to noradrenaline of 4 μM and a specific binding capacity of 178 nM in the immunoglobulin solution prepared, equivalent to about 30 mg/L of anti-catecholamine antibodies in the original goat serum. When 1 mL of this immunoglobulin solution was injected into rats, their metabolic rate was lowered by >10% relative to rats treated with control immunoglobulin solution ($P < 0.05$). These results demonstrate that the lowering of metabolic rate by an immunological approach which targets control points in the sympathetic nervous system is feasible and could offer an immunologically based method of growth promotion for beef cattle.

Additional keywords: immunology, cattle, goats, rats.

Introduction

Decreased energy expenditure in farm animals results in increased efficiency of production because the saved energy can be utilised for growth (Hunter *et al.* 1993). Guanfacin, an α_2-adrenoceptor agonist, lowers the resting energy expenditure of cattle (Hunter 1992), mice (Sillence *et al.* 1992), and rats (Gazzola 1993), and in one experiment, treatment with guanfacin reduced the rate of liveweight loss of undernourished cattle (Hunter 1992). Although guanfacin could conceivably be developed as a growth promotant, the use of an immunological approach to modifying growth rate as an alternative to the use of drugs and hormones is widely recognised as a highly desirable goal (Aston *et al.* 1993).

Gazzola (1993) defined a minimal, functional model in rats for the partial control of energy expenditure via the sympathetic nervous system using α-adrenoceptor agonists and antagonists. The model has been confirmed in cattle using similar methods (Gazzola *et al.* 1994). The model (Fig. 1) consists of pre-synaptic, inhibitory α_2-adrenoceptors, a sympathetic synapse (or neuromuscular junction), and post-synaptic α_1-adrenoceptors. α_2-Adrenoceptor-selective agonists decrease sympathetic activity, and therefore lower metabolic rate, by reducing the tonic release of noradrenaline. α_1-Adrenoceptor-selective antagonists reduce sympathetic activity by blocking the activation of the α_1-adrenoceptors by noradrenaline (see reviews by Ruffolo 1987 and Limbird 1988). Since the effect of these drugs is to decrease the amount of noradrenaline released or to reduce the effectiveness of noradrenaline in transmitting the sympathetic outflow, an alternative approach would be to actively remove noradrenaline from the synapse. Antibodies to noradrenaline, provided they had access to the synapse, could possibly achieve this goal.

With a view to controlling the metabolic rate of cattle (and consequently their nutritional energy requirements and growth rate) using immunological methods, a conjugate of noradrenaline and albumin was produced. This immunogen was used to demonstrate the validity of the approach by raising polyclonal antibodies in goats which were then used to lower the metabolic rates of rats by passive immunisation. Finally, the immunogen was used to actively immunise cattle so that both antibody production and lowering of metabolic rate could be measured.

10.1071/AR98153 0004-9409/99/040441

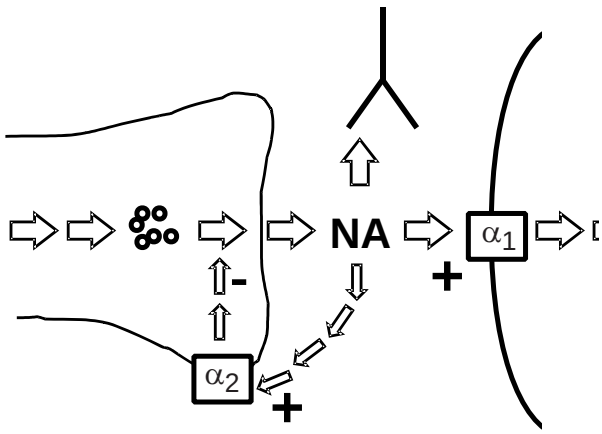

Fig. 1. Minimal functional model for the control of metabolic rate by the sympathetic nervous system. Boxes represent α_1- and α_2-adrenoceptors and circles represent neurotransmitter vesicles. Activation and inhibition are represented by '+' and '–' respectively. The Y-shaped figure represents noradrenaline (NA) specific antibodies.

Materials and methods

All animal work described here was approved by the Animal Ethics Committee of the Tropical Beef Centre, Rockhampton, Australia.

Synthesis of the noradrenaline–ovalbumin immunogen

A noradrenaline–ovalbumin conjugate was synthesised from ovalbumin, noradrenaline, and formaldehyde via the Mannich reaction (for a general description of this reaction see Thompson 1968). A reaction mixture containing 132 mg of ovalbumin (donated by Peptech, Australia), 35 mg of [-]-noradrenaline bitartrate (Sigma, St Louis, MO, USA), and 100 μL of l-[7,8-^{3}H]-noradrenaline acetate (812 kBq; Amersham Australia, North Ryde, NSW) in 10 mL of 3 M sodium acetate (Ajax Chemicals, Sydney) containing 36.5–38% formaldehyde (Ajax) was stirred overnight in the dark at 23°C. The solution was dialysed to a dilution of $1:3 \times 10^6$ with distilled water with all the radioactivity being recovered (106%). After lyophilisation, 90 mg of protein was recovered with a specific activity of 1.3 kBq/mg, which represented an incorporation of approximately 8 noradrenaline molecules per ovalbumin molecule calculated using the supplier's value for the specific radioactivity of noradrenaline.

An ovalbumin control solution, for injection into control animals, was produced in an exactly analogous manner except that no noradrenaline was included in the initial reaction mixture.

Production of goat, polyclonal, anti-noradrenaline antibodies

Adult female crossbred goats (n = 4; liveweight 53–70 kg) were housed in a covered animal house. They were fed, *ad libitum*, with a mixed diet of good quality hay and grain. Primary immunisation was achieved by the intramuscular injection of 1 mg of noradrenaline–ovalbumin conjugate in a 2-mL emulsion of water/Freund's Complete Adjuvant (Sigma). Further intramuscular injections, of 1 mg of conjugate in Freund's Incomplete Adjuvant, were given after 4 weeks, 11 weeks, and 13 weeks. Blood (10 mL) was collected from all goats at regular intervals to monitor noradrenaline binding activity. Large volumes of blood (300 mL) were taken from all goats before immunisation for preparation of control serum. Screening tests identified the goat exhibiting the strongest immunological response and 300 mL of blood was collected from this animal at 12 weeks after the primary immunisation for preparation of immune serum.

Purified goat immunoglobulins were prepared from the pooled, pre-immunisation serum (control immunoglobulin solution) and from the immune serum (immune antibody solution) using recombinant-protein G–Agarose chromatography (Gibco BRL, Gaithersberg, USA). The procedure was as recommended by the manufacturers, except that immunoglobulins were eluted using 0.5 M ammonium acetate (pH 3). The pH of eluate containing immunoglobulins was adjusted to 7 before lyophilisation. The lyophilised immunoglobulins from the pooled control serum and from the immune serum were dissolved in phosphate-buffered saline [0.01 M phosphate (pH 7.2), 0.15 M NaCl, 2.5 mM KCl] and concentrations adjusted so that the antibody solution and control solution had the same absorbance at 280 nm (A_{280} 16.7). For injection into rats, solutions were filtered through 0.45- μm filters (Flow Filtration Systems, Meckenheim, Germany).

Effect of goat, polyclonal, anti-noradrenaline antibodies on the metabolic rate of rats

Female Wistar rats [n = 20; liveweight (mean ± s.e.) 192 ± 3 g] were randomly allocated to control (6) and treated (14) groups. As previously described (Gazzola 1993), rats were housed singly in standard plastic and metal cages at 22–24°C with controlled ventilation, humidity, and lighting (12 h light/12 h dark cycle). Feed (rat pellets, Norco Co-op, Lismore, Australia) and water were freely available. Rats were extensively trained in experimental procedures before experimentation. Resting oxygen consumption was measured as previously described (Gazzola 1993) by measuring the differences in oxygen concentration between the flows of air entering and leaving metabolic rate chambers. Oxygen consumption per rat was corrected to standard temperature and pressure, and metabolic rate was calculated using an equivalence value between oxygen consumption and energy expenditure of 20.3 kJ/L (Jequiver and Felber 1987). Metabolic rates were measured on several days prior to treatment until individual rats yielded consistent results. Rats were injected intraperitoneally with 1 mL of immunoglobulin solution (control or immune preparations, respectively) at 10 00 hours, and oxygen consumption measurements were taken from 10 15 to 10 40 hours.

Active immunisation of Brahman steers

Brahman steers (n = 10) were trained in cattle metabolic rate apparatus for several weeks prior to the experiment (Hunter and Vercoe 1987). Steers were housed in individual pens in a covered animal house with *ad libitum* access to a diet of 1 : 1 *Medicago sativa : Dicanthium aristatum*. At the start of the experiment, steers had a liveweight (mean ± s.e.) of 414 ± 8 kg. During the experiment, liveweight was recorded twice weekly, and voluntary food intake (sufficient food given to allow refusal of 1–1.5 kg/day) was measured. Five days after the start of the experiment, steers were fasted for 72 h, after which the fasting metabolic rate (Hunter and Vercoe 1987) and heart rate (HRM-8A/E, Respironics, Kowloon, HK) were measured. Immediately after these measurements were taken, pre-immunisation serum was collected from the steers, which then received a primary immunisation as an intramuscular injection of 1 mg of conjugate or control ovalbumin as described for the goats. As only 2 calorimeters were available, steers were paired randomly and treated in pairs on 5 consecutive days. Within each pair, 1 steer was randomly injected with the conjugate and the other with the control ovalbumin. Animals were returned to full alimentation with the diet mentioned above over the following 5 days.

The procedure (fasting, fasting metabolic rate measurements, heart rate measurements, serum collection, and realimentation) was repeated 3 weeks after the primary immunisation, still in a staggered manner, at which time the animals received a booster injection as described for the goats. The measurements and serum collection were again repeated 6 weeks after the primary immunisation.

Measurement of noradrenaline binding activity

Noradrenaline binding activity was measured by incubating serum with labelled noradrenaline followed by ammonium sulfate precipitation of protein-bound radioactivity (Hudson and Hay 1989). Diluted serum or antibody solution (300 μL) was mixed with freshly prepared noradrenaline solution (100 μL) in polycarbonate tubes. Noradrenaline solution was prepared in phosphate-buffered saline so that the assay contained about 10 kBq (goat antibody solution assays) or 120 kBq (cattle serum assays) of l-[7-^{3}H]-noradrenaline (l-[7-^{3}H]-epinephrine; DuPont, Wilmington, USA); these were made up to noradrenaline concentrations ranging from 0.1 to 100 μM with unlabelled [-]-noradrenaline (Sigma). After overnight incubation at 5°C, 400 μL of ice-cold saturated ammonium sulfate was added, and the tubes were centrifuged at 1500*G* for 30 min. The supernatant was decanted and the tubes drained for 5 min. The pellet was washed 3 times with 1 mL of ice-cold 50% saturated ammonium sulfate, and finally dissolved in 1 mL of distilled water at room temperature. This solution was quantitatively transferred to counting vials where 5 mL of ACS II (Amersham, Arlington Heights, USA) was added for scintillation counting with external calibration (Wallac, Turku, Finland).

All assays were performed in triplicate. For the goat antibody solutions, the following measurements were made: total added radioactivity; background 'blank', no added immunoglobulin; non-specific binding using control sera; and specific binding using immune sera at each of 5 noradrenaline concentrations. This allowed calculation of the concentration of noradrenaline binding as a result of immunisation, and the ratio of specific bound to specific free for estimation of an apparent K_D (dissociation constant) and binding capacity from a Scatchard plot. Non-specific binding was <20% of total bound. Only one concentration of noradrenaline, 1 μM, was used for the bovine sera where the assay was used as a screening test to determine which animals were responding. Again, binding as a result of immunisation was determined by subtracting the binding activity of serum collected before immunisation from the binding activity of serum collected after immunisation. Serum proteins were measured using the Bio-Rad DC Protein Assay (Bio-Rad, Richmond, USA) with bovine serum albumin (Progen, Darra, Australia) as a standard.

Calculations and statistical analyses

Data from measurements on control and treated animals were compared using the unpaired Student's *t*-test for rat data and the paired *t*-test for cattle data (Snedecor and Cochran 1989). Metabolic rates are presented in watts (W), which are J/s. Data in the text are given as mean ± s.e.

Measurement of fasting metabolic rates in cattle presented difficulties due to the limited number of large calorimeters (only 2), and the long turnover time required between measurements on individual animals (72 h fasting, 1 day for measurements, 5 days for realimentation, and at least 2 weeks for recovery). Consequently, measurements could not be repeated.

Results

Production of goat, polyclonal, anti-noradrenaline antibodies

The antibody solution prepared from goat immune serum gave an approximately linear Scatchard plot (Fig. 2) within the range of noradrenaline concentrations used in these assays (0.1–100 μM). The plot approximated a straight line (r^2 = 0.95, P<0.001) to give an apparent K_D of 4 μM. The binding capacity of these specific antibodies in the immunoglobulin solution was 178 nM, equivalent to about 30 mg/L of noradrenaline-binding antibodies in the original goat serum.

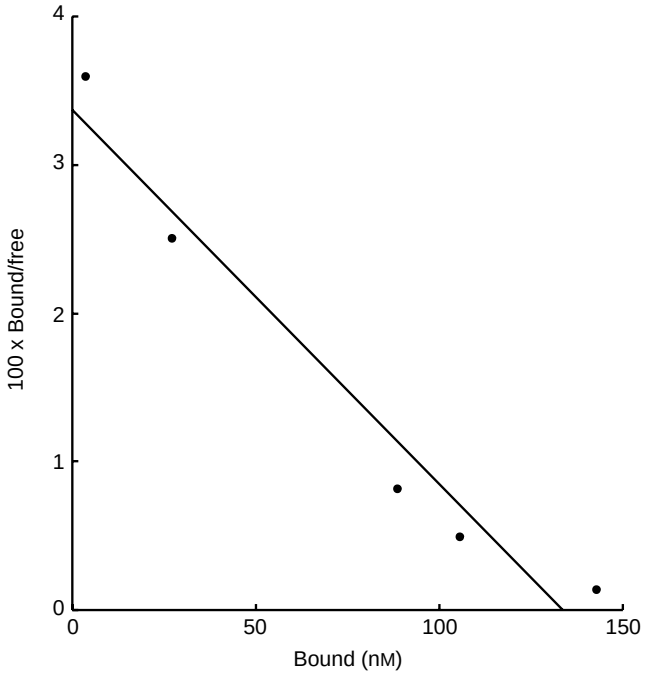

Fig. 2. Scatchard plot for the specific binding of tritiated noradrenaline to purified goat immunoglobulins.

Effect of goat polyclonal anti-noradrenaline antibodies on the metabolic rate of rats

Prior to treatment, mean metabolic rates in female Wistar rats were 2.26 ± 0.12 W in the control group and 2.28 ± 0.08 W in the treatment group. When injected with the immune antibody solution (178 pmol of noradrenaline binding activity) or the control antibody solution, the animals receiving the immune solution had a 10% lower metabolic rate (P<0.05) than the control animals during the following 40 min (Table 1). When metabolic rates were measured again on the following day, the metabolic rates of all animals had returned to normal.

Table 1. Change from pretreatment values in metabolic rates (W) of rats 0.25 h and 24 h after treatment with goat immunoglobulins

	0.25 h	24 h	*n*
Control antibody solution	–0.08	0.15	6
Immune antibody solution	–0.30	0.01	14
s.e.d.	0.11	0.18	
Significance	P < 0.05	n.s.	

n.s., not significant

Active immunisation of Brahman steers

When cattle were immunised with the noradrenaline conjugate, noradrenaline binding could only be detected in some sera. The sera of 2 of the 5 treated animals exhibited noradrenaline binding activity 3 weeks after immunisation (0.24

Table 2. Change from pretreatment values in metabolic rates (W) of steers three weeks and six weeks after immunisation with noradrenaline–ovalbumin conjugate

	3 weeks	6 weeks	*n*
Control group	3.3	–17.8	5
Immunised group	–14.5	–36.6	5
s.e.d.	4.4	7.9	
Significance (paired)	$P<0.02$	$P<0.08$	

and 0.43 nmol noradrenaline bound/g serum protein, respectively) but no activity was detected in any animal after 6 weeks. Before immunisation, fasting metabolic rates were 0.35 ± 0.02 kW in control steers and 0.37 ± 0.02 kW in immunised steers. The changes in fasting metabolic rates at 3 weeks and 6 weeks after the primary immunisation are shown in Table 2. After 3 weeks, the fasting metabolic rates in animals in the immunised group were 17.8 W less than those in the control group. By 6 weeks, the difference was 18.8 W but greater variability in the groups had decreased the significance of the difference. There were no differences in heart rate between these groups throughout the experiment. The mean heart rates at the start, at 3 weeks, and at 6 weeks were 40 ± 1, 35 ± 1, and 34 ± 1 beats/min, respectively.

Discussion

Anti-noradrenaline antibodies, both in a passive immunisation experiment using goat antibodies in rats and in an active immunisation experiment using cattle, lowered metabolic rate in the test animals. This demonstrated that immunological targeting of control points in the sympathetic nervous system is a feasible approach to the control of metabolic rate.

Miwa *et al*. (1977) used the Mannich reaction to synthesise catecholamine–bovine serum albumin conjugates. Their procedure used *N*-maleylated catecholamines to prevent linking of catecholamine molecules. They also used a longer reaction time than that used in the present study, and took precautions against the oxidation of the catecholamines. For convenience, and with a view to scaling up production to industrial quantities, a much simplified procedure was used here without any detrimental effect. This procedure resulted in an incorporation of 8 mol noradrenaline per mol ovalbumin (19 mol lysine, 42% occupied) compared with 15–30 mol noradrenaline per mol bovine serum albumin (58 mol lysine, 26–52% occupied; Miwa *et al*. 1977). There was no evidence of massive noradrenaline polymer formation due to linking of noradrenaline molecules, and only slight darkening of the reaction solution due to oxidation. Any free noradrenaline oligomers formed would have been removed by dialysis, and it is possible that any conjugated oligomers, if they triggered an immune response, would stimulate the production of useful, noradrenaline-binding antibodies.

The expected consequence of an immunisation such as that used here would be a polyclonal response consisting of antibodies to the hapten, the carrier protein, and possibly the linkage used. Total immunoglobulin concentration was not measured directly either before or after immunisation, but from the yields of purified immunoglobulins obtained from the goat exhibiting the strongest response, total serum immunoglobulin concentration was estimated to have increased about 3-fold. That most of this response was directed towards the carrier protein was confirmed by a simple immunoprecipitation using 0.5% ovalbumin solution with diluted, purified antibody solution which resulted in massive amounts of precipitate. The noradrenaline binding assay used in the present study detected only antibodies able to bind isolated noradrenaline molecules. Even this select group of antibodies would be heterogeneous and, consequently, conceptually and technically difficult to analyse in terms of an apparent dissociation constant and total binding capacity. The Scatchard plot presented here is approximately linear and gives an indication of the noradrenaline-binding capacity in the serum of the immune animals.

The immunisation and screening procedures were designed for an experiment aimed at lowering the levels of noradrenaline at synapses. It was not considered necessary to measure serum catecholamines, since previous work, where guanfacin was used to lower metabolic rate, caused no detectable lowering of serum noradrenaline (Hunter *et al*. 1993; D. Lindsay and R. A. Hunter, unpublished data). However, when adrenaline was added to the screening assay, it produced a decrease in apparent specific binding similar to that seen when an equivalent amount of noradrenaline was added, indicating that the catecholamine binding was not specific for noradrenaline. Unfortunately, the serum collected was not suitably stored for catecholamine assays. The hypothesis that the presence of anti-noradrenaline antibodies in the bloodstream reduces sympathetic activity by removing noradrenaline from synapses was supported by the goat/rat experiments. The effect of passively immunising rats with anti-catecholamine antibodies was immediate and transient. The possibility that serum adrenaline was involved in the effect cannot be excluded.

The lower metabolic rate in immunised steers compared with the non-immunised steers could not be directly correlated to the presence of specific antibodies because these could not be readily detected. The lack of noradrenaline binding in sera may have been due to a technical problem with the assay as no anti-oxidant (ascorbic acid) was included in the assay buffer. Also, because of the short duration of this experiment, immunisation and lowered metabolic rate could not be linked to a demonstrable increase in liveweight gain. Hunter *et al.* (1993) calculated that a small lowering of metabolic rate (10%) in pasture-fed cattle could have large effects on animal production provided it could be maintained for a large proportion of the animal's commercial lifespan. The

antibody response and the effect on metabolic rate seen here indicate that immunisation to produce anti-noradrenaline antibodies may be a suitable technology for this purpose. Avoidance of a large carrier protein response to produce a more specific and persistent antibody response might achieve a more useful result. The model for sympathetic control of resting metabolic rate (Fig. 1) indicates a number of other points in the system (e.g. the α-adrenoceptors) amenable to immunological control which could be exploited to produce a more persistent response.

Acknowledgments

The authors gratefully acknowledge the excellent technical support provided by Ms V. Grant (goat and rat experiments), Mr T. Magner (cattle metabolic rate measurements), and Mr W. Sim and the animal house staff (cattle husbandry). This work was partially supported by the Meat Research Corporation, Australia.

References

Aston, R., Pell, J. M., Trigg, T. E., Rathgen, D. A., Bender, V., and Holder, A. T. (1993). Novel immune approaches to the manipulation of growth. *In* 'Animal Health and Production for the 21st Century'. (Ed. K. J. Beh.) pp. 111–21. (CSIRO Publishing: Collingwood.)

Gazzola, C. (1993). α_2-Adrenoceptor-mediated effects on resting energy expenditure. *International Journal of Obesity* **17**, 637–41.

Gazzola, C., Magner, T., Lisle, A. T., and Hunter, R. A. (1995). Effect of α-adrenoceptor agonists and antagonists on metabolic rate in cattle. *Comparative Biochemistry and Physiology* **111A**, 73–7.

Hudson, L., and Hay, F. C. (1989). Ammonium sulphate precipitation (Farr assay). *In* 'Practical Immunology'. pp. 209–12. (Blackwell: London.)

Hunter, R. A. (1992). The effect of the α_2-adrenergic agonist, guanfacin, on the energy metabolism of steers fed on low-quality-roughage diets. *British Journal of Nutrition* **67**, 337–43.

Hunter, R. A., Sillence, M. N., Gazzola, C., and Spiers, W. G. (1993). Increasing annual growth rates of cattle by reducing maintenance energy requirements. *Australian Journal of Agricultural Research* **44**, 579–95.

Hunter, R. A., and Vercoe, J. E. (1987). Reduction of energy requirements of steers fed on low-quality-roughage diets using trenbolone acetate. *British Journal of Nutrition* **58**, 477–83.

Jequiver, E., and Felber, J. (1987). Indirect calorimetry. *Baillière's Clinical Endocrinological Metabolism* **1**, 911–35.

Limbird, L. E. (Ed.) (1988). 'The Alpha-2 Adrenergic Receptors.' (Humana: Clifton, NJ.)

Miwa, A., Yoshioka, M., Shirahata, A., and Tamura, Z. (1977). Preparation of specific antibodies to catecholamines and L-3,4-dihydroxyphenylalanine. I. Preparation of the conjugates. *Chemical Pharmaceutical Bulletin* **25**, 1904–10.

Ruffolo, R. R. Jr (Ed.) (1987). 'The Alpha-1 Adrenergic Receptors.' (Humana: Clifton, NJ.)

Sillence, M. N., Tudor, G. D., Matthews, M. L., and Lindsay, D. B. (1992). Effects of the α_2-adrenoceptor agonist guanfacine on growth and thermogenesis in mice. *Journal of Animal Science* **70**, 3429–34.

Snedecor, G. W., and Cochran, W. G. (1989). 'Statistical Methods. 8th Edn.' (Iowa State University Press: Ames, IA.)

Thompson, B. B. (1968). The Mannich reaction. *Journal of Pharmaceutical Sciences* **57**, 715–33.

Manuscript received 30 September 1998, accepted 21 December 1998