Biochemical Pharmacology, Vol 31, No 6, pp 1039–1047, 1982.
Printed in Great Britain.

0006-2952/82/061039-09 $03 00/0

UPTAKE AND DISPLACEMENT OF [^{3}H]DIHYDROALPRENOLOL, [^{3}H]EPINEPHRINE AND [^{3}H]CLONIDINE IN ISOLATED PERFUSED RABBIT LUNG

RALPH J. ALTIERE,*† JAMES S. DOUGLAS‡ and C. NORMAN GILLIS§‖

Departments of *§Anesthesiology and §Pharmacology, Yale University School of Medicine, New Haven, CT 06510
and ‡The John B. Pierce Foundation Laboratory, New Haven, CT 06510, U.S.A.

(*Received* 12 *May* 1981; *accepted* 20 *August* 1981)

Abstract—Uptake and displacement of three adrenergic receptor ligands, [^{3}H]dihydroalprenolol ([^{3}H]DHA), [^{3}H]epinephrine ([^{3}H]EPI) and [^{3}H]clonidine ([^{3}H]CLON), were examined in isolated rabbit lungs by recirculating perfusion. Removal of [^{3}H]DHA was the most extensive (85% uptake; 6.6 ml/min clearance), [^{3}H]CLON removal was intermediate (50%; 3.8 ml/min), and [^{3}H]EPI removal was the lowest (33%; 1.2 ml/min). Specific displacement of each radioligand from lung was attempted using several competing agents. Both (−)- and (+)-propranolol equally displaced [^{3}H]DHA from lung. Phentolamine, (−)-phenylephrine and (−)-epinephrine were unable to displace 10 nM [^{3}H]EPI from lung, although the latter two agents did produce concentration-dependent increases in perfusion pressure. High concentrations of (−)-epinephrine, which produced near maximal physiological responses, inconsistently displaced 30–40 nM [^{3}H]EPI from lung. [^{3}H]Clonidine was displaced by unlabeled clonidine at concentrations that caused increases in perfusion pressure. Pretreatment of lungs with either 10 μM phentolamine or phenoxybenzamine did not alter the total amount of [^{3}H]CLON displaced by clonidine, suggesting that [^{3}H]CLON was displaced predominantly from non-specific sites, perhaps preventing detection of [^{3}H]CLON displacement from specific (receptor) sites. Alternatively, these results may be interpreted as inhibition of uptake of each radioligand. Thus, both (−)- and (+)-propranolol interfered with [^{3}H]DHA removal, suggesting a common mechanism for uptake and/or retention for these two β-adrenergic receptor antagonists. Inhibition of [^{3}H]EPI removal was observed only at high concentrations of (−)-epinephrine which indicates that pulmonary removal of epinephrine occurs through a low affinity uptake system. [^{3}H]Clonidine removal was effectively inhibited by the same (μM) concentrations of unlabeled clonidine that produced physiological responses. Neither phentolamine nor phenoxybenzamine was able to interfere with pulmonary removal of [^{3}H]CLON. Therefore, uptake and displacement of these adrenergic receptor radioligands showed no correlation with pharmacological effects produced by these agents in isolated perfused rabbit lung. The results are more closely associated with inhibition of removal and/or non-specific retention of the radioligands examined.

It is evident from experiments, both *in vitro* and *in vivo*, that the lung can remove a variety of substances from the pulmonary circulation [1, 2]. Many of these, such as the biogenic amines, norepinephrine and 5-hydroxytryptamine, are rapidly metabolized with subsequent release of the metabolic products from lung [3–5]. Others, such as the β-adrenergic receptor antagonist, propranolol, are extensively removed by lung, but are retained unchanged [6]. Specific sites for transport and/or retention have not been studied in detail, although endothelial cells have been proposed as the locus for uptake and metabolism of many of these substances [7]. Specific binding sites for several adrenergic receptor ligands have been demonstrated, but only in membrane fragments of whole lung [8–11]. One study demonstrated both uptake into whole lung and binding of three adrenergic receptor antagonists to different subcellular fractions of lung [10]. However, specific binding sites, especially those responsible for the pharmacological effects of these substances, have not been demonstrated in the intact organ. Therefore, in an attempt to define these sites, we used rabbit lungs, each perfused simultaneously but independently *in vitro*, to examine removal of three adrenergic receptor ligands, dihydroalprenolol, epinephrine and clonidine, and to assess the correlation between their uptake and release and the pulmonary vascular responses produced by these agents.

MATERIALS AND METHODS

Adult male New Zealand white rabbits (1.9 to 2.6 kg) were anesthetized by intravenous injection of allobarbital (125 mg/kg) and urethane (500 mg/kg) after administration of 500 units heparin/kg. The chest was opened and lungs were removed intact. The trachea and right and left pulmonary arteries were cannulated, and the lungs were statically inflated with 30 ml of room air and suspended in a plexiglass chamber as previously described [12]. Each lung was independently perfused at constant flow (10 or 20 ml/min, see Results) (Masterflex roller pump, Cole–Parmer Instrument Co., Chicago, IL) with Krebs medium containing (mM); NaCl, 118.2; KCl, 4.74; $CaCl_2$, 2.54; KH_2PO_4, 1.19; $MgSO_4$, 1.19;

† Present address: College of Pharmacy, Pharmacodynamics and Toxicology Division, University of Kentucky, Lexington, KY 40506.

‖ Reprint requests should be forwarded to C. N. Gillis at the Department of Pharmacology, Yale University.

$NaHCO_3$, 26.2; dextrose, 11.1; and $CaNa_2EDTA$, 0.027, maintained at 37° and aerated with 95% O_2–5% CO_2. Perfusion pressures were continuously monitored with Statham P23Dc pressure transducers (Hato Rey, PR) and recorded on a Grass polygraph (Grass Instruments, Quincy, MA).

After an initial 15-min equilibration period in which the lungs were perfused with Krebs medium to clear the vascular space, adrenergic receptor radioligands were perfused through the lungs in a recirculating system in which the effluent from each lung was returned to its respective reservoir containing perfusion medium and labeled drug. Experiments were then carried out in one of two ways: (1) radioligand and competing unlabeled agent were simultaneously perfused through one lung, while the contralateral (control) lung was perfused with radioligand alone, or (2) both lungs were perfused with radioligand and, when a steady-state plateau level of uptake was reached, one lung was challenged with increasing concentrations of an unlabeled drug while the other lung served as control. Aliquots (0.1 ml) were taken from each reservoir at time zero for measurements of initial reservoir concentrations and at predetermined time intervals thereafter (every 3 min for control lungs; every 1 min for lungs challenged with unlabeled drug) and transferred directly to scintillation vials. Water (1.9 ml) and Instagel (4 ml) (Packard Instrument Co., Downer's Grove, IL) were added to each aliquot and radioactivity was determined in a Packard Tri-Carb liquid scintillation spectrometer.

Three radiolabeled adrenergic receptor ligands were used in these studies: [^{3}H]dihydroalprenolol ([^{3}H]DHA), [^{3}H]clonidine ([^{3}H]CLON) and [^{3}H]epinephrine ([^{3}H]EPI). For the latter ligand, the perfusion medium also contained 0.1% ascorbic acid, 10 μM pargyline and 1 mM semicarbazide to prevent oxidative degradation. The purity of each radioligand was checked by thin-layer chromatography. During the course of several experiments, the presence of enzymatic metabolites of each radioligand in aliquots of perfusates was also assessed by thin-layer chromatography.

Data analysis. Radioactivity (dpm) present in the entire reservoir at each time of sampling was calculated from aliquot values (cpm) by applying correction factors for counting efficiencies, dead-space volume of the perfusion system, binding of each radioligand to tubing and the reservoir volume, including changes due to successive aliquots removed from the reservoir. These values were then normalized to initial total radioactivity in the reservoir prior to lung perfusion and plotted as log (percent total tritium remaining in the reservoir) versus time of perfusion (min). The slope of the initial linear portion of the curve was used to calculate the rate constant (k, min^{-1}) for uptake, according to the equation [13]:

$$\ln (A_t/A_0) = -kt$$

where A_t/A_0 represents the fraction of radioactivity remaining in the reservoir at time, t. The slope of log A_t/A_0 vs. t then equals $-2.303(k)$. Clearance values for each radioligand were then calculated according to the equation [14]:

$$Cl\ (\text{ml/min}) = V_r\ (\text{ml}) \times k\ (\text{min}^{-1})$$

where V_r represents initial volume of the reservoir. Total uptake was determined from the steady-state plateau level reached in each experiment.

Materials. (−)-[Propyl-1,2,3-^{3}H]dihydroalprenolol hydrochloride (48.6 to 51.1 Ci/mmole), (−)-*N*-[methyl-^{3}H]epinephrine (40.8 Ci/mmole), (±)-[7-^{3}H(N)]epinephrine (15.0 Ci/mmole) and [4-^{3}H]clonidine hydrochloride (22.2 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. (−)-Ascorbic acid (sodium salt), (−)-epinephrine bitartrate, (−)-phenylephrine hydrochloride, pargyline hydrochloride and semicarbazide hydrochloride were obtained from the Sigma Chemical Co., St. Louis, MO. The following drugs were donated: (−)-propranolol hydrochloride and (+)-propranolol hydrochloride (Ayerst Laboratories, New York, NY), phentolamine hydrochloride (CIBA Pharmaceuticals, Summit, NJ), clonidine hydrochloride (Boehringer Ingelheim Ltd., Ridgefield, CT) and phenoxybenzamine hydrochloride (Smith Kline & French Laboratories, Philadelphia, PA). All drugs were dissolved in Krebs medium immediately prior to use, except phenoxybenzamine which was dissolved in a small volume of polyethylene glycol and brought up to volume with Krebs medium. All other reagents were of the highest purity commercially available.

RESULTS

[^{3}H]*Dihydroalprenolol perfusion.* Recirculating perfusion of rabbit lungs with 10 nM [^{3}H]DHA is shown in Fig. 1. Control perfusion of both right and left lungs (Fig. 1A) showed identical uptake of [^{3}H]DHA over a period of 60 min, when expressed as percent total tritium remaining in the reservoir. Both lungs removed approximately 85% of initial [^{3}H]DHA present in the reservoir (see Table 1). When right lung was perfused simultaneously with (−)-propranolol (10 or 100 μM) and [^{3}H]DHA and left (control) lung with [^{3}H]DHA alone, an apparent concentration-dependent inhibition of [^{3}H]DHA removal was observed (Fig. 1, panels B and C). Simultaneous perfusion with (+)-propranolol (10 or 100 μM) produced the same effect (Fig. 1, panels D and E). Several other experiments yielded similar results, indicating a lack of stereospecificity in the inhibition of [^{3}H]DHA removal by propranolol.

Simultaneous perfusion of unlabeled drug with radioligand allows examination of the effects of only a single concentration of unlabeled drug on the removal process. Therefore, the experimental protocol was changed. Both lungs were perfused with radioligand until a steady-state level of uptake was achieved, as determined in control experiments. At that time, one lung was challenged with increasing concentrations of an unlabeled drug, added to the reservoir every 5 min, in an attempt to displace radioligand retained by lung. Such an experiment using [^{3}H]DHA is shown in Fig. 2. Control perfusions with 10 nm [^{3}H]DHA alone (Fig. 2A) again showed identical removal by both right and left lungs, reaching a plateau at approximately 20 min. In a separate experiment, right lung

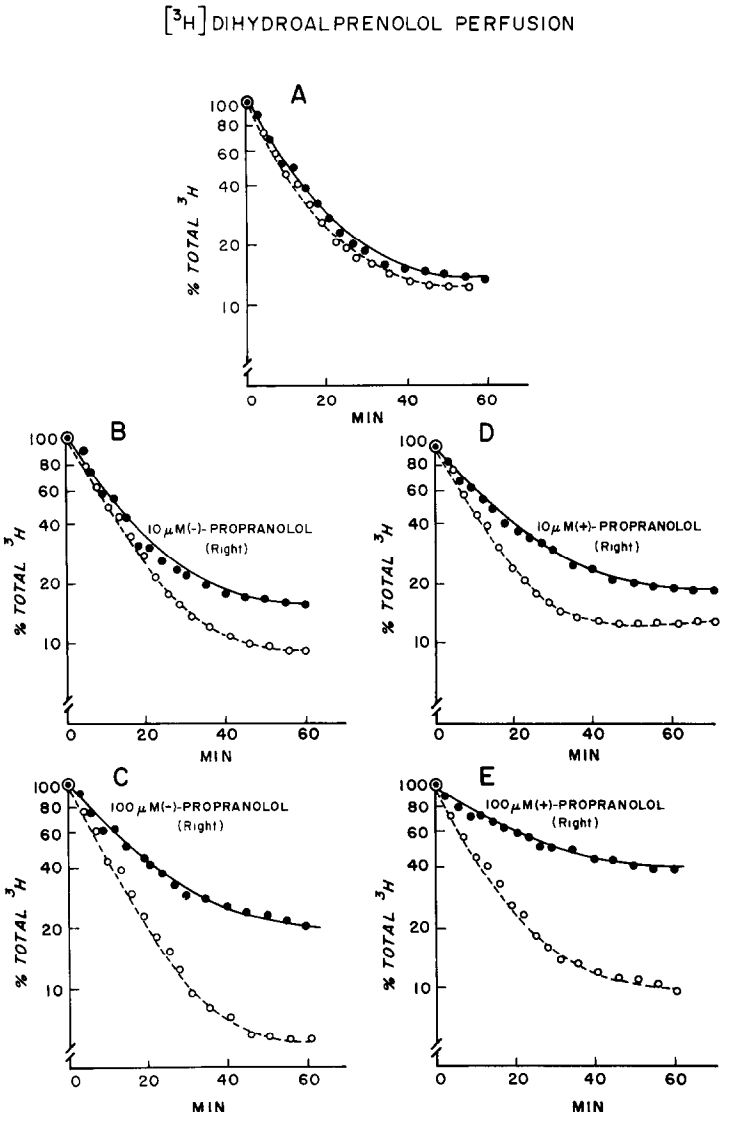

Fig. 1. Inhibition by propranolol of [^{3}H]DHA removal in isolated perfused rabbit lung. Lungs were perfused with 10 nM [^{3}H]DHA. (A) Control perfusions are shown for both right (●——●) and left (○——○) lungs. (B) and (C): The right lung was perfused simultaneously with [^{3}H]DHA and (−)-propranolol (10 and 100 μM); the left lung was the control. (D) and (E): As in (B) and (C), but with (+)-propranolol. Flow rate: 10 ml/min; reservoir volume: 100 ml. Results are from individual experiments. NOTE: in all figures (●——●) represents right lung and (○——○) represents left lung.

was challenged with increasing concentrations of (−)-propranolol starting 30 min after perfusion with [^{3}H]DHA was initiated (Fig. 2B). There was an apparent concentration-dependent displacement of [^{3}H]DHA from lung back into the reservoir (perfusate) (increase in percent total ^{3}H), compared with left control lung perfused with [^{3}H]DHA alone, which remained at the steady-state level of removal. (+)-Propranolol produced similar results over the same concentration range (Fig. 2C).

It should be noted that, in these and all subsequent experiments, the concentrations of unlabeled drug added to the reservoir represented initial reservoir concentrations, uncorrected for either removal by lung or cumulative increase with successive additions.

[^{3}H]*Epinephrine perfusion*. Since [^{3}H]DHA was removed so extensively by lung, perhaps indicating very high non-specific binding and thus preventing detection of specific displacement, we chose to use a ligand removed to a much lesser degree by lung, namely [^{3}H]EPI [12, 15, 16]. Results of representative experiments are shown in Fig. 3. Control perfusions showed similar removal of [^{3}H]EPI (33%) by both right and left lungs (Fig. 3A; Table 1). Perfusion pressures remained constant for the duration of the experiment (Fig. 3A). When the right lung, perfused with 10 nM [^{3}H]EPI, was challenged with increasing concentrations of the α-adrenergic receptor agonist, (−)-phenylephrine, a concentration-dependent increase in perfusion pressure was observed (Fig. 3B). However, there was no apparent displacement of [^{3}H]EPI from lung back

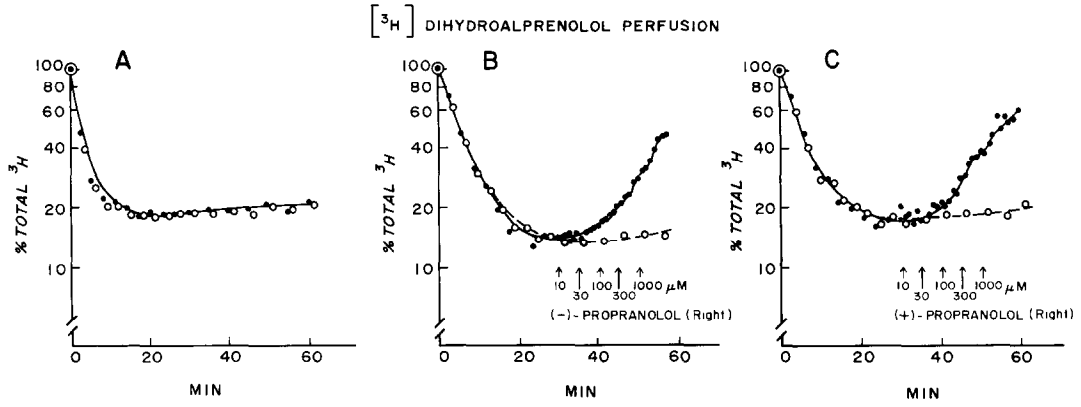

Fig. 2. Displacement by propranolol of [^{3}H]DHA from isolated perfused rabbit lung. (A): Control perfusions with 10 nM [^{3}H]DHA. (B): Challenge of right lung beginning at 30 min with increasing concentrations of (−)-propranolol; left lung was control. (C): As in (B), but with (+)-propranolol. Flow rates: 20 ml/min (A), 10 ml/min (B and C); reservoir volume: 50 ml. Results are from individual experiments.

into the reservoir. Left (control) lung removal of [^{3}H]EPI remained constant, as did perfusion pressure. In other experiments, increasing concentrations of the α-receptor antagonist, phentolamine, also did not displace [^{3}H]EPI from lung (data not shown).

Unlabeled (−)-epinephrine should displace [^{3}H]EPI quite effectively. Challenge of the right lung, perfused with 10 nm [^{3}H]EPI, with increasing concentrations of (−)-epinephrine resulted in a concentration-dependent increase in perfusion pressure, which was reversed by phentolamine, but no apparent displacement of [^{3}H]EPI compared with left control lung was observed (Fig. 3C). Increasing the concentration of [^{3}H]EPI perfusing the lungs to 30–40 nM with subsequent challenge of one lung with (−)-epinephrine resulted in inconsistent displacement of [^{3}H]EPI from the lung. When apparent displacement was observed, it occurred only at high concentrations of (−)-epinephrine which produced near-maximal physiological responses (Fig. 3D).

[^{3}H]*Clonidine perfusion.* The final agent examined was clonidine, a known α-receptor partial agonist in rabbit pulmonary artery [17]. Lung removal of [^{3}H]CLON was found to be intermediate between [^{3}H]DHA and [^{3}H]EPI; total uptake was approximately 50% (Table 1). Pulmonary removal of [^{3}H]CLON in control perfusions (Fig. 4A) reached and remained at a plateau level from 15 min after initiation of perfusion until the experiment was terminated at 60 min. Right and left lung removals were identical. Neither challenge with phentolamine nor (−)-phenylephrine at concentrations up to 100 μM was able to displace 10 nM [^{3}H]CLON from lung (data not shown). When lungs were perfused with 20 nM [^{3}H]CLON, unlabeled clonidine was able to displace [^{3}H]CLON from both right (Fig. 4B) and left (Fig. 4C) lung, compared to contralateral control lung. This effect occurred at the same concentrations of clonidine that produced an increase in perfusion pressure which was antagonized by phentolamine (100 μM) added at the end of the experiment (Fig. 4C). Such results suggested the possible specific displacement of [^{3}H]CLON from lung corresponding to the physiological response produced by clonidine.

In an attempt to characterize these effects, a series of experiments were conducted in which both lungs were perfused with 10 μM phentolamine for 15 min, followed by perfusion with 20 nM [^{3}H]CLON. One lung was then challenged with increasing concentrations of clonidine. As the results in Fig. 4D indicate, there was antagonism of the vasoconstrictor response to low concentrations of clonidine, as expected with an α-receptor antagonist, and a corresponding decrease in the displacement of [^{3}H]CLON from lung. These data suggested that phentolamine may indeed have competitively inhibited [^{3}H]CLON interaction with α-adrenergic receptors, effectively reducing the amount of [^{3}H]CLON to be specifically displaced from lung by clonidine. The results of a number of these experiments are shown in Fig. 5. There was no difference in apparent displacement of [^{3}H]CLON from lung by clonidine between control lungs and those pretreated with 10 μM phentolamine.

Similar experiments were performed with the irreversible α-receptor antagonist, phenoxybenzamine. In these studies, one lung was pretreated, with phenoxybenzamine followed by perfusion of both with 50 nM [^{3}H]CLON. Both lungs were then challenged with increasing concentrations of clonidine. An experiment in which the right lung was pretreated with phenoxybenzamine and left lung served as control is shown in Fig. 6A. Displacement of [^{3}H]CLON from both lungs was identical. The results of several experiments (Fig. 6B) indicated that displacement of [^{3}H]CLON was not altered by the presence of phenoxybenzamine which irreversibly removes a portion of the α-receptor population from the total pool with which [^{3}H]CLON interacts (and which prevented vasoconstriction with clonidine up to 30 μM).

Clearance and total uptake. Values for both clearance and total uptake of each radiolabeled ligand are shown in Table 1. Approximately 85% uptake of [^{3}H]DHA occurred in both right and left lungs; thus, clearance of [^{3}H]DHA was the highest of the three ligands tested. Both pulmonary clearance and total uptake of [^{3}H]EPI were the lowest of the three ligands studied, with the corresponding values for [^{3}H]CLON being of intermediate magnitude. These clearance

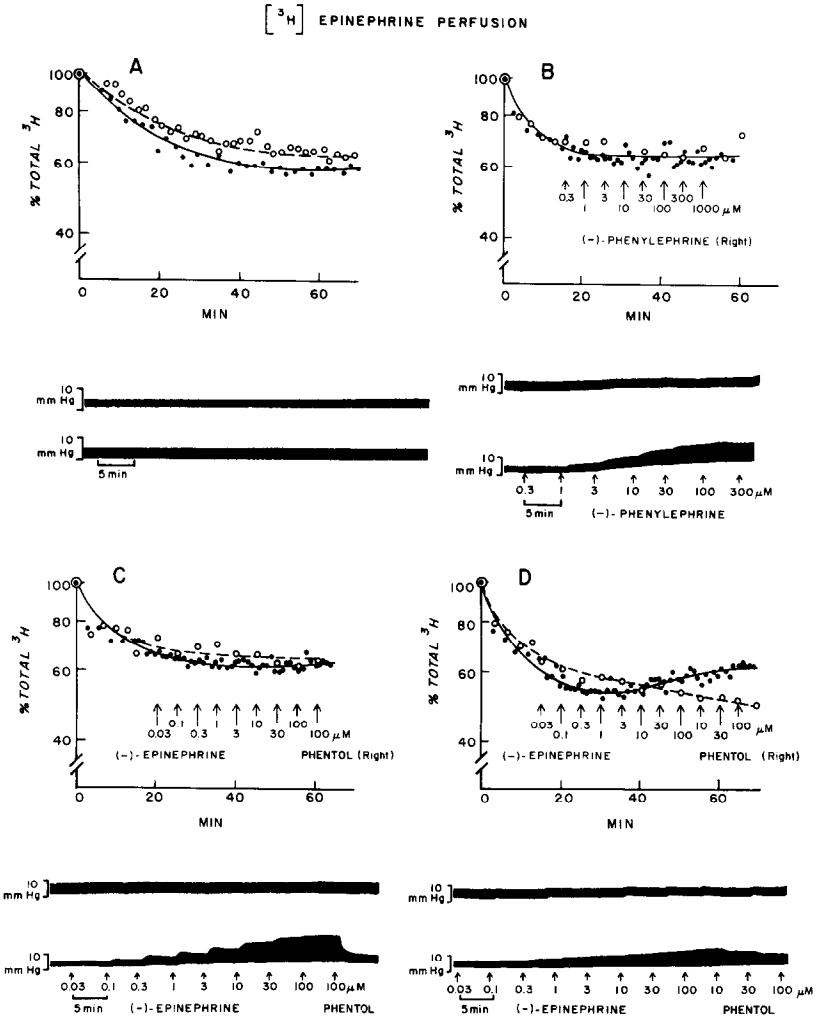

Fig. 3. Uptake and displacement of [^{3}H]EPI. (A): Control perfusions with 40 nM (±)-[^{3}H]EPI showing similar uptake in both right and left lung. Perfusion pressures are below uptake curves; the upper tracing represents left lung and the lower tracing represents right lung in all panels. (B): Both lungs were perfused with 10 nM (−)-[^{3}H]EPI and at 16 min the right lung was challenged with increasing concentrations of (−)-phenylephrine. (C): Both lungs were perfused with 10 nM (−)-[^{3}H]EPI and at 20 min the right lung was challenged with increasing concentrations of (−)-epinephrine, and at 60 min with 100 μM phentolamine (PHENTOL). (D): The concentration of (−)-[^{3}H]EPI perfusing the lungs was increased to 30–40 nM and, beginning at 55 min, 10–100 μM phentolamine was given. Flow rate: 20 ml/min; reservoir volume: 50 ml. Perfusion medium contained 0.1% ascorbic acid, 10 μM pargyline and 1 mM semicarbazide to prevent oxidative degradation of epinephrine. Results are from individual experiments.

Table 1. Removal of [^{3}H]DHA, [^{3}H]EPI and [^{3}H]CLON by isolated perfused rabbit lung*

Ligand	Clearance† (ml/min) Left	Clearance† (ml/min) Right	Total uptake‡ (%) Left	Total uptake‡ (%) Right
[^{3}H]DHA	6.54 ± 0.21 (17)	6.84 ± 0.34 (9)	87.1 ± 1.1 (17)	84.2 ± 1.2 (9)
[^{3}H]EPI	1.12 ± 0.12 (11)	1.20 ± 0.14 (11)	33.6 ± 2.1 (9)	33.2 ± 2.0 (8)
[^{3}H]CLON				
Control	3.35 ± 0.31 (91)	4.14 ± 0.28 (11)	47.2 ± 1.9 (11)	51.8 ± 1.4 (11)
Phentolamine	3.63 ± 0.27 (8)	4.10 ± 0.18 (8)	50.9 ± 1.8 (9)	56.3 ± 1.8 (9)

* Results are from perfusions with 10 nM [^{3}H]DHA, 10 and 30–40 nM [^{3}H]EPI and 20 nm [^{3}H]CLON (control and in the presence of 10 μM phentolamine). Each value is the mean ± S.E. (*n*).

† Clearance values were calculated as described in Materials and Methods.

‡ Total uptake represents combined values from the entire plateau level of uptake in control perfusions and from plateau level of uptake during the control period immediately preceding challenge with unlabeled drug.

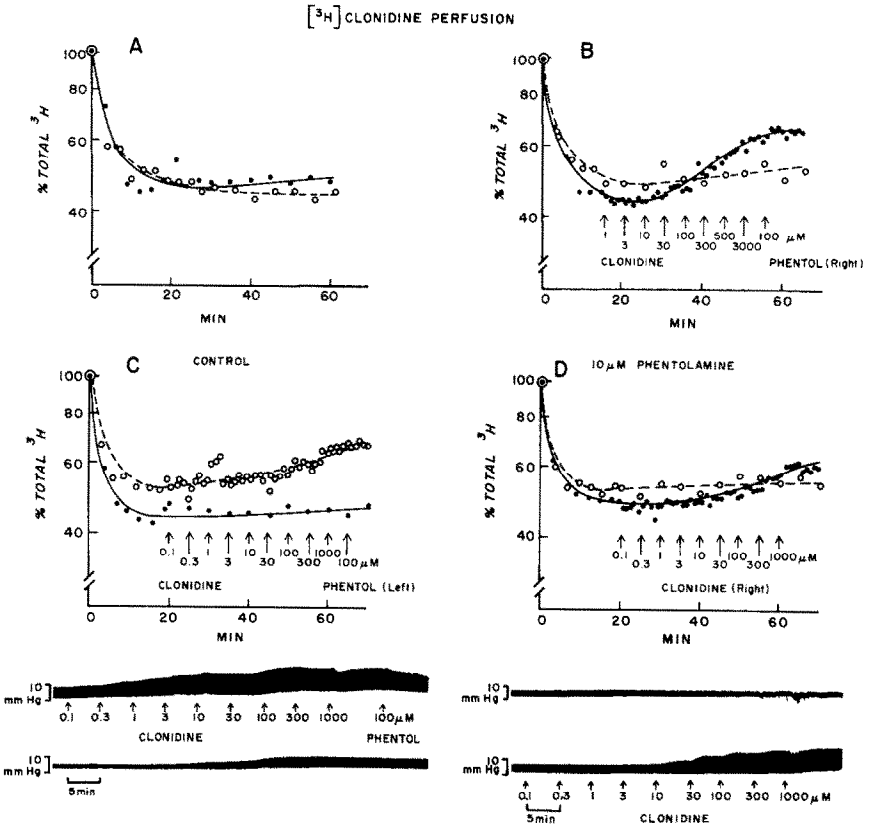

Fig. 4. Uptake and displacement of [^{3}H]CLON. (A): Control perfusions of both right and left lungs with 10 nM [^{3}H]CLON. (B) and (C): Displacement of 20 μM [^{3}H]CLON by clonidine, from right (B) and left (C) lungs, and effects of 100 μM phentolamine (PHENTOL) on perfusion pressure. (D): Pretreatment of both lungs with 10 μM phentolamine for 15 min, followed by perfusion with 20 nM [^{3}H]CLON. Flow rate: 20 ml/min; reservoir volume: 50 ml. Results are from individual experiments.

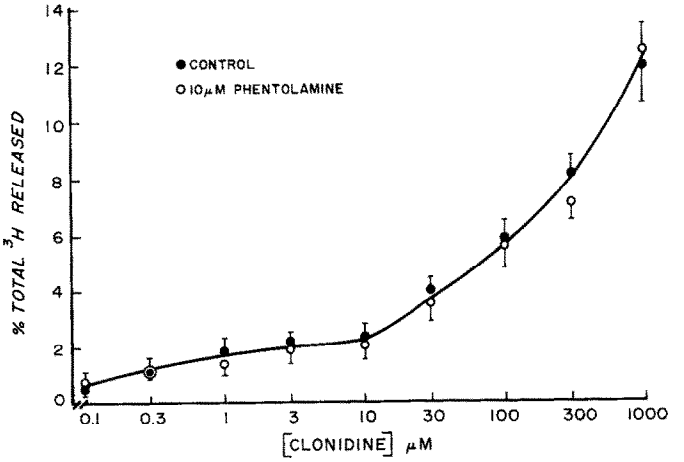

Fig. 5. Effect of phentolamine on [^{3}H]CLON displacement. Lungs were perfused with 20 nM [^{3}H]CLON in the absence (control, ●) or presence of 10 μM phentolamine (○) and then challenged with increasing concentrations of unlabeled clonidine, as in Fig. 4, panels B–D. Data from both right and left lungs were combined. Each point is the mean ± S.E. of four to thirteen determinations of total tritium displaced from both right and left lungs.

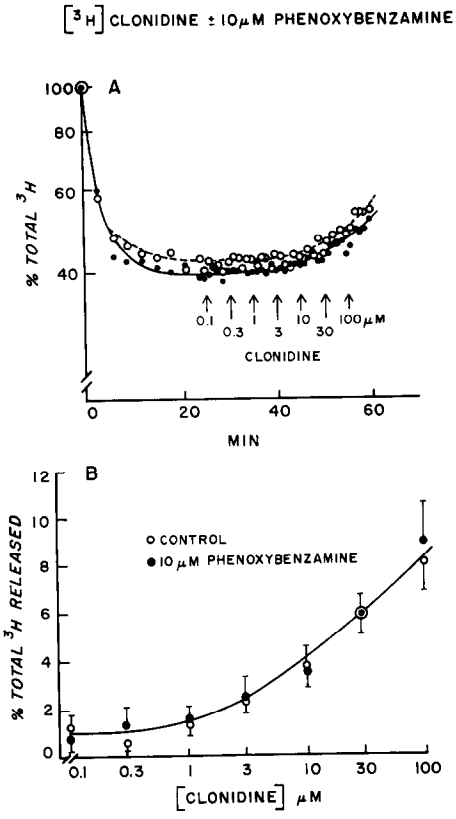

Fig. 6. Effects of phenoxybenzamine on uptake and displacement of [^{3}H]CLON. In (A), only right lung was pretreated with 10 μM phenoxybenzamine for 15 min. Both lungs were then perfused with 50 nM [^{3}H]CLON and challenged with increasing concentrations of unlabeled clonidine. Results from four experiments (combined data from right and left lungs) are summarized in the concentration–effect curve (B).

estimates (Table 1) may be compared with those calculated from earlier experiments using single-pass perfusion of rabbit lungs [12]: 5-hydroxytryptamine, 5.6 ml/min; norepinephrine, 3.89 ml/min; epinephrine, 1.29 ml/ml.

Thin-layer chromatographic analysis of perfusate samples showed no evidence of enzymatic degradation of [^{3}H]DHA, [^{3}H]EPI or [^{3}H]CLON for the duration of perfusion under conditions used in these experiments. This finding was also confirmed by the steady-state plateau level of removal. If metabolites had been formed, they would have been released from lung into the perfusate and the total tritium curve would have risen rather than remain constant. Such an effect has been observed for uptake of a rapidly metabolized amine, 5-hydroxytryptamine, in a recirculating perfusion system [18]. Since this did not occur in our experiments, rapid metabolism and release of metabolites from lung apparently did not take place for [^{3}H]DHA, [^{3}H]EPI or [^{3}H]CLON.

DISCUSSION

Uptake and displacement of three adrenergic receptor radioligands were examined in isolated perfused rabbit lungs. Pulmonary removal of [^{3}H]DHA, a β-adrenergic receptor antagonist, was approximately 85% in both right and left lungs, a value in good agreement with that reported by Blanck and Gillis [10] using a similar preparation. The absence of [^{3}H]DHA metabolism in our study also confirms previous data [10]. Extensive pulmonary removal of DHA, without subsequent metabolism, is similar to the previously reported fate of propranolol [6, 10], which is also a lipophilic β-adrenergic receptor antagonist. In contrast, removal of epinephrine by lung is quantitatively small [12, 15, 16]. In the present study, total uptake of [^{3}H]EPI was somewhat higher than that generally observed, although calculated [^{3}H]EPI clearance was similar to that previously reported during single-pass perfusion of rabbit lungs [12].

Since there was no enzymatic degradation of [^{3}H]DHA, [^{3}H]EPI or [^{3}H]CLON, expression of data as percent total tritium (i.e. unchanged ligand) remaining in the reservoir versus time of perfusion was considered valid. Expressed in this manner, removal of each ligand was similar in both right and left lungs (Table 1); thus our experimental design legitimately involved study of the effects of drug intervention in one lung while the contralateral lung (simultaneously perfused) served as control.

A previous study [10] showed that after a 10-min perfusion [^{3}H]DHA retained by rabbit lung was almost entirely (97%) associated with particulate material. It appeared reasonable then to attempt to characterize specific sites of sequestration for [^{3}H]DHA in intact rabbit lung. We first used the paired lung preparation where one lung was perfused with [^{3}H]DHA alone and the other with both [^{3}H]DHA and propranolol simultaneously. The difference in [^{3}H]DHA retention by the two lungs is taken as an approximation of specific ligand binding, based on the assumption that propranolol would interfere with only [^{3}H]DHA interaction at specific binding sites (presumably β-adrenergic receptors). This assumption is fundamental to radioligand-receptor binding studies in membrane fragments *in vitro* [19], unless interference with non-specific binding is apparent [20]. Our data indicate that both (−)- and (+)-propranolol interfere equally with lung removal of [^{3}H]DHA (Fig. 1, panels B–E). Such lack of stereospecificity is inconsistent with interaction with specific sequestration sites for [^{3}H]DHA.

A shortcoming of this method was that only one concentration of unlabeled competing agent could be examined during each lung perfusion. Therefore, in our second approach, both (−)- and (+)-propranolol (over the same concentration range) were able to displace [^{3}H]DHA from lungs (Fig. 2, panels B and C), again indicating a lack of stereospecificity of the process. Thus, we could not demonstrate specific sequestration of [^{3}H]DHA by lung. This may reflect the fact that non-specifically bound [^{3}H]DHA was displaced to a large extent, and specifically bound [^{3}H]DHA accounted for only a small percentage of the total [^{3}H]DHA displaced by propranolol.

Since [^{3}H]DHA removal was so extensive, we next used epinephrine, which is removed to a limited degree by perfused lung but which acts at both α- and β-adrenergic receptors. Displacement of specifically bound [^{3}H]EPI was also not observed, even with agents such as phenylephrine and epinephrine which

produced concentration-dependent increases in perfusion pressure (Fig. 3, panels B and C). Possibly epinephrine, a full agonist, interacts with only a small percentage of the entire receptor population in order to produce its physiological effects. Therefore, displacement of specifically bound [^{3}H]EPI may be too small to observe. Use of a partial agonist, which is not removed as extensively as the receptor antagonists propranolol, dihydroalprenolol and dihydroergocryptine [10], may circumvent this problem, since the former would be expected to interact with a much larger percentage of the receptor population to produce its physiological effects. Clonidine, a partial α-receptor agonist in rabbit pulmonary artery [17], was chosen.

When the concentration of [^{3}H]CLON perfused was 20 nM, unlabeled clonidine effectively displaced [^{3}H]CLON from lung (Fig. 4, panels B and C). Most importantly, displacement of [^{3}H]CLON occurred over the same concentration range of unlabeled clonidine which produced vasoconstriction (i.e. increases in perfusion pressure). These results suggested that displacement of [^{3}H]CLON from specific sequestration sites, presumably α-adrenergic receptors, was occurring. To test this hypothesis we undertook "receptor protection" experiments. In this approach, lungs were pre-perfused with 10 μM phentolamine followed by perfusion with [^{3}H]CLON; finally one lung only was challenged with unlabeled clonidine. Results of these experiments, such as the one shown in Fig. 4D, suggested that phentolamine antagonized both displacement of [^{3}H]CLON and the α-receptor mediated vasoconstrictor effects of low concentrations of clonidine.

Theoretically, differences in displacement in the absence and presence of phentolamine, i.e. total displacement minus non-specific displacement, respectively, should represent displacement of [^{3}H]CLON from specific (presumably α-receptor) sites in lung. This is based on the assumption that phentolamine would interfere with [^{3}H]CLON interaction only at α-adrenergic receptors and not at non-specific sites of uptake or sequestration. As the results in Fig. 5 indicate, however, phentolamine had no effect on the [^{3}H]CLON displacement curve. Experiments with the irreversible α-receptor antagonist, phenoxybenzamine, yielded similar results (Fig. 6B). Displacement of specifically sequestered [^{3}H]CLON in rabbit lung could not, therefore, be detected, either with the use of agents which caused vasoconstriction (phenylephrine, clonidine) or antagonized these effects (phentolamine, phenoxybenzamine) in the pulmonary vasculature.

We found no correlation between uptake and displacement of sequestered [^{3}H]DHA, [^{3}H]EPI or [^{3}H]CLON and the pharmacological effects of these agents in isolated perfused rabbit lung. Thus, direct determination of specific sites at which these effects occur, presumably α-adrenergic receptors for [^{3}H]DHA, α-adrenergic receptors for [^{3}H]CLON and both for [^{3}H]EPI, was impossible. Perhaps extensive displacement of non-specifically sequestered radioligand prevented detection of the small amount of specifically bound drug.

An alternative interpretation of our data is the following. Both (−)- and (+)-propranolol decreased removal of [^{3}H]DHA when each was perfused simultaneously with [^{3}H]DHA (Fig. 1). These results may also reflect inhibition of [^{3}H]DHA uptake by propranolol, rather than prevention of [^{3}H]DHA binding in lung. This conclusion is supported by the fact that both of these β-adrenergic antagonists were extensively removed by lung (Table 1) [6, 10] and that uptake of propranolol by lung may, in part, be a saturable and energy-dependent process [6, 21, 22] which may involve facilitated transport in addition to its lipid solubility and diffusion through cell membranes [6]. Possibly propranolol and [^{3}H]DHA share a common mechanism of pulmonary removal, and thus they may compete for the same site(s). In this event, high concentrations of propranolol may saturate these sites, thereby inhibiting [^{3}H]DHA uptake. Also, pulmonary removal of other substrates, such as norepinephrine [23], lacks stereospecificity. Therefore, lack of stereospecificity exhibited by propranolol also suggests interference with the [^{3}H]DHA uptake process and, indirectly, that pulmonary removal of propranolol, itself, may not be stereospecific.

Similar conclusions can be drawn from experiments where unlabeled drugs were used to displace radioligand from lung. In the case of [^{3}H]DHA, both (−)- and (+)-propranolol, over the same concentration range, progressively increased the [^{3}H]DHA content of the perfusion medium (Fig. 2, panels B and C). If steady-state level of [^{3}H]DHA removal reflects equilibrium between uptake into and release from lung, then the effect of propranolol can again be interpreted as interference with [^{3}H]DHA uptake. In this experimental situation, propranolol may inhibit [^{3}H]DHA uptake much more than release, the net result being an increase in [^{3}H]DHA content of the perfusate. [^{3}H]DHA release from lung may then continue, unrelated to the decrease in [^{3}H]DHA uptake produced by propranolol. The lack of stereospecificity shown by propranolol, as observed in the simultaneous perfusion studies, again suggests interference with the uptake process. These effects of propranolol are unlikely to be non-specific since a 10 μM concentration of this agent fails to inhibit pulmonary removal of norepinephrine or 5-hydroxytryptamine [24].

These results suggest, therefore, that at least two lipophilic β-adrenergic receptor antagonists, dihydroalprenolol and propranolol, share common removal and/or retention processes in lung (neither can be excluded at this time). This is significant in relation to recent reports on the saturability of propranolol removal in human lung [22]. Increased arterial levels of this β-receptor antagonist were observed in patients on chronic propranolol therapy versus normal control subjects [22]. Possibly, many β-adrenergic receptor antagonists utilize and, therefore, compete for similar uptake and/or sequestration sites, resulting in inhibition of removal or retention by lung with associated increased arterial levels of propranolol.

Similar interpretations may be applied to [^{3}H]EPI and [^{3}H]CLON studies. Removal of [^{3}H]EPI, perfused through the lungs at an initial concentration of 10 nM, was unaffected by increasing concentrations of (−)-phenylephrine, phentolamine or even (−)-epinephrine. However, high concentrations of (−)-epinephrine (> 10 μM) inconsistently displaced

[^{3}H]EPI perfused at initial concentrations of 30–40 nM. Displacement did not correlate with epinephrine-induced vasoconstriction which was near maximal at 10–30 μM. These results do suggest, however, that epinephrine may compete with [^{3}H]EPI uptake at sites having a relatively low affinity without decreasing the rate of release of [^{3}H]EPI from lung. Thus, 10 nM [^{3}H]EPI may have been too low a concentration at which to observe this low affinity uptake (therefore, no apparent displacement was seen) and low concentrations of unlabeled epinephrine were without effect even with 30–40 nM [^{3}H]EPI. Higher concentrations of (−)-epinephrine, which apparently exceeded threshold levels, were required to inhibit [^{3}H]EPI removal by effectively competing for low affinity uptake sites.

Similarly, increasing concentrations of unlabeled clonidine caused a net increase in [^{3}H]CLON content of the perfusate (Fig. 4, panels B and C), which was not correlated with displacement from specific receptor sites (Figs. 5 and 6). Presumably, clonidine was competing with [^{3}H]CLON at uptake sites, so that [^{3}H]CLON uptake was effectively inhibited, while release of retained [^{3}H]CLON from lung continued unaffected. Neither phentolamine nor phenoxybenzamine affected displacement of [^{3}H]CLON (Figs. 5 and 6) or [^{3}H]CLON clearance and total uptake (Table 1). Both of these α-adrenergic receptor antagonists inhibited norepinephrine uptake in rabbit lungs, but did not affect 5-hydroxytryptamine accumulation [24]. Although these latter studies used single-pass perfusion (therefore, a 10 μM concentration of these antagonists perfusing the lungs was constant, whereas in recirculating perfusion there is a decrease in their concentration due to pulmonary removal), they suggest that in our experiments [^{3}H]CLON removal did not proceed by the same mechanism used for norepinephrine uptake. This conclusion is also supported by the fact that phentolamine, phenoxybenzamine [25, 26] and clonidine [27] inhibit extraneuronal uptake of norepinephrine. Since neither 10 μM phentolamine nor 10 μM phenoxybenzamine inhibited [^{3}H]CLON removal, it appears that the uptake of [^{3}H]CLON by lung may have occurred through a different mechanism.

In each series of experiments, micromolar concentrations of unlabeled competing agent were required to effectively displace radioligand from lung. Such concentrations are more closely associated with uptake processes rather than with receptor interactions which are expected to occur at lower concentrations. This was particularly true for epinephrine which causes receptor-mediated physiological effects at relatively low concentrations. In contrast, clonidine is a less potent agonist and produced physiological responses at concentrations associated with uptake processes; thus apparent displacement of [^{3}H]CLON was produced at the same concentrations of clonidine that caused increased perfusion pressures, although the two processes were apparently not related. In conclusion, therefore, our results revealed no correlation between displacement of specifically retained radioligand and receptor-mediated physiological responses. In fact displacement was more closely related to inhibition of uptake and/or non-specific retention of the radioligands tested.

Acknowledgements—We are grateful to Mrs. M. L. Pearson and Mrs. M. Clark for their expert technical assistance. This work was supported by Grants HL 23245 and HL 13315 from the U.S. Public Health Service.

REFERENCES

1. C. N. Gillis and J. A. Roth, *Biochem. Pharmac.* **25**, 2547 (1976).
2. J. D. Catravas and C. N. Gillis, *J. Pharmac. exp. Ther.* **213**, 120 (1980).
3. C. N. Gillis, in *The Mechanism of Neuronal and Extraneuronal Transport of Catecholamines* (Ed. D. M. Paton), p. 281. Raven Press, New York (1976).
4. V. A. Alabaster and Y. S. Bakhle, *Br. J. Pharmac.* **40**, 468 (1970).
5. A. F. Junod, *J. Pharmac. exp. Ther.* **183**, 341 (1972).
6. C. T. Dollery and A. F. Junod, *Br. J. Pharmac.* **57**, 67 (1976).
7. C. N. Gillis, in *Vascular Neuroeffector Mechanisms* (Eds. J. A. Bevan, T. Godfriend, R. A. Maxwell and P. M. Vanhoutte), p. 304. Raven Press, New York (1980).
8. E. L. Rugg, D. B. Barnett and S. R. Nahorski, *Molec. Pharmac.* **14**, 996 (1978).
9. P. Barnes, J. Karliner, C. Hamilton and C. Dollery, *Life Sci.* **25**, 1207 (1979).
10. T. J. J. Blanck and C. N. Gillis, *Biochem. Pharmac.* **28**, 1903 (1980).
11. R. J. Altiere, J. S. Douglas and C. N. Gillis, *Naunyn-Schmiedeberg's Archs Pharmac.* **316**, 278 (1981).
12. C. N. Gillis and Y. Iwasawa, *J. appl. Physiol.* **33**, 404 (1972).
13. H. Steinberg, D. J. P. Bassett and A. B. Fisher, *Am. J. Physiol.* **228**, 1298 (1975).
14. D. A. Wiersma and R. A. Roth, *J. Pharmac. exp. Ther.* **212**, 97 (1980).
15. J. Hughes, C. N. Gillis and F. E. Bloom, *J. Pharmac. exp. Ther.* **169**, 237 (1969).
16. J. C. Boileau, L. Campeau and P. Biron, *Revue Can. Biol.* **30**, 281 (1971).
17. K. Starke, H. Montel, W. Gayk and R. Merker, *Naunyn-Schmiedeberg's Archs Pharmac.* **285**, 133 (1974).
18. L. S. Angevine and H. M. Mehendale, *Am. Rev. resp. Dis.* **122**, 891 (1980).
19. L. T. Williams and R. J. Lefkowitz, *Receptor Binding Studies in Adrenergic Pharmacology*, p. 49. Raven Press, New York (1978).
20. S. R. Nahorski and A. Richardson, *Br. J. Pharmac.* **66**, 469P (1979).
21. D. W. Schneck, J. F. Pritchard and A. H. Hayes, *J. Pharmac. exp. Ther.* **203**, 621 (1977).
22. D. M. Geddes, K. Nesbitt, T. Traill and J. P. Blackburn, *Thorax* **34**, 810 (1979).
23. Y. Iwasawa, C. N. Gillis and G. Aghajanian, *J. Pharmac. exp. Ther.* **186**, 498 (1973).
24. Y. Iwasawa and C. N. Gillis, *J. Pharmac. exp. Ther.* **188**, 386 (1974).
25. A. J. Eisenfeld, J. Axelrod and L. Krakoff, *J. Pharmac. exp. Ther.* **156**, 107 (1967).
26. S. L. Lightman and L. L. Iversen, *Br. J. Pharmac.* **37**, 638 (1969).
27. P. J. Salt, *Eur. J. Pharmac.* **20**, 329 (1972).