Br. J. Pharmac. (1985), **84**, 107–120

Autoradiographic study of the distribution of [^{3}H]- and [^{14}C]-hydrallazine in the rat

John R.J. Baker*, Gillian R. Bullock & Ian H.M. Williamson

Research Centre, Ciba-Geigy Pharmaceuticals Division, Wimblehurst Road, Horsham, Sussex, RH12 4AB

1 The distribution of [^{3}H]-hydrallazine (HP) in the rat was investigated using autoradiography from the whole-body, to the electron microscopic level.

2 Intravenous dosing gave rapid and persistent labelling of blood vessels, particularly arteries, whilst radiolabel from orally administered drug was detectable in the vasculature in modest amounts only at 6 h, the longest interval studied.

3 Light microscopic autoradiographs of blood vessels showed silver grains associated with elastic laminae and the marginal region of smooth muscle cells.

4 Analysis of electron microscopic autoradiographs of arteries from rats dosed intravenously (1 h and 6 h) and orally (6 h) revealed the greatest percentage of radiolabel in each case to be associated with the elastic laminae (34.9 to 40.3%).

5 Significant proportions of total radiolabel (between 12.7 and 34.8%) were ascribed to the smooth muscle cells.

6 It is concluded that radiolabel, possibly in the form of intact HP, is accessible to the vascular smooth muscle cells where the vasodilator action of HP is held to be exerted.

Introduction

The pharmacological properties of 1-hydrazinophthalazine hydrochloride (hydrallazine; HP) which heralded its present-day value as an antihypertensive drug were first described by Gross *et al.* (1950). The therapeutic characteristics of the compound were its gradual onset of action, moderate blood pressure (BP) lowering effect and long duration of action.

While HP received much support in the United States in the treatment of hypertension, it fell rapidly from favour in the majority of European countries where up to 1–2 g per day had been given and had led to a high incidence of headache, tachycardia, angina, some myocardial infarction and systemic lupus erythematosus-like syndrome (Yonkman & Freis, 1952; Taylor *et al.*, 1952; Moyer, 1953; Morrow *et al.*, 1953; Judson *et al.*, 1956).

The shortcomings of HP monotherapy have been largely related to side-effects. Nevertheless, the BP response has been only moderate due to the reflex tachycardia and a compensatory rise in BP (Åblad, 1963). Drugs such as β-adrenoceptor blockers can markedly improve the antihypertensive effect of HP when given concurrently (Zacest *et al.*, 1972), since they suppress reflex tachycardia.

Hence, in recent years there has been renewed interest in HP in the management of hypertension. In combination with β-adrenoceptor blockers and sometimes also diuretics it can be employed effectively to control moderate to severe hypertension at low doses, frequently no more than 200 mg per day. There is thus also a revival of interest in its previously undetermined mode of action. Despite some evidence that HP has a central site of action (e.g. Lim *et al.*, 1955) it has become evident (Åblad, 1963) that its hypotensive effect is exerted directly on the vascular smooth muscle.

Perry *et al.* (1962) discovered that ^{14}C-labelled HP injected into mice produced an intense concentration of radiolabel in arterial walls. Moreover, Moore-Jones & Perry (1966), using intramuscular injections of [^{14}C]-HP in mice, showed autoradiographically that radiolabel was concentrated in arteries of kidney, liver, spleen, heart, lung, brain and skeletal muscle. Wagner & Hedwall (1972) showed that whole-body autoradiography of mice after intravenous administration revealed a rapid and persistent

*Correspondence

association of radiolabel with major and minor blood vessels of several organs.

The absorption and elimination of [^{14}C]-HP in the rat and dog were observed by Wagner (1972, unpublished) who found that the recovery of label in urine and faeces was very similar whether the dose (5 mg kg^{-1}) had been administered intravenously or orally. There have, however, been no comparative studies on tissue levels of orally (p.o.) and intravenously (i.v.) dosed animals and none of the limited autoradiographic studies so far described has employed the oral route.

The purpose of the present study was two fold. Firstly, to use whole-body autoradiography to compare the fate of radiolabel in the rat following [^{3}H]-HP or [^{14}C]-HP administration. The second objective was to use high resolution autoradiography with [^{3}H]-HP up to the quantitative electron microscopic level to elucidate the site and therefore possibly to infer the mode of action of HP in blood vessels after single oral or intravenous administration.

Methods

Radioactive preparations of hydrallazine

Preparation 1 1-Hydrazino 6-[^{3}H](N) - phthalazine hydrochloride prepared at CIBA-GEIGY, Basle, by Dr W. Küng; specific radioactivity 3.2 Ci mmol^{-1}.

Preparation 2 1-Hydrazino 6-[^{3}H](N) - phthalazine hydrochloride prepared at the Research Centre, CIBA-GEIGY Pharmaceuticals Division, Horsham, by Dr D.E. Brundish; specific radioactivity 17.2 Ci mmol^{-1}.

Preparation 3 1-Hydrazino 1-[^{14}C] - phthalazine hydrochloride prepared at CIBA-GEIGY, Basle, by Dr W. Küng; specific radioactivity 2.34 mCi mmol^{-1}.

Whole body autoradiography

After intravenous [^{3}H]-hydrallazine Four male Wistar rats (300–350 g) were used, one each for killing after 5 min, 30 min, 2 h and 6 h. To each animal (all conscious except that to be killed 5 min after dosing) was administered [^{3}H]-HP (Prep. 1) plus unlabelled HP via a tail vein in a total volume of 1 ml sterile saline, amounting to a dose of 7.7 mg kg^{-1} (96 mCi mmol^{-1}). At the prescribed time the animals were anaesthetized with pentobarbitone sodium i.p. (60 mg kg^{-1}) and frozen for 15 min in a hexane/dry ice mixture. Longitudinal vertical sections (20 μm) were cut in a number of planes in a Bright whole-body cryostat (Bright Instrument Co. Ltd, Huntingdon). After freeze-drying for at least 48 h in the cryostat chamber the sections were apposed firmly to Kodirex X-ray film or Ultrofilm for tritium. After exposure for up to 7 weeks (depending upon film type) the autoradiographs were developed in D19 and fixed in Hypam rapid fixer.

After intravenous [^{14}C]-hydrallazine Animals of a similar weight to those used in the above experiments were dosed and sampled at the same times after injection. Each animal received [^{14}C]-HP (Prep. 3) 9.23 mg kg^{-1} (2.34 mCi mmol^{-1}) in 1 ml sterile saline. Further treatment was as above.

After oral [^{3}H]-hydrallazine Duplicate rats (180–220 g) were dosed orally with [^{3}H]-HP (Prep. 2) each dose comprising 128.5 μg kg^{-1} (17.2 Ci mmol^{-1}) in 0.5 ml water without carrier. Further processing was as above with the exception that 5 min samples were omitted and LKB Ultrofilm only was used.

Light and electron microscopic autoradiography

After oral or intravenous [^{3}H]-hydrallazine Identical doses of 128.5 μg kg^{-1} (17.2 Ci mmol^{-1}) in 0.5 ml water without carrier were used for both routes. Duplicate rats (180–220 g) were used and sampled at 10 min, 1 h and 6 h after oral or intravenous administration. Intravenously dosed animals were injected slowly via a tail vein to minimize the osmotic shock of the aqueous injection which was used to avoid further dilution of the isotope in saline. The dose was also administered slowly to obviate the risk of venous-arterial shunting of the radioactivity. Animals to be killed at 1 h and 6 h were anaesthetized as above before death. Tissues sampled were kidney, liver, aorta, renal artery, renal vein, tail artery, tail vein and ear. The primary fixative was a mixture of glutaraldehyde (2.5%) plus freshly made formaldehyde (4%) in 0.1 M cacodylate buffer, pH 7.2.

Triplicate samples of tail artery were taken from one of the 1 h i.v. animals. These samples were processed in a manner identical to those used for microscopy but were passed through 5 ml aliquots of the processing fluids to measure the elution of tritium from the tissue at each stage (Table 1). Following ethanol dehydration the tissues were each totally digested in 1 M sodium hydroxide (5 ml). Aliquots of 50 μl, from each sample were added to Lumigel (2 ml) and counted for 1 min in an LKB 1251 Rackbeta liquid scintillation spectrometer and the counts averaged and expressed as a percentage of the total.

The tissue samples for microscopy were embedded in Epon/Araldite epoxy resin (Mollenhauer, 1964).

For light microscopic autoradiographs, 'thick' resin sections (1 μm) were mounted on clean glass slides and coated with K2 liquid nuclear emulsion

(Rogers, 1979). After an exposure interval of 4 weeks the autoradiographs were developed in D19 (4 min) and fixed in 30% sodium thiosulphate (10 min). Washing and air drying was followed by staining in toluidine blue (0.1% in 0.1% sodium tetraborate). Photomicrographs were produced from these autoradiographs and given a graded visual estimate of relative grain densities based on a scale from 0 to ++++ (see Table 2).

The method used to prepare electron microscopic (EM) autoradiographs has been described previously (Baker *et al.*, 1977). In the present study, exposure was for 12 weeks. The specimens were developed in D19 or Microdol X depending upon the required number of grains per unit area.

Seven tissue/dose/time combinations were examined at the electron microscopic level: after 10 min i.v., the tail and renal arteries, after 1 h i.v., the tail* and renal arteries, after 6 h p.o., the renal artery* and 6 h i.v., the tail* artery and vein. Only the 3 experiments marked * were analysed quantitatively. In these cases, in order to achieve representative micrographic sampling of the highly differentiated vessels, sequential electron micrographs were taken starting at the endothelial cell layer and progressing ultimately to the outer adventitia. This procedure was repeated in sections taken at two levels in each of the duplicate animals per experimental time. The final print magnification was × 25,000.

Analysis of electron microscopic autoradiographs

It is well established that many of the silver grains in EM autoradiographs do not directly overlie the point in the tissue section from which the radioactive decay particles responsible for their formation originated. This is the result of a number of factors, such as variable electron energy, section and emulsion thickness, angle of emission and above all, the ability of the electron microscope to resolve this discrepancy between source of emission and site of silver grain.

A number of approaches have been proposed which are designed to account for this 'cross-fire' whereby a source of decay in organelle *a*, may result in a silver grain over organelle *b*. In the present study we have used the hypothetical grain method of Blackett & Parry (1973; 1977) in which a computer programme is used to compare hypothetical grain distributions with the real grain distribution. Hypothetical sources of radioactivity are established by applying to the autoradiographs a transparent overlay screen which contains computer-predicted source-to-site distances which generate the hypothetical silver grains 'emanating' from these sources. The directions of these hypothetical decays are, of course, random. The distances are derived from range distribution curves from experimentally measured line sources of a given isotope as first described by Salpeter *et al.* (1969)Hence a rectangular matrix for cross-fire is created, there being invariably more sites than sources. Estimates of radioactivity in the various sources derive from systematic modification of the hypothetical source values by a minimizing sub-routine until the hypothetical and real grain distributions fit optimally for the designated sites as assessed by the χ^2 test. The number of degrees of freedom allowed is the difference between the number of sites and sources in the cross-fire matrix.

A feature of this method is that any hypothesis of radiolabel distribution can be tested. In cases where the crude location of radiolabel is difficult to interpret by visual inspection of silver grains it is usual to collect hypothetical grain data by random or regular point sampling of the micrographs so as to include all the possible sources of radioactivity in the analysis. Indeed, this was the approach adopted in the present study despite the apparent concentration of grains over certain areas.

For the 3 experiments presented, the numbers of hypothetical and real grains counted ranged from 1130–1353 and 535–870, respectively.

Other materials

Unlabelled hydrallazine (Ciba-Geigy, Basle), pentobarbitone sodium (May and Baker), hexane (BDH), sterile saline (Travenol), glutaraldehyde (Taab), paraformaldehyde (BDH), sodium cacodylate (BDH), Epon/Araldite (EM Scope), Ultrofilm (LKB), Kodirex (Kodak), K2 and L4 nuclear emulsions (Ilford), D19 and Microdol X developers (Kodak), Hypam (Ilford), sodium thiosulphate (BDH), Lumigel (LKB).

Results

Whole-body autoradiography

Not all of the observations described are depicted in the Figures since it requires at least 3 planes of sectioning to include all major organs.

The distribution of [^{3}H]-HP after i.v. administration is illustrated in Figure 1 (a and b). The urine (bladder contents) and renal pelvis were the most densely labelled areas of the autoradiographs throughout the period studied (5 min–6 h). The kidney cortex and medulla were equally labelled at 5 and 30 min while the cortex was less intensely labelled than the medulla at 2 and 6 h. The initial build-up of label in the submaxillary salivary gland was reduced by 2 and 6 h but the label in the Harderian gland seemed to have increased over the same period. The

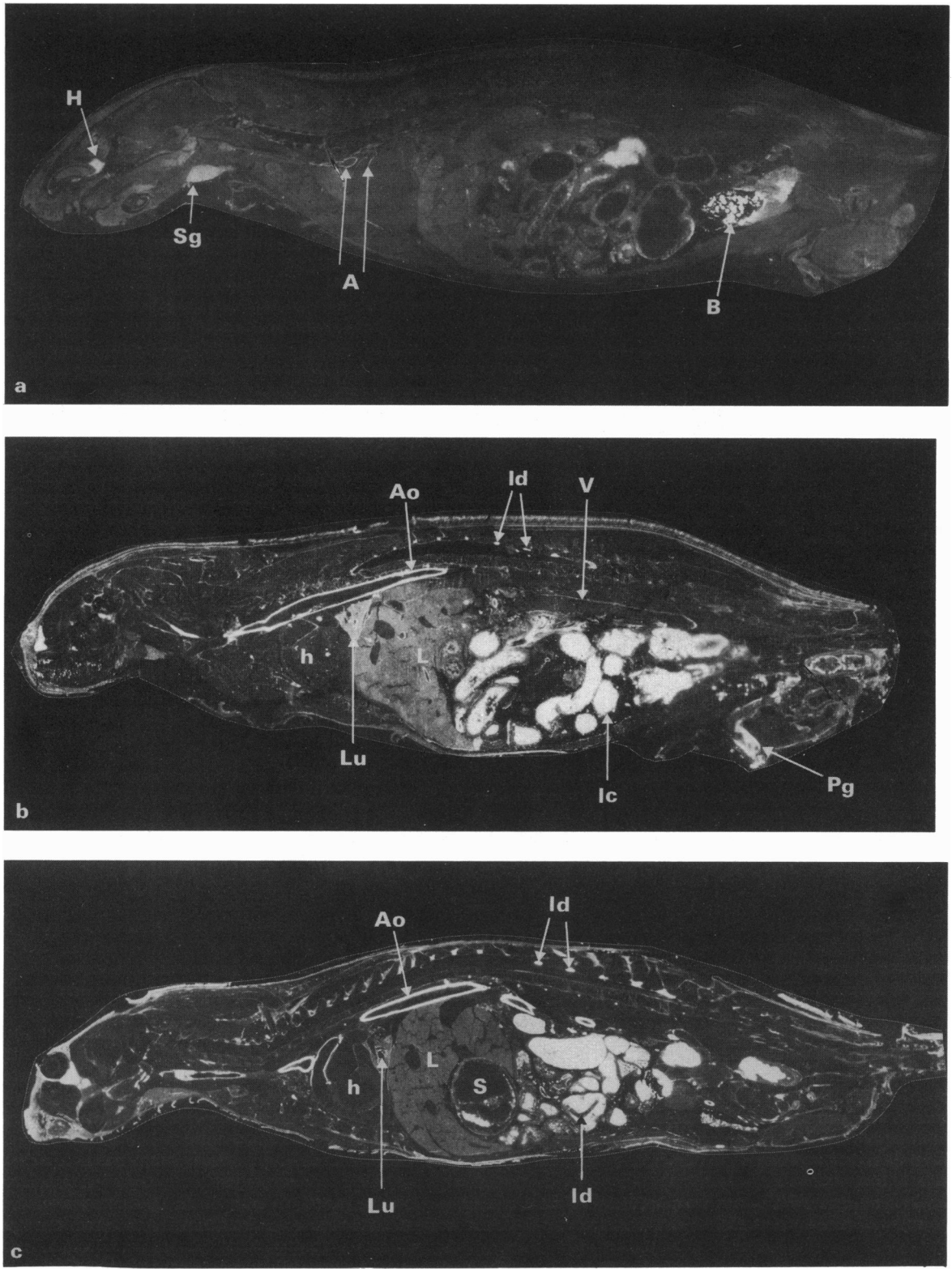

Figure 1 Whole-body autoradiographs of rats following intravenous administration of [^{3}H]-hydrallazine (a and b) or [^{14}C]-hydrallazine (c). (a) Five minutes (b and c) 6 h after injection. Key: A, arterial wall; Ao, wall of aorta; B, bladder content; Ic, intestine content; Id, intervertebral discs; H, Harderian gland; h, heart; K, kidney; L, liver; Lu, lung; Nc, nasal conchae; O, oesophagus; Pg, preputial gland; S, stomach; Sg, salivary gland; V, wall of vena cava.

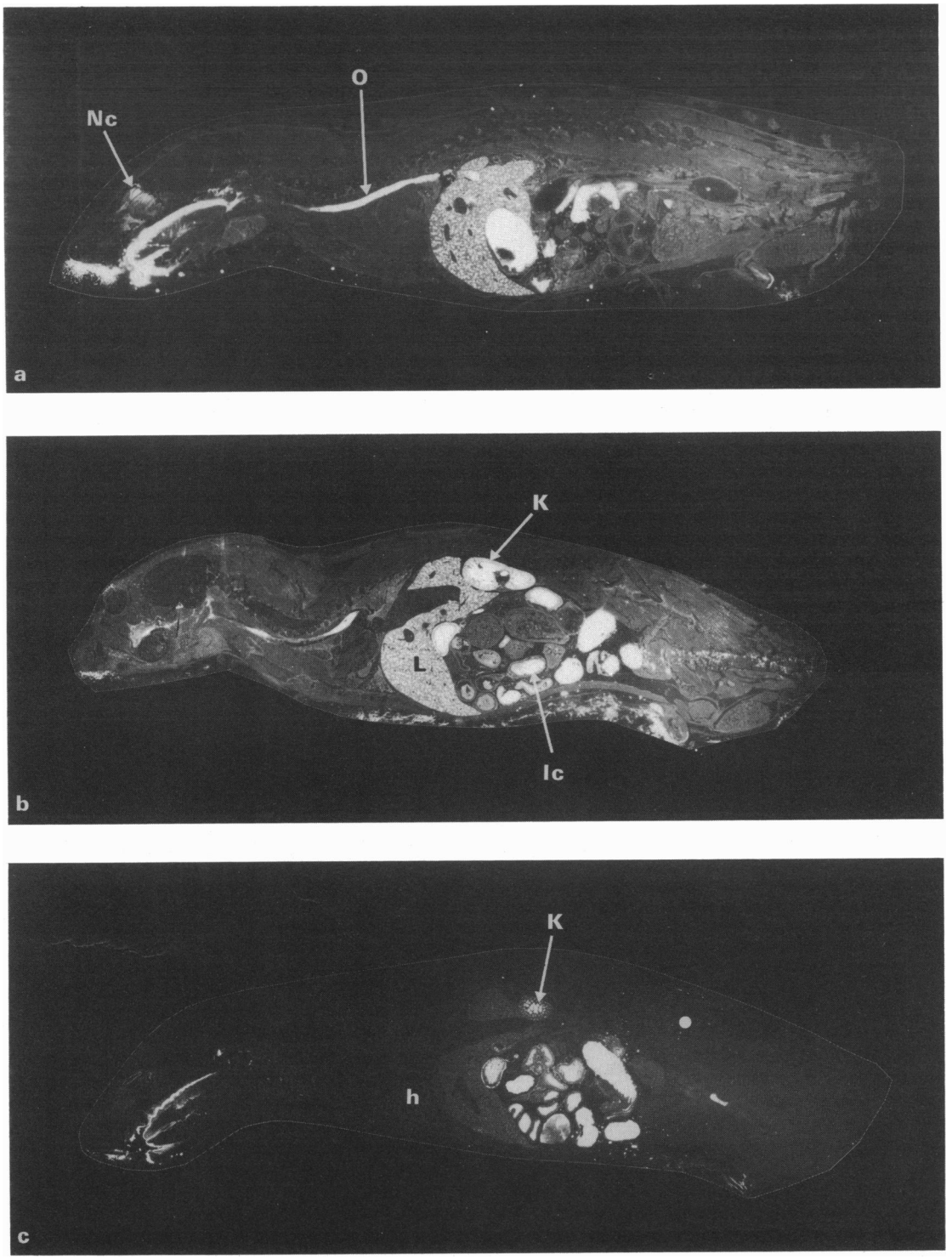

Figure 2 Whole-body autoradiographs of rats (a) 30 min, (b) 2 h and (c) 6 h following oral administration of [^{3}H]-hydrallazine. Key to abbreviations as in Figure 1.

Table 1 Elution of ^3H from rat tail artery (1 h after intravenous administration of [^3H]-hydrallazine) during processing for light and electron microscopy

Solution	*Mean count* ($n = 3$)	*Percentage*
Glutaraldehyde (2.5%) Formaldehyde (4.0%)	4702	26.0
Cacodylate (1)	386	2.1
Cacodylate (2)	78	0.4
Cacodylate (3)	376	2.1
OsO_4 (1% w/v)	36	0.2
Cacodylate	52	0.3
70% EtOH	91	0.5
90% EtOH	41	0.2
Abs EtOH (1)	22	0.1
Abs EtOH (2)	8	0
NaOH digest (1 M)	12259	67.9
Total	18051	99.8

progressive increase with time in the numbers of profiles of the gastro-intestinal tract heavily labelled confirms that significant biliary excretion of HP and/or its metabolites occurred. An intense accumulation of tritium in the walls of the vena cava and dorsal aorta (notably the latter) was a marked feature of the autoradiographs.

No difference in distribution between i.v. administered [^3H]-HP or [^{14}C]-HP was observed (cf. Figure 1 (b and c) except for a slightly higher background level in some of the tritiated preparations.

The distribution of [^3H]-HP after oral administration is illustrated in Figure 2 (a–c). The 30 min autoradiographs showed the expected high labelling of the gastro-intestinal tract as far as, and including some proximal profiles of the small intestine. Nasal conchae in some animals were well labelled presumably due to inhalation or retrograde pharyngeo-nasal passage of part of the dose. The next most marked labelling was in the renal medulla followed by the

Table 2 Light microscopic evaluation of relative intensities of labelling of rat tissues at 10, 60 and 360 min following oral (p.o.) and intravenous (i.v.) administration of [^3H]-hydrallazine

	10 min			
	p.o.		*i.v.*	
	A	*B*	*A*	*B*
Liver	+	0	++	++
Kidney (cortex)	+	+	++	++
(medulla)	++	NE	++	NE
Aorta	0	0	+++	+++
Renal artery	+	+	+++	0
Renal vein	NE	NE	+++	+++
Tail artery	0	0	++++	+++
Ear	0	0	+	0
	60 min			
	p.o.		*i.v.*	
	A	*B*	*A*	*B*
Liver	++	+	++	++
Kidney	+	+	++	+
Aorta	0	0	++	++
Renal artery	0	+	+++	++
Renal vein	NE	+	NE	NE
Tail artery	0	0	++++	++++
Ear	0	0	+	0
Portal vein	0	0	++	++
	360 min			
	p.o.		*i.v.*	
	A	*B*	*A*	*B*
Liver	0	0	+	0
Kidney	+	+	+	+
Aorta	++	+	++	++
Renal artery	++	+	++	++
Renal vein	NE	NE	+	NE
Tail artery	+	+	++++	++
Tail vein	NE	NE	++++	NE
Ear	0	0	+	+

NE = not examined.
A and B = duplicate animals.

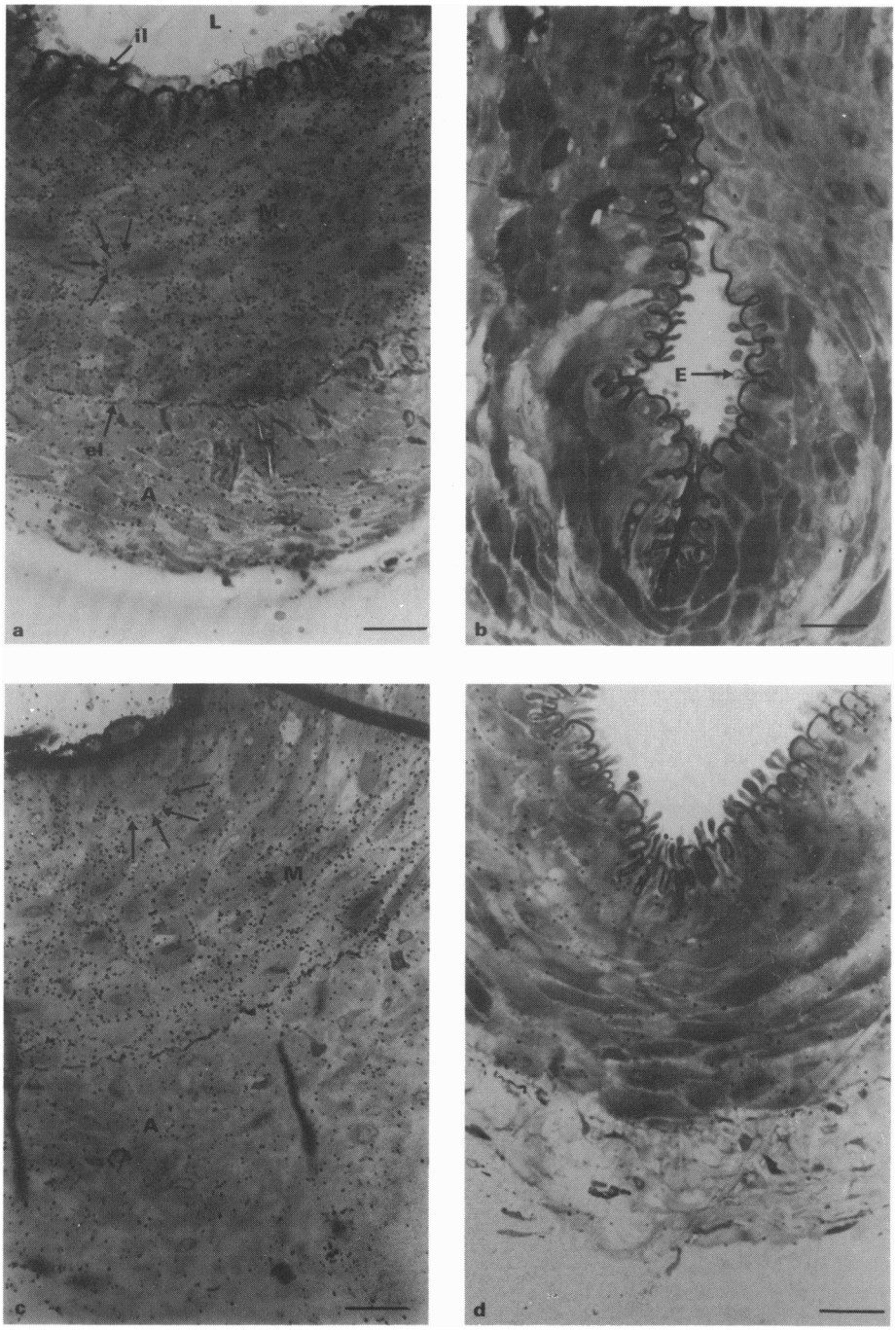

Figure 3 Light microscopic autoradiographs of tail artery. (a) 10 min, (c) 6 h after i.v. administration and (b) 10 min, (d) 6 h after oral administration of [^{3}H]-hydrallazine. Scale bars – 10 μm. (a) Silver grains are evident over the internal elastic lamina (il) but more obvious in the media (M) where many seem to lie close to the margin of the smooth muscle cells (arrows). Note that the heavy toluidine blue staining of the internal elastic lamina prevents the visualization of any silver grains which may be present. L, lumen of artery; el, external elastic lamina; A, adventitia. (b) Very few silver grains are present. E, endothelial cell. (c) There is little obvious change from the situation at 10 min. (d) The silver grain density, particularly over the media has increased notably since 10 min after dosing (cf. b).

liver with patchy label. This pattern changed little at 2 and 6 h with more profiles of the intestine becoming labelled and the diminishing renal label even more concentrated in the renal pelvis. In some sections of animals killed at 2 h there were small densely labelled regions in the lungs, which in transverse section appeared to have lumina and may represent blood vessels or bronchioles which have become labelled by inhalation. Although major blood vessels were sometimes evident in outline, in none of the autoradiographs did they appear to be markedly labelled.

Light microscopic autoradiography

The results shown in Table 1 suggest that most of the [^{3}H]-HP and its major matabolites were retained during the fixation procedure, since 68% of radiolabel was recovered in the digest of tail artery which had been processed identically to samples used for light and electron microscopic autoradiography. Most of the tritium lost was recovered in the primary fixative and may represent material which was not tissue-bound, e.g. in the interstitial fluid.

In general, the tissue samples were less labelled after oral than intravenous administration and the relative intensities are summarized on the basis of visual inspection (scale 0 to ++++) in Table 2. After 10 min, only the renal medulla of one p.o. animal possessed appreciable grain numbers whereas all tissues except the ear from i.v. treated rats were well labelled (Table 2 and Figure 3a). Aorta, renal artery and vein and tail artery were labelled more in the internal elastic lamina and media than in the adventitia and especially in the tail arteries much of the label associated with the smooth muscle cells appeared to be close to the cell margin (Figure 3a).

In the animals sampled after 60 min the situation was much the same (Table 2). However, in one of the p.o. animals there was notable hepatic label, especially in the region surrounding the lobular central veins, presumably corresponding to the patches observed at the whole-body level.

In the aorta of the i.v. animals, the intensity of label seemed lower than in the 10 min animals, although it was still marked in the renal artery where cellular margination was apparent. Portal vein was examined at this time only and was found to have light label in the media in i.v. animals and to be unlabelled where the oral route had been used.

By 6 h (Table 2), in one of the p.o. animals there was an increase of label in the media of aorta, renal and tail arteries (cf. Figure 3b and d). Moreover, the high levels in some of the corresponding vessels of i.v. rats had fallen slightly, although in one of the dupli-

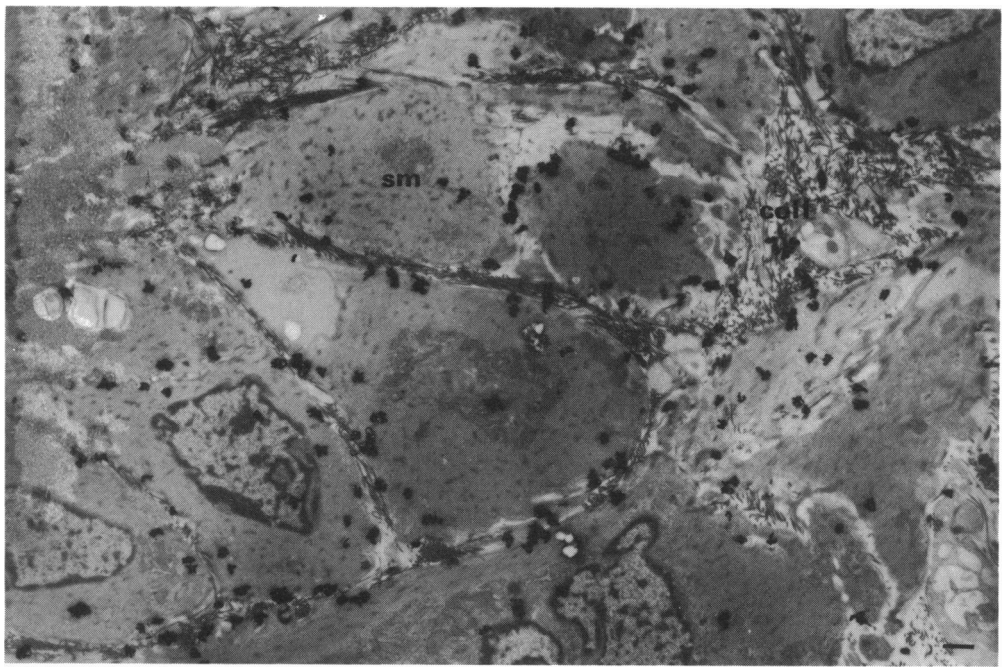

Figure 4 Electron microscopic autoradiograph of tail artery 10 min after i.v. administration of [^{3}H]-hydrallazine. It is clear in the small area of media shown, that most silver grains lie close to the margin of the smooth muscle cells (sm). coll, collagen. Scale bar – 1 μm.

cates the dense labelling of the tail artery (Figure 3c) was maintained with internal elastic lamina and media producing many silver grains but fewer from the adventitia. Grains at the periphery of the tail artery smooth muscle cells were still in evidence. Even in the kidney, where by 6 h after i.v. [^{3}H]-HP there was only background labelling of the cortical tubules, more silver grains could be identified in the walls of arterioles.

Electron microscopic autoradiography of [^{3}H]-hydrallazine following intravenous and oral administration

The most striking observation to be made from examination of EM autoradiographs of arteries from i.v. labelled animals in particular, was the great concentration of silver grains in the region of the internal elastic lamina (e.g. Figure 5). The apparent margination of silver grains around tail artery smooth muscle cells in light microscopic autoradiographs of i.v. labelled animals was confirmed in the EM preparations (e.g. Figures 4 and 5) although it was not possible by visual means to ascribe intra- or extracellular locations to the grains. On rare occasions where smooth muscle cells were well separated it was possible to postulate that silver grains had an affinity for the caveolae-rich cell margins rather than collagen or extracellular space (Figure 6). Where veins had been sampled together with arteries it was noticed that these vessels, which have a lower elastin content than arteries, exhibited a greater grain density within fibrillar collagen (Figure 7). The renal artery of an orally dosed rat after 6 h showed moderate grain numbers which seemed to be associated with the internal elastic lamina and the smooth muscle cell margins.

The results of hypothetical grain analysis of the 3 quantified experiments (see Methods) are shown in Tables 3, 4 and 5. In each case the greatest percentage of radiolabel (35–40%) was in the elastin of the internal elastic lamina. Moreover, where intercellular elastin was retained in the best-fit computer models (i.e. tail artery/i.v./1 h and renal artery/p.o./6 h – Tables 3 and 5) it was deemed to contain 13.5% and 18.3% of total radiolabel, respectively. In both instances, the label was more concentrated in the intercellular than the laminar elastin. In a similar manner when collagen labelling in all 3 experiments was analysed, the concentration factor (grains/grid point) was greater for intracellular than adventitial collagen, irrespective of percentage tritium bound. Smooth muscle cells in each case contained a significant quantity of label ranging from 12.7% (tail artery/i.v./1 h) to 34.8% (renal artery/p.o./6 h).

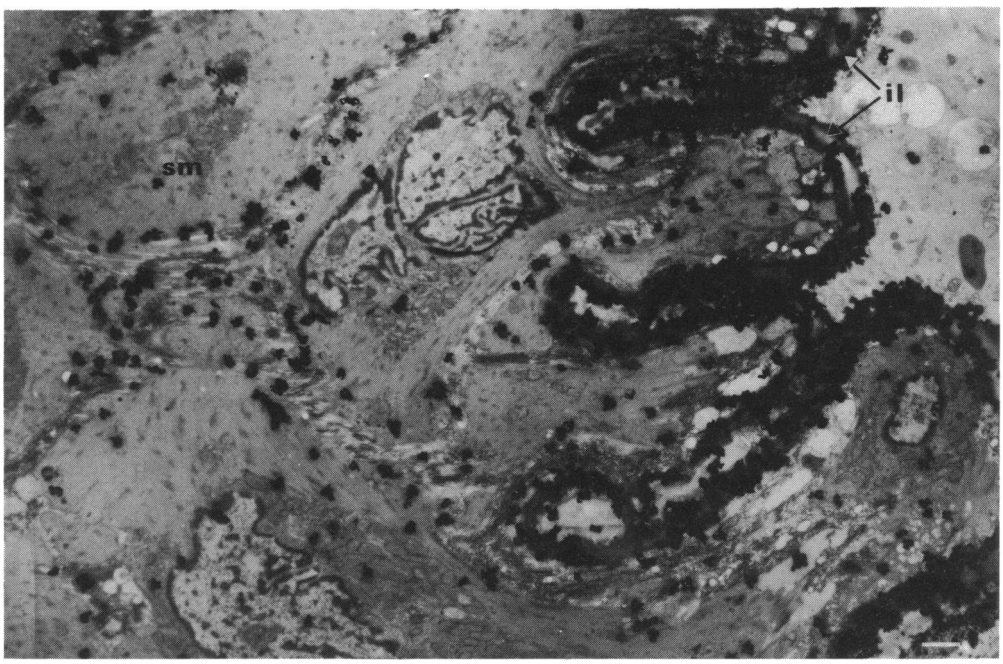

Figure 5 Electron microscopic autoradiograph of tail artery 6 h after i.v. administration of [^{3}H]-hydrallazine. Both the margination of label around smooth muscle cells (sm) and the high label density in internal elastic lamina (il) are shown. Scale bar – 1 μm.

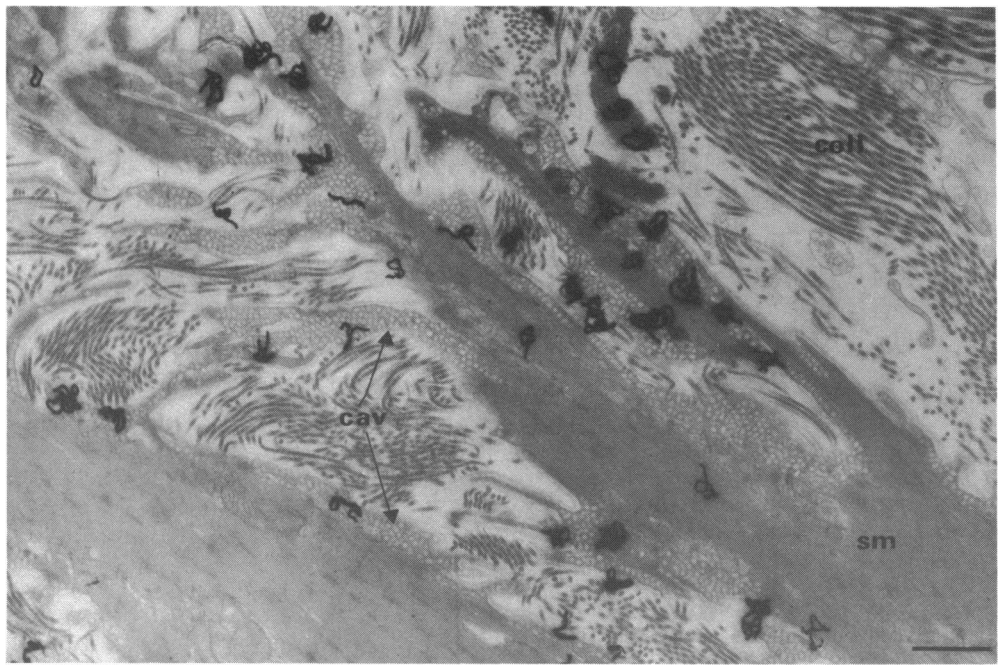

Figure 6 Electron microscopic autoradiograph of tail artery 6 h after i.v. administration of [^{3}H]-hydrallazine. Although the majority of grains are close to the margin of the smooth muscle cells (sm) they do appear in the main to overlie the cell rather than extracellular space or collagen (coll). cav, Caveolae. Scale bar – 1 μm.

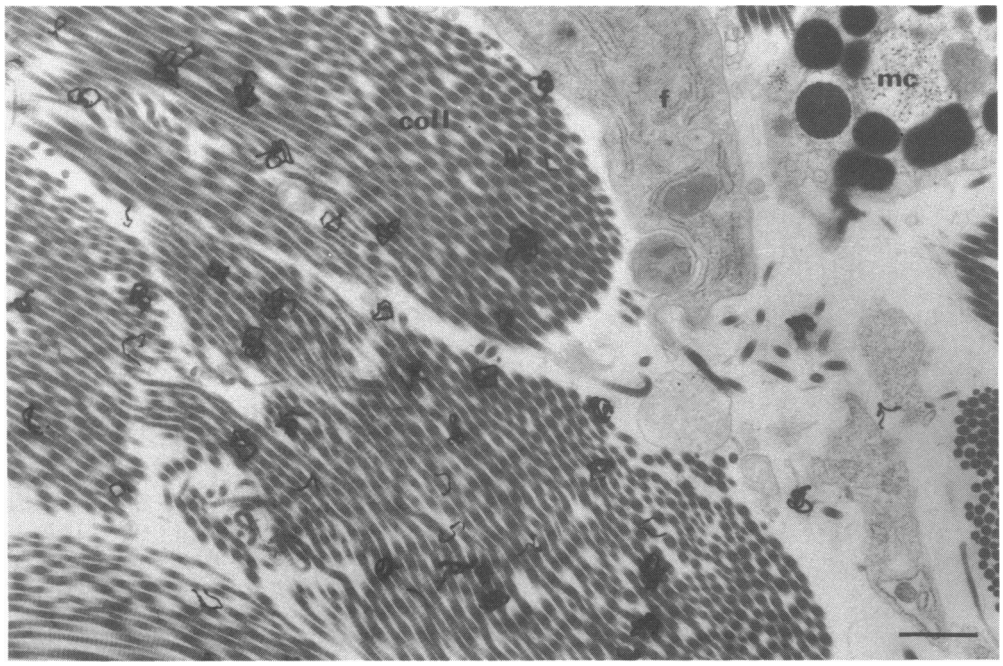

Figure 7 Electron microscopic autoradiograph of outer zone of tail vein 6 h after i.v. administration of [^{3}H]-hydrallazine. The silver grains are highly concentrated over the collagen (coll). f, fibroblast; mc, mast cell. Scale bar – 1 μm.

Table 3 Hypothetical grain analysis of electron microscopic autoradiographs from rat tail artery 1 h after i.v. administration of [^{3}H]-hydrallazine

Source	*Grains/grid point*	*Relative activity*	*Relative area*
Smooth muscle	0.113±0.004	12.7±1.0	43.9±0.2
Intercellular collagen	0.317±0.183	6.2±3.6	7.7±0.6
Intercellular elastin	12.039±4.106	13.5±5.4	0.4±0.1
Collagen	0.013±0.043	0.5±2.2	16.0±0.7
Laminar elastin	4.247±0.262	34.9±3.0	3.2±0.3
Extracellular space	0.440±0.062	32.1±3.0	28.7±0.2

Total χ^2 0.1 (with one degree of freedom), $P > 0.5$.

Table 4 Hypothetical grain analysis of electron microscopic autoradiographs from rat tail artery 6 h after i.v. administration of [^{3}H]-hydrallazine

Source	*Grains/grid point*	*Relative activity*	*Relative area*
Smooth muscle	0.468±0.031	33.0±2.1	51.9±0.6
Intercellular collagen	1.896±0.503	21.8±4.9	8.5±0.8
Collagen	0.210±0.265	2.4±2.1	8.5±1.5
Laminar elastin	7.786±1.557	40.3±2.1	3.8±0.6
Extracellular space	0.068±0.016	2.5±0.6	27.3±1.3

Total χ^2 1.6 (with one degree of freedom), $P > 0.2$.

Table 5 Hypothetical grain analysis of electron microscopic autoradiographs from rat renal artery 6 h after p.o. administration of [^{3}H]-hydrallazine

Source	*Grains/grid point*	*Relative activity*	*Relative area*
Smooth muscle	0.343±0.062	34.8±4.5	49.5±1.8
Intercellular collagen	0.212±0.106	1.5±0.8	3.5±0.9
Intercellular elastin	3.162±1.632	18.3±6.9	2.8±0.3
Collagen	0.110±0.051	3.2±1.5	14.2±1.0
Laminar elastin	2.973±0.327	37.0±4.7	6.1±0.1
Extracellular space	0.105±0.040	5.2±2.3	23.9±0.5

Total 1.7 (with two degrees of freedom), $P > 0.2$.

Discussion

The choice of tritium for this study was governed mostly by its advantages in light and particularly electron microscopic autoradiography. To assess whether the material used was likely to produce spurious patterns of radiolabelling by means of tritium-hydrogen exchange or some other metabolic mechanism, the test study comparing [^{3}H]-HP with [^{14}C]-HP was undertaken. While the whole-body preparations showed a very similar distribution of the two nuclides they could not eliminate the possibility of significant tritium exchange. Nevertheless, there should be few problems for the interpretation of autoradiographs since it may be assumed that the majority of exchanged tritium transfers to water which is eliminated from the tissue sections before contact with film or emulsion.

The reason for inclusion of ear tissue in the present investigation was the observation of elevated levels of radiolabel in ear cartilage after a 28 day chronic oral regime of [^{14}C]-HP (Schmid, 1978, unpublished) and 26 h after a single i.v. dose of [^{14}C]-HP (Curtis *et al.*, 1981). However, no remarkable radiolabelling of ear cartilage or any adjacent tissue was recorded in this study, and we assume that by 6 h

the rate of accumulation in the ear was lower than in vascular tissues and that only later does sequestration of label produce the build-up observed previously.

The portal vein was examined at 1 h to determine whether it could take up large amounts of absorbed radiolabel from the gastro-intestinal (GI) tract after oral dosing, thereby reducing the amount of label reaching the rest of the vasculature. However, the absence of grains from the portal system of orally dosed animals suggests that this was not so. Moreover, if HP metabolites bind less readily to the vascular walls than the parent compound (see below) then biotransformation during GI passage and absorption would prevent the acute accumulation of radiolabel in the portal system.

Only in the case of the whole-body autoradiographs of i.v. dosed animals was unlabelled HP added to give a total dose which was hypotensive (i.e. $7.7\,mg\,kg^{-1}$). In all other experiments, oral or intravenous, the total dose was $0.13\,mg\,kg^{-1}$ which could be expected to have some vasodilator action in i.v. dosed animals but not in p.o. rats (Dr P.R. Hedwall, personal communication). The rationale behind this decision was to enable as many HP binding sites as possible to be labelled in order to render specimens amenable to the relatively insensitive EM autoradiographic technique.

It appears that processing of tail artery for microscopy involved the average loss of 32% of tritium present 'within' the tissue when excised, of which 26% was extracted in the mixed aldehyde primary fixative. Israili *et al.* (1976) similarly observed that the maximum radiolabel extractable from rat vessels following [^{14}C]-HP injection was 24%. Such extracts, when assayed by the method of Jack *et al.* (1975) were found to be present almost entirely in the form of 'apparent HP'. The latter term must be used however, since Zak *et al.* (1977) showed that the major plasma metabolite, pyruvic acid hydrazone, can hydrolyse at the acid pH of the assay to yield HP thus giving spuriously high levels of HP. Although some attempts have been made to overcome the problems of assay specificity (e.g. Smith *et al.*, 1977) there remains much doubt as to the ability of current methods to quantify tissue-bound HP.

Despite the inability of chemical methods to characterize satisfactorily the nature of tissue-bound radiolabel in studies such as this, there are useful biological data which may permit some tentative conclusions. *In vitro* studies (Wagner & Hedwall, 1975) where there was no plasma pyruvic acid with which HP readily conjugates, suggest that HP itself accumulates in mesenteric arteries of the rat whilst eliciting an anti-vasoconstrictor response. Although no correlation could be found between the concentration of HP/metabolites in the vessel walls and the 'hypotensive' effect, the presence of radioactivity in the arteries and arterioles corresponded better to the duration of the BP lowering effect than did the plasma concentration of HP. In further *in vitro* experiments (unpublished) we have found that rat isolated arteries incubated with [^{3}H]-HP at pH 6.4 to inhibit chemical degradation, (Schulert, 1961), give EM autoradiographic labelling patterns similar to those presented here. In other words, it appears that *in vitro* experiments, where metabolism or degradation of the drug are kept to a minimum, can produce a biological response and tracer distribution similar to those observed in comparable *in vivo* experiments. A reasonable conclusion therefore must be that much of the vessel-bound radiolabel in the present study represents intact HP.

The appearance of autoradiographically detectable label in blood vessel walls of one of the orally dosed rats at 6 h may reflect the very early stages of a somewhat slow build-up to a steady state. A 28 day chronic study in the rat by Schmid (1978, unpublished) with [^{14}C]-HP (1 or $5\,mg\,kg^{-1}$) p.o. daily, showed that a radiometrically determined steady state was not reached in the five major arteries studied until between 21 and 28 days. It would thus appear that any difference in tissue distribution between intravenously and orally administered [^{3}H]-HP reflects pharmacokinetic rather than mechanistic differences. Furthermore, although it is known that oral doses of HP are completely absorbed in the rat (Wagner, 1972, unpublished), there are no comparative data for blood, plasma or tissue levels of apparent HP in orally and intravenously dosed rats. Talseth (1976), however, has compared pharmacokinetic parameters in man after identical oral or intravenous doses of HP ($0.3\,mg\,kg^{-1}$) in a cross-over study. This author showed that AUC (area under the plasma concentration curve) values in p.o. subjects were only 26–55% of those when the dose was intravenous – the range depending upon the acetylation phenotype of the subject. It may, therefore, be that presystemic biotransformation of the oral dose leads to a spectrum of products quantitatively and qualitatively different from those resulting from i.v. HP, and that this difference may in part account for the initial low amount and delayed accumulation of radiolabel in vessel walls of p.o. rats.

Although the hypothetical grain analyses have shown in each case that HP and/or labelled metabolites enter the smooth muscle cells, it must be acknowledged that some of the silver grains at the cell periphery are associated with intercellular connective tissue. The proportions of the latter identified as collagen or elastin are not consistent between analyses. These components are usually very closely associated in the narrow intercellular spaces and their ratio may depend upon the vessel and/or the region of the vessel from which the sections are taken.

It is possible that interaction of HP with certain oxidases may account for at least some of the binding sites revealed by high resolution autoradiography. Lyles & Callingham (1982) have shown that HP binds irreversibly with clorgyline-resistant amine oxidase (EC 1.4.3.6.), an enzyme which appears to be associated with smooth muscle cell membranes in arteries in particular (Wibo *et al.*, 1980). Likewise, the association of silver grains with elastin and collagen may relate to the irreversible binding of HP with lysyl oxidase (EC 1.4.1.15.), an enzyme which is essential to the proper function of these connective tissues (Numata *et al.*, 1981).

The possibility must, nevertheless, be considered that at least some of the radiolabel which enters smooth muscle cells may represent intact HP which exerts its vasodilator action by direct contact with some component(s) of the cell. Although for the purpose of this study, the smooth muscle cells were not subdivided into subcellular features in the analyses, there is evidence that most of the cell-associated silver grains were located in the peripheral region just below the sarcolemma. A recent study using electron energy loss spectroscopy (Simon *et al.*, 1982) has shown this sub-sarcolemma region to be an important site of calcium storage within myometrial smooth muscle cells.

The clinical significance of the accumulation of HP and/or its metabolites within the blood vessel walls is not yet clear. HP is normally taken orally except for use in hypertensive emergencies where it can be given by slow intravenous injection or infusion. If the arterial wall content of HP/metabolites plays a role in muscle relaxation, it is evident from the present study that acute single dosing will elevate the non-transformed drug level most rapidly when the intravenous route is adopted which may therefore accelerate the onset of action.

In conclusion, we have demonstrated, by the use of autoradiography from the whole-body to subcellular level, the uptake and distribution of [^{3}H]-HP in arteries of the rat. The results show that a significant quantity of radiolabel, possibly in the form of intact HP, enters or attaches to the smooth muscle cells where it may intervene in the chain of events which brings about excitation-contraction coupling and thereby elicits vasodilatation.

The authors would like to thank Messrs R.A. Christian, B.H. Kemmenoe and P.E. Johnson of these laboratories for expert technical assistance and Drs W. Riess, K. Schmid and P.R. Hedwall of CIBA-GEIGY Pharmaceuticals Division, Basle, for invaluable discussion during the course of this study. Thanks also are due to Dr D.E. Brundish in Horsham and Dr W. Küng in Basle for preparing the radiolabelled hydrallazine.

References

ÅBLAD, B. (1963). A study of the mechanism of the haemodynamic effects of hydralazine in man. *Acta Pharmac. Tox.*, **20** (Suppl. 1), 3–53.

BAKER, J.R.J., BULLOCK, G.R., CRAWFORD, N. & TAYLOR, D.G. (1977). Localization in platelets of sodium [^{51}Cr]-chromate, [^{125}I]-antibody to whole membrane, and [^{3}H]-diisopropylfluoro-phosphate using electron microscopic autoradiography. *Am. J. Path.*, **88,** 277–290.

BLACKETT, N.M. & PARRY, D.M. (1973). A new method for analysing electron microscope autoradiographs using hypothetical grain distributions. *J. cell Biol.*, **57,** 9–15.

BLACKETT, N.M. & PARRY, D.M. (1977). A simplified method of 'hypothetical grain' analysis of electron microscope autoradiographs. *J. Histochem. Cytochem.*, **25,** 206–214.

CURTIS, C.G., CROSS, S.A.M., McCULLOCH, R.J. & POWELL, G.M. (1981). *Whole body autoradiography*, p. 88. London: Academic Press.

GROSS, F.J., DRUEY, J. & MEIER, R. (1950). Eine neue Gruppe blutdrucksenkender Substanzen von besonderem Wirkungskarakter. *Experientia,* **6,** 11–12.

ISRAILI, Z.H., DAYTON, P.G. & BAYER, C.W. (1976). Persistence of hydralazine (HP) in the blood vessels (V) of rats administered HP-[^{14}C].HCl. *Pharmacologist,* **18,** 196.

JACK, D.B., BRECHBUEHLER, S., DEGEN, P.H., ZBINDEN, P. & RIESS, W. (1975). The determination of hydralazine in plasma by gas-liquid chromatography. *J. Chromatog.*, **115,** 87–92.

JUDSON, W.E., HOLLANDER, W. & WILKINS, R.W. (1956). The effects of intravenous apresoline (hydralazine) on cardiovascular and renal-function in patients with and without congestive heart failure. *Circulation,* **13,** 664–674.

LIM, R.K.S., MOFFITT, R.L. & GLASS, H.G. (1955). Observations on the mechanism of central hypotension. *J. Pharmac. exp. Ther.*, **113,** 33–34.

LYLES, G.A. & CALLINGHAM, B.A. (1982). Hydralazine is an irreversible inhibitor of semicarbazide-sensitive, clorgyline-resistant amine oxidase in rat aorta homogenates. *J. Pharm. Pharmac.*, **34,** 139–140

MOLLENHAUER, H.H. (1964). Plastic embedding mixtures for use in electron microscopy. *Stain Technol.*, **39,** 111–114.

MOORE-JONES, D. & PERRY, H.M. Jr., (1966). Radioautographic location of Hydralazine-l-[C^{14}] in arterial walls. *Proc. Soc. exp. biol. Med.*, **122,** 576–579.

MORROW, J.D., SCHROEDER, H.A. & PERRY, H.M. Jr., (1953). Studies on the control of hypertension by Hyphex. II Toxic reactions and side effects. *Circulation,* **8,** 829–839.

MOYER, J.H. (1953). Hydralazine (Apresoline) hydroch-

loride: pharmacological observations and clinical results in the therapy of hypertension. *Arch. intern. Med.*, **91,** 419–439.

NUMATA, Y., TAKEI, T. & HAYAKAWA, T. (1981). Hydralazine as an inhibitor of lysyl oxidase activity. *Biochem. Pharmac.*, **30,** 3125–3126.

PERRY, H.M. Jr., COMENS, P. & YUNICE, A. (1962). Distribution of hydralazine-l-[C^{14}] after injection into normal mice. *J. lab. clin. Med.*, **59,** 456–461.

ROGERS, A.W. (1979). *Techniques of autoradiography*, 3rd Edn. p. 368. Amsterdam, New York, Oxford: Elsevier/North-Holland Biomedical Press.

SALPETER, M.M., BACHMANN, L. & SALPETER, E.E. (1969). Resolution in electron microscopic radioautography. *J. cell Biol.*, **41,** 1–32.

SCHULERT, A.R. (1961). Physiological disposition of hydralazine (l-hydrazinophthalazine) and a method for its determination in biological fluids. *Arch. int. Pharmacodyn.*, **132,** 1–15.

SIMON, G.T., GARFIELD, R.E. & OTTENSMEYER, F.P. (1982). Electron energy loss spectroscopic imaging of calcium in smooth muscle. In *Electron Microscopy*, Vol. 1, ed. Organising committee of the 10th Congress on Electron Microscopy, Hamburg (Deutsche Gesellschaft für Elektronen-mikroskopie e.V.), pp. 595–596, Frankfurt/Main.

SMITH, K.M., JOHNSON, R.N. & KHO, B.T. (1977). Determination of hydralazine in tablets by gas-chromatography. *J. Chromatogr.*, **137,** 431–437.

TALSETH, T. (1976). Studies on hydralazine. III. Bioavailability of hydralazine in man. *Eur. J. clin. Pharmac.*, **10,** 395–401.

TAYLOR, R.D., DUSTAN, H.P., CORCORAN, A.C. & PAGE, I.H. (1952). Evaluation of l-hydrazino phthalazine (apresoline) in treatment of hypertensive disease. *Arch. intern. Med.*, **90,** 734–9.

WAGNER,, J. & HEDWALL, P.R. (1972). Correlation between the pharmacokinetics and the hypotensive action of hydralazine in the rat. *Naunyn-Schmiedebergs Arch. Pharmac.*, **274,** R. 122.

WAGNER, J. & HEDWALL, P.R. (1975). Pharmacokinetics and pharmacological action of vasodilators. In *International Symposium on Hypertension*, Vol. 2, ed. Milliez, P. & Safar, M. p. 331. Reims: Laboratoires Boehringer Ingelheim.

WIBO, M., DUONG, A.T. & GODFRAIND, T. (1980). Subcellular location of semicarbazide-sensitive amine oxidase in rat aorta. *Eur. J. Biochem.*, **112,** 87–94.

YONKMAN, F.F. & FREIS, E.D. (1952). Rational elimination of side reactions and/or tolerance in the development of potentially valuable drugs. *Angiology*, **3,** 36–41.

ZAK, S.B., LUKAS, G. & GILLERAN, T.G. (1977). Plasma levels of real and "apparent" hydralazine in man and rat. *Drug Metab. Disp.*, **5,** 116–121.

ZACEST, R., GILMORE, E. & KOCH-WESER, J. (1972). Treatment of essential hypertension with combined vasodilation and beta adrenergic blockade. *New Engl. J. Med.*, **286,** 617–622.

(Received April 17, 1984.
Revised July 26, 1984.)