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THE FATE OF ^{3}H-*ISO*-PROTERENOL IN THE RAT*

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Abstract—Rats were injected i.v. with ^{3}H-iso-proterenol. Approximately 75% of the administered dose was excreted in the urine over 24 hr. Rats anaesthetized with urethane were injected i.v. with ^{3}H-*iso*-proterenol and their bile and urine collected. It was found that about 92% of the administered dose were excreted over a period of 8 hr, of which about 38% were excreted in the bile and 62% in the urine. By contrast, following the injection of ^{3}H-norepinephrine only 14% of the excreted radioactivity occurred in the bile and 86% in the urine, the total excreted amount being approximately 68% of the administered dose.

If the bile of rats which had received ^{3}H-*iso*-proterenol i.v. was then injected into the small intestine of other rats, increasing amounts of radioactivity were detected over a period of 10 hr in their bile and urine.

The injected ^{3}H-*iso*-proterenol is, as other catecholamines, rapidly O-methylated. The urine contained free ^{3}H-*iso*-proterenol, ^{3}H-*iso*-proterenol glucuronide, free 3-methoxy-^{3}H-*iso*-prenaline and 3-methoxy-^{3}H-*iso*-proterenol glucuronide. No deaminated metabolites could be detected. In the bile only 3-methoxy-*iso*-proterenol glucuronide was found.

In contrast to the findings following administration of ^{3}H-norepinephrine, where relatively large amounts are bound in tissues like heart, spleen and suprarenals, only negligible quantities of ^{3}H-*iso*-proterenol were found bound in these tissues under similar conditions. Most of the activity present in the tissues 10 min after the injection of ^{3}H-*iso*-proterenol was already in the O-methylated form.

It is concluded, that the delay of the urinary excretion of radioactivity following an injection of ^{3}H-norepinephrine is caused by its binding in the tissues and slow release. On the other hand, the delay in the urinary excretion after the injection of ^{3}H-*iso*-proterenol is caused by the excretion of a relatively large amount of activity in the bile, from which it is slowly reabsorbed and finally excreted.

Iso-PROTERENOL is a drug with primarily β-mimetic actions. The *iso*-propyl group attached to the N-atom seemed to suggest that this catecholamine (CA) would not undergo deamination and would be metabolized exclusively by catechol-O-methyl transferase (COMT). Sjoerdsma used the D-isomer of *iso*-proterenol to measure the COMT-activity of hypertensive patients, by giving *iso*-proterenol (ISP) and isolating the 3-methoxy-*iso*-proterenol (MISP).[1] However, no extensive paper on the metabolism of ISP has been published by his group. An important pathway for inactivation of the CA besides enzymatic degradation, conjugation and excretion is the uptake and binding in sympathetically-innervated tissues. The purpose of our work was to study the metabolism and excretion of ISP and to see if this compound was taken up and bound by the tissues.

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METHODS

D1-7-^{3}H-ISP was made on our request by the New England Nuclear Corporation by reduction of *iso*-propyl-noradrenolone with tritium gas. The compound we obtained (sp. act. 2·3 c/mmole) was purified further by paper chromatography in *n*-butanol–acetic acid–water (4 : 1 : 1).

Four male rats (200–300 g) received 64 μc of labeled ISP i.v. into the tail vein. They were placed in small metabolic cages, a steady urine flow was maintained by an i.v. infusion of 0·9% NaCl/5% glucose (1 : 1) and the urine collected for 24 hr. Aliquots of the urine were counted in a Packard liquid scintillation spectrometer;* for identification of the metabolites other aliquots were transferred to Whatman No. 1 paper and chromatographed in different solvent systems (*n*-butanol–acetic acid–water (4 : 1 : 1), *iso*-propanol–ammonia–water (8 : 1 : 1), *n*-butanol–ethanol–water (4 : 1 : 1), 3-methoxy-4-hydroxy-ISP was synthesized enzymatically by incubation of the original compound with the rat-liver enzyme preparation, S-adenosyl-methionine (AMe) and $MgCl_2$ according to the method of Axelrod.[2] The resulting methoxy compound was further purified by extraction into *iso*-amyl alcohol (pH 10) and rechromatography. The final compound gave a colour similar to other O-methyl compounds with diazotized *p*-nitroaniline, the R_f values being different from ISP. Later on we obtained synthetically-prepared MISP† which had R_f values identical to our enzymatically-prepared compound. The MISP and the ISP served as reference compounds on the paper chromatograms. After development, distribution of radioactivity on paper chromatograms of the rat urine was determined using a 4-π-chromatogram scanner (Scanogram II, Atomic Access. Inc., New York).* For identification, aliquots of urine or eluates of radioactive spots from the chromatograms were incubated with β-glucuronidase or glusulase, a mixture of β-glucuronidase and sulfatase.‡ Other aliquots were hydrolysed with HCl (pH 1,100°, 15 min). After enzymatic or acid hydrolysis the urines were chromatographed as described above. Eluates of the radioactive peaks which corresponded to ISP were incubated with the COMT rat-liver preparation and AMe and rechromatographed. For quantitative determination of the excreted compounds the following procedure was used: ascorbic acid and EDTA were added to untreated urine aliquots and aliquots hydrolysed by β-glucuronidase to make a final concentration of 0·1%. The pH was adjusted to pH 8·4 with Na_2CO_3 under control of a glass electrode. Then 0·7 g aluminium oxide (neutral Woelm, Eschwege) was added and stirred for 5 min with a magnetic stirrer. The sample was then brought to a small glass column and the alumina washed with 5 ml 0·2 M acetate buffer (pH 8·4) and 5 ml glass-distilled water. The columns were eluted with 5 ml 0·4 N acetic acid. The effluents and washings were combined, adjusted to pH 6·5 and processed according to Kopin *et al.*[3] on a Dowex 50 column (NH_4-form). The eluates from both columns were evaporated *in vacuo* and counted. Controls were carried through the whole procedure to determine the efficiency of the procedure. Corrections for quenching were made for each sample by adding an internal standard.

* The Packard liquid scintillation spectrometer and the 4π chromatogram scanner were obtained as a gift from the Rockefeller Foundation, New York.

† We are greatly indebted to Messrs. Boehringer/Ingelheim for the synthesis of 3-methoxy-4-hydroxy *iso*-proterenol.

‡ Bacterial β-glucuronidase, Sigma Chemical Company, St. Louis, Missouri. Glusulase, Endo Products Inc., Richmond Hill, New York

In other experiments 1·74 mμmoles/100 g of ^{3}H-*iso*-proterenol (sp. act. as above) or ^{3}H-norepinephrine (sp. act. 7·19 c/mmole) were injected i.v. into the tail vein of groups of rats killed 10 min or 2 hr after the injection. Heart, spleen, liver, kidneys, suprarenals, lung and uterus were removed, blotted, weighed and homogenized in 0·4 N perchloric acid. A blood sample was taken from the right ventricle before the heart was dissected. ^{3}H-*iso*-proterenol (^{3}H-ISP), ^{3}H-methoxy-*iso*-proterenol (^{3}H-MISP), ^{3}H-norepinephrine (^{3}H-NE) and ^{3}H-normethanephrine (^{3}H-NME) were isolated in the respective groups by means of alumina and Dowex-50 columns as described above, and the radioactivity determined.

In a third group of experiments male rats were anesthetized with urethane (1·4 g/kg i.m.) and their bile ducts and ureters catheterized with PE 10 Clay-Adams catheters. 1·74 mμmoles/100 g of labeled ISP or NE (sp. act. ^{3}H-ISP 2·3 c/mmole, ^{3}H-NE 7·19 c/mmole) was injected into the jugular vein and a steady flow of urine and bile maintained by an i.v. infusion of the NaCl–glucose mixture. Samples of urine and bile were taken at 1-hr intervals and treated as described above.

In order to estimate how much of the radioactivity present in the bile was absorbed, the following experiment was performed: bile was collected from rats which had received ^{3}H-ISP and was then injected in an amount of 1·0 ml (4·45 μc/ml) into the duodenum of another group of rats anesthetized with urethane. Their bile and urine was collected for 10 hr and the radioactivity determined.

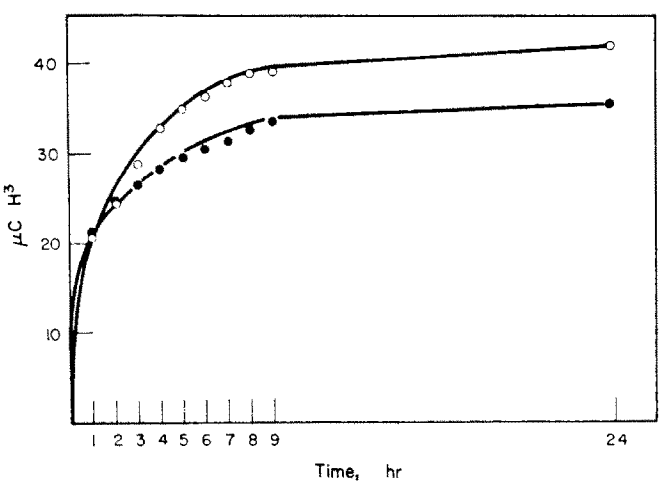

FIG. 1. Urinary excretion of tritium in rat urine following the administration of ^{3}H-*iso*-proterenol. The rats received i.v. 64 μc DL-7-^{3}H-*iso*-proterenol and the urine was collected over a period of 24 hr.

RESULTS

Figure 1 shows the excretion curve of the total radioactivity in the urine of the rats which received ^{3}H-ISP. It can be seen that the major part of the radioactivity is excreted during the first hours after the injection. After 24 hr 67% (rat I) and 62% (rat II) of the injected amount was excreted in the urine.

Figure 2 shows the distribution of the radioactivity in a chromatogram of a urine sample taken in the first hour after the injection of ^{3}H-ISP (*n*-butanol–acetic acid–water, 4 : 1 : 1). The chromatogram shows three peaks. No. 2 corresponded to ISP

which was spotted together with the urine and the colour developed by the Goldenberg reaction.[4] Peak No. 3 corresponded to the reference compound MISP. The radioactive material of peak No. 2 was eluted from a chromatogram and incubated with the COMT preparation and AMe and re-chromatographed. The resulting compound

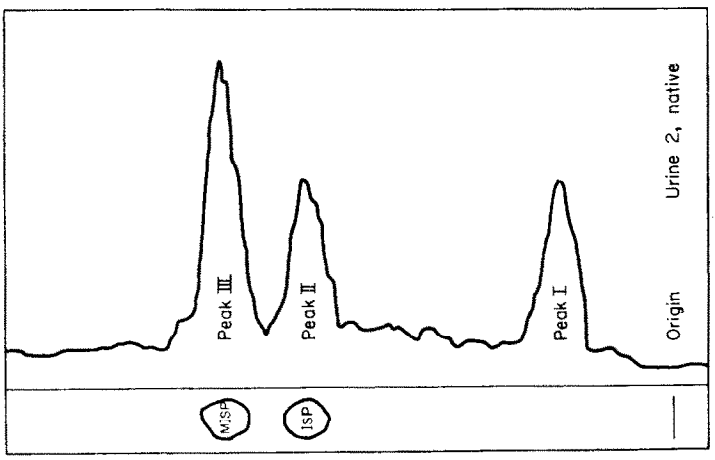

FIG. 2. Radiogram of a paper chromatogram of rat urine collected in the first hour after the injection of DL-7-^{3}H-*iso*-proterenol.
The chromatogram was developed in *n*-butanol–acetic acid–water (4 : 1 : 1). The peaks were identified as follows: Peak 1, a mixture of the glucuronides of *iso*-proterenol and 3-methoxy-*iso*-proterenol; Peak 2, *iso*-proterenol; Peak 3, 3-methoxy-*iso*-proterenol.

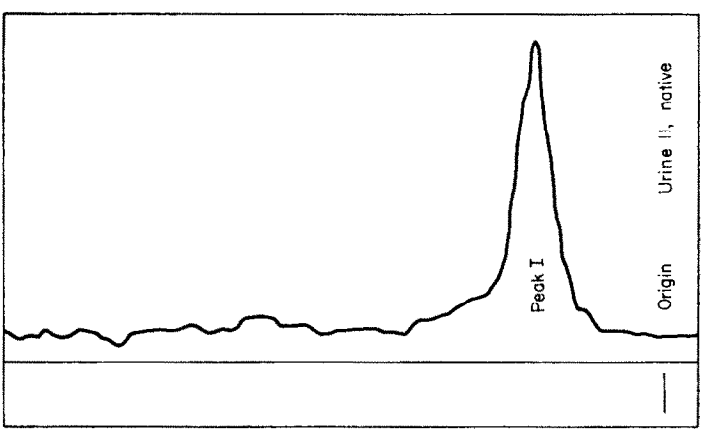

FIG. 3. Radiogram of a paper chromatogram of rat urine collected during the sixth hour after the injection of DL-7-^{3}H-*iso*-proterenol.
The chromatogram was developed in *n*-butanol–acetic acid–water (4 : 1 : 1). Only Peak 1 is now observable.

had R_f values similar to peak No. 3 and the reference compound MISP respectively. During the later periods of the urine collection, first peak No. 2 and then No. 3 disappeared. In all the solvent systems used, R_f values of the reference compounds ISP and MISP corresponded to the R_f values of the respective radioactive peaks.

The R_f values of MISP were: *n*-butanol–acetic acid–water (4 : 1 : 1) 0·68–0·70, *n*-butanol–ethanol–water (4 : 1 : 1) 0·62–0·66, *iso*-propanol–ammonia–water (8 : 1 : 1) 0·86–0·88.

An aliquot of urine of a later sampling period (5–6 hr) was chromatographed in the *n*-butanol–acetic acid–water systems. Only peak No. 1 was observable (Fig. 3). If an aliquot of this urine or an eluate from peak No. 1 of earlier sampling periods was hydrolysed with β-glucuronidase or glusulase and re-chromatographed (Fig. 4), peak No. 1 had disappeared and the peaks 2 and 3 reappeared. Incubation with HCl did not accomplish a total hydrolysis.

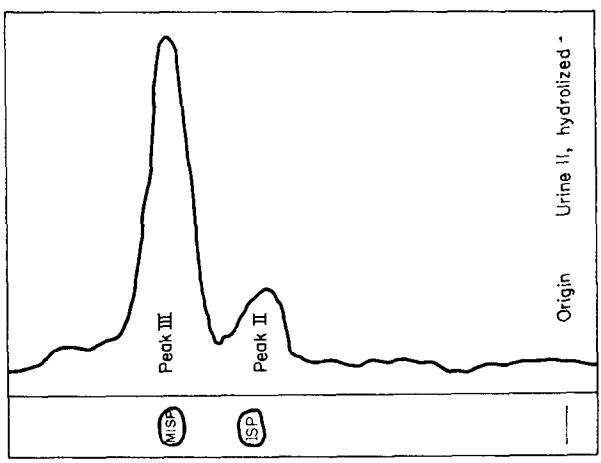

FIG. 4. Radiogram of a paper chromatogram of the same urine as in Fig. 3 after hydrolysis with bacterial β-glucuronidase.
The chromatogram was developed in *n*-butanol–acetic acid–water (4 : 1 : 1). Peak 2, *iso*-proterenol; Peak 3, 3-methoxy-*iso*-proterenol.

It can be concluded that peak No. 1 is a mixture of the glucuronides of ISP and MISP, peak No. 2 is unconjugated ISP and peak No. 3 unconjugated MISP.

Four rats were injected intravenously with 64·0 μc ^{3}H-ISP and the urine collected in three periods during 24 hr. One aliquot of each urine sample was then treated unhydrolysed as described in the methods to determine free ISP and MISP. Other aliquots were hydrolysed with β-glucuronidase and treated in a similar way. The quantity of the conjugated compounds was calculated from the difference between these values. Total hydrolysis was controlled by means of paper chromatography. Table 1 summarizes the results of this experiment.

Groups of rats were injected i.v. with either ^{3}H-ISP or ^{3}H-NE, sacrificed after 10 min or 2 hr and the organs treated as described in the methods. It can be seen in Table 2 that 10 min after injection the concentration of ISP in the organs exceeds its concentration in the blood.

The greatest concentration of unchanged ^{3}H-ISP is present in the kidneys. Most of the radioactivity in the organs is already O-methylated ^{3}H-ISP. Two hr after injection only minute amounts of ^{3}H-ISP can be found in the organs; only in the kidneys there is still a considerable concentration of ^{3}H-ISP. No ^{3}H-ISP was found at all in the suprarenals, uterus and blood.

In contrast to ^{3}H-ISP, ^{3}H-NE can be found in a much higher concentration in the organs 10 min after its injection, the highest amounts being present in the heart and suprarenals. Only in the lung there is more of the O-methylated metabolite than of the original compound. Two hr after its injection the tissue concentrations of ^{3}H-NE decreased by about 50%.

TABLE 1.

Period	mμc ± S.E.M.
I (0–2 hr)	
Total ^{3}H-activity excreted	30,498 ± 1,554
^{3}H-ISP Free	5,604 ± 910
^{3}H-MISP Free	7,241 ± 612
^{3}H-ISP Gluc.	7,782 ± 839
^{3}H-MISP Gluc.	9,871 ± 906
II (2–6 hr)	
Total ^{3}H-activity excreted	12,730 ± 2,780
^{3}H-ISP Free	314 ± 44
^{3}H-MISP Free	1,093 ± 88
^{3}H-ISP Gluc.	1,794 ± 138
^{3}H-MISP Gluc.	9,517 ± 2,638
III (6–24 hr)	
Total ^{3}H-activity excreted	5,964 ± 545
^{3}H-ISP Free	338 ± 44
^{3}H-MISP Free	495 ± 90
^{3}H-ISP Gluc.	1,711 ± 198
^{3}H-MISP Gluc.	3,420 ± 556

^{3}H-*Iso*-proterenol (64·0 μc per animal) was given i.v. to 4 male rats. The urine was collected in 3 periods over 24 hr and the determination carried out as described in the methods.

Administered: 64,000 mμc ^{3}H *iso*-proterenol per animal.

Excreted in the urine during 24 hr: 49,000 mμc ± 3,000 mμc or 76·5% of the administered dose:

Unconjugated ^{3}H-*Iso*-proterenol ^{3}H-ISP Free
Unconjugated ^{3}H-3-Methoxy*iso*-proterenol ^{3}H-MISP Free
Glucuronide of ^{3}H-*Iso*-proterenol ^{3}H-ISP Gluc.
Glucuronide of ^{3}H-3-Methoxy*iso*-proterenol ^{3}H-MISP Gluc.

There is a discrepancy between the fact that ISP was not bound in considerable amounts and on the other hand 24 hr after injection only 60–70% were excreted in the urine. Since it is known that CAs are also excreted in the bile,[5] we injected rats with ^{3}H-ISP or ^{3}H-NE and collected bile and urine.

Table 3 shows the excretion of radioactivity in the bile and urine of these rats. About 34–42% of the radioactivity following the ^{3}H-ISP injection occurred in the bile; in contrast only 12–15% of the radioactivity following the ^{3}H-NE injection was found in the bile. The bile of rats which had received ^{3}H-ISP showed one radioactive peak near to the origin. After hydrolysis with β-glucuronidase a new peak, corresponding to MISP, appeared following chromatography. Figure 5 shows the excretion curves of two rats which received 4·45 μc of radioactive bile into the duodenum. The radioactive compound in the administered bile was identified as MISP-glucuronide. It can be seen from Fig. 5 that after a delay of several hours the radioactivity of urine and bile increased. During the 10-hr observation period 6·7% (rat 1) and 8·0%

TABLE 2. UPTAKE AND BINDING OF ^{3}H-NOREPINEPHRINE AND ^{3}H-*iso*-PROTERENOL IN RAT TISSUES

Time	Heart	Spleen	Liver	Kidneys	Suprarenals	Lung	Uterus	Blood
10 min								
^{3}H-ISP	17·49 ± 3·44	12·70 ± 0·61	14·36 ± 1·35	93·92 ± 3·96	27·32 ± 2·18	18·05 ± 2·44	12·44 ± 0·48	2·57 ± 0·26
^{3}H-MISP	48·85 ± 2·09	89·61 ± 5·96	18·14 ± 3·61	168·82 ± 17·49	101·27 ± 5·44	96·83 ± 15·92	48·59 ± 2·13	11·05 ± 1·91
2 hr								
^{3}H-ISP	1·70 ± 0·26	1·74 ± 0·26	13·49 ± 1·00	53·33 ± 3·39		1·78 ± 0·04		
^{3}H-MISP	1·70 ± 0·61	2·35 ± 0·26	3·70 ± 0·61	9·05 ± 0·78		3·26 ± 0·44		0·78 ± 0·30
10 min								
^{3}H-NE	896·04 ± 111·61	58·16 ± 8·31	16·43 ± 1·99	128·31 ± 4·13	222·14 ± 22·80	59·97 ± 3·99	33·70 ± 4·76	30·46 ± 2·67
^{3}H-NM	53·59 ± 4·90	19·08 ± 2·99	10·96 ± 0·90	42·96 ± 4·13	26·85 ± 0·97	76·27 ± 9·99	14·28 ± 0·90	5·28 ± 0·77
2 hr								
^{3}H-NE	417·93 ± 28·01	28·62 ± 4·41	7·98 ± 0·46	46·38 ± 7·85	167·40 ± 19·24	36·02 ± 5·62	21·91 ± ·70	7·66 ± 1·10
^{3}H-NM	15·93 ± 2·84	3·76 ± 0·43	5·12 ± 0·89	7·84 ± 0·47	9·88 ± 1·21	10·24 ± 0·88	31·38 ± 0·88	0·70 ± 0·10

Each rat received i.v. 1·74 mμmoles ^{3}H-norepinephrine or ^{3}H-*iso*-proterenol per 100 g body weight. Groups of 6 animals were killed 10 min or 2 hr after the injection and the tissues analysed as described in the methods. The results are expressed in mμmoles × 10^{-4} per g tissue ± S.E.M. ^{3}H-norepinphrine = ^{3}H-NE, ^{3}H-normethanephrine = ^{3}H-NM, ^{3}H-*iso*-proterenol = ^{3}H-ISP, ^{3}H-3-methoxy-*iso*-proterenol = ^{3}H-MISP.

TABLE 3.

	Distribution of the total amounts excreted (%)	Administered dose excreted in bile and urine (%)
Rat I: injected 13·77 μc ^{3}H-ISP		91·7
Excreted Bile 5·312 μc	42·06	
Urine 7·316 μc	57·94	
Rat II: injected 14·58 μc ^{3}H-ISP		94·3
Excreted Bile 4·709 μc	34·28	
Urine 9·026 μc	65·62	
Rat III: injected 54·0 μc ^{3}H-NE		67·5
Excreted Bile 5·700 μc	15·6	
Urine 30·752 μc	84·4	
Rat IV: injected 54·0 μc ^{3}H-NE		68·6
Excreted Bile 4·799 μc	12·9	
Urine 32·265 μc	87·1	

Four male rats were narcotized with urethane (1·4 g/kg). Two of the animals were injected i.v. with 1·74 mμmoles/100 g ^{3}H-*iso*-proterenol (ISP sp. act. 2·3 c/mmole) and two animals received 1·74 mμmoles/100 g ^{3}H-Norepinephrine (^{3}H-NE sp. act. 7·19 c/mmole). Urine and bile was collected for 8 hr as described in Methods.

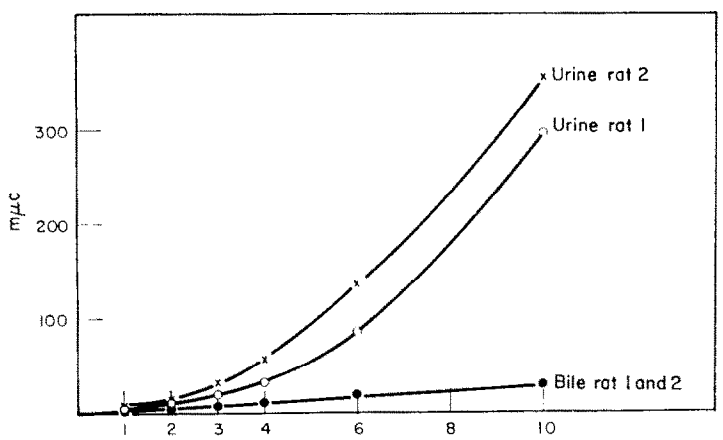

FIG. 5. Urinary and biliary excretion of radioactivity after intraduodenal administration of bile which contained radioactive metabolites of ^{3}H-*iso*-proterenol

Bile of rats which had received ^{3}H-*iso*- proterenol was injected into the duodenum of other rats and their urine and bile collected over a period of 10hr. 1ml of bile (4·45 μc/ml) was administered to each rat.

(rat 2) of the administered amount was excreted in the urine and 0·67% and 0·69% respectively was excreted in the bile.

DISCUSSION

Our results show that ISP is metabolized exclusively by O-methylation. The isopropyl group attached to the nitrogen atom seems to prevent oxidative deamination as has been shown for other nitrogen substituted compounds.[6, 7] The metabolite

MISP as well as the original compound ISP are both excreted in the rat urine in the free state or conjugated as glucuronides. Twenty-four hr after the administration of ISP about 65% of the administered radioactivity was detected in the urine. Because deamination plays no role in the degradation of ISP, this compound should be very suitable for measuring the COMT activity in animals and in man. The availability of the tritium labeled compound largely simplifies the isolation of the metabolite from the urine.

Binding of NE in sympathetically innervated structures plays an important role in the temporary inactivation of this compound. Our results show that the binding of ISP in the tissues does not occur in a manner comparable to NE. Ten min after administration of ^{3}H-ISP, most of the activity present in the tissues is already the O-methylated metabolite. Two hr after the administration of ^{3}H-ISP most of the activity disappeared from all organs except from the kidneys. It is difficult to say if there is any binding in the kidneys or if the relatively high activity present there is the result of its concentration by the kidneys and is already located in the urine. In contrast to these findings relatively high amounts of ^{3}H-NE are bound in the heart, spleen and suprarenals. Two hr after the injection of ^{3}H-NE the concentration in the tissues decreased only moderately. The concentration of ^{3}H-NE in the tissues always exceeds that of the O-methylated metabolite.[8, 9] There is no relationship between the ability of organs to take up NE and the distribution of the two types of receptors. NE has preferably α-mimetic actions but is also taken up and bound to a great extent in organs where the actions of CA's resemble the β-type (e.g. heart). ISP, a compound with primarily β-mimetic actions hardly shows any binding at all in those organs in which it manifests its actions. It is known from previous work that the ability to take up and bind NE depends on the intactness of adrenergic nerve endings.[10] Epinephrine (E) is much less tightly-bound than NE and therefore metabolized more rapidly.[8, 11] The prevention of binding by drugs such as cocaine or by post-ganglionic sympathetic denervation[9, 10, 12, 13] increases the actual concentration of injected CA's on the receptor sites and thereby potentiates their actions. The fact that the action of ISP is not enhanced by cocaine,[14–16] is consistent with our findings that binding of ISP does not play an important role in the inactivation of this compound.

It may be that the substitution on the nitrogen atom interferes with the uptake and binding mechanism. The isopropyl group attached to the nitrogen atom of ISP seems to interfere with the uptake and binding mechanism still more than the methyl group in the E molecule does. It is not possible to conclude from our experiments whether the ISP is incapable of entering the binding sites or whether the ISP which has entered the binding sites is incapable of being bound there. We tend to favour the latter possibility because E is able to penetrate the binding sites but disappears from them more rapidly than the unsubstituted NE.

At this point we were surprised to find that there was hardly any binding of ISP in the tissues and yet twenty-four hours after its administration only about 65% of the administered activity was excreted in the urine. This was clarified by the fact that about 35–40% of the administered activity was excreted in the bile. Relatively little of the activity in the bile is reabsorbed, and excreted in the urine. Contrary to the findings with ISP only 10–15% of the activity after NE administration is excreted in the bile. Hence, the retardation in the urinary excretion of the activity following NE administration results from the binding of this amine in the tissues from which it is

slowly released. ISP on the other hand is not bound to any appreciable extent; about 35% of the administered compound is excreted in the bile and is not primarily excreted via the kidneys. However, the metabolites excreted in the bile are partially reabsorbed from the intestine and finally excreted by the kidneys. This accounts for the delayed excretion in the urine following the injection of ^{3}H-ISP. The delayed urinary excretion of activity following ^{3}H-NE administration mainly arises from its being bound in the tissues. A small amount is derived from the reabsorbed bile. Some of the activity excreted in the bile most probably leaves the body in the faeces.

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