Comparative Metabolic Studies with Natulan®*, Methylhydrazine and Methylamine in Rats

Following the administration of Natulan ® to rats, large amounts of N-isopropylterephthalamic acid were found in the urine[1,2], and the azo compound derived from Natulan ® was identified in blood[2]. Rats and mice given 1-methyl-^{14}C-labelled Natulan ® expire $^{14}CO_2$[3]. In the present study methylamine was found to be a metabolite of Natulan ® in rats.

Results. The following substances were found in the urine of rats after parenteral administration of the compounds listed: from methylamine – methylamine; from methylhydrazine – methylhydrazine and methylamine; from Natulan ® – Natulan® and methylamine. (For quantitative data see the Table, under column E.)

On the other hand no methylhydrazine could be found in the urine of rats given Natulan ®; nor has *p*-(isopropylcarbamoyl)benzylamine, the fragment anticipated from the cleavage of the N–N bond, yet been detected in the urine when rats were given carbamoyl-^{14}C-labelled Natulan®.

With all 3 compounds tested radioactivity was found in the expired air. Only a minor portion of this radioactivity was trapped in acid or at – 80 °C. In the case of methylhydrazine and Natulan ® this radioactivity was represented partially by formaldehyde. The amount was less than 1% of the administered dose. A larger portion of the radioactivity was trapped in the form of CO_2 in ethanolamine-methanol. The rest of the excreted radioactivity was found in the urine, 80% of which appeared in the first 24 h, and only a small amount in the faeces. In spite of similarities, the three compounds tested show differences in their route of excretion: The amount of $^{14}CO_2$ formed in rats from methylamine and Natulan ® is very similar, but about four times less in animals given methylhydrazine. In contrast the total radioactivity excreted in urine was found to be lower after methylamine or Natulan ® administration.

Since methylamine is metabolized to a large extent to CO_2, the question arises as to the possible implication of methylamine as precursor of CO_2 formation from Natulan ®. The pattern of excretion of methylamine or Natulan ® was not altered when SKF 525 A[4] was administered i.v. 30 min prior to these drugs. However, the $^{14}CO_2$ release was slowed down in animals given Natulan ®, but not in those given methylamine. This finding could indicate that CO_2 is formed from these two drugs by different pathways. Experiments are under way to further elucidate this question. DOST, REED and WANG[5] have found methane in the expired air of rats given methylhydrazine. We did not look for methane in our experiment; however, formation of this gas could eventually explain the relatively low recovery we encountered for methylhydrazine.

Formation of methylamine from Natulan ® and methylhydrazine demonstrates that metabolic reductive cleavage of the N–N bond is not confined to aromatic compounds[6,7], but that it can also occur in alkyl or aralkyl compounds.

Methods. 1-methyl-^{14}C-labelled Natulan® hydrochloride (sp. act. 17 μC/mg), ^{14}C-methylamine hydrochloride (sp. act. 20.6 μC/mg) and ^{14}C-methylhydrazine sulphate (sp. act. 3.9 μC/mg) were used[8]. Male albino rats (90–110 g)

* Registered trade mark for *p*-(isopropylcarbamoyl)benzyl methylhydrazine, a cytostatic methylhydrazine derivative.

[1] V. T. OLIVERIO and M. G. KELLY, *Symposium on Chemotherapy of Cancer*, Lugano 29.4.–1.5.1964 (Elsevier, Amsterdam 1964).

[2] J. RAAFLAUB and D. E. SCHWARTZ, Experientia *21*, 44 (1965).

[3] M. BAGGIOLINI, M. H. BICKEL, and F. S. MESSIHA, Experientia *21*, 334 (1965).

[4] Diethylamino-ethylester of α,α-diphenyl-valeric acid. This substance inhibits many enzymatic reactions including demethylation.

[5] F. N. DOST, D. J. REED, and C. H. WANG, Fedn Proc. Am. Socs exp. Biol. *24*, Part I, 547 (1965).

[6] A. T. FULLER, Lancet *1*, 194 (1937).

[7] R. T. WILLIAMS, *Detoxification Mechanisms* (Chapman & Hall, London 1959, 2nd edition), p. 478.

[8] I am indebted to Dr. J. WÜRSCH and Dr. R. BARNER, Chemical Research Department, F. Hoffmann-La Roche, Basle, for the synthesis of labelled methylhydrazine and Natulan®. Labelled methylamine hydrochloride was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England.

Expiration and excretion of radioactivity following a single intraperitoneal dose in rats (radioactivity expressed in % of administered dose)

Substances injected (dose: μmole/kg)	Expired air			Urine			Faeces	Animal homogenate	Total recovery = A+B+C+F+G
	A	B		C	D	E	F	G	
	$^{14}CO_2$ total[a]	Radioactivity trapped both in HCl and at – 80 °C		1st–4th day	1st day	Substances isolated as DNP from 1st day urine[c]	1st–4th day	4th day	
		Total	As formaldehyde						
Methylhydrazine (140)	4.2 9.8	1.3 1.9	0.5 0.8	53.0 53.7	50.7 51.4	Methylhydrazine 16 Methylamine 5	1.5 1.7	3.0 4.4	63.0 71.5
Natulan® (400)	30.4 29.3	0.4 0.5	0.2 0.05	36.2 35.8	32.8 31.8	Natulan® 2–5 Methylamine 1–4	4.0 4.7	11.6 12.9	82.6 83.2
Methylamine (400)	43.6 45.6	< 0.1 < 0.1		28.1 29.4	24.0 24.7	Methylamine 7	0.7 1.2	19.0 11.4	91.4 87.6
SKF[b] (330) + Natulan® (400)	25.9 27.4								
SKF[b] (330) + Methylamine (400)	41.3 44.4	0.1 0.1		33.0 30.1	29.6 25.7		1.2 0.7	17.8 19.1	93.4 94.4

[a] See under 'Methods'. [b] Administered subcutaneously 30 min before the injection of Natulan® or methylamine. [c] Approximate amount assuming the isolation of the DNP was quantitative.

received 400 μmoles/kg Natulan ® or methylamine or 140 μmoles/kg methylhydrazine in one single intraperitoneal injection. For the identification of metabolites in urine, the animals were kept in metabolic cages and the first 24 h urine portion was collected in flasks cooled to 0 °C containing 2 ml of NHCl. The urine was mixed with 9 volumes of chilled ethanol and kept for 15 min at 0 °C and centrifuged. The supernatant was evaporated to dryness at 40 °C under vacuum. Amines and hydrazines present as hydrochlorides in the residue were converted and identified as dinitrophenyl (DNP) derivatives. Urine residues or the synthetic hydrazines or amines were reacted with an excess dinitrofluorobenzene (DNFB) under identical conditions. Because of the relative instability of Natulan ® in solution at alkaline pH[9], the reaction was first conducted for 2 h in a buffer of pH 6.0 (20 volumes 0.1 mol sodium pyrophosphate + 9 volumes 0.3 mol *o*-phosphoric acid). To ensure complete reaction with amines the pH was then raised to 8.5 by addition of a saturated solution of sodium carbonate in 50% ethanol. The reaction was allowed to proceed for 1 h more and the excess DNFB was removed with glycine according to LOCKART[10]. The identity of DNP derivatives obtained from urine was ascertained by thin layer radiochromatography in 5 chromatographic systems using the synthetic ^{14}C-labelled DNP derivatives as reference compounds.

For experiments on the rate and route of excretion of the drugs, animals were put in closed glass metabolic cages provided with an inlet and an outlet for the air. The expired air was aspirated with a water pump, a pressure difference of 45 mm Hg between the air inlet and outlet being used, first through NHCl to absorb bases, then through a trap at − 80 °C, finally through two wash bottles containing 25 ml each of 12% ethanolamine in methanol for the absorption of CO_2. CO_2 was collected continuously up to 14 h after administration, then intermittently. Values obtained after 14 h were interpolated. The total amount of ^{14}C-formaldehyde was determined both in the HCl absorption solution and in the water trapped at − 80 °C. After addition of weighted amounts of carrier formaldehyde, its dimedone derivative was precipitated and recrystallized to constant activity in 50% ethanol.

The radioactivity of CO_2 absorbed in 12% ethanolamine in methanol was measured after addition of one volume scintillation solution consisting of 4 g PPO (2,5-diphenyloxazol) and 100 mg POPOP (1,4-bis-2-(-5-phenyloxazol)benzene) in 1 l toluene. The radioactivity of the other samples was determined after combustion according to the technique of KALBERER and RUTSCHMANN[11]. However, the presence of volatile metabolites made it necessary to omit the drying of samples prior to burning. Radioactivity was measured with a Tricarb-liquid scintillation spectrophotometer (Packard, Mod. 3000).

Zusammenfassung. Nach intraperitonealer Verabreichung von Natulan ®, Methylamin oder Methylhydrazin an Ratten konnte im Urin neben den verabreichten Stoffen Methylamin nachgewiesen werden, dagegen wurde Methylhydrazin als Metabolit von Natulan ® nicht gefunden. Das Auftreten von Methylamin nach Gabe der erwähnten Hydrazinverbindungen zeigt, dass der Rattenorganismus nicht nur, wie bisher bekannt, aromatische Azoverbindungen an der N–N-Bindung spalten kann, sondern auch aliphatisch-araliphatische.

D. E. SCHWARTZ

Research Department of F. Hoffmann-La Roche & Co. Ltd., Basel (Switzerland), January 7, 1966.

[9] K. BERNEIS, M. KOFLER, W. BOLLAG, P. ZELLER, A. KAISER, and A. LANGEMANN, Helv. chim. acta *46*, 2157 (1963).
[10] I. M. LOCKART, Nature *177*, 394 (1956).
[11] F. KALBERER and J. RUTSCHMANN, Helv. chim. acta *40*, 1956 (1961).

Combined Autoradiography and Fluorescence Microscopy. Localization of Labelled 5-Hydroxytryptophan in Relation to Endogenous 5-Hydroxytryptamine in the Gastrointestinal Tract

In a previous paper[1] concerning the distribution of radioactive 5-hydroxytryptophan (5-HTP) and 5-hydroxytryptamine (5-HT) in mice, it was found of value to be able to relate the uptake of the labelled substance to the endogenous monoamines. The present paper describes a combination of autoradiographic techniques with a histochemical method developed by FALCK and HILLARP[2–5] for localization of catecholamines and indolamines.

Since the enterochromaffin cells of the intestine are known to contain large amounts of 5-HT[6–9], these cells have been chosen for methodologic studies of the combination of autoradiographic and fluorescence techniques.

1 mC ^3H-*dl*-5-hydroxytryptophan (G) with a specific activity of 3.6 C/m*M* (Radiochemical Centre, Amersham, England) was given intravenously to an adult female mouse. The dose given was 3 mg/kg body weight. 4 h after injection specimens from the gastric and intestinal walls were freeze-dried and treated with formaldehyde gas[10]. Paraffin sections were mounted under coverslips in liquid paraffin and examined and photographed in the fluorescence microscope. The coverslips were then removed in xylene and the sections were passed down an alcohol series before application of a stripping film emulsion (Kodak AR 10). After exposure (8–10 weeks) and photographic processing some sections were stained, while others were dehydrated and examined unstained in

[1] M. RITZÉN, L. HAMMARSTRÖM, and S. ULLBERG, Biochem. Pharmac. *14*, 313 (1965).
[2] B. FALCK, N.-Å. HILLARP, G. THIEME, and A. TORP, J. Histochem. Cytochem. *10*, 348 (1962).
[3] B. FALCK, Acta physiol. scand. *56*, Suppl. 197 (1962).
[4] H. CORRODI and N.-Å. HILLARP, Helv. chim. Acta *46*, 2425 (1963).
[5] H. CORRODI and N.-Å. HILLARP, Helv. chim. Acta *47*, 911 (1964).
[6] V. ERSPAMER and B. ASERO, Nature *169*, 800 (1952).
[7] R. BARTER and A. G. E. PEARSE, J. Path. Bact. *69*, 25 (1955).
[8] E. P. BENDITT and R. L. WONG, J. exp. Med. *165*, 509 (1957).
[9] V. ERSPAMER, Fortschr. Arzneimittelforsch. *3*, 151 (1959).
[10] B. FALCK and C. OWMAN, Acta univ. lund. *2*, 7 (1965).