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THE EFFECT OF NANDROLONE, AN ANABOLIC STEROID ON PUTRESCINE METABOLISM IN THE MOUSE

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1 The catabolism of injected ^{14}C-putrescine was studied in mice treated with nandrolone phenpropionate, an anabolic steroid.

2 The putrescine was rapidly metabolized; almost 50% of the injected radioactivity was recovered within 2 h as $^{14}CO_2$ in the expired air.

3 Considerable amounts of radioactive γ-aminobutyric acid (GABA) and an unidentified compound were found in the kidney and in the urine in addition to radioactive putrescine, spermidine and spermine both in controls and nandrolone-treated mice.

4 Nandrolone elevated the concentration of endogenous putrescine in the kidney and urine, eightfold and twentyfold, respectively, and the concentrations of spermidine and spermine were also increased.

5 After the injection of ^{14}C-putrescine the incorporation of ^{14}C into spermidine was significantly increased in the kidney of mice receiving nandrolone.

Introduction

Evidence is accumulating to show that putrescine and its metabolites spermidine and spermine are ubiquitously present in animal tissues. It is known that putrescine can be metabolized to spermidine and spermine and it has been proposed that putrescine formation is the rate-limiting step in the synthesis of these polyamines (Jänne & Raina, 1968; Williams-Ashman, Pegg & Lockwood, 1969). Relatively little is known about the oxidation of putrescine in animal tissues (Raina & Jänne, 1975). Seiler and his coworkers were the first to show that [^{14}C]-putrescine could be incorporated into γ-aminobutyric acid (GABA) in mammalian tissues (Seiler, Wiechmann, Fischer & Werner, 1971; Seiler & Al-Therib, 1974). GABA was first shown to be a constituent of non-neural tissue in the late sixties (Zachmann, Tocci & Nyhan, 1966; Whelan, Schriver & Mohyuddin, 1969). However, the kidney is the only non-neuronal organ in which its concentration is considerable (Lancaster, Mohyuddin, Schriver & Whelan, 1973). GABA is alleged to stimulate protein synthesis (Baxter, 1970). This is of particular interest since it has been suggested that polyamines might be implicated in growth processes and in RNA metabolism (see e.g. Cohen, 1971; Russell, 1973; Raina & Jänne, 1975). Thus GABA formed from putrescine may have a similar regulatory function in growth processes.

A distinct sex difference in putrescine formation has been disclosed (Henningsson & Rosengren, 1975). Putrescine formation by mouse kidney was low in both sexes up to 3 weeks of age. Thereafter the amine formation in the male increased conspicuously, whereas that of the female kidney remained low. Testosterone administration resulted in an elevated biosynthesis of putrescine in the mouse kidney and caused a striking increase in the urinary excretion of free putrescine (Grahn, Henningsson, Kahlson & Rosengren, 1973). Nandrolone, an anabolic steroid with low androgenic activity produced a hundredfold elevation of putrescine formation (to be published).

This significantly increased formation of putrescine in the mouse treated with nandrolone led us to investigate the metabolic fate of [^{14}C]-putrescine in mice. Special attention was paid to the kidney as its growth is stimulated by this anabolic steroid. The present paper also describes changes in the concentrations of endogenous putrescine and polyamines in the kidney and urine after administration of nandrolone.

Methods

Castrated adult mice (30 g, Naval Medical Research Institute strain) were used. They were fed a standard pellet diet and water *ad libitum* except when urine was collected in which case the mice were given 4 g daily of a partly synthetic diet, the composition of which was given in an earlier paper (Kahlson, Rosengren & Westling, 1958). The putrescine content of this diet

was 2 nmol/g. The mice were kept individually in metabolic cages, which provided facilities for collecting urine and faeces separately. The beaker in which urine was collected contained 10 drops of 5 M HCl.

Nandrolone phenpropionate (the phenylpropionate ester of 19-nortestosterone; Durabolin, Organon) was suspended in arachis oil; controls were given arachis oil only. 1,4[^{14}C]-putrescine (New England Nuclear) was dissolved in 0.9% NaCl solution (saline). All injections were given s.c.

Preparation of samples for analysis

Urine samples were prepared as follows: sulphosalicylic acid (30 mg) and ethylenediamine tetraacetic acid (EDTA, 1 mg) were added to each ml of urine and the mixture was kept cold (4°C) at least 3 h before the pH was adjusted to 2.0–2.5 with NaOH. The solution was then filtered through a filter with a pore size of 0.22 μm (Millipore Corp., Bedford, Mass. 01730). Extracts of homogenized kidney were prepared in a similar way.

Determination of putrescine and its metabolites

The separation and quantitative estimation of the relevant compounds in urine and kidney extracts were carried out at 60°C on a column of Durrum DC 6 A (7.0 × 0.6 cm) using the automatic amino acid analyser, LKB-BIOCAL 3201; the principle of the method has been published (Hatano, Sumizu, Rokushika & Murakami, 1970). In our modification of the method for separation of putrescine, spermidine and spermine four buffers made up of sodium citrate and sodium chloride were used in sequence with a flow rate of 80 ml/hour. The composition and the run time of the buffers were: Buff. A: 0.20 M Na^+, pH 3.5, 44 min; Buff. B: 0.35 M Na^+, pH 6.3, 44 min; Buff. C: 0.9 M Na^+, pH 6.3, 48 min; Buff. D: 2.8 M Na^+, pH 5.8, 40 minutes. The amines were quantified by measuring the peak areas of the curve representing the ninhydrin coloured complex.

For analysis of radioactive compounds the same amino acid analyser was used. After the stream had passed the column, it was split according to the method of Lou (1973). One half of the effluent was used for ninhydrin reaction in the usual way and the other half proceeded to a fraction collector by means of which a total of 70 fractions (2.0 ml each) were collected. To 1 ml of these fractions 9 ml of the liquid scintillator Instagel (Packard) was added and the radioactivity was measured in a liquid scintillation spectrometer (Packard TRI-CARB, 2002).

The final identification of the compounds in relevant fractions of eluates from the amino acid analyser was achieved as follows. Putrescine, spermidine and spermine were isolated by electrophoresis and determined as described in an earlier paper (Henningsson & Rosengren, 1975). The identity of GABA was confirmed by rechromatography on the column of the amino acid analyser but in a different buffer system (0.127 M Na^+, 0.025 M citrate, pH 2.88) as described by Hamilton (1962) for separation of amino acids. Further, co-chromatography with authentic GABA showed a coincident peak.

Determination of expired $^{14}CO_2$

Mice were injected with [^{14}C]-putrescine (2.5 μCi, 50 μg) and immediately placed in a cylindrical plastic chamber. A flow system provided air through the chamber in a constant stream. This air-stream was finally directed to a series of vials containing 5 ml of hyamine 10-X for quantitative absorption of the $^{14}CO_2$ excreted. Aliquots (0.1 ml each) of the hyamine were transferred to counting vessels containing 10 ml of Bray scintillation solution (Bray, 1960) and the radioactivity was determined.

Results

Radioactivity in the kidney and urine after administration of [^{14}C]-putrescine

Castrated mice were injected with nandrolone (0.1 mg daily for 3 days); controls received arachis oil only. A subcutaneous injection of [^{14}C]-putrescine was given on the 3rd day and the amount of [^{14}C]-compounds in the kidney was determined 1 h and 24 h later and in the urine 24 h later.

The results of the determinations of [^{14}C]-compounds found in the kidney are presented in Figure 1 where for the sake of simplicity the radioactivity is given as nCi/g. The combined weight of the two kidneys of castrated mice was about 260 mg and of mice given nandrolone (0.1 mg daily for 3 days) 360 mg. In both groups of mice 40% of the total radioactivity found in the kidneys 1 h after the injection of [^{14}C]-putrescine was unchanged [^{14}C]-putrescine. After 24 h this portion was reduced to 5% of the total radioactivity.

A major part of the radioactivity found in the mouse kidney after the injection of [^{14}C]-putrescine was apparently incorporated into the spermidine skeleton; this was particularly noticeable in determinations made 1 h after the injection. Further, it was found that at 1 h after administration of [^{14}C]-putrescine, the concentration of labelled spermidine was twice as high in the kidney of the nandrolone-treated animals as it was in the kidneys of the controls while at 24 h no difference was found between the two groups.

Appreciable amounts of [^{14}C]-spermine were found in both groups of mice 1 h and 24 h after the injection of [^{14}C]-putrescine. The highest concentrations were noted at 24 hours. As seen from Figure 1, the

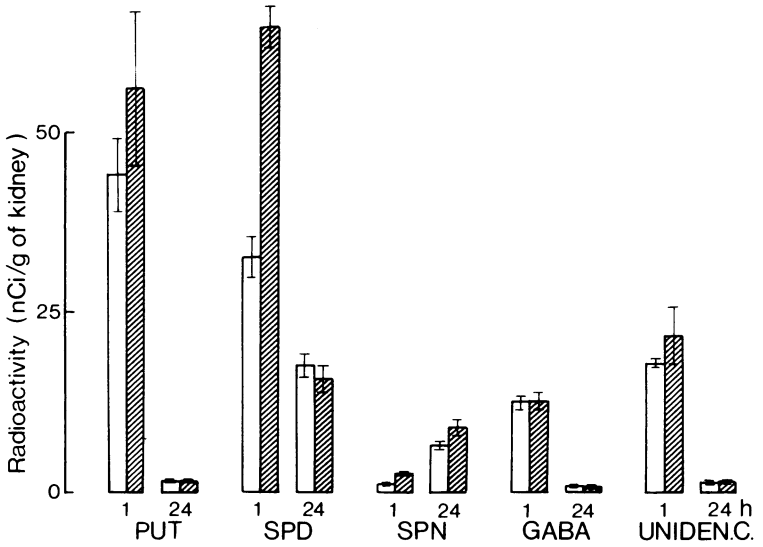

Figure 1 Radioactivity (nCi/g) in the kidney 1 h and 24 h after [^{14}C]-putrescine (2.5 μCi s.c.). Open columns: controls; hatched columns: mice injected with nandrolone (0.1 mg daily for 3 days). PUT = putrescine, SPD = spermidine, SPN = spermine, GABA = γ-aminobutyric acid, UNIDEN. C. = unidentified compound. Each column is the mean ± s.e. mean of 4 observations. Total kidney weights: controls 260 mg, nandrolone-treated animals 360 mg; no significant difference in body weight.

spermidine concentrations were reduced and those of spermine elevated 24 h after the injection; this observation may be due to the fact that the labelled carbon of putrescine is incorporated into spermidine before it can be transferred to the spermine molecule.

In the mouse kidney considerable amounts of radioactive carbon were recovered as GABA following the subcutaneous injection of [^{14}C]-putrescine (Figure 1). In addition to the above mentioned radioactive compounds found in the kidney extract after injection of [^{14}C]-putrescine, an unidentified radioactive compound was eluted from the column. This substance appeared before GABA in the eluate and was distinctly separated from the latter. The amount of this unknown compound was greater than that of GABA and it appeared that the concentration of the unidentified compound as well as that of GABA was several times higher at 1 h after putrescine administration than at 24 hours. No quantitative differences were seen in the amounts of GABA or the unidentified compound between the kidneys of animals treated with nandrolone or the controls.

From the mice in which [^{14}C]-putrescine metabolites were determined in the kidneys 24 h after [^{14}C] were incorporated into GABA. An unidentified examined its [^{14}C]-compounds. Of the total radioactivity injected 20% appeared in the urine in the untreated controls and 39% in the group of mice given nandrolone. Table 1 shows that about two-thirds of the total urinary radioactivity was excreted as unmetabolized putrescine. Significant amounts of the injected [^{14}C] was incorporated into GABA. An unidentified carbon-labelled compound was also present in the urine and appeared in the same fraction of the column

Table 1 Radioactivity (nCi/24 h) in urine after injection of [^{14}C]-putrescine in 6 controls and 6 mice injected with nandrolone (0.1 mg daily for 3 days). Means ± s.e. means and the degrees of significance between nandrolone-treated animals and controls are given. For abbreviations see Figure 1.

	Total radioactivity	*Putrescine*	*Spermidine*	*Spermine*	*GABA*	*Unidentified compound*
Controls	490.8 ± 118.05	300.5 ± 94.33	2.5 ± 0.38	0.4 ± 0.12	17.2 ± 2.65	128.2 ± 13.11
Nandrolone	975.4 ± 167.75*	694.9 ± 126.70*	8.0 ± 1.00**	0.8 ± 0.20	21.8 ± 0.90	178.4 ± 22.23

* $P < 0.05$, ** $P < 0.01$.

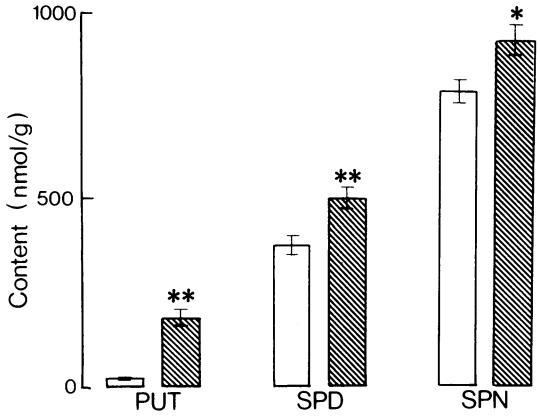

Figure 2 Concentrations of endogenous putrescine (PUT), spermidine (SPD) and spermine (SPN) in the kidneys in controls (open columns) and in mice injected with nandrolone (0.1 mg daily for 3 days; hatched columns). The content is expressed in nmol/g; each column is the mean ± s.e. mean of 8 observations. The degree of significance between nandrolone-treated animals and controls is given. * $P < 0.02$, ** $P < 0.01$.

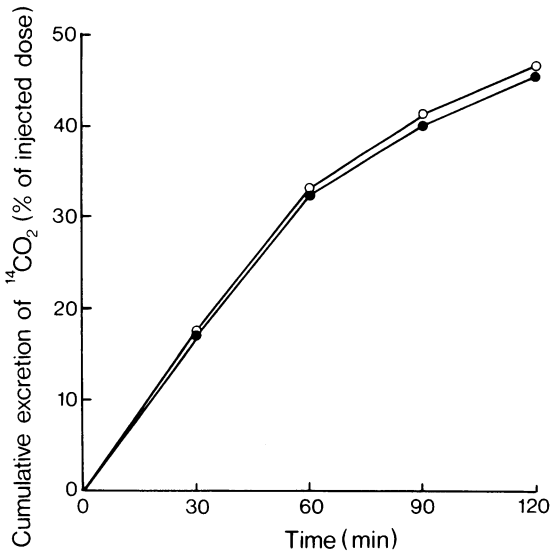

Figure 3 Cumulative $^{14}CO_2$ excretion following injection of [^{14}C]-putrescine in mice injected with nandrolone (0.1 mg daily for 3 days; ●) and in controls (○). Mean values of 3 experiments.

eluate in which we had observed the unidentified radioactive substance in the kidney extracts. The amount of this substance in the urine was almost 10 times that of GABA. Less radioactivity was found in the urinary polyamines spermidine and spermine. The excretion of [^{14}C]-spermidine was significantly higher in mice treated with nandrolone.

Content of endogenous putrescine, spermidine and spermine in the kidney and urine after nandrolone administration

The content of endogenous putrescine, spermidine and spermine in mouse kidney and urine after nandrolone injections was determined and compared with that of castrated control mice. The analysis was done on the same mice which were used in the experiments with [^{14}C]-putrescine (see above). In the kidney the content of each of the amines determined was elevated after nandrolone administration, particularly that of putrescine which was increased eight times (Figure 2). Still greater differences were found between controls and nandrolone-injected animals in the urinary excretion; the nandrolone-treated group excreted 20 times more putrescine and 5 times more spermidine than the controls (Table 2). The excretion of spermine was low and not significantly different in the two groups.

Radiorespirometric recording of $^{14}CO_2$ after injection of labelled putrescine

Figure 3 shows the time course of the occurrence of $^{14}CO_2$ in the expired air during the 2 h period

Table 2 Urinary excretion (nmol/24 h) of putrescine, spermidine and spermine in 6 controls and 6 mice injected with nandrolone (0.1 mg daily for 3 days). Means ± s.e. means and the degrees of significance between nandrolone-treated animals and controls are given. The volumes of the 24 h urinary samples were 4 to 5 ml in both groups.

	Putrescine	*Spermidine*	*Spermine*
Controls	198.4 ± 34.02	29.3 ± 3.96	2.2 ± 1.40
Nandrolone	4307.9 ± 457.60*	137.1 ± 26.64*	4.6 ± 2.48

* $P < 0.01$.

following the injection of [^{14}C]-putrescine (2.5 μCi). Nearly 50% of the injected dose was expired as $^{14}CO_2$ within 2 hours. No difference was seen between the control group and the group treated with nandrolone. In mice pretreated with the diamine oxidase inhibitor aminoguanidine (5 mg/kg) half an hour before the [^{14}C]-putrescine injection the production of $^{14}CO_2$ was lowered to 3% of the injected radioactivity.

Discussion

A rather conspicuous finding in this study was the presence of radioactive GABA in the kidney and urine after injection of [^{14}C]-putrescine. The incorporation of putrescine carbon into GABA in rat liver and brain has been reported (Seiler *et al.*, 1971). During the preparation of this report Seiler and his group reported also the urinary excretion of radioactive GABA after [^{14}C]-putrescine injection in mice but only after pretreatment with an inhibitor of γ-aminobutyrate aminotransferase, an enzyme that hastens the degradation of GABA (Seiler & Eichentopf, 1975). The discrepancy between Seiler's and our results cannot yet be explained. It may be due to differences in the methods used to assay GABA. We have found the automatic amino acid analyser very useful for determinations of amines and, when combined with the split-stream technique as in this study, also for determinations of radioactivity in various amine metabolites, such as GABA.

Jänne (1967) showed in rats that one-third of the injected dose of [^{14}C]-putrescine was expired as $^{14}CO_2$ within 2 h, an observation which we have confirmed in mice. [^{14}C]-GABA administered to rats is rapidly degraded to $^{14}CO_2$ via the Krebs' tricarboxylic acid cycle (Wilson, Hill & Koeppe, 1959). The present results, together with the reports of Seiler's group (Seiler *et al.*, 1971; Seiler & Eichentopf, 1975), show that GABA is an intermediate in the catabolism of putrescine to carbon dioxide.

One hour after injection of putrescine, [^{14}C]-spermidine was present in the kidney in amounts similar to that of the parent compound. Further, in mice treated with nandrolone [^{14}C]-spermidine was elevated at 1 h and the radioactive spermidine disappeared faster, indicating an accelerated turnover rate. These observations appear useful in attempts to explore possible functions of the amine.

As mentioned in the Introduction recent studies on rapidly growing cells and tissues suggest a connection between synthesis of polyamines on the one hand and nucleic acid and protein metabolism on the other. We report an increased accumulation of endogenous putrescine and polyamines in the kidney under the influence of an anabolic agent which is reflected in the urinary excretion. Besides, the urinary excretion of radioactive carbon incorporated into spermidine was elevated indicating an increased formation of this amine. The elevation of the total urinary radioactivity owing to an increased [^{14}C]-putrescine excretion in the anabolic steroid treated mice may be explained by a heightened renal blood flow which is reported to occur in mice treated with testosterone propionate (Broulik, Kochakian & Dubovsky, 1969).

Evidence is accumulating that in patients with tumours and leukaemia the excretion of polyamines is elevated; determination of the rate of polyamine excretion has been suggested as a test in the early detection of cancer (Russell, Levy, Schimpff & Hawk, 1971). The present study showed that more urinary radioactive carbon was found in the fractions of GABA and in the unidentified compound than in the polyamine fractions pointing out the relative importance of the different pathways in putrescine metabolism. If it were possible to determine endogenous amounts of the as yet unidentified putrescine metabolite the estimation of this compound in the urine may be a more sensitive tool in revealing early cancer.

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