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Isocarboxazid Metabolism in Rat Liver Homogenate. (26704)

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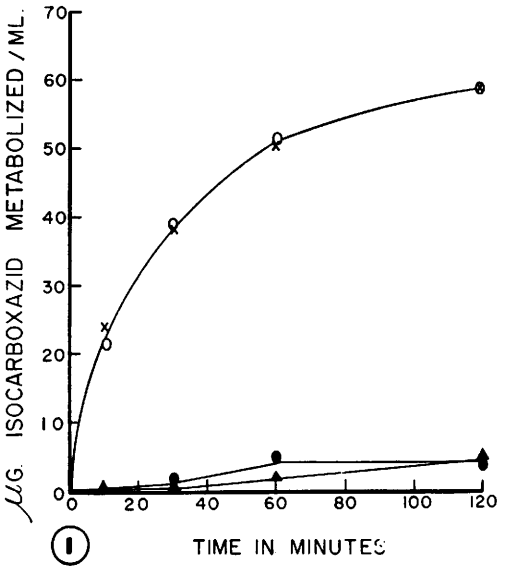
Isocarboxazid (Marplan®), 1-benzyl-2-(5-methyl-3-isoxazolylcarbonyl) hydrazine is a potent monoamine oxidase inhibitor(1) being used in treatment of various depressions(2,3, 4) and in management of angina pectoris(5, 6). In rats 75% of the radioactivity of an intraperitoneal dose of C^{14}-isocarboxazid labeled in the benzyl moiety was recovered in the urine in 24 hours(7). By reverse isotope dilution, virtually all radioactivity was found to reside in hippurate indicating that the benzyl moiety of isocarboxazid was oxidized to benzoate prior to formation and excretion of hippurate. The present study was designed to gain information concerning this biological conversion of the benzyl moiety to benzoate.

Methods. All incubations of liver homogenates were performed in a Dubnoff metabolic shaker at 38°C. Isocarboxazid was solubilized in an aqueous medium *via* Tween 80 as described by Meier *et al.*(8). For its determination, isocarboxazid was quantitatively extracted from an aliquot of the incubation medium by 2 volumes followed by 1 volume of chloroform. The combined chloroform extracts were backwashed with ½ volume of water and evaporated to dryness. The residue was then dissolved in acetone and the isocarboxazid content measured in a Beckman DU spectrophotometer by the method of Colarusso *et al.*(9). Synthesis of C^{14}-isocarboxazid with the methylene carbon of the benzyl moiety labeled has been described(7). Radioactivity was measured in a Nuclear of Chicago Corp. D-47 flow gas counter and corrected for self-absorption.

A solvent system of tertiary butanol:methanol:water (50:40:10) run by the ascending method on Whatman #1 paper pretreated with McIlvaine's phosphate-citrate buffer, pH 3.7, was employed for separation of labeled benzylhydrazine from other labeled components in extracts of the incubation medium. "Carrier" benzylhydrazine (technical grade containing 2% benzylamine) was added to the extract before it was applied to the paper and its position was determined by spraying the paper with a 1% solution of p-dimethylaminobenzaldehyde in 1N HCl. Radioactivity on the chromatograms was located by putting the papers through a Nuclear of Chicago Actigraph II chromatogram scanner.

Labeled benzylhydrazine was determined by a reverse isotope dilution technic involving the synthesis of benzylhydrazine monooxalate. For this purpose, an extract of the incubated material was prepared by adding an equal volume of absolute ethanol to an aliquot of incubation medium and filtering off the proteins after 15 minutes in an ice bath. Benzylhydrazine hydrochloride (300 mg as carrier) was dissolved in an aliquot of this extract which was then made alkaline with dilute NaOH solution, and following the addition of anhydrous sodium sulfate was extracted with 2 volumes of chloroform. The chloroform layer was concentrated to an oil after the complete removal of water by further addition of sodium sulfate. Oxalic acid (200 mg) in alcohol was added to precipitate benzylhydrazine monooxalate and this product was recrystallized from ethanol-water to constant specific activity. Furthermore, the final product caused no depression in the melting point of authentic benzylhydrazine monooxalate.

Results. 1. Enzymatic destruction of isocarboxazid. The aerobic disappearance of isocarboxazid in presence of rat liver homo-



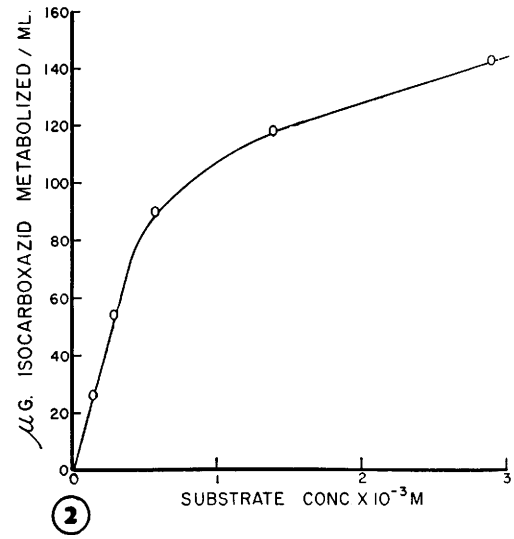


FIG. 1. Metabolism of isocarboxazid as a function of time. Each flask contained 400 μg of isocarboxazid dispersed in Krebs-Ringer phosphate buffer, pH 7.4 (1 ml). Symbols refer to addition of rat liver homogenate as follows: (○) and (×), duplicate flasks containing 2 ml of 1:4 liver homogenate in Krebs-Ringer phosphate buffer; (●), 2 ml of boiled homogenate (3 min. in boiling water bath); and (▲), no homogenate added. Volume in each flask was brought to 6 ml with buffer, making final isocarboxazid concentration 67 μg/ml.

FIG. 2. Effect of substrate concentration on isocarboxazid metabolism. Duplicate flasks containing 2 ml of 1:4 liver homogenate in Krebs-Ringer phosphate buffer, pH 7.2, and 4 ml of buffer containing graded amounts of isocarboxazid were incubated aerobically for 2 hr at 38°C. Values for isocarboxazid metabolized were corrected for the slight loss of isocarboxazid observed in incubated control flasks containing just isocarboxazid and buffer.

genate and boiled rat liver homogenate is presented in Fig. 1. After 2 hours about 90% of the isocarboxazid was destroyed by the liver homogenate while the breakdown in presence of boiled liver homogenate occurred to the same small extent as found in the buffer control. This apparently enzymatic destruction of isocarboxazid was checked by using ethyl alcohol instead of heat to denature the enzymes. Furthermore, the effect of a casein suspension on the disappearance of isocarboxazid was investigated to ascertain whether this disappearance was catalyzed by specific enzymes or by non-specific proteins. Also included in this experiment was a study of the effect of anaerobiosis on liver homogenate-catalyzed isocarboxazid destruction (Table I). While there was some destruction of isocarboxazid in the buffer alone (9 and 29%), 92% disappeared in presence of liver homogenate. Denaturation of enzymes with ethanol resulted in a decrease in isocarboxazid destruction to the buffer control levels, and casein could not replace the homogenate as a catalyst. Furthermore, the anaerobic incubation of isocarboxazid with liver homogenate resulted in a breakdown almost identical to that seen aerobically. It is concluded from these experiments that in the system employed isocarboxazid was metabolized by liver enzymes and that the first step in this metabolism was not an oxidation.

The effect of substrate concentration on the metabolism of isocarboxazid is shown in Fig. 2. It is apparent that the reaction is linear with respect to substrate concentration up to approximately 0.5 x 10^{-3} M (116 μg/ml).

2. Isolation of intermediates in isocarboxazid metabolism using C^{14}-isocarboxazid (labeled benzyl moiety). Since enzymatic hydrolysis of isocarboxazid might lead to the formation of benzylhydrazine, its presence in incubation medium extracts was investigated by paper chromatography and reverse isotope dilution. Radioactive material in the same position as the "carrier" benzylhydrazine was found in chloroform and ethanol

TABLE I. Effect of Liver Homogenate and Casein Suspension on Isocarboxazid Destruction; Effect of Alcohol and Anaerobiosis on Homogenate-Induced Destruction.

Sample	Contents	% isocarboxazid destruction after 2 hr at 38°C
1—Aerobic	Isocarboxazid + buffer	29
2 "	*Idem*	9
3 "	Isocarboxazid + homog.	92
4 "	*Idem*	92
5 "	Isocarboxazid + homog. + EtOH	8
6 "	*Idem*	12
7 "	Isocarboxazid + casein	28
8 "	*Idem*	15
1—Anaerobic	Isocarboxazid + homog.	91
2 "	*Idem*	91

Isocarboxazid concentration in each flask was 3.6×10^{-4} M; final volume was 3 ml. Isocarboxazid, rat liver homogenate and casein were prepared in Krebs-Ringer phosphate buffer, pH 7.3. One ml of homogenate (1:4), 1 ml of absolute ethanol, and 1 ml of a 5% suspension of casein were employed where noted in the table. These additions replaced an equal volume of buffer. Evacuated Thunberg tubes were used for anaerobic incubation.

extracts of the incubation medium when these extracts were chromatographed as described under *Methods*. The use of "carrier" benzylhydrazine made identification of labeled benzylhydrazine possible in spite of the fact that there was a wide variation in R_f values of benzylhydrazine (R_f .30-.47). In any one sample the difference in R_f values between labeled component and added benzylhydrazine did not exceed 0.02. The variation of the benzylhydrazine R_f values might be due to the effects of other components in these extracts or to sensitivity of this chromatographic system to factors which were not adequately controlled.

The presence of labeled benzylhydrazine in the incubation medium was confirmed by reverse isotope dilution (Table II). Approximately 80% of the radioactivity was extracted with alcohol following both aerobic and anaerobic incubation (column a) while 91% was extracted from the control flask containing boiled homogenate (flask 5). From the specific activities of the isolated benzylhydrazine monooxalate, amount of benzylhydrazine hydrochloride used as carrier, and radioactivity of the alcohol extract aliquot to which carrier was added, the percentage of the total extracted radioactivity residing in benzylhydrazine was calculated (column b). These values ranged from 48.3% to 52.0% and were independent of the mode of incubation. Furthermore, in the control flask only an insignificant fraction of the extracted radioactivity (0.4%) was calculated to be benzylhydrazine. The values of column (b) can be taken as the percentage conversion of isocarboxazid to benzylhydrazine by assuming the benzylhydrazine content of that radioactivity not extracted by alcohol (100%-column a) to be the same as the percentage benzylhydrazine in the radioactivity which was extracted (column b). This asumption appears valid since in the control flask (flask 5) 9% of the radioactivity was not extracted with alcohol while when benzylhydrazine was formed (flasks 1-4) approximately 20% of the radioactivity was not extracted, indicating that roughly half of this unrecovered radioactivity was benzyl-

TABLE II. Percentage of C^{14}-isocarboxazid Converted to Labeled Benzylhydrazine after 2 Hour Incubation with Rat Liver Homogenate.

Flask No.	Incubation	% incubation medium radioactivity in alcohol extract (a)	Specific activity of isolated benzylhydrazine monooxalate (cpm/mg)	% alcohol extract radioactivity present in benzylhydrazine* (b)
1	Aerobic	77	416	48.3
2	Aerobic	78	444	52.0
3	Anaerobic	82	453	50.0
4	Anaerobic	83	467	49.5
5†	Aerobic	91	2	0.4

* These values also represent the % conversion of isocarboxazid to benzylhydrazine (see text).

† Flask 5 contained boiled rat liver homogenate and served as a control.

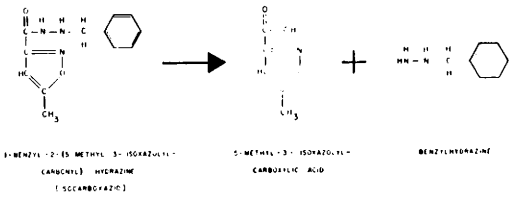


FIG. 3. Primary reaction in *in vitro* metabolism of isocarboxazid.

hydrazine. This agrees with the roughly 50% of the alcohol extract radioactivity recovered as benzylhydrazine (column b).

Discussion. It has been demonstrated *in vitro* that rat liver homogenate enzymatically catalyzes the breakdown of isocarboxazid and that one major product of this metabolism is benzylhydrazine. These findings can most simply be interpreted as reflecting a hydrolytic cleavage of isocarboxazid to yield 5-methyl-3-isoxazolylcarboxylic acid and benzylhydrazine (Fig. 3). The isolation of benzylhydrazine from animal tissues following administration of pharmacological doses of isocarboxazid has not been reported. However, Roth and Rieder† have detected benzylhydrazine in rat and guinea pig tissues following massive doses of isocarboxazid. This, in conjunction with the present finding that *in vitro* there is extensive metabolism of isocarboxazid to benzylhydrazine and the previous finding(7) that *in vivo* 75% of the drug was cleaved with the benzyl moiety further oxidized to benzoate and excreted as hippurate in 24 hours, indicates that benzylhydrazine is an intermediate in the metabolism of isocarboxazid *in vivo*. Since benzylhydrazine is a more potent MAO inhibitor than isocarboxazid, the role of benzylhydrazine as an agent responsible for various aspects of the pharmacological activity of isocarboxazid becomes of prime importance. This problem is currently being studied.

Summary. Isocarboxazid was extensively metabolized under aerobic or anaerobic conditions in an *in vitro* system containing rat liver homogenate as the enzyme source. Benzylhydrazine was a major product of this metabolism as determined by paper chromatography and reverse isotope dilution in studies with C^{14}-isocarboxazid. The possible occurrence of this pathway *in vivo* was discussed.

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† Personal communication from Hoffmann-La Roche, Inc., Basel, Switzerland.

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Development of Serum Haptoglobins in Man.* (26705)

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Twenty years ago Polonovski and Jayle described a fraction of serum protein capable of combining with hemoglobin to form a stable complex which possessed peroxidase activity. This fraction they called haptoglobin (1). Smithies, using the technic of starch gel electrophoresis, has demonstrated that several haptoglobins may be present in a sin-

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