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STUDIES ON PHARMACOGENETICS—I. THE ENZYMIC ACETYLATION OF ISONICOTINIC ACID HYDRAZIDE (INH)*†

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Abstract—The transfer reaction of the acetyl group of acetyl-CoA (using ^{14}C-Acetyl-CoA) to INH by an INH-acetylating enzyme from liver is described. Moreover a suggested polymorphism of INH-inactivation in *Cercopithecus aethiops* corresponding to man is reported.

INTRODUCTION

AFTER administration of a standard dose INH per kg body weight to a group of individuals different plasma levels of INH could be detected; a bimodal distribution is obtained.[1] This variability is genetically determined and caused by different inactivation of INH, i.e. different acetylating rates of INH. The differences of INH-inactivation have not yet been investigated on the enzyme level. Enzymic acetylation reactions are known to occur by the production of citric acid via the Krebs cycle, in the fatty acid metabolism and by the formation of cholesterol, steroids and acetylcholine. The differences in INH-acetylation believed to be caused by a polymorphism of the acetylating enzyme protein which is observed within one population as well as between various populations.[2] A simple genetic model is established for this polymorphism.[3] Recent investigations let suppose that acetyl-CoA may be the acetyldonor of INH-acetylation.[4, 5] In the present paper the transfer reaction of the acetyl group of acetyl-CoA to INH is described. Acetyl-INH is formed by an INH-acetylating enzyme from liver which is checked using ^{14}C-acetyl-CoA resp. ^{14}C-INH + ^{14}C-acetyl-Coenzyme A → ^{14}C-acetyl-INH + Coenzyme A. Furthermore, investigations are reported on a possible polymorphism of the INH-acetylating enzyme in the liver of *Cercopithecus aethiops*.

EXPERIMENTAL AND RESULTS

1. *Determination of* INH *blood plasma-level of* Cercopithecus aethiops in vivo

Three hours after i.m. administration of 20 mg/kg body weight, 8–10 ml blood were taken from the vena femoralis, mixed with 0,2 ml Heparin and centrifuged 10 min at 450 g. INH was determined using the following method.

A modified method for estimation of INH *according to* Maher.[6] 3,2 g of anhydrous ammonium sulfate was added to 3,0 ml blood plasma, stirred and shaken for 30 min

* Dedicated to Prof. Schoepf for his 65th birthday.

† Abbreviations: INH = isonicotinic acid hydrazide; CoA = coenzyme A; ATP = adenosine triphosphate; EDTA = ethylenediamine tetra-acetic acid.

‡ Essential parts of this publication will be submitted as part of the thesis of D. Fleischmann to the Medical Faculty of the University of Freiberg, i.Br., Germany.

with 40 ml isoamyl-alcohol/ethylene-dichloride (3 : 7). After centrifugation at 4000 *g* for 15 min an aliquot (30 ml) of the supernatant was shaken with 4,0 ml of 0,1 M HCl and centrifuged for 10 min at 600 *g*. 3 ml of the supernatant was mixed with 0,2 ml of a 4% solution of vanillin in alcohol. After 5 min the optical density in the photometer Eppendorf was recorded at 366 mμ (1-cm cuvette). The concentration of INH per ml plasma was estimated using calibration curves.

2. *Determination of the* INH *turnover by measurement of* INH *decrease*

Freshly removed livers from *C. aethiops* were used for preparation of acetone powder. 2 g of acetone powder were ground for 15 min in a mortar with 10 ml of 2×10^{-2}M phosphate buffer, pH 6, 8 at 4°C and centrifuged at 37,000 g (0°C). 1,5 ml of the supernatant were used for each of the following incubation mixtures. The INH turnover was calculated in mμ moles per mg protein.

Reaction mixture (volume 3,0 ml) : $6,6 \times 10^{-3}$M phosphate buffer, pH 6,8; $3,3 \times 10^{-3}$M $MgCl_2$; $1,0 \times 10^{-3}$M ATP; $1,0 \times 10^{-3}$M acetyl-CoA (prepared according to Simon and Shemin[7]); $1,94 \times 10^{-3}$M INH; 1,5 ml enzyme solution (about 60 mg protein/ml); incubation for 3 hours at 37°C. The enzymic reaction was stopped by addition of ammonium sulfate and organic solvent mixture. INH and acetyl-INH were extracted as described above. The non acetylated INH was estimated in an aliquot (0,2 ml) of the acidified supernatant (cf. 1).

3. *Determination of the activity of the* INH-*acetylating enzyme by measurement of* ^{14}*C-acetyl*-INH *formed.*

The acetyldonor was acetyl-CoA. Reaction mixtures, as described above, were incubated with ^{14}C-INH (carbonyl-^{14}C) or ^{14}C-acetyl-CoA (2-^{14}C-acetyl). Figure 1 shows the amount of c.p.m. of ^{14}C-INH and ^{14}C-acetyl-CoA, resp. present in the beginning of the experiment. The reaction was stopped and extracted as described above, but the organic solvent mixture was evaporated to dryness. The residue was dissolved in 0,2 ml water and used for chromatography.

Descending paper-chromatography of INH *and acetyl*-INH. Solvent system: Isoamylalcohol/acetone/glacial acetic acid/water (224:96:24:56), chromatographic paper, Schleicher and Schüll 2043.b.Mgl. The papers were run for 20 hr at 20°C. The positions of INH and acetyl-INH were determined by u.v. light, using as detecting reagents benzidine and cyanogen bromide. The radioactivity was recorded with the radiopaperchromatograph FH 452 and methane-flowcounter FH 407 (Frieseke and Höpfner, Erlangen, Germany). This identified radioactivity of INH and acetyl-INH was estimated by counting in a Packard liquid scintillation spectrometer (model 63) H.V.: Coarse 4; Fine 303; Window 70–1000; Gain 28 per cent. The chromatographic paper was divided into pieces and placed in specimen bottles containing 10 ml of the following scintillation liquid: 800 ml Toluene (p.a.); 400 ml Ethanol (p.a.); 4,8 g PPO, 2,5-Diphenyloxazole; 0,12 g Dimethyl-POPOP, 1,4-bis-2-(4-Methyl-5-Phenyloxazolyl)-Benzene. Small amounts of isonicotinic acid formed in the reaction mixtures were identified by thin layer chromatography (silica gel G, according to Stahl) in n-butanol/glacial acetic acid (10:1), running for 5 hr at 20°C. The separation of isonicotinic acid and acetyl-INH could not be realized by paperchromatography employing the solvent system described above.

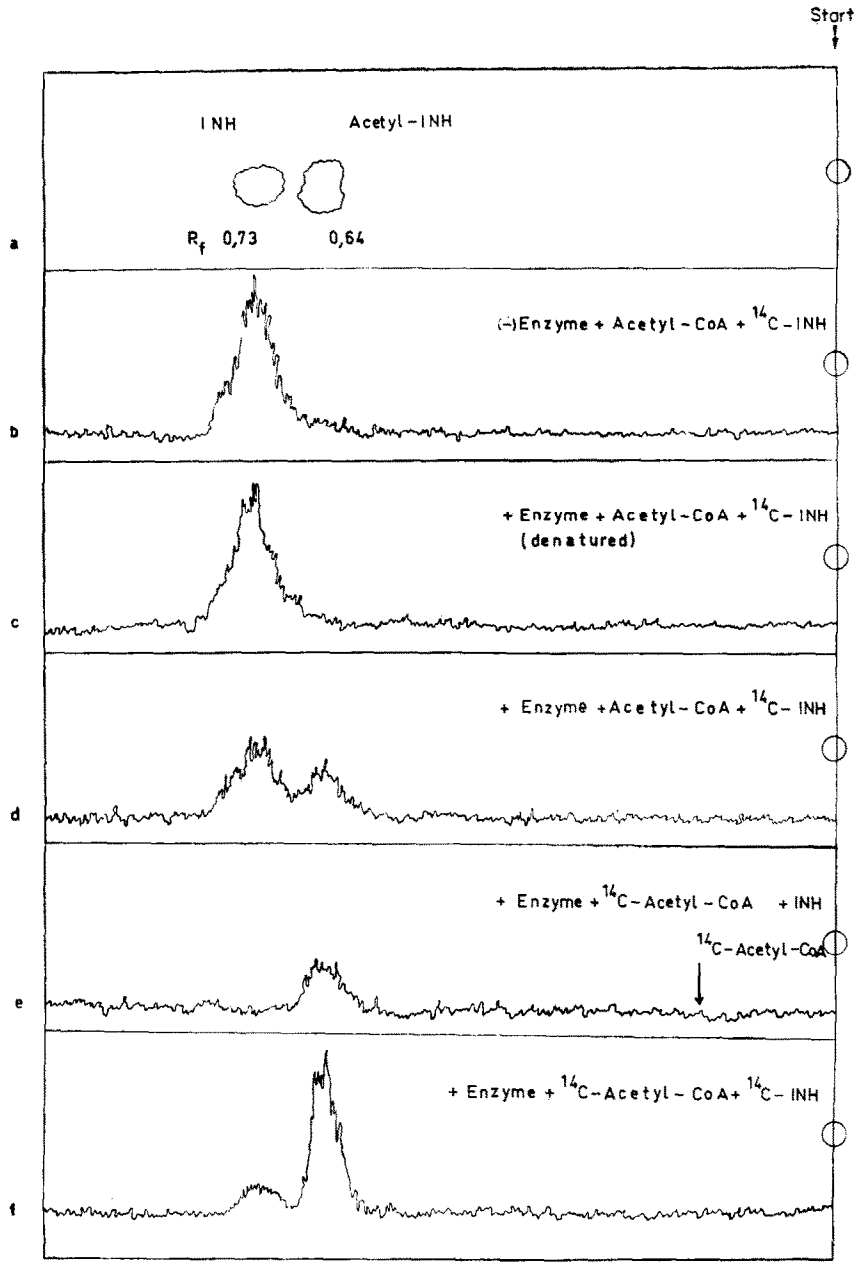

FIG. 1. Acetylation of ^{14}C-INH by INH-acetylating enzyme from liver of *C. aethiops* with acetyl-CoA (or ^{14}C-acetyl-CoA) as acetyldonor. Incubation mixtures cf. experimental. All reaction mixtures but e contained labeled INH (^{14}C-carbonyl, 67, 500 cpm.). Labeled acetyl-CoA (2-^{14}C-acetyl, about 55,000 cpm.) is only used in the reaction mixtures e and f.

4. *Spectrophotometric measurement of the activity of the* INH-*acetylating enzyme.*[8] Reaction mixture: $6{,}6 \times 10^{-1}$M pyrophospoate buffer pH 9,5; 5×10^{-4}MEDTR; $3{,}0 \times 10^{4}$M acetyl-CoA; $1{,}66 \times 10^{-3}$M INH; 5×10^{-4}M sodium thioglycolate; enzyme protein (II); 313 mμ (1-cm cuvette); volume 3 ml, 37°C. This method can be used to measure the kinetics of the INH-acetylating enzyme (acetyl-CoA as acetyl-donor). Preliminary results obtained with this method shall be published elsewhere.

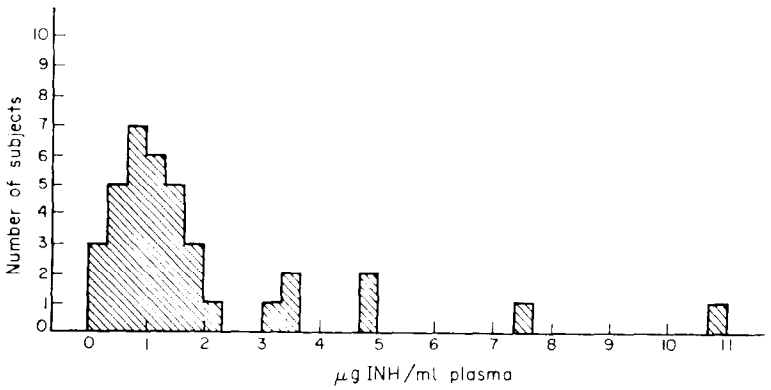

FIG. 2. INH-plasma-levels of *C. aethiops* (test conditions see experimental 1).

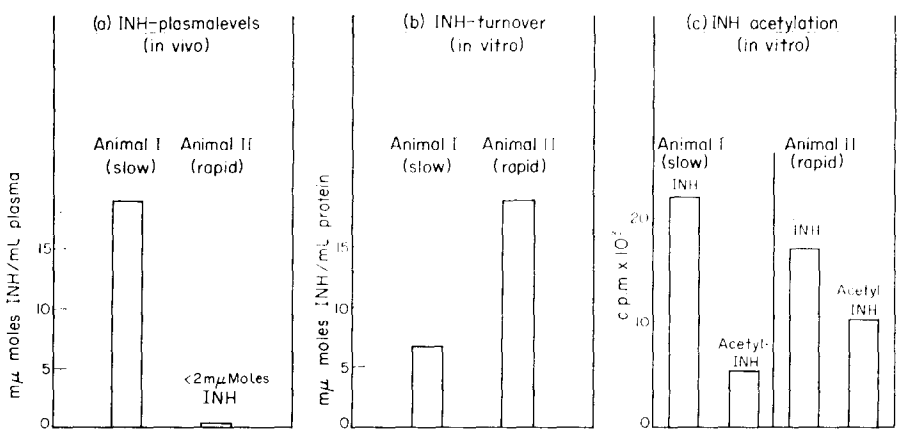

FIG. 3. Comparison of INH plasma levels *in vivo* with the enzymic INH turnover *in vitro* and the rates of INH-acetylation *in vitro* in two *C. aethiops* (Animal 1 = slow inactivator of INH, Animal 2 = rapid inactivator of INH). a, b and c see experimental 1, 2, 3.

The radiopaperchromatograms of Fig. 1 show experiments on the mechanism of enzymic INH-acetylation. For Rf-values of INH and acetyl-INH cf. a ; the controls b and c (without enzyme, with denatured enzyme resp.) show that no acetyl-INH is formed in comparison with the standard assay d. Evidence for the transfer of the acetyl group from acetyl-CoA to INH is given by the fact that the radioactivity from ^{14}C-acetyl-CoA appears only in the acetyl group of acetyl-INH (see e, Rf = 0,64); in this incubation mixture INH was not labeled. The Rf-values of acetyl-CoA in the solvent system used is 0,17. A confirmation of this experiment is given by incubation

of labeled acetyl-CoA plus labeled INH in the same assay (f); as expected a higher peak is found at the Rf-value of acetyl-INH. The formation of acetyl-INH is dependent on the concentrations of enzyme and of acetyl-CoA resp.

Studies on a likely polymorphism of the INH-acetylating enzyme are shown in Fig. 2. In 37 subjects (*C. aethiops*) the INH plasma levels were estimated under similar conditions. In seven of the tested animals the levels are significantly higher than in the others; therefore no unimodal distribution appears. This variability together with the following results suggest a polymorphism of the acetylating enzyme in *C. aethiops*: Fig. 3, part a shows different INH plasma levels in two *C. aethiops*. Incubation of liver enzyme preparations from animals with high INH plasma levels leads to a slow disappearance of INH; the reverse is true for incubations of liver enzyme preparations from animals with low INH plasma levels (cf. Fig. 3, part b). Furthermore the activity of INH-acetylating enzyme in animals with a low INH plasma level is higher than in those with a high plasma level: the rate of acetylation of INH was estimated by counting the labelled acetyl-INH on the chromatograms in a Packard liquid scintillation spectrometer (cf. Fig. 3, part c).

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REFERENCES

1. R. BÖNICKE and W. REIF, *Arch. exp. Path. Pharmak.*, **220**, 321 (1953).
2. R. S. MITCHELL, D. K. RIEMENSNIDER, J. R. HARSCH and J. C. BELL, in: Transactions of the conference of the Chemoth. of Tuberc., Vol. 17, p. 17, Washington D.C. Veterans Administration (1958).
3. MOTULSKY, A. G., in: *Progress in Med. Gen.* Vol. III, p. 49 (1964).
4. S. SUNAHARA, M. URANO and M. OGAWA, *Science* **134**, 1530 (1961).
5. D. A. P. EVANS, *Amer. J. Med.*, **34**, 639 (1963).
6. J. R. MAHER, J. M. WHITNEY, J. S. CHAMBERS and D. J. STANONIS, *Am. Rev. Tuberc.*, **76**, 852 (1957).
7. E. J. SIMON and D. SHEMIN, *J. Amer. chem. Soc.* **75**, 2520 (1953).
8. J. W. JENNE and P. D. BOYER, *Biochim. biophys. Acta*, **65**, 121 (1962).