Acetylation of Serotonin *in vitro* by a Human *N*-Acetyltransferase

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(Received 11 February 1969)

1. There is a well-recognized genetic polymorphism for the *N*-acetylation of isoniazid and sulphamethazine by human livers. 2. Serotonin was found to be acetylated by human liver enzyme preparations and the *N*-acetylserotonin formed was identified and determined quantitatively. 3. In 13 livers examined there was a wide variability in the capacity to acetylate serotonin that did not correlate with the capacity of the same livers to acetylate isoniazid and sulphamethazine. The results suggest that serotonin is not a natural substrate for the polymorphic *N*-acetyltransferase and that it may be acetylated by a different enzyme.

Oxidative deamination to 5-hydroxyindol-3-yl-acetic acid was the only known biotransformation of serotonin (5-hydroxytryptamine) until McIsaac & Page (1959) investigated the metabolism of the ^{14}C -labelled compound. They found in rats and rabbits that 50–98% of the administered radioactivity appeared in the urine within 24 hr. of serotonin administration as the following metabolites: 5-hydroxyindol-3-ylacetic acid, 5-hydroxyindol-3-ylaceturic acid, serotonin, *N*-acetylserotonin (*N*-acetyl-5-hydroxytryptamine) and serotonin glucuronide (5-hydroxytryptamine glucuronide). Quantitative determination of these metabolites obtained by scanning radiochromatograms revealed that 35–83% of the dose was metabolized by oxidative deamination and 5–25% by *N*-acetylation.

The supernatant fractions of livers from rats pretreated with iproniazid, a potent inhibitor of monoamine oxidase, were shown to acetylate serotonin in the presence of an acetyl-CoA-generating system by Weissbach, Redfield & Axelrod (1960). Further information on the enzymology of the *N*-acetyltransferase involved was published by Weissbach, Redfield & Axelrod (1961).

Goedde, Schloot & Valesky (1967) measured K_m and K_i values for various substrates and inhibitors of the *N*-acetyltransferase from Rhesus monkeys of 'rapid'-acetylator phenotype. The K_i for serotonin was $1.1 \times 10^{-4}\text{M}$. Goedde, Altland & Schloot (1968) also described the acetylation of serotonin by the monkey enzyme preparation with recognition of the product by radio-t.l.c.

A technique of injecting [^{14}C]serotonin into the portal-vein blood of perfused rat liver, with assay of metabolites in blood, bile and liver, was used by Tyce, Flock & Owen (1968). They found that

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conjugates of *N*-acetylserotonin accounted for 12% of the dose. On addition of β -phenylisopropylhydrazine (JB 516), a potent monoamine oxidase inhibitor, to the perfusate, the conjugates (glucuronide and sulphate) of serotonin and *N*-acetylserotonin increased and the 5-hydroxytryptophol and 5-hydroxyindol-3-ylacetic acid diminished in plasma and in bile.

A genetic polymorphism of *N*-acetyltransferase has been described in human liver by Evans & White (1964) and Jenne & Orser (1965). The substrates known at present are the drugs isoniazid, sulphamethazine and hydralazine. The object of the present study was to test whether serotonin is a naturally occurring substrate for this polymorphic enzyme.

MATERIALS

Human post-mortem livers were excised from cadavers within 3–5 hr. of death and immediately placed at 4°. Later they were transferred to a deep-freeze at –20°, where they were stored until required for experiment. All livers obtained in this way were from individuals who had died from extrahepatic diseases and were macroscopically normal.

CoA (trilithium salt) was obtained from Koch-Light Laboratories Ltd., Colnbrook, Bucks. Serotonin creatinine sulphate and *N*-acetylserotonin were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Serotonin [$3\text{-}^{14}\text{C}$]creatinine sulphate was obtained from The Radiochemical Centre, Amersham, Bucks.

Ilford Industrial G X-ray film was used for radio-t.l.c.

METHODS

Phenotyping of livers. Each liver used in these experiments was phenotyped by two different procedures: (1) by the spectral-shift assay procedure of Jenne & Orser (1965); (2) by the sulphamethazine acetylation procedure of Evans & White (1964).

The spectral-shift assay of isoniazid acetylation developed by Jenne & Orser (1965) depends on an increase in extinction as the acetylated compounds are formed. The assay was performed at 303 nm. at pH 9.0 and 25° in a Unicam SP.500 spectrophotometer. Sample and blank (acetyl-CoA omitted) contained 0.6 ml. of 0.01 M-isoniazid, 0.3 ml. of '(NH₄)₂SO₄ preparation' of enzyme (see below) and 75 mM-boric acid-15 mM-tris buffer, pH 8.6, to a total volume of 2.7 ml. The reaction was started by adding 0.3 ml. of 4.0 mM-acetyl-CoA solution (for details of preparation see below). The pH was adjusted to 9.0, the contents were thoroughly mixed and after equilibration for 3 min. extinction measurements were taken at 1 min. intervals for 20–30 min. depending on the activity. The reaction was linear for the first 0.2 *E*₃₀₃ unit increase, then it slowly levelled off owing to depletion of acetyl-CoA. Velocity of reaction has been previously found to be proportional to enzyme concentration (Jenne & Orser, 1965). Protein determination was performed on the '(NH₄)₂SO₄ preparation', a portion of which had been used for the above assay of enzymic activity.

The sulphamethazine phenotyping procedure depends on the acetylation of sulphamethazine by acetyl-CoA with a crude liver homogenate as the enzyme source. Approx. 300 mg. of human liver was homogenized in a small hand-operated glass homogenizer standing in crushed ice and containing the following: 1.0 ml. of 0.2 M-sodium phosphate buffer, pH 7.5, 0.5 ml. of 0.5 M-tris-HCl buffer, pH 7.5, 0.2 ml. of 1.0 M-KF, 0.2 ml. of 1.0 M-KCl, 0.2 ml. of 0.1 M-MgCl₂ and 0.8 ml. of aq. 0.02% sulphamethazine. After homogenization the mixture was centrifuged for 5 min. at 1000 *g* to remove gross cellular debris. Two 1.0 ml. portions of the supernatant were taken. To the first was added 0.3 ml. of acetyl-CoA (prepared as described below) and to the second was added 0.3 ml. of 0.2 M-sodium phosphate buffer, pH 7.5. Two 0.2 ml. portions of each incubation mixture were removed immediately after mixing and were added to a mixture of 2.8 ml. of water and 1 ml. of 25% (w/v) trichloroacetic acid. After incubation for 2 hr. at 37° two further 0.2 ml. portions were removed from each incubation mixture and added to trichloroacetic acid solution. The protein-trichloroacetic acid mixtures were filtered and the free sulphamethazine concentrations of the protein-free filtrates were determined by the method of Bratton & Marshall as given by Varley (1967). The remainder of the incubation mixtures were used for protein assay by the method of Lowry, Rosebrough, Farr & Randall (1951).

Preparation of partially purified enzyme. This followed the general procedure developed by Jenne & Orser (1965). A 20 g. portion of human post-mortem liver was homogenized with 80 ml. of deionized water in a Sunbeam blender for 90 sec. Then 75 ml. of the homogenate was centrifuged for 90 min. at 70500 *g*_{av.} in an MSE Superspeed 40 centrifuge. The supernatant from the centrifugation was brought to 50% saturation with (NH₄)₂SO₄ and kept for 30 min. at 4° with intermittent shaking. The mixture was centrifuged again for 15 min. at 19500 *g*_{av.} The sediment was resuspended in 2.5 ml. of 0.01 M-sodium phosphate buffer, pH 7.0, containing EDTA (final concn. 3.65 mM). This preparation, referred to as the '(NH₄)₂SO₄ preparation', was used without further purification in the subsequent procedures. All '(NH₄)₂SO₄ preparations' were used on the same day of preparation, as it had been established that there occurred a considerable diminution in activity with respect to serotonin acetylation when these '(NH₄)₂SO₄ preparations'

were stored for 24 hr. at -20°. Similar preparations showed no diminution of activity for the acetylation of isoniazid even after 3 days at -20°.

Preparation of acetyl-CoA. Acetyl-CoA was prepared by a modification of the method of Ochoa (1955), which was based on the procedure of Simon & Shemin (1953). A 19.4 mg. portion of CoA (trilithium salt) was dissolved in 2 ml. of ice-cold deionized water, 0.2 ml. of 1.0 M-KHCO₃ was added and then 0.5 ml. of freshly prepared ice-cold diluted acetic anhydride was added [the diluted acetic anhydride was prepared by diluting 0.94 ml. of A.R. grade (97% pure) acetic anhydride to 100 ml. with ice-cold deionized water]. After the addition of the acetic anhydride the mixture was kept at 0° for 30 min. At the end of this time 1.6 ml. of ice-cold 0.1 M-HCl was added, the pH was adjusted to 6.0 and the volume was adjusted to 5.0 ml. The acetyl-CoA thus prepared was kept for 2 hr. at 4° to allow hydrolysis of any residual acetic anhydride and was then stored at -20° until used. If the CoA is assumed to have been 85% pure then the concentration of the acetyl-CoA solution was 4.0 mM.

Enzymic acetylation of serotonin. The '(NH₄)₂SO₄ preparations' were shown to possess monoamine oxidase activity by the method of Weissbach, Smith, Daly, Witkop & Udenfriend (1960); it has previously been shown by Evans (1964) that iproniazid at a concentration of 1 mM will inhibit all monoamine oxidase activity in human liver homogenates. Iproniazid was therefore incorporated into the incubation mixture to block oxidative deamination of serotonin. The omission, individually, of serotonin, acetyl-CoA or '(NH₄)₂SO₄ preparation' resulted in failure to produce *N*-acetylserotonin (see Table 2 legend).

Fig. 1. Photograph of typical t.l.c. plate stained with 3% *p*-dimethylaminobenzaldehyde in conc. HCl. The five spots on the left are *N*-acetylserotonin standards; the spots on the right are derived from six unknowns. The identities of the spots in the unknowns other than *N*-acetylserotonin have not been established apart from that due to serotonin. The slight differences in the *R_F* between the authentic *N*-acetylserotonin and the *N*-acetylserotonin in the unknowns may be accounted for by the presence of electrolytes in the unknowns. The latter would compete for the binding sites on the support medium and give rise to a 'surfing effect'.

Assay of protein concentration. Protein concentrations were determined by the method of Lowry *et al.* (1951). The protein standard used was Lab-Trol (Dade Reagents Inc., Miami, Fla., U.S.A.). The total protein concentration of this standard as determined by the manufacturers by a macro-Kjeldahl technique is 6.95 g./100 ml. A range of

standard concentrations was set up each time an unknown was processed, and the protein concentration in the unknown was obtained by calculation by using the Beer's Law curve obtained from the standards.

Purification of N-acetylserotonin formed. The whole of the incubation mixture (6 ml.) was treated with 12 vol. of ethanol (72 ml.). The precipitated proteins, salts etc. were filtered off and the residue and the filter paper were washed with a further three 10 ml. portions of ethanol. The whole of the ethanolic extract was evaporated to dryness under a partial vacuum at 50° in a Büchi rotary evaporator. The residue was then extracted by three 20 ml. portions of A.R. diethyl ether. The extracts were pooled, filtered and extracted with three 10 ml. portions of water. The aqueous extracts were pooled, filtered and evaporated to dryness in a Büchi rotary evaporator *in vacuo* at 60°. The residue in the flask was dissolved in 1 ml. of ethanol and kept in the dark at -20° to await further analysis.

Thin-layer chromatography and measurement of N-acetylserotonin formed. Samples (50 µl.) of each unknown were spotted on to Merck precoated Kieselgel F₂₅₄ t.l.c. plates. An array of standards of 1, 2, 3, 4 and 5 µg. of N-acetylserotonin was included on each t.l.c. plate set up. The t.l.c. plates were developed at 4° in the dark in butan-1-ol-acetic acid-water (12:3:5, by vol.) for approx. 5 hr. Previous work had indicated that serotonin and its derivatives were somewhat labile, both to heat and to light, hence the low temperature and the darkness. After development the plates were dried in cold air in the dark at room temperature. The plates were next sprayed with 3% (v/v) *p*-dimethylaminobenzaldehyde in conc. HCl; N-acetylserotonin yields a bright-blue spot with this reagent. The plate was next

Table 1. *Colour reactions given by both presumed and authentic N-acetylserotonin*

Reagent	Colour produced in daylight	Interpretation
3% <i>p</i> -dimethylamino-benzaldehyde in conc. HCl	Bright blue	Indole derivatives substituted with -OR or -OH at position 5
1% 2,6-dichloro- <i>p</i> -benzoquinone-4-chloroimine in ethanol	Bright blue	Indole derivative
Sulphanilic acid reagent	Light brown	Phenolic compound, i.e. 5-hydroxy position not substituted
Ninhydrin-pyridine reagent	Bright yellow	Gives purple colour with serotonin; amino group on side chain substituted

Table 2. *Results from human post-mortem livers*

The table summarizes results for 13 human post-mortem livers. Column 2 indicates the acetylator phenotype (R, rapid; S, slow) derived from the data in columns 3 and 4. Column 5 gives the results obtained for the enzymic acetylation of serotonin. The primary mixture for the enzymic acetylation of serotonin consisted of: 3 ml. of '(NH₄)₂SO₄ preparation', 1 ml. of 7 mM-iproniazid in 0.1 M-sodium phosphate buffer, pH 7.5, 1 ml. of 4.83 mM-serotonin creatinine sulphate in 0.1 M-sodium phosphate buffer, pH 7.5, and 2 ml. of 0.1 M-sodium phosphate buffer, pH 7.5. A 5 ml. portion of this primary mixture was mixed with 1 ml. of acetyl-CoA solution and incubated for 1 hr. at 37°. The incubation mixture was stored at -20° to await extraction and analysis. The remainder of the primary mixture was stored at -20° for measurement of protein concentration. Both the spectral-shift assay of isoniazid acetylation and the acetylation of serotonin were carried out with fresh '(NH₄)₂SO₄ preparations', but not necessarily on the same day. The sulphamethazine phenotyping procedure was performed with a freshly prepared crude liver homogenate.

Human post-mortem liver no.	Phenotype	Isoniazid acetylation (ΔE ₃₀₃ /10 min./mg. of protein)	Sulphamethazine acetylation (µg./g. of protein)	N-Acetylserotonin production (µg./g. of protein)
1	R	1140	848	1228
2	R	497	876	1399
3	R	367	860	1091
4	S	25	63	831
5	S	42	Nil	826
6	S	132	101	< 100
7	S	36	33	1162
8	S	145	59	742
9	R	2640	1644	580
10	S	30	Nil	1072
11	S	81	84	< 100
12	R	340	530	651
13	R	2070	1288	144

dried with warm air and placed in a vacuum desiccator at 4° in the dark overnight for colour development. The following day the plate was bathed in aqua regia fumes, to intensify the colour of the *N*-acetylserotonin spot, and then scanned in a Chromoscan recording densitometer with matched red filters (maximum transmission at 620 nm.).

An approximate estimate of peak areas (in mm.²) was obtained by multiplying peak height by half the width of the peak base. When the peak area, *y*, determined in this way was plotted against weight of *N*-acetylserotonin, *x*, in 'standard' spots, straight lines were obtained. Weights of *N*-acetylserotonin in unknowns were calculated from the standard line set up at the same time (see Table 3).

Fig. 1 is a photograph of a typical t.l.c. plate, stained by *p*-dimethylaminobenzaldehyde.

Proof of identity of N-acetylserotonin formed. (1) It was found that serotonin reacted chemically with acetic anhydride and the resulting product had the same properties with regard to *R_F* on t.l.c., appearance under and reaction to u.v. light and colour production with spray reagents as the product of the enzymically catalysed reaction between acetyl-CoA and serotonin.

(2) When an equimolecular amount of *N*-acetylserotonin was placed in the incubation mixture instead of serotonin it was almost completely recovered, indicating that in this system *N*-acetylserotonin is the end product of metabolism.

(3) The presumed and authentic *N*-acetylserotonin both showed the same bright-purple fluorescence on t.l.c. plates when irradiated with u.v. light at 254 nm.; on re-examination after this in daylight both spots had become greyish brown, whereas they had initially been invisible in daylight.

(4) Both presumed and authentic *N*-acetylserotonin spots on t.l.c. plates gave the same colours with four spray reagents (Table 1).

(5) When ¹⁴C-labelled serotonin was incorporated into the incubation mixture described in the legend to Table 2, radioautography of a t.l.c. plate after two-dimensional development in butan-1-ol-acetic acid-water (12:3:5, by vol.) and then 20% (w/v) KCl yielded a radioactive spot at the same location as *N*-acetylserotonin. Similar radioactive spots coinciding with those obtained by staining were obtained by one-dimensional t.l.c. in each of the same two solvent systems.

RESULTS

Table 2 summarizes the results obtained from 13 human post-mortem livers. The acetylator phenotype derived from the results shown in columns 3 and 4 is indicated in column 2.

Where the result for serotonin acetylation is given as '<100' it means that insufficient *N*-acetylserotonin was formed to be determined quantitatively by the procedure described. However, in such instances the presence of a very small spot at the position of *N*-acetylserotonin on the t.l.c. plate could be detected by eye.

N-Acetylserotonin standard lines. The parameters of six *N*-acetylserotonin standard lines are presented in Table 3. These show the reliability of the procedure developed for determining quantitatively the *N*-acetylserotonin produced from the incubations.

DISCUSSION

From the series of livers examined there was no obvious correlation between acetylator phenotype and ability to acetylate serotonin. The results are interpreted as indicating that serotonin is not a substrate for the polymorphic *N*-acetyltransferase of human liver.

The authors thank The Medical Research Committee of the United Liverpool Hospitals (Chairman, Professor C. A. Clarke), The Nuffield Foundation (via Professor C. A. Clarke) for generous financial support, and Dr Olof Borge, Department of Pharmacology, Karolinska Institutet, Stockholm, for helpful suggestions during the preparation of the manuscript.

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Table 3. *Statistical parameters of six standard lines of N-acetylserotonin*

Peak areas (y mm.²) were obtained by multiplying densitometer peak height by half the width of the peak base and plotted against weight of *N*-acetylserotonin (x μg.).

Regression coefficient (b)	Intercept (a) (in units of y)	S.D. of points about regression line (Sr) (in units of y)	S.E.M. of determination (Sr/b) (μg.)
334.0	191.2	54.45	0.163
224.4	175.8	21.86	0.097
401.3	172.5	57.40	0.143
177.8	60.6	42.38	0.238
246.6	182.6	56.82	0.230
185.7	192.7	30.86	0.166

Means ± S.E.M. of parameters of six standard lines obtained during determination procedures

261.6 ± 36.1

162.6 ± 20.7

43.96 ± 6.10

0.173 ± 0.022

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