Acetyl Transfer in Arylamine Metabolism

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1. *N*-Hydroxyacetamidoaryl compounds (hydroxamic acids) are metabolites of arylamides, and an enzyme that transfers the acetyl group from these derivatives to arylamines has been found in rat tissues. The reaction products were identified by thin-layer chromatography and a spectrophotometric method, with 4-aminoazobenzene as acetyl acceptor, was used to measure enzyme activity. 2. The acetyltransferase was in the soluble fraction of rat liver, required a thiol for maximum activity and had a pH optimum between 6·0 and 7·5. 3. The soluble fractions of various rat tissues showed decreasing activity in the following order: liver, adrenal, kidney, lung, spleen, testis, heart; brain was inactive. 4. With the exception of aniline and aniline derivatives all the arylamines tested were effective as acetyl acceptors but aromatic compounds with side-chain amino groups were inactive. 5. The *N*-hydroxyacetamido derivatives of 2-naphthylamine, 4-aminobiphenyl and 2-aminofluorene were active acetyl donors but *N*-hydroxyacetanilide showed only slight activity. Acetyl-CoA was not a donor. 6. Some properties of the enzyme are compared with those of other acetyltransferases.

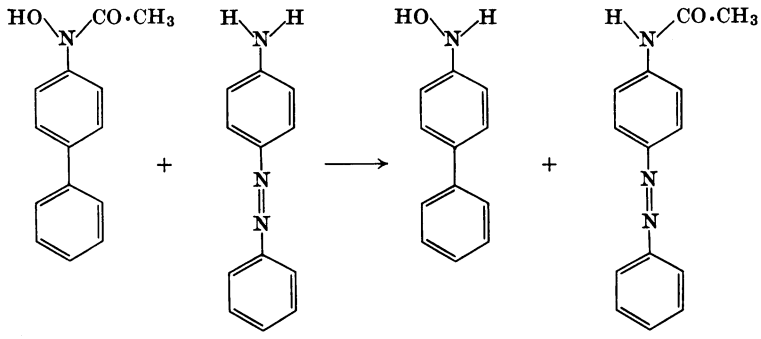
The characterization of metabolites of arylamines or arylamides in urine has shown that deacetylation and acetylation occur *in vivo*. In experiments *in vitro* the deacetylation of 2-acetamidofluorene by rat-liver microsomes (Seal & Gutmann, 1959), of 4-acetamidobiphenyl by rabbit-liver microsomes (Booth & Boyland, 1964) and of acetanilide and 2-acetamidonaphthalene by rat-liver homogenates (Weisburger, 1955) has been demonstrated. Acetanilide is deacetylated by extracts of liver or kidney from guinea pig, rat, mouse, horse, dog, cat and rabbit (Bray, James, Thorpe & Wasdell, 1950). Krisch (1963) has isolated an acetanilide deacetylase from the microsomes of hog liver. These reactions are presumably due to hydrolysis catalysed by aryl-acylamide amidohydrolase (EC 3.5.1.13). Most studies on the acetylation of arylamines have been concerned with an enzyme in pigeon liver that transfers acetyl from acetyl-CoA to a variety of arylamines (Chou & Lipmann, 1952; Tabor, Mehler & Stadtman, 1953) although Sekeris & Herrlich (1964) have reported that acetyl-CoA–arylamine *N*-acetyltransferase (EC 2.3.1.5) is also present in some mammalian tissues such as hog and ox liver. This enzyme also catalyses acetyl transfer between arylamines without acetyl-CoA (Bessman & Lipmann, 1953).

Since the report that 2-acetamidofluorene is converted into 2-(*N*-hydroxyacetamido)fluorene (Cramer, Miller & Miller, 1960) by the rat it has been established that other arylamides are also metabolized by many animals to hydroxamic acids. The present paper described an acetyltransferase in rat tissues that transfers acetyl from these metabolites to arylamines and has several properties that are different from those of EC 2.3.1.5.

Enzyme activity is measured by the rate of acetyl transfer from 4-(*N*-hydroxyacetamido)-biphenyl to 4-aminoazobenzene to produce 4-(hydroxyamino)biphenyl and 4-acetamidoazobenzene as shown in Scheme 1.

MATERIALS AND METHODS

Materials. NAD^+ and the sodium salts of NADH, $NADP^+$ and NADPH were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany; pyridoxyl 5-phosphate monohydrate was from California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A.; thiamine pyrophosphate was from Roche Products Ltd., Welwyn Garden City, Herts. CoA was purchased from Sigma Chemical Co., St Louis, Mo., U.S.A., and acetylated by the method of Stadtman (1957). Acetone-dried powders of rat and pigeon liver were purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A., and extracts prepared by the method of Jacobson (1961). L-Cysteine hydrochloride solutions were prepared and neutralized with NaOH immediately before use. *N*-Acetylbenzidine was prepared by the method of Bülow & Baur (1925). 4-Aminoazobenzene (for chromatographic standardization) was purchased from British Drug Houses Ltd., Poole, Dorset,



Scheme 1.

and 4-acetamidoazobenzene was prepared by the method of Schultz (1884).

Tissue preparations. Tissues from male adult rats of the Chester Beatty strain were homogenized in 4 vol. of 0·25 M-sucrose with a Potter & Elvehjem (1936) type homogenizer with a Teflon pestle. The homogenates were centrifuged in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd.) at 1480 $g_{av.}$ for 20 min. and the supernatant phases centrifuged at 105400 $g_{av.}$ in a Spinco model L preparative ultracentrifuge with rotor no. 40 for 1 hr. The supernatant phase is referred to as the soluble tissue fraction and could be stored at −15° for 1 month without loss of activity. All operations were carried out at approx. 8°. For the subcellular location of the enzyme, rat livers were homogenized as above in 9 vol. of 0·25 M-sucrose and fractionated by the method of Schneider & Hogeboom (1950), except that the microsomal fraction was separated from the soluble fraction in the Spinco model L preparative ultracentrifuge as described above.

Dialysis. Dialysis was against 800 vol. of 0·05 M-pyrophosphate buffer, pH 7·0, for 16 hr. at 8°.

Identification of reaction products by thin-layer chromatography. Standard incubation mixtures contained (final concns. given): pyrophosphate buffer, pH 7·0 (50 mM), cysteine (30 mM), soluble fraction from 90 mg. of tissue, and acetyl donor and acetyl acceptor, each dissolved in ethanol (0·1 ml.). The total volume was 2·5 ml. and incubations were in air for 15 min. at 37°. At the end of the incubation period the reaction mixtures were shaken with ethyl acetate (10 ml.) and centrifuged at 2000 g for 10 min. A sample of ethyl acetate (8 ml.) was removed and evaporated to dryness at 30°. The residue was dissolved in ethyl acetate (0·05 ml.) and applied to thin-layer chromatograms. Thin-layer chromatography, solvent systems and colour reactions were as described by Booth & Boyland (1964) except that the plates were spread with silica gel HF 254 + 366 (E. Merck A.-G., Darmstadt, Germany). To identify *N*-hydroxyarylamines it was essential to use short incubation times (15 min.) and to run the chromatograms immediately, owing to the instability of these compounds. 4-(Hydroxyamino)biphenyl appeared to be more stable than the other *N*-hydroxyarylamines.

Enzyme assay. The rate of transacetylation was measured with 4-aminoazobenzene as acceptor amine because this compound has a characteristic absorption maximum at 497 mμ in acid solution. The absorption maximum of the acetylated derivative is at 348 mμ, and at 497 mμ it has a molecular extinction coefficient that is 5·7% that of the free amine (Handschumacher, Mueller & Strong, 1951). Thus the rate of decrease in $E_{497\,m\mu}$ could be used as a direct measure of the rate of transacetylation. The method used was essentially as described by Handshumacher *et al.* (1951) for the assay of CoA. Convenient standard incubation mixtures, which did not require dilution, contained 4-(*N*-hydroxyacetamido)biphenyl (1 mM) as acetyl donor and 4-aminoazobenzene (0·1 mM) as acetyl acceptor added to the mixtures described for qualitative experiments. These incubation mixtures were used throughout except where stated otherwise. Incubations were in air for 15 min. at 37°. The reactions were stopped by addition to the incubation mixtures of an equal volume (2·5 ml.) of 20% (w/v) trichloroacetic acid in 50% (v/v) ethanol. The solutions were filtered and $E_{497\,m\mu}$ was read on a Unicam SP. 500 spectrophotometer. If the extinction was greater than 0·8 the solutions were suitably diluted with 10% (w/v) trichloroacetic acid in 25% (v/v) ethanol. In 10% (w/v) trichloroacetic acid in 50% (v/v) ethanol at 497 mμ the molecular extinction coefficient, $\epsilon_{max.}$, of 4-aminoazobenzene was 13200. Thus, with the standard reaction mixtures, acetylation of 0·1 μmole of 4-aminoazobenzene produced a decrease in $E^{1\,cm.}_{479\,m\mu}$ of 0·264. Recovery of various amounts (0·05–0·25 μmole) of 4-aminoazobenzene added to incubation mixtures at zero time was 101 ± 6% (S.D.) in 12 experiments. The activity is expressed as μmoles of 4-aminoazobenzene acetylated/g. of tissue/hr.

4-(*N*-Hydroxyacetamido)biphenyl was estimated as described by Booth & Boyland (1964).

RESULTS

Identification of the reaction products of transacetylation. The reaction products were identified by a comparison of their R_F values and colour reactions with those of authentic specimens. The chromatographic properties of some of these compounds have been reported by Booth & Boyland (1964), and those of others are shown in Table 1. The use of silica gel HF 254 + 366 made the compounds visible as dark spots on a yellow-fluorescent background when viewed under u.v. light.

Some examples of metabolites identified in transacetylation reactions are listed in Table 2. Expts. 1–3 demonstrated that when 4-(*N*-hydroxy-

acetamido)biphenyl was incubated with a suitable acceptor amine both the acetylated amine and the deacetylated donor were identified. These reaction mixtures also contained 4-aminobiphenyl, which had probably arisen by the reduction of 4-(hydroxyamino)biphenyl since it was also present when this compound was incubated alone under the same conditions (Expt. 4). Identification of 4-acetamido-4′-hydroxybiphenyl and 4-acetamido-3-hydroxybiphenyl after incubation of the amines with 2-(*N*-hydroxyacetamido)naphthalene as acetyl donor (Expts. 5 and 6) showed that transacetylation could also occur with other arylamines. Benzidine and 4-aminoazobenzene were used as

Table 1. *Thin-layer chromatography of some reaction products of transacetylation reactions*

Thin-layer chromatography was on glass plates spread with a 0·25 mm. layer of silica gel HF 245 + 366. Solvent systems: 1, light petroleum (b.p. 40–60°)–acetone (7:3, v/v); 2, chloroform–ethyl acetate–acetic acid (6:3:1, by vol.). Spray reagents: (*a*) *p*-dimethylaminocinnamaldehyde [1% (w/v) in 50% (v/v) ethanolic 3N-HCl]; (*b*) 2,6-dichloroquinonechloroimide [0·5% (w/v) in ethanol] followed by Na_2CO_3 (10%, w/v); (*c*) sodium aminoprusside (0·5%, w/v); (*d*) Folin & Ciocalteu's reagent (British Drug Houses Ltd.) followed by Na_2CO_3 (10%, w/v). The chromatographic properties of 4-acetamido-3-hydroxybiphenyl, 4-acetamido-4′-hydroxybiphenyl, 4-(hydroxyamino)biphenyl and 4-aminobiphenyl have been reported by Booth & Boyland (1964).

	R_F		Colours with spray reagents			
Compound	Solvent 1	Solvent 2	(*a*)	(*b*)	(*c*)	(*d*)
2-(Hydroxyamino)naphthalene	0·39	*	Red	None	Mauve	Grey
2-Naphthylamine	0·47	0·28	Red	Brown	None	Grey
4-Acetamidoazobenzene	0·34	0·43	Yellow†	Yellow†	Yellow†	Yellow†
N-Acetylbenzidine	0·07	0·07	Red	Brown	None	Grey

* Decomposed. † Before spraying.

Table 2. *Some examples of reaction products identified by thin-layer chromatography in transacetylation reactions*

Substrates were added to the standard incubation mixtures to give the following final concentrations: 4-(*N*-hydroxyacetamido)biphenyl (1 mM), 2-(*N*-hydroxyacetamido)naphthalene (1 mM), 4-acetamidoazobenzene (0·25 mM) and acetyl acceptors (0·1 mM). Incubations were in air for 15 min. at 37°. Ethyl acetate extracts of the incubation mixtures were examined by thin-layer chromatography, and the R_F values and colour reactions described in Table 1 and by Booth & Boyland (1964) were used to identify the reaction products.

Expt. no.	Acetyl donor	Acetyl acceptor	Reaction products identified
1	4-(*N*-Hydroxyacetamido)biphenyl	None	None
2	4-(*N*-Hydroxyacetamido)biphenyl	4-Amino-3-hydroxybiphenyl	4-Acetamido-3-hydroxybiphenyl, 4-(hydroxyamino)biphenyl, 4-aminobiphenyl
3	4-(*N*-Hydroxyacetamido)biphenyl	4-Amino-4′-hydroxybiphenyl	4-Acetamido-4′-hydroxybiphenyl, 4-(hydroxyamino)biphenyl, 4-aminobiphenyl
4	None	4-(Hydroxyamino)biphenyl	4-Aminobiphenyl
5	2-(*N*-Hydroxyacetamido)naphthalene	4-Amino-3-hydroxybiphenyl	4-Acetamido-3-hydroxybiphenyl, 2-(hydroxyamino)naphthalene, 2-naphthylamine
6	2-(*N*-Hydroxyacetamido)naphthalene	4-Amino-4′-hydroxybiphenyl	4-Acetamido-4′-hydroxybiphenyl, 2-(hydroxyamino)naphthalene, 2-naphthylamine
7	4-(*N*-Hydroxyacetamido)biphenyl	4-Aminoazobenzene	4-Acetamidoazobenzene, 4-(hydroxyamino)biphenyl, 4-aminobiphenyl
8	4-(*N*-Hydroxyacetamido)biphenyl	Benzidine	*N*-Acetylbenzidine, 4-(hydroxyamino)biphenyl, 4-aminobiphenyl
9	None	4-Aminoazobenzene	None
10	4-Acetamidoazobenzene	4-(Hydroxyamino)biphenyl	4-Aminobiphenyl

Table 3. *Conditions for maximum acetyltransferase activity*

Reaction mixtures contained (final concns.): pyrophosphate buffer, pH 7·0 (50 mM), soluble fraction from 90 mg. of liver, 4-(*N*-hydroxyacetamido)biphenyl (1 mM) and 4-aminoazobenzene (0·3 mM) in a total volume of 2·5 ml. Incubations were for 30 min. in air at 37°.

Expt. no.	Treatment	Activity (μmoles/g. of liver/hr.)
1	None	5·16
2	Dialysed	3·66
3	Dialysed and cysteine (30 mM) added	6·32
4	Cysteine (30 mM) added	7·20
5	Cysteine (60 mM) added	7·32
6	GSH (30 mM) added	7·16
7	Sodium thioglycollate (30 mM) added	5·66
8	Cysteine (30 mM) and EDTA (100 mM) added	7·32
9	Cysteine (30 mM) and NaF (100 mM) added	7·32
10	Cysteine (30 mM) and KF (100 mM) added	7·32
11	Cysteine (30 mM) added and soluble liver fraction boiled	0
12	Cysteine (30 mM) added and 4-(*N*-hydroxyacetamido)biphenyl omitted	0

acceptors (Expts. 7 and 8) to demonstrate acetyl transfer to unsubstituted arylamines because *N* - acetylbenzidine and 4 - acetamidoazobenzene could be readily detected as reaction products on chromatograms. The reaction used in quantitative experiments was the transfer of acetyl from 4-(*N*-hydroxyacetamido)biphenyl to 4-aminoazobenzene and no metabolites were identified when either of these compounds was incubated alone (Expts. 1 and 9). Products that would be expected if the reverse reaction had occurred were not seen after incubation of 4-acetamidoazobenzene and 4-(hydroxyamino)biphenyl (Expt. 10).

Conditions required for maximum rate of transacetylation. With 4-(*N*-hydroxyacetamido)biphenyl as acetyl donor and 4-aminoazobenzene as acceptor amine, preliminary experiments at various pH values with tris–hydrochloric acid, sodium phosphate or sodium pyrophosphate (all at final concn. 50 mM) buffer solutions indicated that the highest rate of transacetylation was achieved in pyrophosphate at pH 7·0, and this buffer was used throughout. Table 3 shows that dialysis of the soluble liver fraction against this buffer caused a loss of some activity (Expt. 2), which could be more than restored by the addition of cysteine (30 mM) (Expt. 3), and that (Expts. 4 and 5) when added to undialysed soluble fraction this concentration was sufficient for maximum activity. GSH produced the same amount of activation as cysteine (Expt. 6) but sodium thioglycollate had little effect (Expt. 7). Addition of EDTA (0·1 M), which stimulated the acetyltransferase of pigeon liver (Tabor *et al.* 1953), and sodium fluoride or potassium fluoride (0·1 M), which inhibit the deacetylation of 2-acetamidofluorene (Seal & Gutmann, 1959), had no effect (Expts. 8–10). Expts. 11 and 12 showed that there was no metabolism of 4-aminoazobenzene when the soluble liver fraction was boiled before incubation, or when the acetyl donor was omitted. The recovery of 4-(*N*-hydroxyacetamido)biphenyl, however, in the absence of acceptor amine was 84% after 15 min. incubation and 75% after 30 min. (see the Discussion section). The following cofactors at final concn. 0·2 mM had no effect on the rate of transacetylation: NAD^+, NADH, $NADP^+$, NADPH, pyridoxyl 5-phosphate and thiamine pyrophosphate.

The pH–activity curve (Fig. 1) showed that maximum activity is maintained between pH 6·0 and 7·5 but decreases on either side of these values.

The initial reaction velocity was linear with enzyme concentration up to soluble fraction from 90 mg. of liver and with incubation time up to 15 min. with 0·1 mM-4-aminoazobenzene or 30 min. with 0·3 mM-4-aminoazobenzene.

A determination of K_m values was not attempted since a small loss (16% after 15 min. incubation) of 4-(*N*-hydroxyacetamido)biphenyl in the absence of acceptor occurs and it was not possible to saturate the enzyme completely because of the limited solubility of the substrates. However, Fig. 2 indicates that the acetyl acceptor (4-aminoazobenzene) has a considerably higher affinity for the enzyme than the acetyl donor [4-(*N*-hydroxyacetamido)biphenyl].

Irreversibility of the reaction. The ultraviolet spectra of reaction mixtures after acidification with the trichloroacetic acid reagent (Fig. 3) show that transacetylation proceeds in one direction only. Under the conditions shown in Fig. 3 the 4-aminoazobenzene was completely converted into 4-acetamidoazobenzene after incubation for 1 hr. with 4-(*N*-hydroxyacetamido)biphenyl and soluble liver

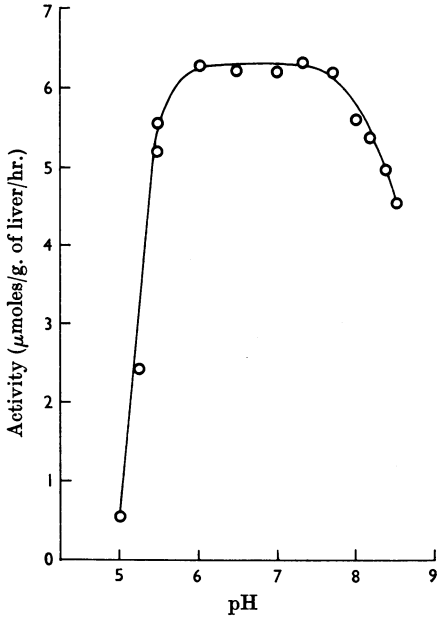


Fig. 1. pH–activity curve for acetyltransferase. Standard incubation mixtures contained pyrophosphate buffer (final concn. 50mM), the final pH of the incubation mixtures being shown.

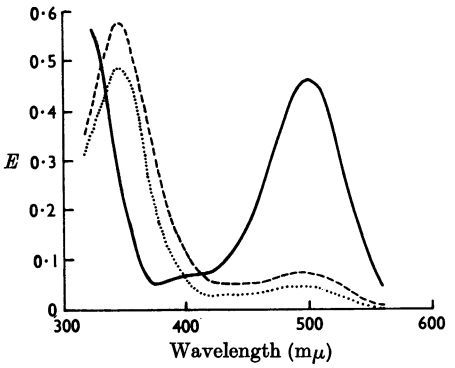


Fig. 3. Ultraviolet spectra of acetyltransferase reaction mixtures after the addition of trichloroacetic acid reagent. Reaction mixtures contained (final concns.) pyrophosphate buffer, pH7·0 (50mM), cysteine (30mM), and acetyl donor 4-(*N*-hydroxyacetamido)biphenyl (0·25mM) and acetyl acceptor 4-aminoazobenzene (0·07mM) incubated with boiled soluble liver fraction from 90mg. of liver (——) or with fresh soluble liver fraction from 90mg. of liver (----), or acetyl donor 4-acetamidoazobenezne (0·25mM) and acetyl acceptor 4-(hydroxyamino)biphenyl (0·07mM) incubated with fresh soluble liver fraction from 90mg. of liver (·····) (diluted fourfold with trichloroacetic acid reagent after the incubation). Incubations were in air for 1hr. at 37°.

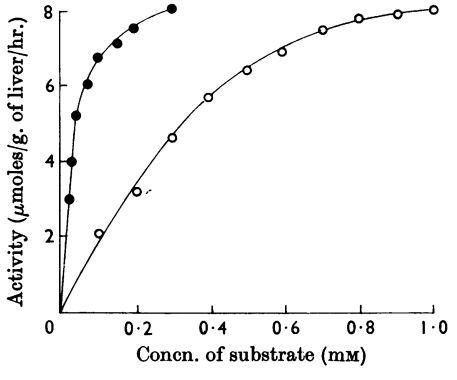


Fig. 2. Effect of substrate concentration on acetyltransferase. Standard incubation mixtures contained: ○, 4-aminoazobenzene (0·3mM) and various 4-(*N*-hydroxyacetamido)biphenyl concentrations; ●, 4-(*N*-hydroxyacetamido)biphenyl (1mM) and various 4-aminoazobenzene concentrations. Incubations were in air at 37° for various times (5–15min.).

Table 4. *Subcellular distribution of acetyltransferase*

Subcellular fractions were prepared from a 1:10 homogenate of six male adult rat livers in 0·25M-sucrose and samples equivalent to 90mg. of liver were incubated in the standard reaction mixtures.

Subcellular fraction	Activity (μmoles/g. of liver/hr.)
Whole homogenate	6·75
Nuclei	0·34
Mitochondria	0
Microsomes	0
Soluble fraction	7·25

fraction, although no reaction took place if the soluble liver fraction was boiled before the incubation. On the other hand, 4-acetamidoazobenzene was not deacetylated when incubated with 4-(hydroxyamino)biphenyl under the same conditions.

Distribution of acetyltransferase. Estimations of activity in the intracellular fractions of a rat-liver homogenate in 0·25M-sucrose showed that all the activity was present in the soluble fraction of the liver (Table 4). The activities of the soluble fractions of several rat tissues are listed in Table 5, which shows that although liver is the most efficient tissue tested there is considerable activity in adrenal, kidney and lung, some activity in spleen, heart and testis but none in brain.

Table 5. *Distribution of acetyltransferase in the soluble fractions of rat tissues*

Samples of the soluble fractions corresponding to 90mg. of the various tissues were incubated in the standard incubation mixtures. The result from adrenals is from one experiment with pooled adrenals from 26 rats. All other results are the mean values (±S.D.) of three experiments, each with the pooled tissues from five rats.

Tissue	Activity (μmoles/g. of tissue/hr.)
Liver	6·82±0·47
Adrenal	2·85
Kidney	2·09±0·23
Lung	1·92±0·13
Spleen	1·72±0·36
Testis	1·24±0·22
Heart	0·89±0·17
Brain	0

Specificity. (*a*) Acetyl acceptor. Qualitative experiments with thin-layer chromatography demonstrated that several arylamines or their ring-hydroxylated metabolites could serve as acetyl acceptors (Table 6). The addition of these compounds to incubation mixtures showed that they inhibited the rate of acetyl transfer from 4-(*N*-hydroxyacetamido)biphenyl to 4-aminoazobenzene. The degree of inhibition was dependent on the concentration of 4-aminoazobenzene, indicating a competitive type of inhibition, e.g. addition of 4-amino-3-hydroxybiphenyl (0·2mM) to incubation mixtures containing 0·03mM-, 0·06mM-, 0·1mM- or 0·2mM-4-aminoazobenzene caused 69, 62, 57 or 47% inhibition respectively. Therefore the degree of inhibition of acetylation of 4-aminoazobenzene caused by various compounds was used as an indication of their ability to serve as acetyl acceptors. The compounds tested and the percentage inhibition that they produce are listed in Table 6. Expts. 1–6 demonstrated that, except for aniline, all the arylamines tested can serve as acceptors, although 1-naphthylamine was not as efficient in this respect as 2-aminofluorene, benzidine, 2-naphthylamine or 4-aminobiphenyl. 4-Aminobiphenyl is metabolized by animals by hydroxylation in the 3- and 4′-positions and benzidine in the 3-position (Bradshaw & Clayson, 1955; Bradshaw, 1957), and Expts. 7–9 showed that this introduction of the hydroxyl group into the aromatic ring had little effect on the ability of the arylamine to serve as an acetyl acceptor. All the tested derivatives of aniline were like the parent compound since they caused no inhibition of 4-aminoazobenzene acetylation (Expts. 10–13). Some naturally occurring compounds with side-chain amino groups such as histamine, tryptamine and 5-hydroxytryptamine (Expts. 14–16), glucosamine (Expt. 17) and the hydrolysis products of yeast RNA (Expt. 18) were also ineffective.

Table 6. *Comparison of acetyl acceptors for the acetyltransferase reaction*

The ability of compounds to serve as acceptors is indicated by the percentage inhibition that they produce on the rate of acetyl transfer from 4-(*N*-hydroxyacetamido)biphenyl to 4-aminoazobenzene. Inhibitors (final concn. 0·2mM) were added to standard reaction mixtures and incubated in air for 15min. at 37°. Activity without inhibitor was 6·80 μmoles/g. of liver/hr.

Expt. no.	Inhibitor	Inhibition (%)
1	2-Aminofluorene	80
2	Benzidine	79
3	2-Naphthylamine	67
4	4-Aminobiphenyl	60
5	1-Naphthylamine	30
6	Aniline	2
7	3-Hydroxybenzidine	84
8	4-Amino-3-hydroxybiphenyl	58
9	4-Amino-4′-hydroxybiphenyl	57
10	2-Aminophenol	6
11	4-Nitroaniline	2
12	4-Aminobenzoic acid	2
13	Sulphanilamide	0
14	Histamine	0
15	Tryptamine	0
16	5-Hydroxytryptamine	0
17	Glucosamine	0
18	RNA hydrolysis products*	0

* Yeast RNA hydrolysed at 100° in N-HCl for 1hr., neutralized with NaOH and 0·25ml. containing 0·4mg. of hydrolysed RNA added to the incubation mixture.

(*b*) Acetyl donor. The activities with various donors are listed in Table 7. When concentrations less that 1mM were necessary, because of limited solubility of the donor, the activity with equivalent concentrations of 4-(*N*-hydroxyacetamido)biphenyl was also estimated. Table 7 shows that 4-(*N*-hydroxyacetamido)biphenyl was the most active of the hydroxamic acids tested although 2-(*N*-hydroxyacetamido)naphthalene and 2-(*N*-hydroxyacetamido)fluorene had only slightly lower activities (Expts. 1, 2, 6 and 7). On the other hand, activity with *N*-hydroxyacetanilide was considerably lower (Expt. 3). The possibility of an arylamide serving as acetyl donor was investigated with 4-acetamidobiphenyl (Expts. 8 and 9). In this case the activity was 20% of that produced by the same concentration of 4-(*N*-hydroxyacetamido)biphenyl.

Evidence that the present enzyme was different from the arylamine acetyltransferase (EC 2.3.1.5) in pigeon liver was obtained by comparing the donor

Table 7. *Comparison of acetyl donors for the acetyltransferase reaction*

Standard incubation mixtures contained 4-aminoazobenzene (0·1 mM) as acetyl acceptor and various donors at the concentrations indicated. When concentrations less than 1 mM were used, because of limited solubility, the activity with equivalent concentrations of 4-(*N*-hydroxyacetamido)biphenyl is included for comparison. Extracts of acetone-dried powders (A.P.) of rat and pigeon liver were prepared as described by Jacobson (1961). Incubations were in air for 15 min. at 37°.

Expt. no.	Liver preparation	Acetyl donor	Activity (μmoles/g. of liver/hr.)	Activity (μmoles/g. of A.P./hr.)
1	Rat soluble fraction	4-(*N*-Hydroxyacetamido)biphenyl (1 mM)	7·30	—
2	Rat soluble fraction	2-(*N*-Hydroxyacetamido)naphthalene (1 mM)	6·43	—
3	Rat soluble fraction	*N*-Hydroxyacetanilide (1 mM)	0·81	—
4	Rat soluble fraction	4-Nitroacetanilide (1 mM)	0	—
5	Rat soluble fraction	Acetyl-CoA (1 mM)	0	—
6	Rat soluble fraction	4-(*N*-Hydroxyacetamido)biphenyl (0·5 mM)	6·40	—
7	Rat soluble fraction	2-(*N*-Hydroxyacetamido)fluorene (0·5 mM)	5·18	—
8	Rat soluble fraction	4-(*N*-Hydroxyacetamido)biphenyl (0·25 mM)	4·01	—
9	Rat soluble fraction	4-Acetamidobiphenyl (0·25 mM)	0·80	—
10	Pigeon A.P.	4-(*N*-Hydroxyacetamido)biphenyl (1 mM)	—	7·74
11	Pigeon A.P.	4-Nitroacetanilide (1 mM)	—	7·74
12	Pigeon A.P.	Acetyl-CoA (1 mM)	—	263
13	Rat A.P.	4-(*N*-Hydroxyacetamido)biphenyl (1 mM)	—	5·50
14	Rat A.P.	4-Nitroacetanilide (1 mM)	—	0
15	Rat A.P.	Acetyl-CoA (1 mM)	—	0

specificity of the two enzymes by using liver acetone-dried powders extracted by the method of Jacobson (1961). With various aniline derivatives as acceptors the pigeon-liver enzyme can utilize 4-nitroacetanilide as acetyl donor but the reaction is very much faster with acetyl-CoA (Jacobson, 1961). Similar results were obtained with the pigeon-liver enzyme and 4-aminoazobenzene as acceptor (Table 7, Expts. 11 and 12) but neither rat-liver soluble fraction nor extracts of rat-liver acetone-dried powder showed any activity with these donors (Expts. 4, 5, 14 and 15). However, extracts of acetone-dried powders of rat and pigeon liver showed comparable activity with 4-(*N*-hydroxyacetamido)biphenyl as acetyl donor (Expts. 10 and 13).

DISCUSSION

N-Hydroxylated metabolites are more active carcinogens than the parent arylamides (Miller, Miller & Hartmann, 1961; Miller, Wyatt, Miller & Hartmann, 1961; Anderson, Enomoto, Miller & Miller, 1963; Boyland, Dukes & Grover, 1963; Gutmann, Galitski & Foley, 1966) and therefore there is considerable interest in their further metabolism. They are deacetylated by various liver preparations, e.g. 2-(*N*-hydroxyacetamido)-fluorene by rabbit-liver microsomes (Irving, 1964), by rat-liver soluble fraction (Grantham, Weisburger & Weisburger, 1965) and by guinea-pig- and hamster-liver homogenates (Lotlikar, Miller, Miller & Margreth, 1965) and 4-(*N*-hydroxyacetamido)-biphenyl by rabbit-liver microsomes (Booth & Boyland, 1964). These reactions are partly inhibited by fluoride and the products are probably formed by hydrolysis. The present paper shows that the acetyl groups can also be removed from hydroxamic acids by an acetyltransferase that uses arylamines as acceptors and is not effected by fluoride. In the reaction used in quantitative experiments, when the acetyl donor [4-(*N*-hydroxy-acetamido)biphenyl] was omitted from the incubation mixtures, all the acceptor (4-aminoazobenzene) could be recovered after 15 min. incubation. In the presence of donor, however, 4-aminoazobenzene concentration decreased as it was converted into 4-acetamidoazobenzene. Similarly, 4-(hydroxy-amino)biphenyl formed from 4-(*N*-hydroxyacet-amido)biphenyl was only identified when the acceptor was also present. However, it is possible that small undetected amounts were also formed by hydrolysis in the absence of acceptor since in these experiments only 84% of added 4-(*N*-hydroxy-acetamido)biphenyl was recovered after 15 min. incubation and the fate of the remainder was not established. By analogy with the metabolism of 2-(*N*-hydroxyacetamido)fluorene reduction, de-acetylation or deacetylation followed by oxidation to nitroso derivatives may have occurred (Irving, 1964; Grantham *et al.* 1965; Lotlikar *et al.* 1965).

Studies on substrate specificities indicated that the present enzyme is different from previously described acetyltransferases. An acetyl exchange

between arylamines without acetyl-CoA has been demonstrated with 4-acetamidoazobenzene-4-sulphonate or 4-nitroacetanilide as donor in the presence of pigeon-liver extracts and a variety of arylamines as acceptors. However, it was considered that this transfer was catalysed by the same enzyme that uses acetyl-CoA (Bessman & Lipmann, 1953), the reaction being much faster with this donor (Jacobson, 1961). Similar results were obtained in the present work with crude extracts of acetone-dried pigeon liver as the enzyme source, but similar preparations from rat liver were inactive with either acetyl-CoA or 4-nitroacetanilide as donor. Further, some of the arylamines that have been used as acceptors by the pigeon-liver enzyme, such as 4-aminobenzoic acid (Chou & Lipmann, 1952), 4-nitroaniline (Tabor *et al.* 1953) and aniline (Jacobson, 1961), were ineffective in the present system as judged by their inability to inhibit the acetylation of 4-aminoazobenzene. The present enzyme also differs in substrate specificity from the acetyltransferases that are in the soluble fraction of both rat liver (Weissbach, Redfield & Axelrod, 1961) and human liver (Jenne, 1965).

The transfer of acetyl from 4-(*N*-hydroxyacetamido)biphenyl to 4-aminoazobenzene proceeded to completion, and thin-layer chromatography and ultraviolet spectra of reaction mixtures failed to detect any evidence for the occurrence of the reverse reaction. This is in contrast with the finding by Bessman & Lipmann (1953) that the purified enzyme from pigeon liver catalysed the acetyl transfer between 4-acetamidoazobenzene-4-sulphonate and sulphanilamide in either direction towards the same equilibrium point. However, when studying acetyl transfer from 4-nitroacetanilide to aniline Jacobson (1961) could not detect the reverse reaction unless a purified enzyme preparation was used and found that reaction rates were greatly influenced by ring substituents in either substrate. Thus purification of the present acetyltransferase is required to confirm the difference in substrate specificity observed with crude liver soluble fractions.

Although it is established that *N*-hydroxylation increases the carcinogenic potency of arylamines the effect of *N*-acetylation is not clear. However, the dog, which is very susceptible to bladder tumour formation by certain arylamines such as 2-naphthylamine, excretes metabolites of this chemical in the unacetylated form (Boyland, Kinder & Manson, 1961), whereas the rabbit, which is resistant to 2-naphthylamine, excretes mainly acetylated derivatives (Boyland, Manson & Nery, 1963).

The acetyltransferase using the hydroxamic acid metabolites as acetyl donors may play an important role in the carcinogenic activity of arylamides by releasing the reactive arylhydroxylamines and hence nitroso derivatives. There is increasing evidence for the carcinogenicity of these derivatives (Miller, Cooke, Lotlikar & Miller, 1964; Miller, McKechnie, Poirier & Miller, 1965) and they react with many compounds of biological importance. For example, 2-(hydroxyamino)naphthalene reacts with phosphoric acid, sulphuric acid, L-cysteine, *N*-acetyl-L-cysteine and GSH (Boyland, Manson & Nery, 1962), 2-(hydroxyamino)fluorene with DNA (Kriek, 1965) and 2-nitrosofluorene with GSH (Lotlikar *et al.* 1965).

It is hoped that a study of enzyme distribution in tissues of susceptible and resistant species may indicate whether it is concerned in the carcinogenic activity of arylamines and arylamides, and determine to what extent acetylation and deacetylation reactions that occur *in vivo* are due to this enzyme.

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