A NOTE ON THE DEACYLATION OF THE CARCINOGEN 2-ACETAMIDOFLUORENE AND RELATED COMPOUNDS BY RAT LIVER AND INTESTINE*

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In the course of work on the metabolism of the carcinogen 2-acetamidofluorene (AAF) *in vitro* we have investigated the deacetylation of this compound with the aid of radioactive tracer methods. Although both rat liver slices and rat intestinal sections hydrolyzed the amide rapidly to 2-aminofluorene (AF), the intestine appeared to be particularly active. This finding was quite unexpected, since it has recently been reported[1] that deacetylation of AAF by intestinal homogenates is negligible. Intestinal sections also readily deacylated a number of other 2-acylaminofluorenes to AF and, in every instance, hydrolysis by intestine was more rapid than by liver. It has been suggested[2,3] that liberation of AF is required for carcinogenesis by AAF and related compounds. The remarkably high deacylase activity displayed by intestine may therefore play a significant role in the mechanism of action of these carcinogens when they are administered orally to the rat.

MATERIALS AND METHODS

Animals, tissue preparations and incubation media

Adult male rats of the Sprague-Dawley strain** were used in these investigations. The details concerning the preparation of the liver slices and the composition of the incubation media have been reported previously[4]. The small intestine was thoroughly rinsed with cold saline, cut into approximately 1 cm squares and the sections were suspended in the incubation medium. The wet weight of liver slices or intestinal sections was 1.0 g per flask and the total volume of incubation medium was 11.9 ml per flask. The labeled substrates were added to the medium in 0.10–0.15 ml of a 1:1 acetone: 95% ethanol solution, with the exception of N-2-fluorenyl-9-^{14}C-succinamic acid (SAF), which was added in 0.15 ml of 0.1 *M* phosphate buffer. The exact quantities of substrate per flask are listed in Table II. The mixtures were incubated for 4 hours at 37° with oxygen as the gas phase. Enzymically active extracts of the intestine were obtained by gently agitating the intestinal sections in cold incubation medium for ½ hour. After filtration, suitable aliquots of the filtrate were incubated with substrate under standard conditions. In other experiments, the intestinal extract was passed through a sterilizing Seitz filter (1 μ pore diameter) prior to incubation. In the experiments with intestinal extracts, samples of the incubation mixture were taken for bacteriological assay at the end of the experiments, but no bacterial growth was observed at any time.

Preparation of labeled and unlabeled compounds, and analytical methods

The following labeled substrates were prepared on the semi-micro scale by treatment of AF-9-^{14}C with the appropriate acylating agent: N-2-fluorenyl-9-^{14}C-acetamide (AAF-9-^{14}C), m.p. 196–198°[5,***]; N-2-fluorenyl-9-^{14}C-succinamic acid (SAF-9-^{14}C), m.p. 233°[6]; N-2-fluorenyl-9-^{14}C-benzamide (BAF-9-^{14}C), m.p. 219–221°[7]; N-2-fluorenyl-9-^{14}C-*p*-toluenesulfonamide (TSAF-9-^{14}C),

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** Obtained from the Holtzman Company, Madison, Wisconsin.

*** All melting points are corrected.

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m.p. 163–164°. N-2-Fluorenylbutyramide (BuAF), a new compound, was synthesized by acylation of 3.62 g (20 millimole) of AF in 100 ml of anhydrous benzene with 2.24 g (21 millimole) of *n*-butyryl chloride and 2.27 g (30 millimole) of pyridine. The mixture was heated under reflux for one hour, cooled and diluted with 50 ml of water. The precipitate was collected and recrystallized from benzene (2.14 g, m.p. 177–178°). Additional material was obtained by diluting the reaction mixture and benzene filtrate with ligroin. The compound was purified by passing a solution of the combined products in 1:1 chloroform:ethyl acetate through a 2 × 22 cm column of activated alumina. The purified product, 3.62 g, was recrystallized from ethyl acetate, m.p. 178–179°.

Anal. Calculated for $C_{17}H_{17}ON$: C, 81.2; H, 6.82; N, 5.57.

Found: C, 81.1; H, 6.75; N, 5.76.

BuAF-9-^{14}C was prepared in a similar manner on the semi-micro scale. AF, m.p. 128–129°, was synthesized by the standard procedure[9]. All labeled substrates were examined for contamination with AF-9-^{14}C by paper ionophoresis[10]. No radioactive peaks were detected in the region to which AF-9-^{14}C migrates. Control experiments showed that contamination in excess of 1% was readily detectable by this method.

The release of AF-9-^{14}C from the labeled 2-acylaminofluorenes by the action of the tissues was estimated by the inverse isotope dilution technique. This method proved invaluable in experiments with intestine, since in these cases the spectrophotometric measurement of AF is liable to appreciable error on account of turbidities caused by accompanying fat. Following incubation, the proteins were precipitated with acetone and separated by centrifugation as previously described[4]. Carrier AF was added to the protein-free mixtures and the acetone removed by vacuum distillation. The mixtures were then rendered alkaline and the carrier was isolated by ether or chloroform extraction, followed by evaporation of the solvent. The crude material was purified to constant specific radioactivity by one or more vacuum sublimations and by recrystallizations from appropriate solvents. Radiochemical purity was proved by conversion of the carrier to suitable derivatives with no change of the specific radioactivity. The data of Table I illustrate a typical purification procedure and the method of calculation of deacylation. For radioactivity measurements, suitable aliquots of solutions and appropriate samples of the isolated compounds were converted to barium carbonate by wet combustion and the radioactivities of the precipitates were measured as previously described[11]. In the experiments with aqueous extracts of intestine, fat was absent and the liberation of AF was determined spectrophotometrically by the modified R-salt test[9].

TABLE I

PURIFICATION OF ISOLATED AF-9-^{14}C AFTER INCUBATION OF BuAF-9-^{14}C* WITH INTESTINAL SECTIONS

Purification procedure	*Specific activity of isolated carrier***, *** c.p.m./mg × 10^{-4}*	*Calculated deacylation*§ %
Sublimation; 0.001 mm, < 90°	8.22	
Recrystallization; EtOH H_2O (1:2)	8.24	
Recrystallization; hexane	9.36	
Sublimation; 0.001 mm, < 90°	9.69	
Recrystallization; EtOH-H_2O (1:2)	9.61	
Derivative, BAF-9-^{14}C; recrystallization, benzene	9.53	72.3

* Conditions: Each of 4 incubation flasks contained 1.0 g of tissue, 11.9 ml of medium and 259 μg of BuAF-9-^{14}C ($4.5 \cdot 10^5$ c.p.m.). Temperature, 37°; gas phase, oxygen; incubation time 4 hours.

** Weight of carrier, 210 mg.

*** Specific activity is expressed in c.p.m./mg of labeled C_9.

§ Calculated deacylation = $\frac{\text{(c.p.m./mg of purified carrier) (weight of carrier) (100)}}{\text{total substrate radioactivity}}$.

RESULTS AND DISCUSSION

The relative rates of hydrolysis of several carbon-14-labeled 2-acylaminofluorenes are presented in Table II. Intestinal sections were uniformly more effective in cleaving these substrates than were liver slices. Thus, intestinal sections deacylated 72.3 and

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TABLE II

DEACYLATION OF CARBON-14-LABELED 2-ACYLAMINOFLUORENES BY INTESTINE AND LIVER *in vitro*

Substrate	*Per cent of substrate deacylated in 4 hours*		*Micromoles of substrate incubated/g of*	
	by intestine	*by liver*	*intestine*	*liver*
BuAF-9-^{14}C	72.3	58.7	1.03	1.03
AAF-9-^{14}C	38.1	23.0*	0.97	1.42*
SAF-9-^{14}C	30.9	0.25	1.06	1.01
BAF-9-^{14}C	14.0	2.1*	0.55**	0.63*
TSAF-9-^{14}C	0.67	0.68	0.76**	0.76**

* Data taken from previous publications[14,15].
** The insolubility of the compound prevented the use of larger amounts of substrate.

38.1% of BuAF-9-^{14}C and AAF-9-^{14}C, while liver slices hydrolyzed only 58.7 and 23.0% of BuAF-9-^{14}C and AAF-9-^{14}C respectively. Similarly, SAF-9-^{14}C and BAF-9-^{14}C were cleaved at appreciable rates by intestinal sections. In contrast, SAF-9-^{14}C proved to be resistant to hydrolysis by liver slices; likewise, the hydrolysis of BAF-9-^{14}C proceeded at only a very slow rate. The stability of TSAF-9-^{14}C toward deacylation by intestine *in vitro* is in accord with *in vivo* experiments in which 90% of orally administered T^{35}SAF were found unchanged in the feces[3].

In view of the conflicting evidence between the data of WEISBURGER[1] and the present results the question was raised whether the hydrolytic activity of intestinal strips could possibly be due to the action of intestinal bacteria rather than to the activity of an enzyme of the intestine proper. The experimental evidence makes this extremely unlikely, since rapid deacylation of AAF and BuAF was observed with aqueous extracts of intestine under sterile conditions (Table III). It appears to us that the small intestine contains a highly active deacylase which, like liver deacylase, acts on acylaminofluorenes, but which is less specific in its substrate requirements than is the liver enzyme.

TABLE III

LIBERATION OF AF BY EXTRACT* OF RAT INTESTINE

Substrate	*Amount incubated* μg	*AF formed*** μg	*Estimated deacylation* %
BuAF	254	130	71
AAF	226	30	16
BuAF (control)***	269	0§	0

* The volume of extract was 11.9 ml per flask.
** From the appearance of diazotizable groups as determined by the R-salt test.
*** The extract was boiled for 3 minutes, cooled and incubated with substrate.
§ The optical density was at the lower limit of sensitivity of the method.

The present experiments permit a fairly precise description of the fate of orally administered AAF. The carcinogen is in large part deacetylated to AF in the small intestine. The free base is rapidly absorbed and transported to the liver[13]. The metabolism of AF and AAF by rat liver has already been described[4,14,15].

It is of interest that a relation appears to exist between the liberation of AF from

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2-acylaminofluorenes in the intestine and the carcinogenicities of these compounds after oral feeding to the rat. Thus, the relative rates of formation of AF follow the same order as the relative carcinogenicities of 2-acylaminofluorenes (AAF > SAF > BAF > TSAF)[16,*]. These observations support the view that AF is a key compound in carcinogenesis by AAF and related derivatives and that the small intestine is the principal site for its release. The ready liberation of AF in the intestine would also explain the carcinogenicity of compounds, such as SAF, which are not attacked by liver deacylase.

SUMMARY

The deacylation of the carcinogen 2-acetamidofluorene and several related 2-acylaminofluorenes to 2-aminoflorene by rat liver slices, intestinal strips, and aqueous extracts of intestine, was studied. Intestinal strips deacylated all substrates at faster rates than did liver slices. Aqueous extracts of intestine likewise cleaved 2-acetamidofluorene at appreciable rates. Evidence is presented that the observed hydrolyses were due to a deacylase of the intestine and not to bacterial action. The relative rates of release of 2-aminofluorene by intestinal strips from various 2-acylaminofluorenes closely parallels the relative carcinogenicities of these compounds.

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QUANTITATIVE DETERMINATION OF 0.5–5 μg OF AMINO ACID NITROGEN ON PAPER CHROMATOGRAMS AND IN SOLUTION

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Numerous methods have been evolved for the quantitative determination of amino acids on paper chromatograms and in solution[1]. Most of them depend on measurements of the intensity of the blue colour produced on treatment with ninhydrin. In the application of this technique to paper chromatograms, the intensity of colour of the blue

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