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MODIFIED SYSTEM *IN VITRO* FOR THE METABOLISM OF 2-ACETYLAMINOFLUORENE

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Abstract—The development of a modified system *in vitro* for the metabolism of 2-acetylaminofluorene (2-AAF) is described. The system depends upon utilization of an NADPH-generating system, low amounts of microsomal protein and substrate, and utilization of *N*-2-hydroxyethylpiperazine-*N*-2 ethanesulfonic acid (HEPES) buffer. With these modifications, metabolism of 2-AAF *in vitro* was found to be linear with time and at 30 min formation of *N*-hydroxy-2-acetylaminofluorene (N-OH-2-AAF) represented 30 per cent or greater of the 2-AAF added originally. Differences in 2-AAF metabolism were determined in mice fed different diets. Using this system *in vitro*, mice on Purina chow metabolized, *in vitro*, 30 per cent more of the added 2-AAF to N-OH-2-AAF than mice receiving a synthetic diet. Data on the effects of protein concentration, perfusion and sources of NADPH are presented. The modified system is discussed also with respect to its differences from previous 2-AAF systems *in vitro*.

A NUMBER of investigators have studied the conversion *in vitro* of 2-AAF to *N*-hydroxy-2-AAF (N-OH-2-AAF) and to various ring-hydroxylated metabolites* by liver homogenates and by liver microsomal preparations.[1–5] In these studies, 2-AAF converted to *N*-hydroxy-2-AAF has varied from 0 per cent in the guinea pig to about 12 per cent in rabbits; in mice and rats, conversion rates were about 3 and 1 per cent respectively.

Primary attention has been given to *N*-hydroxy-2-AAF in most studies *in vitro*, since this metabolite has been postulated to be a proximate carcinogen. However, it has also been shown that the systems *in vitro* commonly employed are capable of converting 2-AAF to the 5- and 7-hydroxy metabolites; in the absence of added fluoride, 2-AAF is also converted to 2-AF and the *N*-hydroxy metabolite is deacetylated to form *N*-hydroxy-AF and other derivatives.[6]

The limited formation of *N*-hydroxy-2-AAF and of the various ring-hydroxylated metabolites of 2-AAF by systems *in vitro* presents difficulties if one wishes to study factors such as diet, age and chemical agents which might inhibit the metabolism of 2-AAF by liver microsomes. The present studies were undertaken to elucidate conditions required for increased metabolism *in vitro* of 2-AAF, with respect to percentage of the substrate metabolized, in a liver microsomal system. Preliminary work in our laboratory indicated that a system *in vitro* consisting of low amounts of substrate, when compared with other studies, an NADPH-generating system, low amounts of

* Abbreviations used are: 1-, 3-, 5- and 7-OH-2-AAF corresponding to 1-, 3-, 5- and 7-hydroxy-2-acetylaminofluorene; AF, 2-aminofluorene; N-OH-AF, *N*-hydroxy-2-aminofluorene.

protein, and HEPES buffer promoted efficient metabolism of 2-AAF. This paper describes the exact composition of the system *in vitro* and results obtained to establish the effect of perfusion, protein concentration and time of incubation. A comparative study, involving the effects of different diets on the metabolism of 2-AAF in male BALB/c mice, was undertaken after the development of the modified system *in vitro* to test its applicability to an experimental situation involving possible differences in microsomal metabolism of 2-AAF. These data are also discussed.

EXPERIMENTAL

Chemicals. 2-AAF-9-^{14}C, sp. act. 31·7 mCi/m-mole, was purchased from Mallinckrodt Radiochemicals. Authentic standards of N-OH-2-AAF, 1-OH-2-AAF, 3-OH-2-AAF, 5-OH-2-AAF, 7-OH-2-AAF and 2-AF were supplied through the courtesy of Dr. C. C. Irving, Veterans Hospital, Memphis, Tenn. Glucose 6-phosphate, NADPH, NADP, and Normal Protein Test Diet for Rat and Mouse were from Nutritional Biochemicals Co.; nicotinamide was purchased from Aldrich Chemical Co.; HEPES (*N*-2-hydroxyethylpiperazine-*N*-2 ethanesulfonic acid) and D-glucose 6-phosphate dehydrogenase from the Sigma Chemical Co.; precoated Silica gel and alumina TLC plates from Brinkman Instruments; and PCS scintillation fluid from Amersham/Searle Co.

Animals. Male BALB/c mice, 4–6 weeks of age, were obtained from the animal production facilities of the National Center for Toxicological Research.

Microsomal preparation. All procedures prior to incubation of the final microsomal preparations were carried out at 3–5° with materials, solutions and equipment precooled to 5° prior to the time of sacrifice. Animals were sacrificed by cervical dislocation and the abdominal cavity was quickly opened as the heart continued to beat. Livers were removed, after perfusion *in situ* with chilled HEPES buffer (0·1 M, pH 7·4, containing 2×10^{-3} M $CaCl_2$), weighed and homogenized in 3 vol. of HEPES buffer with a Potter–Elvehjem type tissue grinder fitter with a Teflon pestle. The homogenates were centrifuged at 10,000 *g* for 30 min. The middle layer of the supernatant was recentrifuged at 105,000 *g* for 60 min and the supernatant was drained from the microsomal pellet which was then resuspended in a volume of HEPES buffer equal to the original volume of the 10,000 *g* supernatant. After resuspension, the protein content of each microsomal suspension was determined by the method of Lowry *et al.*[7]

Incubation procedures and identification of metabolites. Various amounts of the microsomal suspensions (as determined by experimental design) were added to incubation vials containing one or both of the following systems *in vitro*: (1) system I: D-glucose 6-phosphate, 0·1 ml (29 mg disodium salt/ml of HEPES buffer); NADP 0·1 ml (10 mg disodium salt/ml of HEPES buffer); D-glucose 6-phosphate dehydrogenase, 0·1 ml (1000 units/30 ml of HEPES buffer); and HEPES, 2·6 ml, 0·1 M, pH 7·4, containing 2×10^{-3} M $CaCl_2$; 2-AAF-9-^{14}C, 0·1 ml (0·66 mg/100 ml of distilled water), and (2) system II: nicotinamide, 0·1 ml (2·9 g/10 ml of HEPES); NADPH, 0·1 ml (0·3 g/5 ml of HEPES); D-glucose 6-phosphate, 0·1 ml (2 g disodium salt/4 ml of HEPES); and 2-AAF-9-^{14}C, 0·1 ml of solution (as in system I); HEPES (as in system I).

All incubations were carried out for 30 min at 37° in a Dubnoff metabolic shaking

bath unless otherwise specified. The reactions were stopped by placing the incubation vials in a boiling water bath for 5 min.

AAF and metabolites thereof were extracted three times from the incubation vials with 3-ml portions of ethyl ether; the three extracts were pooled. Recovery of radioactivity averaged 90 per cent (range, 85-100 per cent).

The ether extracts were analyzed for hydroxylated metabolites by thin-layer chromatography according to the method of Irving[3] utilizing the solvents 97% chloroform–3% methanol (v/v). Quantitation of the parent compound and its respective metabolites was accomplished by scraping spots from the TLC plate and counting in a liquid scintillation counter (Packard model 3380). R_f values for 2-AAF and metabolites thereof in this system based on TLC of authentic standards were found to be: 2-AF, 0·55; 2-AAF, 0·33; 1-OH-2-AAF, 0·27; 3-OH-2-AAF, 0·21; 5-OH-2-AAF and 7-OH-2-AAF, 0·10; and N-OH-2-AAF, 0·03. The 1- and 3-OH metabolites were never found present in extracts of the incubation mixtures and are not listed in the results. Since the 1-OH-2-AAF chromatographs to a point near to 2-AAF in this system, confirmation of the absence of 1-OH-2-AAF and 3-OH-2-AAF was accomplished in a second TLC system utilizing alumina plates and the solvents chloroform–cyclohexane–*t*-butanol–acetic acid–water (30:50:20:5:5, v/v). R_f values of authentic standards in this TLC system are: 2-AF, 0·90; 2-AAF, 0·75; 1-OH-2-AAF, 0·65; 3-OH-2-AAF, 0·62; 5-OH-2-AAF, 0·51; 7-OH-2-AAF, 0·47; and N-OH-2-AAF, 0·28. Analyses of incubation samples *in vitro* prepared from liver tissue of mice did not reveal the presence of 1-OH-2-AAF or of 3-OH-2-AAF when the latter TLC system was employed.

In both the Silica gel TLC system and in the alumina TLC system, the low R_f values for N-OH-2-AAF are obtained with solutions which contain at least 1000 ng N-OH-2-AAF, an amount which was never exceeded in the analyses of 2-AAF metabolism. Although the precise amount of N-OH-2-AAF which would result in migration of the metabolite up the plate was not established, it was noted that spotting of N-OH-2-AAF in amounts which were visible under u.v. light led to migration of the metabolite to an area corresponding to 2-AAF in both TLC systems. During routine analyses, labeled N-OH-2-AAF standard in an amount not exceeding 1000 ng was utilized to monitor the migration of this metabolite on the TLC plates. Additional confirmation that the material which remained near the origin was N-OH-2-AAF in incubation mixtures from mice was obtained by liquid–liquid chromatography according to the method of Gutmann.[8] The peak corresponding to N-OH-2-AAF was collected during liquid–liquid chromatography of a sample *in vitro* and counted in a liquid scintillation counter. The amount of N-OH-2-AAF determined in this manner was 90 per cent of the amount of N-OH-2-AAF determined by TLC on Silica gel plates. Complete details of the liquid chromatographic procedure will appear elsewhere.[8]

RESULTS

Added NADPH versus an NADPH-generating system. Three experimental groups were prepared to resolve the question of whether an NADPH-generating system supported the metabolism of 2-AAF *in vitro* to a greater extent than by simply adding exogenous NADPH. Group I contained an NADPH-generating system; group II

contained NADPH; and group III contained both NADPH and an NADPH-generating system.

Addition of an NADPH-generating system provided a more efficient environment for the metabolism of 2-AAF by liver microsomes of mice than did addition of NADPH alone. In group I, the formation of N-OH-2-AAF and of the 5-OH plus the 7-OH metabolites was 84 ± 10 and 50 ± 9 ng/mg of protein/30 min respectively. Corresponding values for groups II and III were: N-OH-2-AAF, 51 ± 11 and 63 ± 15 ng/mg of protein/30 min; and 5-OH-2-AAF plus 7-OH-2-AAF, 24 ± 3 and 30 ± 11 ng/mg of protein/30 min. These results were obtained with a total of 4·5 mg protein/each incubation mixture. Incubation systems I and II were compared subsequently at a lower protein concentration (0·5 mg protein/3 ml) and again the NADPH-generating system was found to be superior based on the formation of larger amounts of N-OH-2-AAF and of the 5- and 7- hydroxy metabolites. Since the ratio of N-OH-2-AAF to the 5- and 7-OH metabolites was similar in the three groups, it appears that the generating system simply increased over-all metabolism of 2-AAF without altering the relative ratios of metabolites.

Metabolism in perfused vs non-perfused liver. The metabolism of 2-AAF by liver microsomes isolated from perfused liver and by microsomes isolated from non-perfused liver was compared utilizing the NADPH-generating system *in vitro* (system I). Examination of the data (not shown) for conversion of 2-AAF to its respective metabolites revealed no differences in metabolism between the two groups.

Effect of microsomal protein content on the metabolism of 2-AAF in vitro. Microsomal pellets were prepared as described (Materials and Methods), pooled and divided among three experimental groups each consisting of three samples. One group contained a total of 1·0 mg microsomal protein; a second group contained 0·5 mg microsomal protein; and a third group contained 0·1 mg microsomal protein. All incubation mixtures contained the NADPH-generating system.

With the above three levels of microsomal protein, the quantities of 2-AF, N-OH-2-AAF, and 7- and 5-hydroxy-2-AAF formed (ng/mg protein/30 min) were: 65 ± 8, 189 ± 22 and 282 ± 33, respectively (1·0 mg protein); 81 ± 8, 167 ± 22, and 263 ± 22, respectively (0·5 mg protein); and 119 ± 41, 215 ± 0 and 215 ± 0, respectively (0·1 mg protein).

No statistically significant differences ($P = 0·05$) were found in either the over-all metabolism of 2-AAF or in the formation of individual metabolites between the 1·0 mg protein level and the 0·5 mg protein level. Formation of metabolites by the 0·1 mg protein level was significantly different ($P < 0·05$) from the 0·5 mg level with respect to the N-OH metabolite and the 5- and 7-OH metabolites; formation of AF at the 0·1 mg level was significantly different ($P < 0·05$) from the 1·0 mg level.

In group I (1·0 mg protein), the conversion rate of 2-AAF was 543 ng/mg of protein/30 min based on substrate disappearance which represents 76 per cent of the 2-AAF added originally. Since this approaches maximum conversion, 1·0 mg protein/incubation mixture would appear to be undesirable in studies involving stimulation of hydroxylase activity. Conversely, 0·1 mg protein/incubation resulted in an over-all conversion rate of 55 ng/0·1 mg of protein/30 min or about 8 per cent of the 2-AAF added originally. This level of conversion makes it difficult to quantitate accurately the amounts of the various metabolites formed and may explain the statistical differences found between the 0·1 mg protein level and the 0·5 or the 1·0 mg

protein level. At the 0·5 mg level, the conversion rate of 2-AAF was 512 ng/mg of protein/30 min or 256 ng/0·5 mg of protein/30 min which represents about 39 per cent of the 2-AAF added originally. The 0·5 mg protein level, therefore, appears to be most appropriate for studies involving either inhibition or stimulation of microsomal hydroxylase activity if the conditions discussed previously for the remainder of the system *in vitro* are maintained. However, 1·0 mg microsomal protein would also appear to be suitable in mouse studies involving inhibition of microsomal hydroxylase activity.

Effect of incubation time on metabolism of 2-AAF. The optimal incubation time was determined for the modified system *in vitro* in the following manner. Incubation mixtures were prepared as described in the previous section, utilizing the 0·5 mg microsomal protein level. Three samples were incubated at each of five time periods, 10, 15, 20, 30 and 45 min. Results are summarized in Fig. 1.

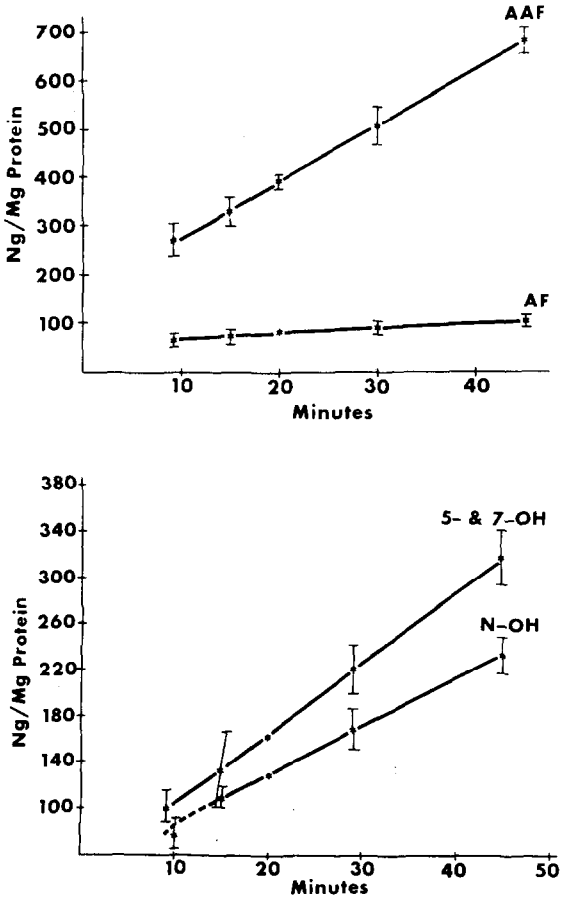

FIG. 1. Effect of incubation time on the metabolism of 2-AAF *in vitro*. The system *in vitro* was system 1 (Table 1); 0·5 mg microsomal protein. Mice were male BALB/c, 4 to 6-weeks-old. Total ng 2-AAF metabolized, total ng formed of each metabolite. Four determinations using the same microsomal solution for each of the five points.

With 0·5 mg microsomal protein, metabolism of 2-AAF and of 2-AF was linear with time (Fig.1A). A linear response was observed in formation of the *N*-hydroxy and the 5- and 7-hydroxy metabolites as well, although formation of the *N*-hydroxy metabolite did not appear to become linear until the 15-min incubation period (Fig. 1B). At the 30-min end-point, the amount of 2-AAF metabolized (α 500 ng/mg of protein) represented 38 per cent of the amount added originally (0·5 mg protein × 500 ng/mg of protein ÷ 600 ng 2-AAF). Formation of *N*-hydroxy and of the 5- and 7-hydroxy metabolites was 12 and 15 per cent respectively, of the 2-AAF added originally. Based on these results and those presented previously on the comparison of microsomal protein levels, a decision was made to utilize the 30-min incubation period and two levels of microsomal protein (0·5 mg and 1·0 mg) in future experiments. At 0·5 mg protein and a 30-min incubation period, determinations of inhibited microsomal activity appear feasible. Other combinations of incubation time and levels of microsomal protein may, of course, be useful, but the combinations decided upon were believed to be reasonable and practical for studies of 2-AAF metabolism *in vitro*.

Effect of diet on metabolism of 2-AAF by liver microsomes. The following study was carried out to evaluate the usefulness of the modified system *in vitro*, with respect to its application to an investigation of comparative metabolism.

Male BALB/c mice, 4 to 6-weeks-old, were divided into two groups and placed into metabolism cages, three animals/cage. One group consisting of a total of six animals was maintained on Purina laboratory chow for 2 months; the second group of six animals was maintained on a synthetic diet during the same period. At the end of 2 months, five animals from each group were sacrificed and the metabolism of 2-AAF *in vitro* was compared between the two groups utilizing system I.

TABLE 1. EFFECT OF DIET ON THE METABOLISM OF 2-ACETYLAMINOFLUORENE BY LIVER MICROSOMES OF MALE BALB/C MICE*

Group†	Total microsomal protein (mg)	AAF and metabolites (ng/mg protein/30 min)‡			
		AAF	AF	N-OH	5-7-OH
Synthetic	0·5	201 ± 32	57 ± 10	90 ± 19	56 ± 14
	1·0	197 ± 35	37 ± 4	84 ± 16	73 ± 29
Purina	0·5	274 ± 43	36 ± 4	129 ± 16	120 ± 28
	1·0	275 ± 32	28 ± 4	120 ± 26	120 ± 15

* The system *in vitro* was system I (see Materials and Methods). Five animals for each protein concentration. The *t*-test shows a difference between synthetic and Purina groups in formation of all metabolites. There was no difference in metabolism between the 0·5 and 1·0 mg protein level within each group ($P = 0·05$).

† Synthetic, Normal Protein Test Diet for Rat and Mouse; Purina, Purina Mouse Chow (50-10-C). Animals were obtained at 4–6 weeks of age and maintained in metabolism cages on the respective diets for 2 months.

‡ Total ng AAF metabolized; ng formed for all others.

Table 1 shows that a significant difference in the formation of all metabolites was found between the two treatment groups ($P \leq 0·05$). At the 0·5 mg protein level, for example, the amount of N-OH-2-AAF formed by the synthetic diet group was 69 per cent of the amount formed by the Purina-fed animals. Identical results were

obtained at the 1·0 mg protein level. Formation of the 5- and 7-OH metabolites by synthetic diet-fed animals was 53 per cent of the amount formed by the Purina group (average of both protein levels). It is apparent from these data that the modified system *in vitro* can be utilized to document differences in 2-AAF metabolism between groups of animals treated in different ways.

DISCUSSION

The results reported herein represent an attempt to refine the techniques utilized in an over-all approach to studies on the metabolism of 2-AAF and in which *N*-hydroxylase activity represents a major step. A modified system *in vitro* is described which is capable of efficiently metabolizing 2-AAF to *N*-hydroxy-2-AAF and to other metabolites, and one which is applicable to comparative studies of the induction or inhibition of the microsomal system. Mice on different diets, for example, were shown to metabolize 2-AAF at different rates; mice on synthetic diets formed 30 per cent less N-OH-2-AAF than those maintained on Purina chow. Presumably, induction of 2-AAF metabolism could be studied with the modified system, if one does not exceed a concentration of 0·5 mg microsomal protein/3 ml (0·16 mg/ml). At higher microsomal protein concentrations, marked microsomal induction would lead to depletion of substrate.

In previous studies,[1–5] it has been customary to utilize a substrate level of 0·2 μmole to 0·5 μmole of 2-AAF. At these levels, about 2 per cent of the 2-AAF was metabolized to *N*-hydroxy-2-AAF in mice. The present report shows that with a modified system containing 3 nmoles, or approximately 160 times less 2-AAF than the amounts used previously, about 12 per cent of the 2-AAF substrate is metabolized to *N*-hydroxy-2-AAF, at a protein level of 0·5 mg/3 ml. This substrate level would appear to offer a better means of relating data *in vitro* to data *in vivo*, since in the modified system 2-AAF can be added in an endogenous vehicle (water) whereas methanol or other exogenous chemicals were required for the 2-AAF concentrations used in previous studies.[1–5] Further, the possibility exists that high concentrations of 2-AAF result in a swamping of the enzyme systems involved in the metabolism of 2-AAF which may restrict comparisons of data *in vivo* and *in vitro*.

The modified system *in vitro* differs in a number of other respects from sytems utilized in the past to investigate 2-AAF metabolism. Sodium fluoride was not added to inhibit deacetylase activity and the system should, therefore, reveal a more complete picture of 2-AAF metabolism, particularly since it has been reported recently that sodium fluoride inhibited the formation of N-OH-2-AAF by as much as 50 per cent in rat preparations *in vitro*.[9] Moreover, the mouse apparently has low deacetylase activity,[10] and this was confirmed in the current study by deleting sodium fluoride and observing the small amounts of 2-AF formed. The amount of microsomal protein was reduced, when compared with previous studies, and an NADPH-generating system was utilized in preference to added NADPH. Each of these factors, and the utilization of the HEPES buffer, formed the basis of a modified, effective system *in vitro* for the study of 2-AAF metabolism.

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