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THE BIOLOGICAL ARYLATION OF PROTEINS *IN VITRO* BY A METABOLITE OF THE CARCINOGEN N-2-FLUORENYLACETAMIDE*

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Abstract—The sequential metabolic pathway leading to the arylation of proteins by the carcinogen, N-2-fluorenylacetamide, has been studied in rat liver slices *in vitro*. The differential binding of ^{14}C-labeled N-2-fluorenylacetamide, N-(1-hydroxy-2-fluorenyl) acetamide, and 2-amino-1-fluorenol to the proteins of the various liver cell fractions indicated that metabolism to the *o*-hydroxy derivative was required for protein binding. Comparison of the protein-labeling pattern of acetyl-^{14}C- and 1-^{14}C-labeled N-(1-hydroxy-2-fluorenyl) acetamide conclusively demonstrated that deacetylation preceded the protein arylation reaction. The microsomal and mitochondrial proteins were more selectively labeled than the soluble proteins, although the soluble proteins were likewise radioactive when liver slices were incubated with 1-^{14}C-labeled N-(1-hydroxy-2-fluorenyl) acetamide.

The binding to mitochondrial proteins was very likely catalyzed by mitochondrial cytochrome c-cytochrome oxidase, since, in a model system, cytochrome c-cytochrome oxidase catalyzed the oxidative arylation of 2-amino-1-fluorenol to exogenously added bovine serum albumin.

THE BINDING to tissue proteins at the site of tumor formation by chemical carcinogens or their metabolites has, until recently, been considered one of the major etiological factors in the induction of neoplasms by these agents.[1–3] With the discovery of N-hydroxylation, a new drug-metabolizing reaction[4, 5] mediated by hepatic microsomes[6] and giving rise to acetylated N-hydroxy aromatic amines having equal or greater carcinogenic potencies than the parent acetylated aromatic amines,[7, 8] and the recent observation that certain carcinogens bind to intracellular nucleic acids,[9] this protein-binding phenomenon has been relegated to secondary importance. Nevertheless, the metabolic transformations of carcinogenic, cytotoxic, pharmacologic, or other agents which lead eventually to their covalent† binding to tissue proteins are of intrinsic interest, since alterations effected by such substitutions on natural constituent proteins would be expected, *a priori*, to have significant effects on cellular systems—morphologically, immunologically, or biochemically. Discussion of the latter aspects are outside the scope of this paper, the purpose of this study being to demonstrate that biochemical mechanisms exist in the cell, indeed are required, for transforming these agents to reactive metabolites which ultimately interact with

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† As distinguished from ionic, coordinate covalent, charge-transfer, or van der Waal's type of attractive binding.

protein constituents. It is possible that metabolism may likewise be necessary for binding these agents to nucleic acids.

Previous studies with model enzyme systems by Gutmann *et al.*[10–13] from this laboratory have established that several metabolic steps were very likely prerequisite for protein binding by the acetylated carcinogenic aromatic amine, N-2-fluorenylacetamide.* The postulated sequential metabolic path taken by FAA can be summarized by the biochemical reactions: (a) hydroxylation; (b) deacetylation; (c) oxidation to a quinoneimine; and (d) binding of the quinoneimine to protein. According to this scheme, the aromatic residue becomes attached in covalent linkage to the amino acid side chains of the protein at step (d) and, by analogy with biological alkylations of proteins by aliphatic agents,[14] protein binding by benzenoid compounds with the attachment of protein residues directly on the aromatic ring can be referred to as protein *arylation*, and the binding agent, an *arylating agent*.† Thus the binding to mouse skin proteins *in vivo* by the carcinogenic dibenz (a, h) anthracene via possible intermediates described by Bhargava *et al.*,[15, 16] the oxidative binding of catechol to N-terminal proline of the protamine salmine catalyzed by phenolase *in vitro*,[17] and the oxidative binding of the bladder carcinogen 2-amino-1-naphthol to mouse bladder protein[18] represent some chemically well-defined examples of biochemical protein arylations by compounds having other aromatic ring structures.

We wish to present the results of further studies with the aid of isotopic tracers which verify, in a cellular system of rat liver slices, the essential correctness of the sequential metabolic steps hypothesized for protein arylation by FAA.

MATERIALS AND METHODS

Preparation of labeled fluorene derivatives

N-2-Fluoren-9-^{14}C-ylacetamide and N-(1-hydroxy-2-fluoren-1-^{14}C-yl)acetamide, both prepared by published procedures,[19, 20] were available from previous studies.‡ N-(1-Hydroxy-2-fluorenyl)acet-1-^{14}C-amide was prepared by acetylation of unlabeled 2-amino-1-fluorenol hydrochloride with acetic-1-^{14}C anhydride (dilute aqueous $NaHCO_3$ as the acid acceptor). The crude product was purified by column chromatography on acid-washed activated alumina with ethyl acetate as eluent, and then recrystallized from ethanol–water; yield, 67%; m.p. 210–212° uncorr. (reported:[21] 208°). Each of the above samples moved as a single radioactive component when chromatographed independently on Whatman No. 1 paper in the cyclohexane–t-butanol–glacial acetic acid–water (16:4:2:1) system of Weisburger *et al.*[22] In the case of the hydroxylated compounds the radioactive peak on the chromatogram coincided with the the chromogenic spot which appeared with the Folin–Ciocalteau phenol reagent.

Calculated quantities of unlabeled carrier compounds were added to and intimately dissolved with the labeled compounds when it was necessary to adjust the specific radioactivities of the fluorene derivatives. The calculated activities were checked by actual radioassay in all cases. The specific activities of the samples used were:

* Consistent with previous assignment and usage[12, 13] the following abbreviations are used throughout: N-2-fluorenylacetamide (FAA); N-(1-hydroxy-2-fluorenyl)acetamide (OFAA); 2-amino-1-fluorenol (OFA); 2-imino-1, 2-fluorenoquinone (FQI); trichloroacetic acid (TCA). Their structural formulas (except for TCA) appear under Results.

† This term has been used in similar connection by Miller *et al.*[7]

‡ We are grateful to Dr. H. R. Gutmann for kindly making these available.

FAA-9-^{14}C, 2·65 × 10^5 cpm/μmole; OFAA-1-^{14}C, 2·48 × 10^5 cpm/μmole, and 2·11 × 10^5 cpm/μmole; OFAA-acetyl-^{14}C, 2·31 × 10^5 cpm/μmole. 2-Amino-1-fluoren-1-^{14}C-ol (spec. act. 6·14 × 10^5 cpm/μmole, and 2·33 × 10^5 cpm/μmole) was prepared by dilution of chromatographically pure OFAA-1-^{14}C with carrier and hydrolysis of the acetyl group with aqueous ethanolic hydrochloric acid followed by recrystallization of the product from 4 N hydrochloric acid as described by King *et al.*[23]

Enzyme preparation, incubation systems, radioactive counting, and tissue fractionations

Crystalline bovine serum albumin was purchased from Nutritional Biochemicals Corporation* and for calculations, the molecular weight of 69,000 was used. Hog heart cytochrome oxidase[24] prepared on several different occasions was assayed according to the procedure of Smith.[25] Preparation A (used in Method A below) had an activity of 6·08 × 10^{-1}/sec per μg protein N, while preparation B (used in Method B) assayed 1·90 × 10^{-2}/sec per μg protein N. Cytochrome c† was standardized spectrophotometrically.[26] OFA was oxidized with cytochrome c–cytochrome oxidase in the presence of bovine serum albumin as described previously[12] except that the ^{14}C-labeled compound was used here. Control flasks contained albumin, OFA-1-^{14}C and cytochrome oxidase in 0·1 M phosphate buffer, but no cytochrome c. The albumin was isolated and analyzed for radioactivity by one of two procedures as follows.

Method A. After incubation (30 min) the reaction was quenched and the albumin precipitated by addition of an equal volume of 10% aqueous TCA solution. The protein was sedimented, washed twice with 5% TCA, suspended (mostly dissolved here) in ice-cold acetone, then reprecipitated by addition of an equal volume of cold ether and collected and air dried to constant weight. After analysis for radioactivity, the protein was dissolved in 5% aqueous NaOH by treatment at room temperature for 18 hr and then reprecipitated with an equal volume of 50% aqueous TCA. A further wash with 5% TCA followed by trituration with cold acetone–ether (1:1) flocculated the protein which was sedimented by centrifugation, washed with acetone, resedimented, and dried to constant weight. The protein samples were combusted in duplicate to $^{14}CO_2$ and collected[27] as $Ba^{14}CO_3$ for counting in a gas-flow counter. Counting times were sufficiently long to maintain counting statistics within the standard error of ±5%, and corrections were applied for self-absorption. Only the radioactivities of the proteins determined at this stage are recorded. In general, about 10% of the radioactivity initially bound to the albumin was removed by this treatment.

Method B. Alternatively, the incubation mixture was chromatographed on a mixed-bed column of Amberlite XE-64:diethylaminoethyl cellulose ion-exchange column as previously described[28] and subsequently modified.[23] Time lapse from the end of the incubation to charging on the column was held below 2 to 3 min. The fractions containing the effluent protein were combined and dialyzed against distilled water for 24 hr, the protein isolated by lyophilization and then counted in a Packard Tri-Carb liquid scintillation spectrometer‡ in the system described by Alexander *et al.*[29]

Pooled liver slices from four to six male albino rats§ weighing 200 to 350 g were incubated with the labeled fluorene derivatives essentially as described by Gutmann

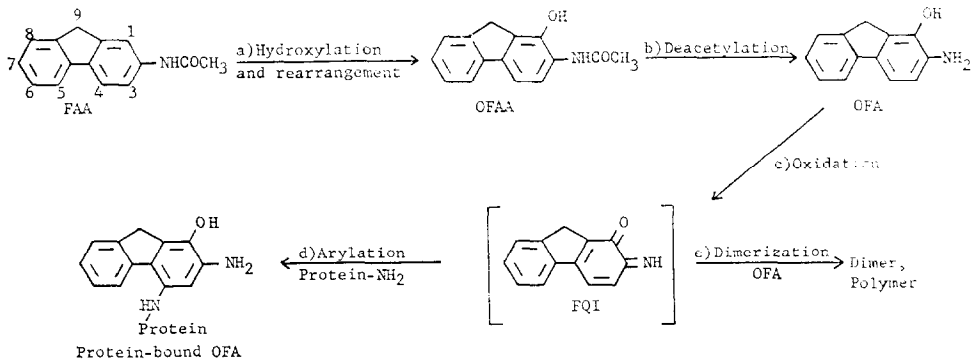
* Cleveland, Ohio.
† Sigma Chemical Co., St. Louis, Mo.
‡ Packard Instrument Co., LaGrange, Ill.
§ Holtzman Rat Co., Madison, Wis.

and Peters.[30] Incubation time was 1 hr, and the labeled fluorene derivatives (0·52 μmole/g of slices) were added as solutions in ethylene glycol monomethyl ether, the use of ethanol being avoided, since ethanol is known to inhibit both the microsomal hydroxylase and the amidase.[11] After incubation the slices were combined, sedimented by centrifugation, washed twice with 0·1 M phosphate buffer (pH 7·4), sedimenting the slices after each wash, and the slices homogenized as a 13% suspension in phosphate buffer with a perfluorocarbon-on-glass homogenizing pestle and tube. The individual cell fractions were then isolated by centrifugation at centrifugal forces prescribed by Hogeboom.[31] Each pelletized centrifuge sediment was washed twice with buffer by alternate homogenization and sedimentation, the first wash liquids being always added to the succeeding lighter fractions, but the second washes discarded. The liver proteins from each fraction were precipitated by first homogenizing the washed pelletized sediment in water and then adding an equal volume of cold 10% TCA. A final treatment of the isolated proteins with cold acetone (twice) served to remove lipid material as well as the excess TCA. The protein-bound radioactivity was determined at this stage (after drying) by using the combustion procedure described above. The liver proteins were then subjected to successive hot extractions with acetone and ethanol followed by dissolution in alkali (10%NaOH), reprecipitation with 50% TCA, and a final hot extraction with ethanol, a procedure patterned after Gutmann and Peters.[30] After each extraction step the proteins were analyzed for the radioactive content.

In one experiment noted in the text, the liver proteins were precipitated by addition of 10 volumes of cold acetone instead of TCA, and the proteins were not subjected to dissolution in alkali or to hot solvent extractions. In this same experiment the microsomes were sedimented by centrifugation at 105,000 *g* for 1 hr.

RESULTS

The generalized metabolic pathway[10,23] leading to protein arylation by acetylated aromatic amines applied to the particular case of FAA follows thus:



As alluded to earlier, each step of this sequence has been experimentally observed. FAA is metabolized *in vivo*[32] and *in vitro*[11, 33] to hydroxylated derivatives, one of which is the *o*-hydroxylated OFAA shown. The formation of the latter in the rat has been found to proceed via the rearrangement of an intermediate N-hydroxylated metabolite of FAA, viz. N-hydroxy-N-2-fluorenylacetamide.[34] An amidase widely

distributed in rat tissue found also in the liver microsomes[11, 13] deacetylates OFAA, while rat kidney or liver mitochondrial cytochrome c–cytochrome oxidase can catalyze the oxidative step (c), leading, in the presence of protein, spontaneously to the protein arylation step (d)[12] provided that the protein acceptors have isoelectric points in the range of 4 to 6.[23] The intermediate *o*-quinoneimine, FQI, can also dimerize or polymerize by combination with OFA, as in step (e).[12] It is known from ^{15}N tracer studies that the amino nitrogen of OFA is not lost during this oxidative binding to proteins.[23]

From considerations of steps (c), (d), and (e) it follows that increasing the protein concentration relative to OFA should result in the trapping of more of the intermediate FQI as a protein-bound conjugate and should prevent the competitive dimerization reaction (e). Indirect evidence that this is the case has been shown previously in a model system of cytochrome c–cytochrome oxidase and bovine serum albumin by measuring the color of the dimeric product formed, which diminished as protein concentration was increased.[12] This study has now been repeated with OFA labeled in the one-position with carbon-14.

Oxidative binding of OFA to bovine serum albumin

Oxidation of OFA-1-^{14}C with cytochrome c–cytochrome oxidase in the presence of bovine serum albumin resulted in the binding of the radioactive label to the protein (Fig. 1). As the albumin concentration relative to OFA was increased, the protein-bound radioactivity likewise increased and then leveled off at higher albumin:OFA mole ratios. The data for curves A and B, Fig. 1, were obtained by two independent

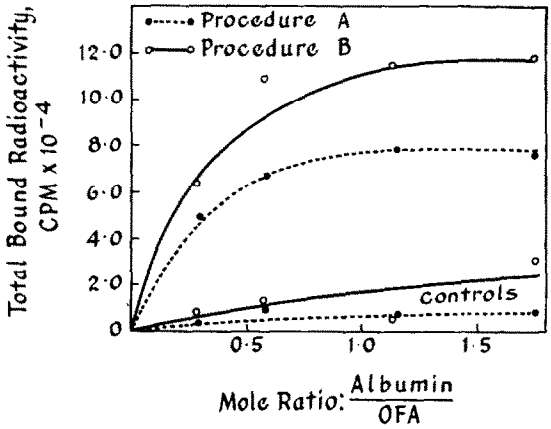


FIG. 1. The oxidative binding of OFA to bovine serum albumin catalyzed by cytochrome c–cytochrome oxidase. Experimental conditions described in Methods.

procedures differing in the manner of workup of the radioactive albumin and the activities of the enzyme preparations. In curve A the protein was precipitated with TCA, extracted with solvents, etc., and the radioactivity determined as $Ba^{14}CO_3$ (Method A); in curve B the radioactive albumin–OFA conjugate was purified by ion-exchange chromatography and then counted directly in a liquid scintillation spectrometer (Method B). This latter chromatographic procedure separates and removes polymers of OFA.[23] From Fig. 1 it is evident that as the albumin concentration relative to OFA was increased, more label became attached to the albumin

irrespective of the method used for its isolation and assay. The slight labeling of the control proteins can be ascribed to air oxidation of OFA and, even here, protein binding increased at higher albumin concentrations.

Whereas total binding of OFA increased as the mole ratio of albumin:OFA was raised, the specific binding to the albumin decreased (Fig. 2). This is to be expected

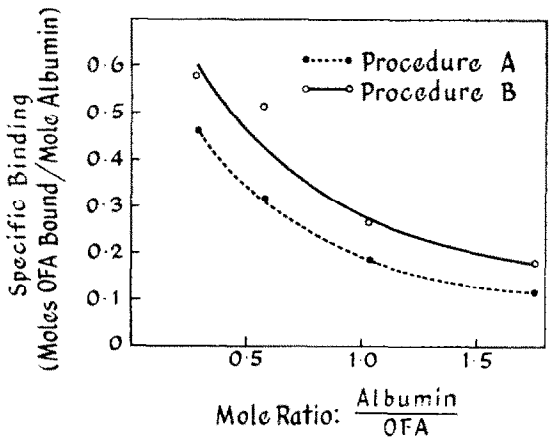


FIG. 2. The specific binding of oxidized OFA to bovine serum albumin.

under the conditions of the experiment, since initial OFA concentrations were kept the same while albumin concentrations alone were varied, and only a limited quantity of substrate would therefore be available for binding to the added protein. The reasonable quantitative agreement of the two curves of Fig. 2, determined by independent procedures, attests to the validity of the interpretation and discounts any possibility that coprecipitated polymeric radioactive contaminents were responsible for the observed results with Method A. These tracer studies serve to reinforce previous conclusions that enzymic oxidation of OFA in a protein environment results in binding of an *oxidized* fragment, viz. FQI, to the protein, and that this trapping of FQI by protein increases with increasing protein concentration relative to OFA.

Protein binding of fluorene derivatives to rat liver cell fractions

To study the sequence of metabolic events leading to protein arylation as outlined above in a more dynamic cellular system, rat liver slices were incubated with equimolar quantities of ^{14}C-labeled FAA, OFAA, and OFA having comparable specific activities. FQI could not be included, owing to its extreme lability.[35] It is assumed here that the closer the metabolite is to the actual arylating specie (FQI), the greater will be the protein labeling, since one or more enzymic steps will have been by-passed by supplying the system with a metabolite in place of the metabolite precursor. The molar specific activities of these labeled compounds were adjusted and equalized with carrier to within 5% of their average values in order that differential labeling of the protein fractions could be compared directly without recourse to recalculation, a necessary step had the specific activities been unequal. After incubation the slices were thoroughly washed, homogenized, the cellular components separated

into their ultracentrifuge fractions, and the proteins isolated and processed as described under Methods. As can be seen from Table 1, which represents a typical result,

TABLE 1. THE BINDING OF N-(1-HYDROXY-2-FLUOREN-1-^{14}C-YL)ACETAMIDE TO RAT LIVER PROTEINS *in vitro*

Cell fraction	Dry weight before extraction (mg)	Specific activities of protein (cpm)			
		Before extraction	After extraction, 95% ETOH, 20 hr	After extraction, acetone, 20 hr	After dissolution in alkali, precipitation, and extraction, ETOH
Mitochondria	193	141	131	146	130
Microsomes	113	486	476	430	490
Soluble	396	151	147	134	170

exhaustive solvent extractions in attempts to remove all the noncovalently bound radioactivity did not appreciably alter the specific activities of the liver proteins initially isolated. In order to insure uniformity of treatment, however, this protocol was used throughout this study. Manipulative losses being inevitable during these extraction procedures, the total radioactivity on the protein of each cell fraction was calculated by multiplying the initial weight of the isolated protein before extraction by its specific radioactivity after the final treatment.

Incubation of rat liver slices with FAA-9-^{14}C gave rise to a definite but low-level binding of radioactivity to the various liver protein fractions (Table 2). The specific radioactivity of the microsomal proteins was twice that of the mitochondrial proteins. With OFAA (the *o*-hydroxylated metabolite of FAA which is presumed to be two

TABLE 2. COMPARATIVE BINDING OF ^{14}C-LABELED FLUORENE DERIVATIVES TO RAT LIVER PROTEINS *in vitro*

Fluorene derivative	Specific activity of protein fractions isolated (cpm/mg)			Total protein-bound radioactivity (cpm × 10^{-4})		
	Mitochondria	Microsomes	Soluble	Mitochondria	Microsomes	Soluble
FAA-9-^{14}C	15·7	30·6	16·4	0·235	0·543	1·07
OFAA-1-^{14}C	130	490	170	2·50	5·54	6·73
OFAA-1-^{14}C (N_2)	84·0	292	130	1·41	3·18	4·48
OFAA-1-^{14}C (5×10^{-3} M CN^-)	85·2	98·0	34·3	1·32	1·27	0·704
OFA-1-^{14}C	219	245	49·7	2·91	4·04	2·53
OFA-1-^{14}C (5×10^{-3} M CN^-)	94·1	134	25·3	1·96	2·99	1·12

metabolic steps removed from FQI), the binding to all fractions increased considerably, the specific activities of the mitochondrial, microsomal, and soluble proteins increasing by factors of 8, 16, and 10 respectively. Incubation of liver slices with OFA, the metabolite which is the immediate precursor of FQI and therefore only one step

removed from the protein arylation reaction (step d), similarly resulted in increased labeling of the mitochondrial, microsomal, and soluble proteins; and relative to FAA, the values were higher by factors of 14, 8 and 3 respectively.

The number of moles of bound metabolite per unit weight of the cell protein fractions is directly proportional to the specific activities tabulated in Table 2 with the result just described but curiously the pattern of total binding of OFA or OFAA to mitochondria or microsomes (also Table 2) did not parallel this specific binding. This was due to the variation in the weights of the isolated mitochondrial and microsomal proteins. With FAA or OFA as substrates, the weight ratio of mitochondrial:microsomal protein remained relatively constant in the range 0·84 to 0·93. In contrast, this weight ratio was reversed with OFAA as substrate and ranged from 1·71 to 1·20 (added cyanide) in the three instances recorded. The combined weights of the mitochondrial–microsomal proteins isolated were, as would be expected, nearly the same irrespective of the fluorene derivative incubated (range 277 to 320 mg); likewise, the total amount of binding to the combined mitochondrial–microsomal fractions with OFAA was 4·0% of the total activity incubated, whereas the comparable value with OFA was 3·5%. This suggests that OFAA may have caused an aggregation of the microsomal proteins such that they were now sedimented with the mitochondrial fraction. It is noteworthy also that the soluble proteins were especially highly labeled when OFAA was the substrate. The significance of these observations is yet not apparent. It must be emphasized, however, that the morphological and biochemical integrity of the liver cytoplasmic fractions isolated by ultracentrifugal fractionation after incubation of slices in an *in-vitro* system are not established.

Controls of the type generally used, e.g. incubation of the liver slices at high or low pH values or utilizing heat-denatured slices, could not be applied here as these procedures would surely alter the sedimentation characteristics of the cell fractions, and other means were required to show that these sequential steps were enzyme mediated.* Incubation of OFAA-1-^{14}C under nitrogen inhibited the protein binding by approximately 40%. Blocking the cytochrome system[37] by addition of 5×10^{-3} M cyanide† decreased the binding of OFAA-1-^{14}C considerably—by 80% to microsomes; likewise cyanide depressed the binding of OFA-1-^{14}C to all the liver protein fractions by approximately 50%, but these values did not approach the very low figures for binding by FAA. Gutmann *et al.*[10] have reported that cyanide reduces even further the binding of FAA-9-^{14}C to rat liver slices, while Hultin[38] has observed that the binding of isotopically labeled 2-aminofluorene to fortified rat liver microsomes was not inhibited by cyanide, although equivalent concentrations of 8-hydroxyquinoline depressed binding by 82%.

These results offer strong evidence that FAA does not itself bind to liver proteins without prior metabolism, but do not differentiate between the binding of OFAA and OFA—i.e. whether OFAA is first deacetylated to OFA before being bound; nor do

* Weisburger *et al.*[38] have reported that ^{14}C-labeled OFA as well as the isomeric 3- and 7-hydroxy-2-fluorenamines bind to the proteins of rat liver homogenates by a nonspecific, nonenzymic reaction not further elaborated. While such 'artifact binding' cannot be entirely excluded and in fact must occur to some extent, owing to the ready oxidizability of these derivatives in air (cf. Fig. 1), it is difficult to ascribe the protein binding of OFA observed here in viable liver slices as being due wholly to nonenzymic oxidative mechanisms.

† Cyanide does not inhibit the hydroxylation or deacetylation of FAA by fortified rat liver microsomes.[11]

they serve to distinguish the primary oxidation site as the mitochondria or microsomes, since both cell fractions were labeled extensively.

Comparative protein arylation by ring-labeled and acetyl-labeled OFAA

In the interaction of chemically oxidized OFAA, viz. 1, 2-fluorenoquinone-2-acetimide, with bovine serum albumin in a model system, it has been shown that a ^{14}C-labeled acetyl group is retained in the protein-bound adduct.[39] The possibility that OFAA *in its oxidized form* can bind to proteins with its acetyl group intact, contrasted to the observation that OFAA is not oxidizable by cytochrome c even though the latter's oxidation potential is sufficiently high to oxidize OFA[12] as well as the naphthalene analog 2-amino-1-naphthol,[18] enhanced the need for a definitive study at the cellular level.

Accordingly, OFAA labeled in the aromatic ring (1-position) and OFAA labeled in the acetyl group, both of comparable specific radioactivities, were incubated with rat liver slices under identical conditions in parallel experiments, and the labeling of the mitochondrial and microsomal proteins was compared. In this study the proteins were precipitated with cold acetone instead of with TCA and were not subjected to hot solvent extractions or to dissolution in alkali, in order to eliminate any possibility of solvolysis of the acetyl group during the isolation procedure. The results are shown in Fig. 3. It can be seen that the binding of radioactivity to liver proteins by OFAA

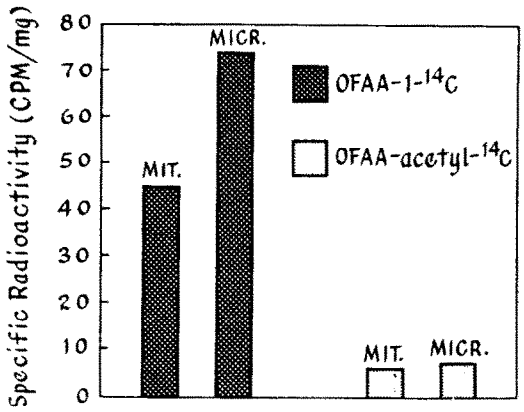


FIG. 3. Comparative binding of ring-labeled and acetyl-labeled OFAA to rat liver proteins *in vitro*.

labeled in the acetyl group was negligible at only 6 to 7 counts bound per mg of protein. In marked contrast, ring-labeled OFAA was protein bound in significant amounts to both the mitochondrial and microsomal proteins, the specific binding to the proteins of the latter being nearly twice that of the former. It follows that protein arylation by OFAA is still a multistage enzymic process with deacetylation a prerequisite step.

The similarities of the binding by OFA and OFAA, which differ by an order of magnitude to the binding of FAA itself (Table 2), indicate that neither deacetylation of OFAA to OFA nor the oxidation of the latter to FQI can be the rate-limiting step in the protein arylation reaction.

DISCUSSION

The present study together with previous results from this laboratory lends strong support to the thesis earlier alluded to that biological arylation of proteins by benzenoid compounds, as here exemplified by FAA, proceeds by a series of sequential biochemical reactions, the hepatic microsomes playing an important role in the metabolic transformations leading the active arylating specie. The oxidative binding to mitochondria of exogeneously added or enzymically liberated OFA in liver slices is likely mediated by cytochrome c–cytochrome oxidase as observed in the model system with albumin, but the oxidative enzyme that catalyzes the binding of OFA to microsomal proteins cannot yet be identified. Since microsomal cytochrome has been shown not to be involved,[12] the possible implication of a NADP-linked microsomal enzyme in this oxidative step may be suggested. Of pertinence in this regard are the observations by Seal and Gutmann[11] and of Hultin[38] that FAA as well as 2-aminofluorene is metabolized by, and becomes protein bound to, fortified rat liver microsomes in the absence of mitochondria.

The technique of comparing the utilization of isotopically labeled metabolites, possible metabolites, and/or metabolite precursors by the cell, a procedure routinely used by investigators studying the biosynthesis of complex nitrogenous material in plants,[40] and successfully applied by Roberts and Warwick in elucidating the sequential metabolic fate of the alkylating agent, Myleran,[41] has much to recommend it in studies of this type.

The hazards involved in translating results obtained *in vitro* to actual metabolic events as they may occur *in vivo* have been thoroughly discussed by Hultin and Arrhenius.[38, 42] Discrepancies arise from the fact that normal conjugation reactions and other mechanisms for eliminating the metabolite from the primary metabolic site are either absent or operating submaximally *in vitro*. Thus, while labeled OFAA administered intraperitoneally to rats or guinea pigs binds to liver proteins much more extensively than similarly administered FAA, quite in accord with the *in vitro*-data presented here, it is also further, hydroxylated, conjugated, and eliminated in the urine.[43] Difficult to reconcile, but nevertheless acceptable as facts, are the observations that OFAA is weakly or not carcinogenic to rats when fed in the diet or on repeated intraperitoneal administration,[7, 44] and that OFA is not carcinogenic to mouse bladder on implantation.[45]

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