THE DISTURBANCE OF OXIDATIVE PHOSPHORYLATION BY N-ACETOXY-N-ACETYL-2-AMINOFLUORENE, A MODEL ULTIMATE CARCINOGEN*

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Currently N-acetoxy-N-acetyl-2-aminofluorene is favored by many investigators to be a model of the ultimate electrophilic carcinogenic agent derived metabolically from the carcinogen N-acetyl-2-aminofluorene. The model induced *in vitro* a delayed ATP energized increase in mitochondrial volume as indicated by the decrease in absorbancy at 520 nm. The ATP energized decrease in absorbancy was inhibited by rutamycin, 2,4-dinitrophenol and a high level of antimycin known to induce ATPase activity. The ATP energized effect was not altered either by rotenone or by a low level of antimycin known to inhibit respiration without inducing ATPase activity. Malate or potassium ion did not affect the phenomenon, however, sulfate ion which has been implicated in liver carcinogenesis shortened the induction period. Showdomycin stimulated the phenomenon. N-Acetoxy-N-acetyl-2-aminofluorene interacts with the machinery of oxidative phosphorylation. N-Acetoxy-N-acetyl-2-aminofluorene was enzymically converted by the mitochondria to N-hydroxy-N-acetyl-2-aminofluorene. These findings extend the experimental confluence of oxidative phosphorylation with carcinogenesis.

A comprehensive examination of the action of carcinogens, metabolites of carcinogens and related derivatives of carcinogens with tissue constiuents should lead to meaningful data regarding the mechanism of chemical carcinogenesis. As a result of this general approach we synthesized a variety of K-region derivatives of the potent carcinogen 7,12-dimethylbenz (a) anthracene over a decade ago[1]. We have also examined the interaction of several carcinogens and their metabolites with mitochondria by means of a convenient *in vitro* system based upon the ability of an agent or a combination of agents to induce an ATP-energized mitochondrial volume change. The change was indicated by a decrease in absorbancy at 520 nm. While the three parent carcinogens, N-acetyl-2-aminofluorene (AAF), dibenz (a, h) anthrancene and N-acetyl-4-aminobiphenyl were inactive in this *in vitro* system; their appropriate acidic metabolites, N-hydroxy-N-acetyl-2-aminofluorene (N-OH-AAF), 4′,8′-dihydroxy-1,2,5,6-dibenz-9,10-anthraquinone and N-hydroxy-N-acetyl-4-aminobiphenyl were active in this *in vitro* test[2,3,4]. The studies with N-OH-AAF were extended to three other isomers in which constituents were not only in position two of the fluorenyl moiety as in N-OH-AAF but also in positions one, three and four[5]. The four N-hydroxy derivatives induced (when combined with showdomycin) ATP-energized mitochondrial changes whose magnitudes were parallel to their carcinogenicity in the rat. These *in vitro* mitochondrial studies illustrated a confluence of experimental data dealing with oxidative phosphorylation and chemical carcinogenesis and directed attention to our unitary hypothesis

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of carcinogenesis[2,6]. This hypothesis postulated that when a carcinogen interferes with a flux of energy in the mitochondria, mitochondrial membrane damage becomes feasible and such damage could result in the release of mitochondrial genetic material. This released mitochondrial genetic material could then behave like an oncogenic virus and enter the genome of the cell.

On the other hand MILLER and MILLER have suggested for some time[7,8] that the critical reaction which engenders carcinogenesis was between a nucleophilic site of the informational macromolecules (proteins and nucleic acids) and an electrophilic site of the original carcinogen or appropriate metabolite derived from the carcinogen. The crucial electrophilic species was named the ultimate carcinogen while intermediates between the parent carcinogen and the ultimate carcinogen were designated as proximate carcinogens. MILLER and MILLER and associates[9,10,11,7,8] and others[12,13] designated in rat liver, N-OH-AAF to be the proximate carcinogen and N-OSO_2OH-AAF to be the ultimate carcinogen derived from AAF. Because of the considerable lability of N-OSO_2OH-AAF much experimental work had been carried out with the more stable and more lipid-soluble anhydride N-acetoxy-N-acetyl-2-aminofluorene (N-OAc-AAF)[14,9,15,10,16,7,17,18,19,20]. Other workers have also considered the possibility that N-OAc-AAF may be a metabolite of N-OH-AAF because (a) the slow nonenzymic acetylation of N-OH-AAF by acetyl-CoA[21] and (b) the enzymic conversion of N-OH-AAF to N-OAc-AAF and 2-nitorsofluorene by certain mammalian peroxidases[22]. MILLER and MILLER also reported that N-OAc-AAF was a more potent carcinogen than N-OH-AAF[23].

In order to further explore the confluence of experimental observations dealing with oxidative phosphorylation and carcinogenesis we have examined the *in vitro* interaction of N-OAc-AAF with mitochondria by means of our ATP energized mitochondrial volume change system and also by means of oxygen uptake and ATPase measurements.

Materials and Methods

General procedures, methods and purification of the water and preparation of mitochondria has been described[24,25,26,3] for the mitochondrial volume change experiments.

Mitochondrial Swelling

Incubation was at 27°C in standard rectangular glass cuvettes with a 1-cm light path. The basic reaction mixture for the volume change experiments had a final volume of 3 ml and contained 0.75 mg mitochondrial protein (prepared fresh daily from male rat liver), 75 mM sucrose and 75 mM tris-HCl buffer of pH 7.4. A decrease in absorbancy at 520 nm was taken as a measure of mitochondrial swelling[24]. A model 2000 automatic spectrophotometer manufactured by Gilford Instrument Laboratories, Inc., Oberlin, Ohio, was used. All cations were added in the form of chloride salts and anions were added in a form of tris salts neutralized to pH 7.4. N-OH-AAF and N-OAc-AAF were dissolved in acetone (dried with sodium sulfate and distilled from potassium permanganate) or dimethylsulfoxide (distilled from calcium hydride *in vacuo* and stored under nitrogen) when added to the incubation mixture. These solutions were prepared fresh for each day of experimentation. All controls contained the appropriate amount of carrier solvent. The adding-mixing device has been described[25]. The charts and legends provide additional experimental details.

Oxygen Uptake

Oxygen consumption was measured at 27°C in an oxygen monitor which utilized a CLARK electrode. The instrument (Model 535A) was manufactured by Yellow Springs Instrument Company, Yellow Springs, Ohio. A ten inch recorder (Model 100S) manufactured by Beckman Instruments, Fullerton, California, was used in conjunction with the oxygen monitor.

ATPase Measurement

In the ATPase experiment, orthophosphate was measured as previously described[24,31]. Aliquots of 1 ml, were removed at 3-minute intervals from the specially designed optical cuvettes by means of a spring loaded Cornwall glass syringe fitted with a 6″, 20 gauge stainless steel needle.

Metabolism of N-OAc-AAF

To study the enzymic metabolism of N-OAc-AAF by rat liver mitochondria, 3 mg N-OAc-AAF (10.7 μmole) in acetone (0.1 ml) was added to the basic reaction mixture in a 16×150 ml test tube which contained 9 mg mitochondrial protein, 75 mM sucrose and 75 mM tris-HCl buffer in a final volume of a 3 ml. The reaction temperature was 27°C; shaking was at the rate of 27 excursions per minute. Following the addition of N-OAc-AAF aliquots of 1 ml were removed at 0.5 and 30 minutes with a spring loaded Cornwall glass syringe fitted with a 6″, 20 gauge stainless steel needle. The contents of the syringe were expelled into a 16×150 mm test tube which contained 1 ml of 1 M phosphoric acid to which was then added 7 ml of a mixture of *t*-butanol - water (75 : 25). The contents of the tube were mixed. A volume of 8 ml of 1,2-dichloroethane - dichloromethane (50 : 50) was added and the contents were mixed. Low speed centrifugation separated the two layers. An aliquot of 11 ml (syringe) was removed from the lower (organic layer) and dried with 2 g of anhydrous sodium sulfate. An aliquot (10 ml) of the organic phase was transferred to a 50-ml round bottom flask and taken to dryness *in vacuo* at 40°C (rotary evaporator). Acetone (0.3 ml) was added, the residue dissolved and an aliquot of 10 μl was spotted on silica gel TLC plates (QF1) 250 μ thick, with phosphor purchased from Quantum Industries, Fairfield, New Jersey. The plates were developed by a mixture of chloroform - methanol (97 : 3). The various spots were located by means of fluorescent quenching.

Synthesis of N-OAc-AAF

The procedure was a modification of that reported by GUTMANN and ERICKSON[27]. A solution of 250 mg (1.04 nmole) of N-OH-AAF in a mixture of 17 ml of freshly distilled pyridine and 17 ml of acetic anhydride (distilled) stood at room temperature for 24 hours under an atmosphere of nitrogen. The volatile reagents were then removed *in vacuo* on a rotary evaporator at 40°C. The residue was co-evaporated six times with benzene (purified by having been shaken with sulfuric acid, washed with water, dried with sodium sulfate and distilled). The off-white solid was washed with ether (analytical grade) and cyclohexane (purified by having been shaken with sulfuric acid, washed with water, dried with sodium sulfate and distilled) and then recrystallized from cyclohexane-acetone (dried with sodium sulfate and distilled from potassium permanganate). The crystals had a melting point of 110~111°C (corrected microscope hot stage). Calculated for $C_{17}H_{15}NO_3$: C, 72.58; H, 5.37 Found: C, 72.61; H, 5.40 [Analysis carried out by Galbraith Laboratories, Knoxville, Tennessee, 37291, U.S.A.]. GUTMANN and ERICKSON[27] reported a melting point of 112~114°C; LOTLIKAR, SCRIBNER, MILLER and MILLER[14] reported a melting point of 112~114°C.

Other samples of N-OAc-AAF were kindly provided by Dr. HELMUT GUTMANN of Minneapolis, Minnesota and by the Drug Development Branch, Division of Drug Treatment, National Cancer Institute, Washington, D.C., U.S.A. This agency also kindly provided us with samples of N-OH-AAF.

The experiments reported were carried out with the sample provided by the National Cancer Institute and this sample showed only a single spot when examined by T.L.C. as described in Fig. 11.

Results

It is seen in Fig. 1 that N-OAc-AAF at a level of 300 μM when combined with 333 μM ATP did elicit a delayed but significant increase in mitochondrial volume. Two possible cleavage products of N-OAc-AAF *viz.* N-OH-AAF and acetate each at the level of 300 μM either alone or when combined with 333 μM ATP did not elicit any volume change. Similar data

was obtained with two different samples of N-OAc-AAF kindly provided to us by Dr. HELMUT GUTMANN and the National Cancer Institute. The effect was identical when dimethylsulfoxide rather than acetone was used as the carrier solvent (data not shown). The onset of the swelling phenomenon varied two to three minutes from day to day (*i.e.* with different mitochondrial preparations) but was constant throughout any one day with the same mitochondrial preparation in any single set of parallel controlled experiments.

The ATP energized effect with N-OAc-AAF was barely detectable at a level of 200 μM N-OAc-AAF but was readily induced by 300 μM N-OAc-AAF (data not shown).

It is seen in Fig. 2 that the onset of the ATP energized swelling induced by N-OAc-AAF was hastened by sulfate ion while potassium ion and malate ion had no influence.

In Fig. 3 it is seen that the ATP energized effect was not inhibited by the respiratory inhibitors rotenone and antimycin at respective levels of 3 μM and 0.1 μg per 3 ml. These are concentrations known to inhibit mitochondrial respiration[28] with minimal ATPase activity. When the concentration of antimycin was increased tenfold to 1 μg per 3 ml the volume change effect was blocked. It is known that antimycin at high concentrations induces ATPase activity[28].

Fig. 1. N-OAc-AAF, N-OH-AAF and acetate alone and combined with ATP.

The basic medium (see "Materials and Methods") was used. Either N-OAc-AAF (in acetone) or N-OH-AAF (in acetone) or an aqueous solution of tris acetate was added in a volume of 0.03 ml by means of the adding-mixing device as indicated by arrows. The adding-mixing device also carried 0.03 ml of acetone when tris acetate was added. The final volume of system was 3 ml. The ATP (tris salt), where indicated, was present in the cuvette before the mitochondria were added. The top trace is a mitochondrial control with the addition of solvent. The designations in the lower right-hand corner of this and subsequent figures are for internal reference at our laboratory.

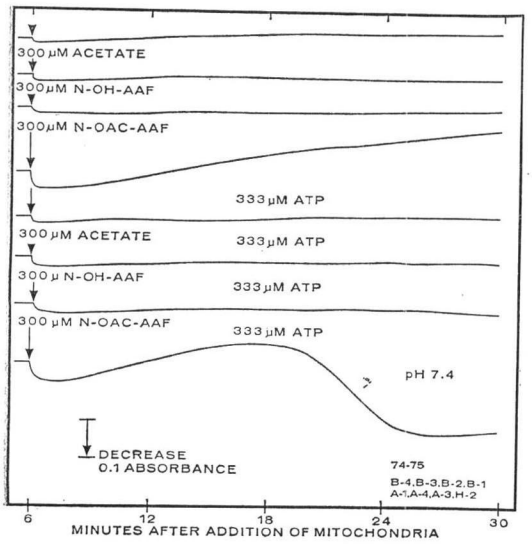


Fig. 2. The effect of various ions.

See legend of Fig. 1 for conditions. The various ions, where indicated, were present in the cuvette before the mitochondria were added.

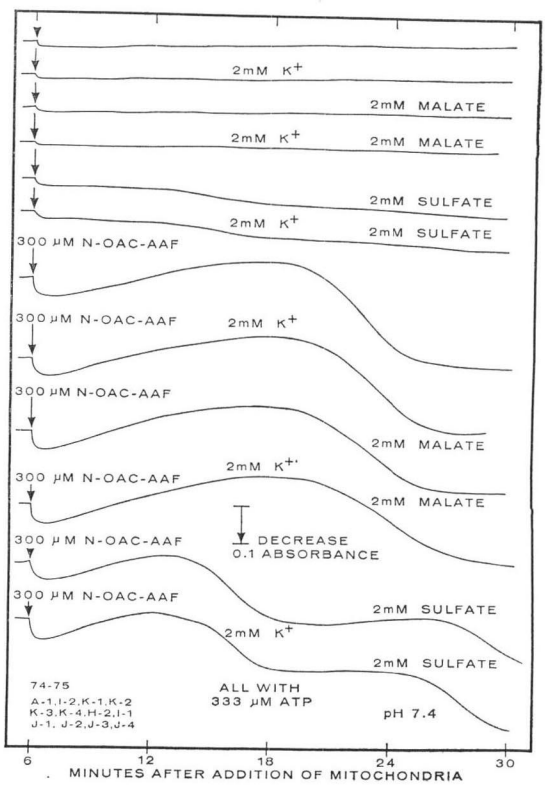


Fig. 3. The effect of various inhibitors.
See legend of Fig. 1 for conditions. The various inhibitors, where indicated, were present in the cuvette before the mitochondria were added.

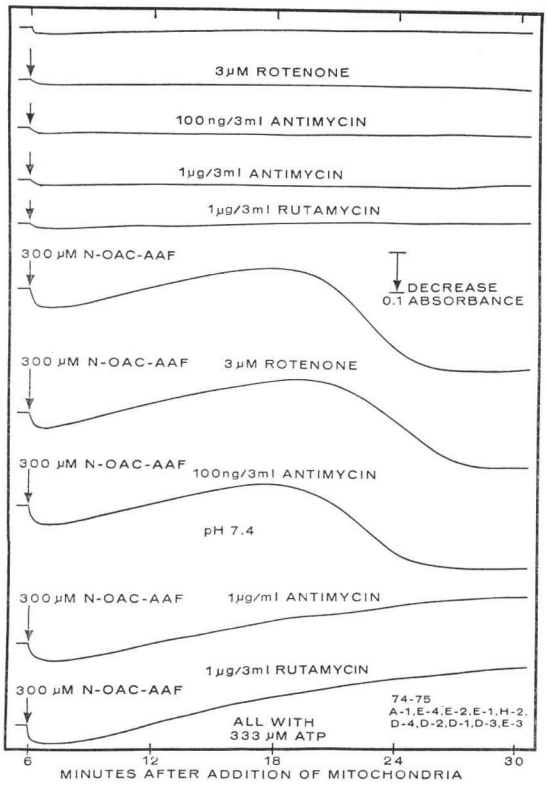
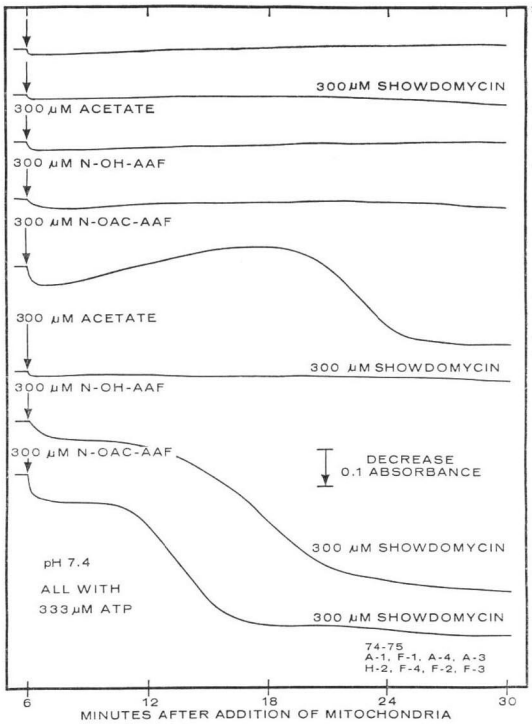


Fig. 4. The effect of showdomycin.
See legend of Fig. 1 for conditions. Showdomycin, where indicated, was present in the cuvette before the mitochondria were added.

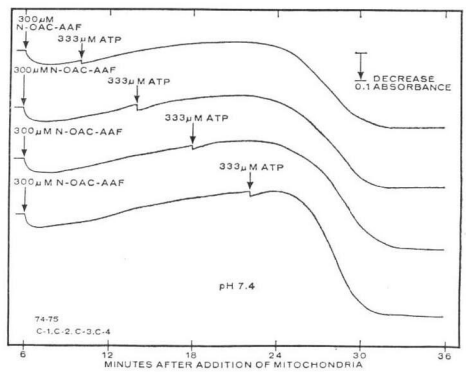


It is also seen in Fig. 3 that the ATP energized effect was inhibited by rutamycin.

The classical uncoupler of oxidative phosphorylation 2,4-dinitrophenol (DNP), also inhibited the ATP energized volume change (data not shown).

The addition of the thiol reagent showdomycin (Fig. 4) enhanced and hastened the onset of the ATP energized effect dependent on N-OAc-AAF. Acetate plus showdomycin plus ATP did not induce an ATP energized mitochondrial change. In contrast N-OH-AAF the other possible cleavage product of N-OAc-AAF when combined with showdomycin produced an ATP energized mitochondrial volume change as previously reported[2].

Fig. 5. Variation in the time of ATP addition.
The conditions were those described by the legend of Fig. 1 except as indicated below. The addition of N-OAc-AAF is indicated by the first arrow of each graph. The addition of ATP (tris salt) in a volume of 0.05 ml by means of the adding-mixing device is indicated by the second arrow.



In Fig. 5, N-OAc-AAF was added to the mitochondrial suspension before ATP was added. The induction period required for the onset of swelling was not influenced by varying the

interval of time between the addition of N-OAc-AAF and the addition of ATP. The later the addition of ATP the larger the mitochondrial change.

N-OAc-AAF induced less initial ATPase activity than N-OH-AAF (Table 1). Showdomycin stimulated the ATPase activity and ATP energized volume change induced by N-OAc-AAF (Fig. 4 and Table 1). Although showdomycin was a requirement for the ATP energized volume change induced by N-OH-AAF (Fig. 4) showdomycin did not appreciably alter the ATPase activity induced by N-OH-AAF (Table 1). Sulfate ion stimulated the ATP energized mitochondrial volume change induced by N-OAc-AAF (Fig. 2) but did not alter the ATPase activity induced by N-OAc-AAF (Table 1).

State 3 mitochondrial respiration and DNP uncoupled respiration were inhibited by 100 μM

Table 1. ATPase activity of N-OAc-AAF (in nmoles Pi/ml/min)

Additions	ATP (333 μM)	N-OAc-AAF (300 μM)	N-OH-AAF (300 μM)
	23	50	84
Showdomycin (300 μM)	24	90	82
Sulfate (2 mM)	22	50	

The assay procedure for phosphate employing the specially designed cuvettes is described in "Materials and Methods." The final volume of the system was 15 ml. The initial velocity of ATP hydrolysis was taken from the first linear portion of the curve.

Fig. 6. Effect of N-OAc-AAF on mitochondria respiration.

The final concentrations were; sucrose 225 mM, tris chloride pH 7.4 20 mM, tris phosphate pH 7.4 10 mM, tris β-hydroxybutyric acid pH 7.4 12 mM, potassium chloride 20 mM, magnesium chloride 5 mM. Either ADP, DNP or N-OAc-AAF (in acetone) was added in a volume of 0.02 ml via a micro syringe fitted with a stainless steel needle (20 gauge) as indicated by arrows. The final volume of the system was 3 ml. The mitochondrial protein concentration was 1.0 mg/ml.

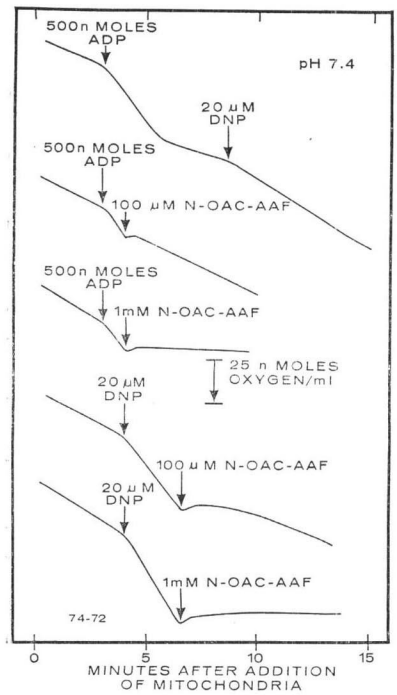
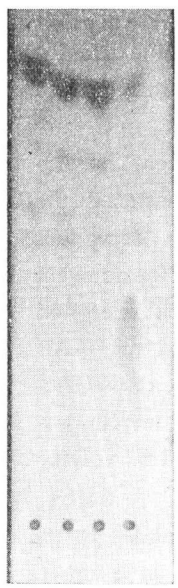


Fig. 7. Thin-layer chromatographic analysis of N-OAc-AAF incubated with mitochondria.

See "Materials and methods" for conditions of incubation and extraction.

The spots from left to right are as follows:

1. Boiled mitochondria, 0.5-min incubation.
2. Boiled mitochondria, 30-min incubation.
3. Native mitochondria, 0.5-min incubation.
4. Native mitochondria, 30-min incubation.



The prominent spots were those associated with N-OAc-AAF (Rf 0.90) and N-OH-AAF (Rf 0.46 with tailing). In order to quantify the prominent spots, the absorbant (1 cm) was scraped off, the developed plate, about each spot. The organic materials were eluted from the absorbant with 2 ml of methanol (distilled). After centrifugation the absorbance at 275 nm was read in quartz cuvettes with a 1-cm light path. 275 nm corresponds to or is close to an absorption maxima of the two compounds. The absorbance of spots of N-OAc-AAF from left to right are: 508, 481, 437, 81. The absorbance of spots of N-OH-AAF from left to right are: 11, 13, 8, 141. The N-OH-AAF always had characteristic trailing in the solvent system used. The other spots, which may be metabolic or artifactual, remain unidentified, and were not present in the N-OAc-AAF.

N-OAc-AAF and respiration was completely blocked by 1 mM N-OAc-AAF when the mitochondrial concentration was 1.25 mg mitochondrial protein per ml (Fig. 6). Inhibition was immediate and sharp. The data does not reveal which coupling sites were blocked.

Rat liver mitochondria mediated an enzymic cleavage of N-OAc-AAF to yield N-OH-AAF (Fig. 7).

In all instances (Figs. 1 and 3) when N-OAc-AAF was present either alone or in combination with other reagents (*e.g.* DNP or antimycin) so that no ATP energized increase in mitochondrial volume was observed there was a decided decrease in volume as indicated by an increase of absorbance at 520 nm.

Discussion

Neither ATP alone nor N-OAc-AAF alone was capable of inducing an increase in mitochondrial volume, however, such an effect was observed when these two agents were used in combination (Fig. 1). This observation held true whether the N-OAc-AAF was prepared as described by GUTMANN and ERICKSON[27] or by the method of LOTLIKAR *et al.*[14] or by the method described in this report. Since the ATP energized effect was inhibited by rutamycin (Figs. 1 and 3) and such inhibition is characteristic of ATP energized mitochondrial processes involving the machinery of oxidative phosphorylation[29,30,31] it follows that N-OAc-AAF interacted with the machinery of oxidative phosphorylation.

The induction period of the ATP energized phenomenon (Fig. 5) did not vary when the time between the addition of mitochondria and the subsequent addition of ATP was progressively increased. This suggests that the induction period prior to the observable decrease in absorbancy was due to a reaction between mitochondria and N-OAc-AAF. Once this reaction had taken place to an adequate extent, ATP was able to energize the volume change. Furthermore, ATP is not required for the reaction to take place during the induction period. As N-OAc-AAF set up an ATPase activity in the mitochondria (Table 1) the amount of ATP present at the end of the induction period, presumably was greater the greater the delay between the addition of mitochondria and the addition of ATP. This would explain the graded increase in response seen in Fig. 5. This also explains why reagents (*e.g.* DNP, the high level of antimycin) which set up substantial ATPase activity prevent the ATP energized volume change phenomenon induced by N-OAc-AAF.

Whenever N-OAc-AAF was added to the mitochondria and no ATP energized mitochondrial expansion took place (*i.e.* N-OAc-AAF alone, N-OAc-AAF plus ATP plus one of rutamycin, a high concentration of antimycin, or DNP) a definite delayed mitochondrial contraction was observed. A slight contraction was also characteristic of the induction period previously discussed. These observations are all compatible with a reaction between N-OAc-AAF and mitochondria occurring during the induction period.

Showdomycin stimulated both the ATP energized mitochondrial volume change (Fig. 4) and ATPase activity (Table 1) induced by N-OAc-AAF. In contrast while showdomycin was a requirement for the ATP energized mitochondrial volume change induced by N-OH-AAF (Figs. 1 and 4), showdomycin had little effect on the ATPase activity induced by N-OH-AAF (Table 1). Acetate alone (Fig. 1) or in combination with showdomycin (Fig. 4) did not induce an ATP energized mitochondrial volume change. Thus the reaction of N-OAc-AAF during the induction period could not be attributed to two of its possible products *viz.* N-OH-AAF and acetate. Nevertheless the enzymic conversion of N-OAc-AAF to N-OH-AAF was demonstrated (Fig. 7). Thus in addition to N-OAc-AAF undergoing a cleavage reaction which yielded N-OH-AAF there is some other reaction involving N-OAc-AAF and the machinery of oxidative phosphorylation.

Like N-OH-AAF[2] N-OAc-AAF was a respiratory inhibitor (Fig. 6). The sharp response reflected the easy penetration of the mitochondria by a non-polar, reactive substance.

Sulfate ion increased the rate of the ATP independent reaction between mitochondria and the electrophile N-OAc-AAF which took place during the induction period as the induction period was decrease but the ATPase activity remained unaltered (Fig. 2, Table 1). It is cogent that sulfate ion has been implicated as a requirement for liver carcinogenesis induced by N-OH-AAF[10,11,12,13].

The sulfotransferase which converts N-OH-AAF to N-OSO_2OH-AAF has been located in the cytosol of the rat liver[11,32,13,33]. When N-OSO_2OH-AAF was generated *in vitro* it did not bind to nuclear nucleic acid enclosed by the nuclear membrane[13]. If binding to nucleic acid is considered essential for carcinogenesis the route whereby N-OSO_2OH-AAF affects the nuclear genome is moot. One should recognize, however, the data of WEISBURGER *et al.*[13] does not eliminate all *in vivo* possibilities. On the other hand, if mitochondrial disturbance is significant for carcinogenesis the *in vitro* data with N-OAc-AAF presented in this report supports such a thesis and directs attention to our unitary theory for carcinogenesis[2,6].

While both AAF and N-OH-AAF produced tumors in rat ZYMBAL's and mammary glands as well as liver, N-OH-AAF sulfotransferase was found in only in liver[11,32,33]. Although rat embryo fibroblasts were transformed by N-OH-AAF the sulfotransferase was not detected in these cells[34]. The macromolecular binding theory accordingly requires the replacement of N-OSO_2OH-AAF by another electrophilic agent[7] in certain instances. On the other hand, because N-OH-AAF as well as N-OAc-AAF interacted with the machinery of oxidative phosphorylation it is our alternative suggestion, that N-OH-AAF itself may be the ultimate carcinogen in rat ZYMBAL's and mammary glands and embryo fibroblasts. YOST, GUTMANN and RYDELL[35] found that while both N-hydroxy-N-acetyl-3-aminofluorene and N-acetoxy-N-acetyl-3-aminofluorene were carcinogenic in the rat the N-acetoxy compound did not behave like a model ultimate electrophilic carcinogenic agent and generate the arylamidonium moiety. This is compatible with our suggestion that the ultimate carcinogen may in certain circumstances be the hydroxamic acid.

The search for an alternative electrophile to N-OSO_2OH-AAF has recently been extended to include N-acetoxy-2-aminofluorene (N-OAc-AF)[36,37,38]. N-OAc-AF is believed to be a potent electrophile derived enzymically from N-OH-AAF via a transacylase reaction. Our data with N-OAc-AAF certainly raises the possibility that N-OAc-AF could disturb the machinery of oxidative phosphorylation. No direct experimentation with N-OAc-AF is yet possible as the successful synthesis of N-OAc-AF remains to be accomplished[36,38].

The assignment of the role of model ultimate carcinogen to N-OAc-AAF rather than to N-OH-AAF depends strongly upon the greater local carcinogenic potency of N-OAc-AAF (subcutaneous injection in the rat) relative to N-OH-AAF[23,7]. This conclusion is not unequivocal because the reported greater potency of N-OAc-AAF could be due to the lesser polarity of N-OAc-AAF (an anhydride) relative to N-OH-AAF (a hydroxamic acid). Thus N-OAc-AAF could penetrate the cell more readily. Our observed Rf values for N-OAc-AAF, AAF, and N-OH-AAF, 0.92, 0.60 and 0.46 respectively, are in agreement with these relative polarities. The assignment to N-OH-AAF, the role of metabolite involved in carcinogenesis is also based upon the greater local carcinogenic potency of N-OH-AAF (by subcutaneous injection in the rat, mouse, hamster, rabbit and guinea pig) relative to AAF[23,7]. This conclusion is strengthened by the type of argument used above, because even though N-OH-AAF (a hydroxamic acid) is more polar and, therefore, presumably less permeant through cellular membranes than AAF (an acetamide), N-OH-AAF has a greater carcinogenic potential. In this instance, a chance correlation between the ability of an agent to penetrate the cell and carcinogenicity does not exist.

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