THE INDUCTION OF ATP ENERGIZED MITOCHONDRIAL VOLUME CHANGES BY CARCINOGENIC N-HYDROXY-N-ACETYL-AMINOFLUORENES WHEN COMBINED WITH SHOWDOMYCIN. A UNITARY HYPOTHESIS FOR CARCINOGENESIS[*,**,***]

HERBERT I. HADLER, BARBRA G. DANIEL
and RAYMOND D. PRATT

Department of Chemistry, Southern Illinois University,
Carbondale, Illinois 62901, U.S.A.

N-Hydroxy-N-acetyl-2-aminofluorene, a well-known carcinogenic meta-bolite (MILLER and MILLER) derived from the carcinogen N-acetyl-2-amino-fluorene induced an ATP energized mitochondrial volume change when com-bined with the thiol reagent showdomycin. Neither N-acetyl-2-aminofluorene nor showdomycin by themselves induced an ATP enegized mitochondrial volume change. Showdomycin could be replaced by other thiol reagents. The ATP energized mitochondrial volume change induced by gramicidin was partially inhibited by N-hydroxy-N-acetyl-2-aminofluorene and the inhibition was relieved by showdomycin. N-Hydroxy-N-acetyl-2-aminofluorene inhibited mitochondrial respiration during a state of active phosphorylation. In accor-dance with the reasoning in our previous publications it was deduced that N-hydroxy-N-acetyl-2-aminofluorene inhibited the cycle which meshed with the respiratory chain and exposed a strategically located pivotal mitochondrial thiol group.

The following data suggested a confluence of experimentation in oxidative phosphorylation and carcinogenesis. The parent carcinogen N-acetyl-2-amino-fluorene plus a thiol reagent did not induce an ATP energized mitochondrial volume change in keeping with a requirement for metabolic conversion of the parent carcinogen in order to engender carcinogenesis. N-Acetyl-3-amino-fluorene (a marginal carcinogen in the rat as observed by GUTMANN) did not induce an ATP energized mitochondrial volume change in combination with a thiol reagent. N-Hydroxy-N-acetyl-3-aminofluorene which could be derived synthetically but not metabolically from the parent marginal carcinogen was carcinogenic (GUTMANN) and did induce an ATP energized mitochondrial volume change when combined with showdomycin.

It was hypothesized that when the mitochondria were damaged, genetic material could be released from the mitochondria. The released genetic material could behave like an oncogenic virus and enter the nuclear genome. Thus carcinogenesis resulted from a disruption of the symbiotic relationship between the mitochondria and the host cell previously established during the course of evolution. In effect, carcinogenesis represented a *partial reversal of evolution.*

During the past several years, we have been using antibiotics and other exogenous agents of known structure to identify the functional groups of mitochondria involved in the related phenomena of oxidative phosphorylation, ion transfer and energized mitochondrial volume change.[1~8] Irrespective of the mechanism of energized mitochondrial volume changes, the occurrence of an ATP energized mitochondrial volume change dependent upon an agent or group of agents clearly indicated an interaction of the agent or group of agents with the mitochondria. As the exogenous agent or agents were of known structure, it was possible to recognize the nature of the mitochondrial functional groups which were involved.

It was deduced that a pivotal mitochondrial thiol group was strategically located between two catalytic cycles[6]. One of the cycles meshed with the respiratory chain and the other cycle meshed with ATP, ADP, and Pi. The pivotal thiol group was exposed (that is, made reactive) either by the addition of an uncoupling agent such as DNP[5] or by the addition of a respiratory inhibitor such as antimycin[4]. When the exposed pivotal thiol group became conjugated with a thiol reagent such as the antibiotic showdomycin or the mercurial thiol reagent PHMB an ATP energized mitochondrial volume change was induced.[4,5]

The interaction of the nucleophilic terminal hydroxyl group of the antibiotic gramicidin with a non-phosphorylated electrophylic center located in the cycle which meshes with ATP, ADP and Pi had been previously inferred[3]. It was shown that gramicidin was not inactivated when the terminal hydroxyl group was acetylated but was inactivated when the terminal hydroxyl group was methylated. Also when the gramicidin effect was energized by ATP, oligomycin was inhibitory[1] and when the gramicidin effect was energized by respiration oligomycin was not inhibitory while a low level of DNP (50 μM) was inhibitory[2].

These studies were extended to other well known agents of known structure which interacted with mitochondria[8]. Dicoumarol and pFCCP mimicked DNP while the nonmercurial thiol reagent NEMI resembled showdomycin. The antibiotic valenomycin behaved like the antibiotic gramicidin. The concept of a strategically located pivotal mitochondrial thiol group thus rationalized the behavior of many exogenous agents and hence was of general significance.[8] The action of the lipophilic hydroxyquinone lapachol (an antitumor agent) resembled DNP. This observation was in keeping with our hypothesis for oxidative phosphorylation[9] which involved quinones, hydroxyquinones, and hydroquinones.

It has been unpopular for some time to link the phenomena of experimental carcinogenesis with either an impairment of respiration[10,11] or with damage to the

mitochondria as suggested by WARBURG[12] many years ago. For some time research dealing with the mechanism of carcinogenesis has emphasized the binding of the carcinogen or one of its metabolite to either protein or nucleic acid[13~30]. The evaluation of binding to nucleic acids is currently under intensive investigation[25].

Fig. 1. Structural formulae.

The thesis that binding to an intracellular macromolecule is an essential interaction leading to carcinogenesis does not rule out, however, other possible decisive interactions. Accordingly, we have examined on an empirical basis the possibility that target sites relevant to the process of carcinogenesis, exist in mitochondria. Our search for such an interaction was stimulated by our success with the antitumor agent lapachol[7] as a similarity in properties of antitumor agents and carcinogens has often been noted[31]. Lapachol is a vinylogue of a carboxylic acid and accordingly is an acidic molecule.

The structures of the many other agents (antimycin, DNP, dicoumarol, pFCCP, lawsone) which we have postulated to interact with the catalytic cycle which meshes with the respiratory chain also suggested that a compound with an acidic functional group could interact with this cycle. Much of the most recent[25] exciting work in carcinogenesis has been done with the neutral carcinogenic amide AAF (Fig. 1) and with the hydroxamic acid N-OH-AAF (a carcinogenic metabolite of AAF (Fig. 1), and with acid anhydride derivatives of N-OH-AAF, e.g. N-OAc-AAF (Fig. 1). These considerations suggested that the hydroxamic acid N-OH-AAF might interact with the catalytic cycle which meshes with the respiratory chain. If such an interaction did indeed take place, then the interaction could be visualized as an ATP energized mitochondrial volume change on the further addition of a thiol reagent such as showdomycin.

It is the purpose of this report to present data showing that N-OH-AAF interacts with the catalytic cycle which meshes with the respiratory chain and that this interaction is relevant to the mechanism of carcinogenesis.

Methods

The procedures and methods have been previously described[1,4,8]. The pH of the tris-chloride buffer is indicated on the diagrams. Incubations were at 27°C in standard rectangular quartz cuvettes with a 1-cm path. The basic reaction mixture had a final volume of 3 ml and contained 0.75 mg of mitochondrial protein (prepared from rat liver); 333 μM ATP (tris salt) which was added in 0.05 ml by means of the adding-mixing device[2] as indicated by an arrow on the diagrams; 75 mM sucrose; and 75 mM tris-chloride buffer. A decrease in absorbancy at 520 nm was considered to be a measure of mitochondrial swelling. A Model 2000 automatic spectrophotometer manufactured by Gilford Instrument Laboratories, Incorporated, Oberlin, Ohio, was used. All cations were added in the form of chloride salts and all anions were added in the form of tris salts, neutralized to pH 7.4. Solutions of AAF, AAF-3, N-OH-AAF and N-OH-AAF-3 were prepared in 95 % ethanol (distilled) and added to the incubation mixtures in a volume of 0.06 ml. All controls contained the appropriate amount of alcohol. The figures and legends provide further experimental details.
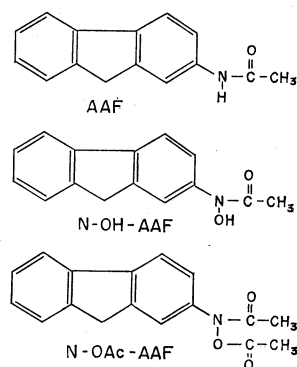
Oxygen consumption was measured in an oxygen monitor which utilized a Clark electrode. The instrument (Model 53 SA) was manufactured by Yellow Springs Instrument Company, Yellow Springs, Ohio. A 10″ recorder, Model 1005, manufactured by Beckman Instruments, Fullerton, California was used in conjunction with the oxygen monitor.

Commercial gramicidin, obtained from Mann Research Laboratories, Inc. (New York, N. Y., U.S.A.) was used. The activity of such a mixture of gramicidins had been previously shown to be indistinguishable from gramicidin A[3]. The molecular weight of the mixture was arbitrarily taken as 1,870. We wish to thank Dr. KEN'ICHI TAKEDA of Shionogi and Company, Osaka, Japan for the showdomycin used in this work. Dr. HELMUT GUTMANN (Minneapolis, Minn., U.S.A.) generously provided samples of AAF-3 and N-OH-AAF-3 and Dr. ELIZABETH WEISBURGER (Washington D. C., U.S.A.) kindly provided N-OH-AAF. AAF was purchased from Mann Research Laboratories (New York, N. Y., U.S.A.), and crystallized four times from ethanol and water.

Results

It is seen in Fig. 2 that the combination of 300 μM showdomycin with 300 μM N-OH-AAF induced an ATP energized mitochondrial volume change at pH 7.4. The phenomena was inhibited by the antibiotic oligomycin and thus was related to oxidative phosphorylation.[32] The enhancement of the phenomena by graded increases in the concentration of N-OH-AAF is shown in Fig. 3. In Fig. 4, the enhancement of the phenomena by increasing the pH from 7.4 to 7.8 is shown and thus the data supports the involvement of a ionizable nucleophilic thiol group. The addition of 2 mM potassium ions had a minimal effect (Fig. 5). The addition of 2 mM malate ions increased the period of the oscillation in a characteristic empirical manner (Fig. 5) observed in other experiments.[6,7] The similar effectiveness of 300 μM of the thiol reagent[33] showdomycin with appropriate amounts of other thiol reagents viz., 30 μM NEMI and 3 μM PHMB was observed (Fig. 6). Again, with all three thiol

Fig. 2. N-OH-AAF combined with showdomycin and effect of oligomycin.

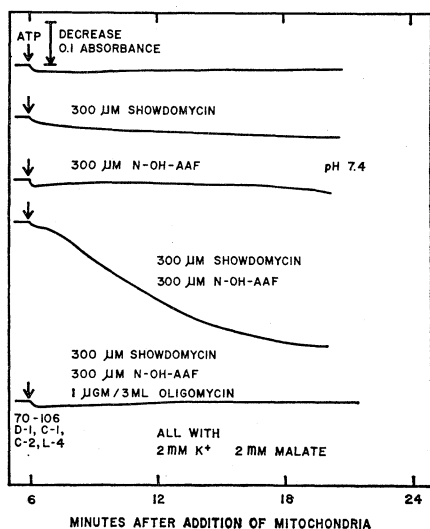Basic medium, see methods.

Fig. 3. Various concentrations of N-OH-AAF.

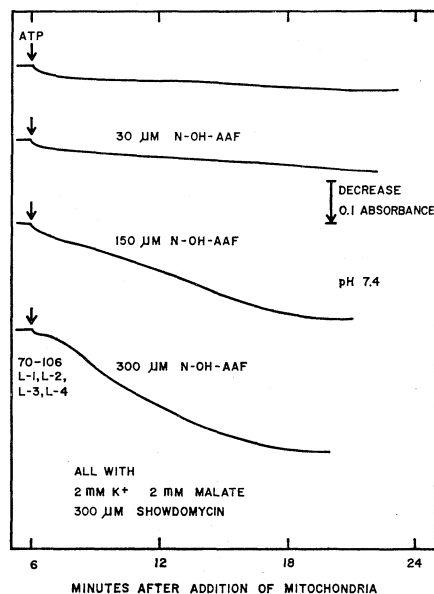Basic medium, see methods.

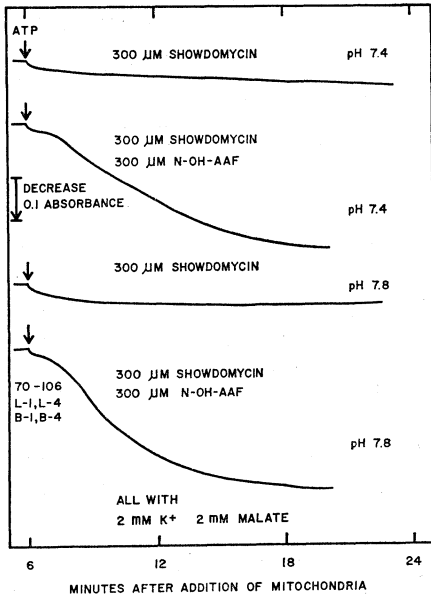Fig. 4. Effect of pH.

Basic medium, see methods.

Fig. 5. Role of potassium ion and L malate.
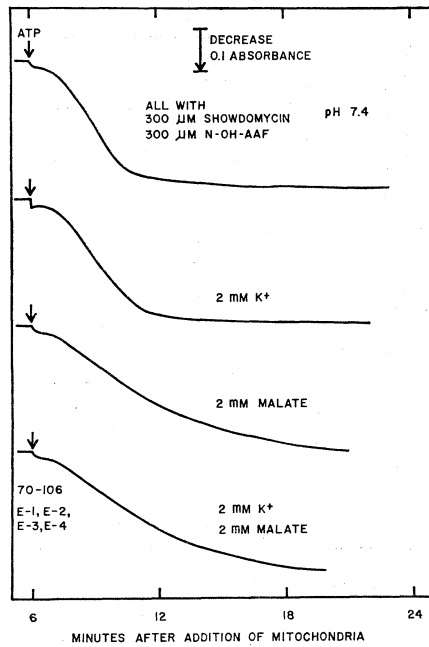
Basic medium, see methods.

Fig. 6. Various thiol reagents.
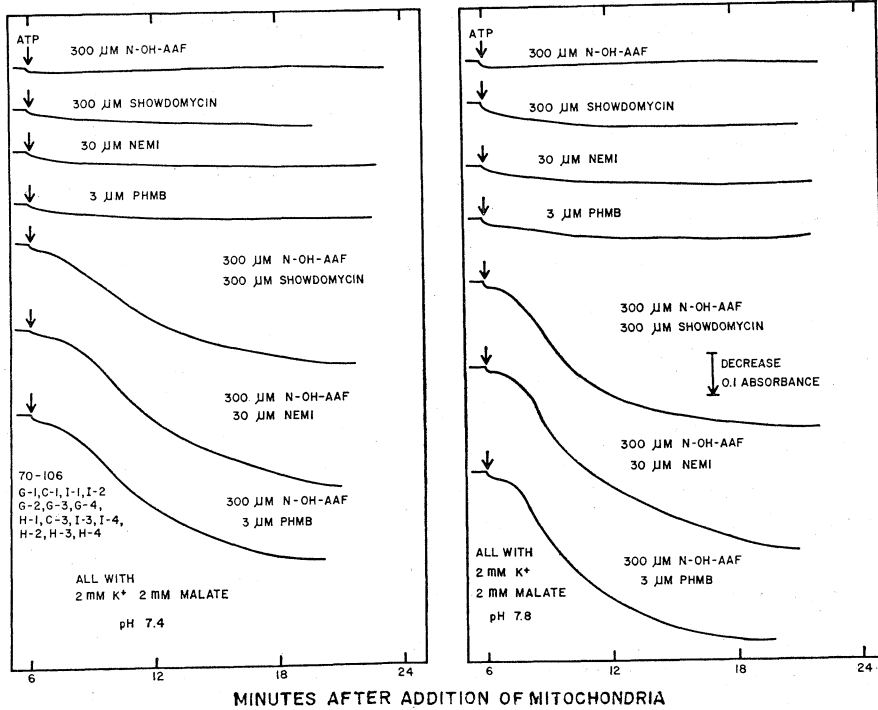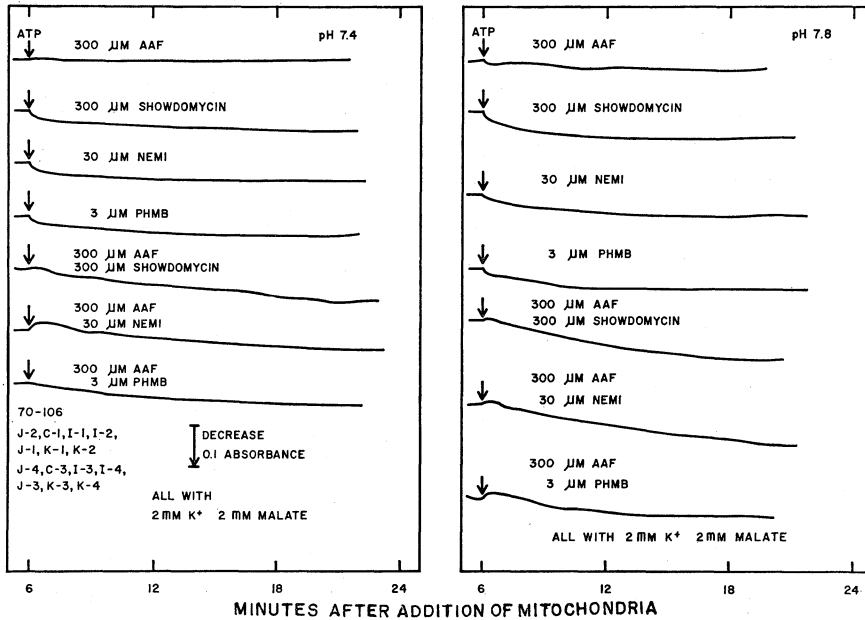
Basic medium, see methods.

Fig. 7.   AAF combined with various thiol reagents.
Basic medium, see methods.

reagents the phenomena was enhanced when
the pH was raised from 7.4 to 7.8.   In Fig. 7
the ineffectiveness of the carcinogen AAF
when combined with the thiol reagents showdo-
mycin, NEMI, and PHMB is observed at pH
7.4 and 7.8.   It is seen in Fig. 8 that the ATP
energized mitochondrial volume change induced
by the antibiotic gramicidin in combination
with the permeant ions potassium and malate
was partially inhibited by the addition of 300
μM N–OH–AAF.   The inhibition was relieved
by the further addition of showdomycin to
the combination of gramicidin plus N–OH–AAF
(Fig. 9).   In all the responsive systems contain-
ing gramicidin (Fig. 10) $i.e.$, gramicidin alone,
gramicidin plus N–OH–AAF plus showdomycin,
and gramicidin plus showdomycin, the addition
of potassium ion was a requirement or had a
decided effect in enhancing the phenomena.
This is in agreement with our previous observa-
tions[6,7] and it follows that the gramicidin
system remained intact in the relieved system
of gramicidin plus N–OH–AAF plus showdo-
mycin.   It is also seen that the addition of

Fig. 8.   Inhibition of gramicidin
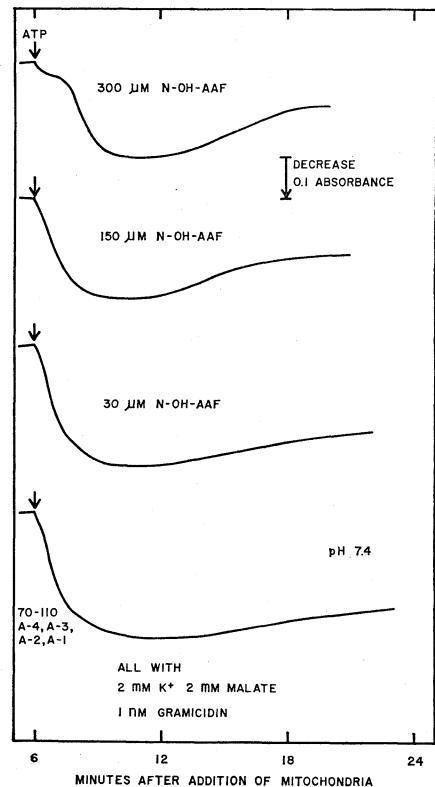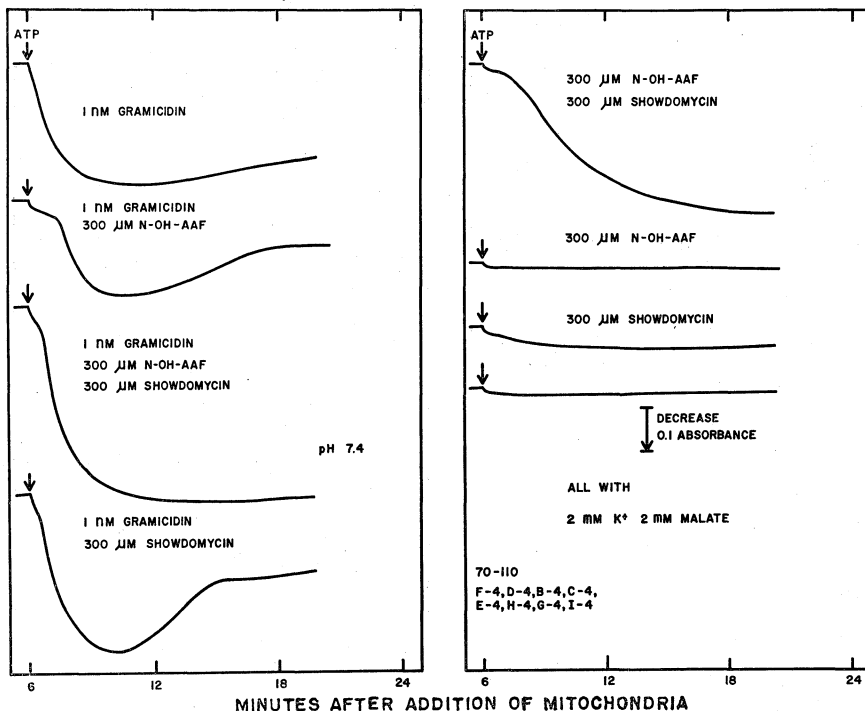system by N–OH–AAF.
Basic medium. see methods.

Fig. 9. Showdomycin plus the inhibited system.
Basic medium, see methods.

malate to the relieved system which contained showdomycin (that is, N–OH–AAF
plus showdomycin plus gramicidin) caused an increase in the amplitude of oscillation.
This increase is characteristic of active systems dependent upon showdomycin.[6,7] It
is seen in Fig. 11 that AAF–3 when combined with showdomycin was inactive while
N–OH–AAF–3 when combined with showdomycin was active in inducing an ATP
energized mitochondrial volume change and the phenomenon was enhanced when the
pH was raised from 7.4 to 7.8.

The inhibition of respiration by N–OH–AAF of an active coupled mitochondrial
system, is clearly shown in Fig. 12.

Discussion

N–OH–AAF when combined with one of the reagents viz, showdomycin, NEMI or
PHMB induced an ATP energized mitochondrial volume change. The effect was enhanced
by raising the pH from 7.4 to 7.8. The ability of showdomycin[33] and the other thiol
reagents to conjugate with thiols and the effect of raising the pH implicated the exposure
of an ionizable mitochondrial thiol group by N–OH–AAF. The volume change data did
not determine whether N–OH–AAF was an uncoupling agent such as DNP or a respiratory
inhibitor such as antimycin. The experiment with the oxygen electrode established N–
OH–AAF as a respiratory inhibitor.

In accordance with our previous rationalizations regarding the respiratory inhibitors
rotenone[5] and antimycin[4] N–OH–AAF interacted with the cycle which meshed with the
respiratory chain. Because of this interaction a strategically located mitochondrial thiol
group became unable to conjugate with its normal electrophilic acceptor located in the
cycle which meshed with the respiratory chain. The thiol group thus became exposed

Fig. 10.   Role of ions in the reinstated system.
Basic medium, see methods.

MINUTES AFTER ADDITION OF MITOCHONDRIA
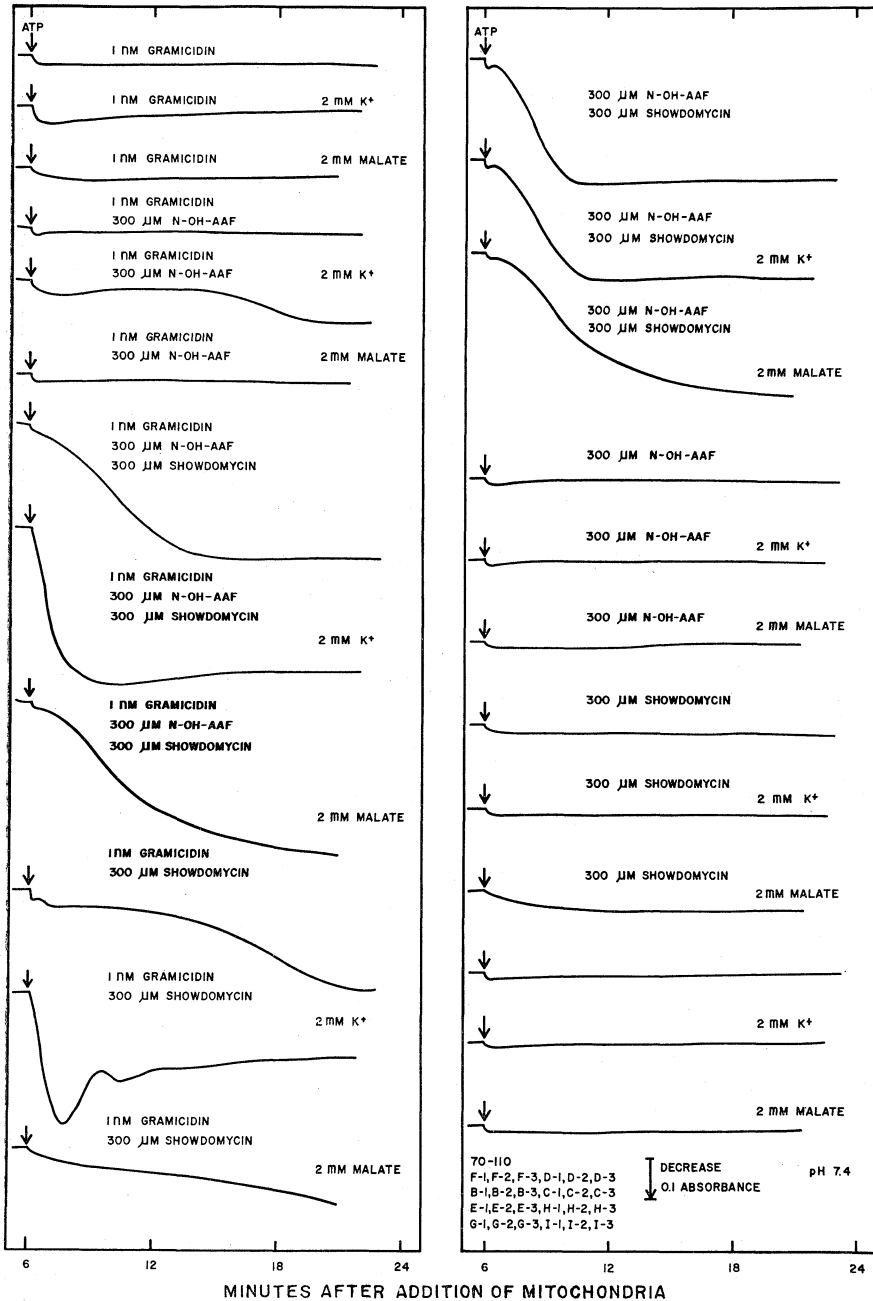
i.e. capable of conjugating with an exogenous thiol reagent such as showdomycin, NEMI, or PHMB. This conjugation resulted in an ATP energized mitochondrial volume change to be induced by the combination of N-OH-AAF plus a thiol reagent.

The pivotal nature of the strategically located mitochondrial thiol group was revealed as follows: Firstly, the exposed nucleophilic thiol group was able to compete with the nucleophilic hydroxyl of gramicidin for the nonphosphorylated electrophilic center in the cycle which meshed with ATP, ADP and Pi. As a result of this competition, the ATP

Fig. 11. AAF-3 and N-OH-AAF-3 combined with showdomycin.
Basic medium, see methods.
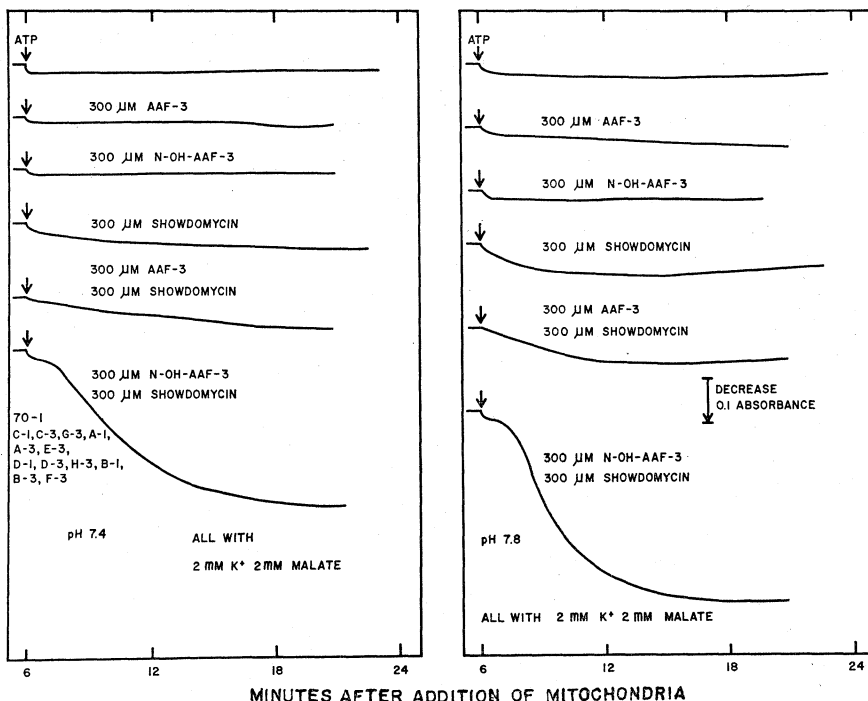
MINUTES AFTER ADDITION OF MITOCHONDRIA

energized mitochondrial volume change induced by grami-cidin was partially inhibited by N-OH-AAF. Secondly, the partial inhibition of the gramicidin system was re-lieved by adding showdomycin which in our explanation essentially tied up and thus eliminated the exposed com-petitive thiol group responsible for the partial inhibition. The pivotal thiol group thus was able to communicate with the cycle which meshed with the respiratory chain and the cycle which meshed with ATP, ADP, and $Pi^{6)}$.

The magnitude of the mitochondrial volume change induced by the combination of N-OH-AAF plus showdo-mycin closely resembled that previously reported for the combination of antimycin plus showdomycin.[4,5] It is likely that N-OH-AAF inhibits the second coupling site of the respiratory chain. The volume change data does not however reveal this information. Other types of future experiments will be necessary to determine the precise locale of the respiratory chain which is inhibited by N-OH-AAF.

The carcinogen AAF, unlike its carcinogenic metabolite N-OH-AAF[34~37] did not induce a significant ATP energized mitochondrial volume change when combined with any of the thiol reagents showdomycin, or NEMI, or PHMB.

The in vivo carcinogenic and N-hydroxylation[34~37] data and the in vitro mitochondrial volume change data may be related as follows: When the carcinogen AAF is

Fig. 12. Effect of N-OH-AAF on mitochondrial respiration.

The total volume of the system was 3.0 ml. The final concen-trations were sucrose 0.25 M; KCl 20 mM; $MgCl_2$ 5 mM; potas-sium phosphate buffer (pH 7.2), 10 mM; Tris chloride (pH 7.4), 20 mM; β-hydroxybutyric acid 13 mM; N-OH-AAF was added in 0.1 ml 95 % ethanol. The 95 % ethanol by itself did not effect the rate of respiration in other control experiments. There were 5 mg of mitochondrial pro-tein in the 3 ml system.
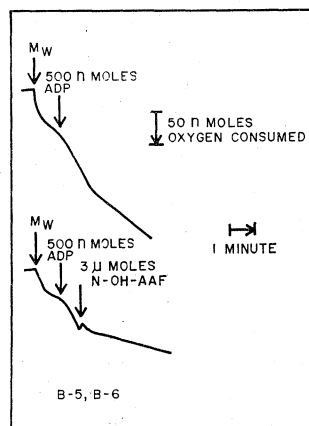
fed to a rat, the carcinogen becomes hydroxylated at the endoplasmic reticulum of a liver cell (as has been observed by others *in vitro*).[38] The generated N-OH-AAF then leaves the endoplasmic reticulum, interacts with the mitochondria and exposes the strategically located pivotal mitochondrial thiol group. A mechanistic requirement for N-hydroxylation is thus established. These data point to a useful experimental confluence of the fields of oxidative phosphorylation and carcinogenesis.

The observations with AAF-3 and N-OH-AAF-3 provided further support for the experimental confluence of carcinogenesis with oxidative phosphorylation. GUTMANN et al.[39] observed that AAF-3 was a marginal carcinogen in the rat and was not converted by the rat *in vivo* to N-OH-AAF-3. When N-OH-AAF-3 was prepared synthetically, the synthesized hydroxamic acid proved to be carcinogenic in the rat.[39] Accordingly, N-OH-AAF-3 like N-OH-AAF should interact with mitochondria and expose the strategically located pivotal mitochondrial thiol group while AAF-3 should not interact with the mitochondria in this manner. It was observed that AAF-3 in combination with showdomycin did not induce an ATP energized mitochondrial volume change while N-OH-AAF-3 in combination with the thiol reagent showdomycin or NEMI or PHMB did induce an ATP energized mitochondrial volume change. Thus the *in vitro* mitochondrial data are in agreement with the *in vivo* carcinogenic and metabolic data from GUTMANN's laboratory.

These observations do suggest a relationship between an interference with the flux of energy through the mitochondria and chemical carcinogenesis. There are reports which have focused attention on changes in mitochondrial structure as a consequence of chemical carcinogenesis. CLERICI and CUDKOVICZ[40] fed the carcinogenic azo dye 4-dimethylaminazobenzene and observed by means of electron microscopy an increased rigidity in rat liver mitochondria. EMMELOT and BOS[41] fed the carcinogen 4-monomethylaminoazobenzene for five months to rats and observed that the swelling of rat liver mitochondria induced by thyroxine was reduced. ARCOS and coworkers[42,43] also used mitochondrial swelling as an index of structural alteration in the mitochondria during chemical carcinogenesis. They observed drastic oscillatory changes in rat liver mitochondria when they fed carcinogenic azo dyes, AAF, and ethionine. Non carcinogens did not produce drastic effects on the mitochondrial swelling index. These workers also observed a decided decrease in the mitochondrial content (as measured by mitochondrial protein) of rat liver during azo dye carcinogenesis[42] and thus confirmed the earlier work of PRICE et al.[44,45] and STRIEBICH et al.[46] Other investigators drew the broad conclusion that neoplastic cells have less mitochondria than normal cells.[11,47~50]

There are therefore several pieces of evidence linking an alteration of mitochondrial function or structure with carcinogenesis. Nevertheless, in view of current research which associates changes in the genome with viral carcinogenesis[51,52] the thesis that an alteration of mitochondrial machinery or structure has a causal relationship to carcinogenesis is not very satisfying. Accordingly, the following hypothesis which relates a disturbance in oxidative phosphorylation to nuclear genetic alteration and carcinogenesis is proposed.

Many investigators agree that during the course of evolution mitochondria was a procaryotic cell which invaded a host cell. As a consequence of evolution the mitochondria and the host cell developed a symbiotic existence with each other. (There are several recent excellent reviews on this subject.)[53,54,55] We have noted with interest that the mitochondria is an organelle with its own relatively simple unique compliment of DNA and RNA. It is thus possible that the mitochondrial genetic material which is normally separated by a membrane system from the remainder of the cell may not necessarily be compatible with the normal genetic material in the nucleus of the cell. It is also known that when an appropriate exogenous oncogenic virus invades a host cell and alters the nuclear genome,[51,52] cancer develops.

We do hypothesize that when the mitochondrial machinery for oxidative phosphorylation is disturbed in a subtle fashion by an agent which is not present in adequate

concentration to kill the cell, the mitochondrial membrane is altered. It then becomes possible for genetic material to leak through the mitocondrial membrane (in perhaps a single mitochondrion) and act like an exogenous oncogenic virus which has penetrated the cellular membrane. The mitochondrial genetic material may thus eventually alter the genome of the cell. In this way, a cancerous cell may be generated. Essentially the symbiotic relationship that existed between mitochondria and the host cell has been disrupted and *evolution has been partially reversed*. In this suggested scheme the chemical carcinogen engendered a process which released a latent oncogenic virus which was part of the normal genetic makeup of the mitochondria.

There also could be other means of damaging the membrane of the mitochondria (*e. g.* radiation injury or heat). Our hypothesis is accordingly a unitary hypothesis for carcinogenesis. Perhaps future research will implicate the release of other oncogenic genetic material from other organelles.

We are currently examining other classes of carcinogens and plan to report that appropriate metabolites of other classes of carcinogens also interfere with the normal flux of energy in the mitochondria.

The energized mitochondrial volume change phenomena might eventually become a useful rapid *in vitro* test which can help detect environmental carcinogenic hazards. Basic principles and generalizations are necessary to begin to cope with the potential biological hazards of the multitude of new compounds which have become part of the modern environment.

References

1) FALCONE, A. B. & H. I. HADLER: Action of gramicidin on mitochondria. I. Ion-dependent mitochondrial volume changes energized by adenosine-5'-triphosphate. Arch. Biochem. Biophys. 124 : 91~109, 1968

2) HADLER, H. I. & A. B. FALCONE: Action of gramicidin on mitochondria. II. Ion-dependent mitochondrial volume changes energized by substrate oxidation. Arch. Biochem. Biophys. 124 : 110~114, 1968

3) FALCONE, A. B. & H. I. HADLER: Action of gramicidin on mitochondria. III. Activity of gramicidin derivatives. Arch. Biochem. Biophys. 124 : 115~121, 1968

4) HADLER, H. I.; B. E. CLAYBOURN & T. P. TSCHANG: Mitochondrial volume changes induced by the antibiotic showdomycin. Biochem. Biophys. Res. Commun. 31 : 25~31, 1968

5) HADLER, H. I.; B. E. CLAYBOURN & T. P. TSCHANG: The mode of action of dinitrophenol revealed by mitochondrial volume changes requiring rotenone or antimycin or dinitrophenol with showdomycin. J. Antibiotics 21 : 575~581, 1968

6) HADLER, H. I.; B. E. CLAYBOURN, T. P. TCHANG & T. L. MOREAU: The pivotal position of the mitochondrial thiol group exposed by dinitrophenol located by means of ATP energized mitochondrial volume changes requiring gramicidin, showdomycin, and dinitrophenol. J. Antibiotics 22 : 183~188, 1969

7) HADLER, H. I. & T. L. MOREAU: The induction of ATP energized mitochondrial volume changes by the combination of the two antitumor agents showdomycin and lapachol. J. Antibiotics 22 : 513~520, 1969

8) HADLER, H. I.; B. E. CLAYBOURN & T. P. TSCHANG: Combinations of agents which induce ATP energized mitochondrial volume changes. J. Antibiotics 23 : 276~287, 1970

9) HADLER, H. I.: An hypothesis for oxidative phosphorylation. Experientia 17 : 268~269, 1961

10) LEPAGE, G. A. & F. J. HENDERSON: Biochemistry of tumors. Progress in Experimental Tumor Research 1 : 440~476, 1960

11) WENNER, C. B.: Progress in tumor enzymology. Advances in Enzymology 29 : 321~390, 1967

12) WARBURG, O.: The metabolism of tumors. R. R. Smith, New York, 1931.

13) MILLER, E. C. & J. A. MILLER: The presence and significance of bound aminoazo dyes in the livers of rats fed *p*-dimethylaminoazobenzene. Cancer Research 7 : 468~480, 1947

14) MILLER, E. C. & J. A. MILLER: *In vivo* combinations between carcinogens and tissue constituents and their possible role in carcinogenesis. Cancer Research 12 : 547~556, 1952

15) HEIDELBERGER, C. & M. G. MOLDENHAUER: The interaction of carcinogenic hydrocarbons with tissue constituents. IV. A quantitative study of the binding to skin proteins of several ^{14}C-labeled hydrocarbons. Cancer Research 16 : 442~449, 1956

16) BROOKES, P. & P. D. LAWLEY: Evidence for the binding of polynuclear aromatic hydrocarbons to the nucleic acids of mouse skin. Relationship between carcinogenic power of hydrocarbons and their binding to deoxyribonucleic acid. Nature 202 : 781~784, 1964

17) MARROQUIN, F. & E. FARBER: The binding of 2-acetylaminofluorene to rat liver ribonucleic acid in vivo. Cancer Research 25 : 1262~1269, 1965

18) BROOKES, P.: Quantitative aspects of the reaction of some carcinogens with nucleic acids and the possible significance of such reactions in the process of carcinogenesis. Cancer Research 26 : 1994~2003, 1966

19) MILLER, E. C. & J. A. MILLER: Mechanism of chemical carcinogenesis: Nature of proximate carcinogens and interactions with macromolecules. Pharmacol. Rev. 18 : 805~838, 1966

20) DINGMAN, C. W. & M. B. SPORN: The binding of metabolites of aminoazo dyes to rat liver in vivo. Cancer Research 27 : 938~944, 1967

21) WARWICK, G. P. & J. J. ROBERTS: Persistent binding of butter yellow metabolites to rat liver DNA. Nature 213 : 1206~1207, 1967

22) GOSHMAN, L. M. & C. HEIDELBERGER: Binding of tritium labelled polycyclic hydrocarbons to DNA of mouse skin. Cancer Research 27 : 1678~1688, 1967

23) SWANN, P. F. & P. N. MAGEE: Nitrosamine-induced carcinogenesis. The alkylation of nucleic acids of the rat by N-methyl-N-nitrosourea, dimethylnitrosoamine, dimethylsulphate, and methyl methanesulfonate. Biochem. J. 110 : 39~47, 1968

24) SZAFARZ, D. & J. H. WEISBURGER: Stability of binding of label from N-hydroxy-N-2-fluorenyl-acetamide to intracellular targets, particularly deoxyribonucleic acid in rat liver. Cancer Research 29 : 962~968, 1969

25) MILLER, J. A.: Carcinogenesis by chemicals: An overview—G.H.A. CLOWES memorial lecture. Cancer Research 30 : 559~576, 1970

26) DeBAUN, J. R.; E. C. MILLER & J. A. MILLER: N-Hydroxy-2-acetylaminofluorene sulfotrans-ferase: Its probable role in carcinogenesis and in protein-(methion-s-yl)-binding in rat liver. Cancer Research 30 : 577~595, 1970

27) MAHER, Sr. V. M.; J. A. MILLER, E. C. MILLER & W. C. SUMMERS: Mutations and loss of trans-forming activity of Bacillus subtilis DNA after reaction with esters of carcinogenic N-hydroxy aromatic amides. Cancer Research 30 : 1473~1480, 1970

28) HEIDELBERGER, C.: Chemical carcinogenesis, chemotherapy. Cancer's continuing core challenges-G.H.A. CLOWES memorial lecture. Cancer Research 30 : 1549~1569, 1970

29) GRUNBERG, D. & I. B. WEINSTEIN: Effect of 2-acetylaminofluorene (AAF) binding to guanosine on coding properties of ribopolynucleotides and triplets. Abstracts Tenth International Cancer Congress, May 22~29, 1970, Houston, Texas (U.S.A.). Abstract number 613, page 378

30) LIJINSKY, W.; K. Y. LEE & C. H. GALLAGHER: Interaction of aflatoxins b_1 and g_1 with tissues of the rat. Cancer Research 30 : 2280~2283, 1970

31) CLAYSON, D. B.: Chemical Carcinogenesis. Little Brown and Company, Boston, Massachusetts. pp. 385~386, 1962

32) LARDY, H. A.; D. JOHNSON & W. C. McMURRAY: Antibiotics as tools for metabolic studies. I. A survey of toxic antibiotics in respiratory phosphorylation and glycolytic systems. Arch. Biochem. Biophys. 78 : 587~597, 1958

33) WATANABE, S.: Equimolecular reaction of showdomycin with thiols. J. Antibiotics 23 : 313~314, 1970

34) CRAMER, J. W.; J. A. MILLER & E. C. MILLER: A new metabolic reaction observed in the rat with the carcinogen 2-acetylaminofluorene. J. Biol. Chem. 235 : 885~888, 1960

35) MILLER, J. A.; J. W. CRAMER & E. C. MILLER: The N and ring hydroxylation of 2-acetylamino-fluorene during carcinogenesis in the rat. Cancer Research 20 : 950~962, 1960

36) MILLER, E. C.; J. A. MILLER & H. A. HARTMANN: N-Hydroxy-2-acetylaminofluorene: A meta-bolite of 2-acetylaminofluorene with increased carcinogenic activity in the rat. Cancer Research 21 : 815~824, 1961

37) MILLER, J. A. & E. C. MILLER: The metabolic activation of carcinogenic aromatic amines and amides. Progress in Experimental Tumor Reseach 11 : 273~301, 1969

38) IRVING, C. C.: Enzymic N-hydroxylation of the carcinogen 2-acetylaminofluorene and the metabolism of N-hydroxy-2-acetylaminofluorene-9-C14 *in vitro*. J. Biol. Chem. 239 : 1589~ 1596, 1964

39) GUTMANN, H. R.; D. S. LEAF, L. YOST, R. E. RYDELL & C. C. CHEN: Structure activity relationships of N-acylarylhydroxylamines in the rat. Cancer Research 30 : 1485~1498, 1970

40) CLERICI, E. & G. CUDKOVICZ: Certain effects of *p*-dimethylaminoazobenzene, deficient diet and hypotonic media, on mitochondrial enzymes in experimental rat-liver carcinogenesis. J. Nat. Cancer Inst. 16 : 1459~1470, 1956

41) EMMELOT, P. & C. J. BOS: The effect on thyroxine on the swelling of mitochondria isolated from normal and neoplastic livers. Exp. Cell Research 12 : 191~195, 1957

42) ARCOS, J. C.; G. W. GRIFFITH & R. W. CUNNINGHAM: Fine structural alterations in cell particles during chemical carcinogenesis. J. Biophys. & Biochem. Cytol. 7 : 49~60, 1960

43) ARCOS, J. C.; H. H. GOSCH & D. ZICKAFOOSE: Fine structural alterations in cell particles during chemical carcinogenesis. III. Selective action of hepatic carcinogens other than 3'-methyl-4-dimethylaminoazobenzene on different types of mitochondrial swelling. Effect of stimulated liver growth. J. Biophys. & Biochem. Cytol. 10 : 23~36, 1961

44) PRICE, J. M.; E. C. MILLER, J. A. MILLER & G. M. WEBER: Studies on the intracellular composition of livers from rats fed various aminoazo dyes. I. 4-Aminoazobenzene, 4-dimethyl-aminoazobenzene, 4'-methyl and 3'-methyl-4-dimethylaminoazobenzene. Cancer Research 9 : 398~402, 1949

45) PRICE, J. M.; E. C. MILLER, J. A. MILLER & G. M. WEBER: Studies on the intracellular composition of livers from rats fed various aminoazo dyes. II. 3'-Methyl-2'-methyl and 2-methyl-4-dimethylaminoazobenzene, 3-methyl-4-monomethylaminoazobenzene, and 4'-fluor-4-dimethylaminoazobenzene. Cancer Research 10 : 18~27, 1950

46) STREIBICH, M. J.; M. SHELTON & W. C. SCHNEIDER: Quantitative morphological studies on the livers and liver homogenates of rats fed 2-methyl- or 3'-methyl-4-dimethyl-aminoazobenzene. Cancer Research 13 : 279~284, 1953

47) SCHNEIDER, W. C.: Intracellular distribution of enzymes. II. Distribution of succinic dehydrogenase, cytochrome oxidase, adenosinetriphosphatase and phosphorous compounds in normal rat liver and rat hepatomas. Cancer Research 6 : 685~690, 1946

48) SCHNEIDER, W. C. & G. HOGEBOOM: Intracellular distribution of enzymes. VI. The distribution of succinoxidase and cytochrome oxidase activities in normal mouse liver and in mouse hepatoma. J. Nat. Cancer Inst. 10 : 969~975, 1950

49) WENNER, C. E. & S. WEINHOUSE: Metabolism of neoplastic tissue. IV. Diphosphopyridine nucleotide requirements for oxidations by mitochondria of neoplastic and non neoplastic tissues. Cancer Research 13 : 21~26, 1953

50) AISENBERG, A.: The glycolysis and respiration of tumors. Academic Press, New York, 1961

51) DULBECCO, R.: Cell transformation by virusus. Science 166 : 962~968, 1969

52) BALUDA, M. A. & D. P. NAYAK: DNA complementary to viral RNA in leukemic cells induced by avian myeloblastosis virus. Proc. Nat. Acad. Sci. U.S.A. 66 : 329~336, 1970

53) NASS, M.M.K.: Mitochondrial DNA: Advances, problems, and goals. Science 165 : 25~35, 1969

54) NASS, S.: The significance of the structural and functional similarities of bacteria and mitochondria. Int. Rev. Cytol. 25 : 55~129, 1969

55) COHEN, S. S.: Are/were mitochondria and chloroplasts microorganisms? American Scientist 58 : 281~289, 1970