destroyed 85–91% of the total charcoal-adsorbable acid-labile phosphate of lysed and restored cells, and 98.5% of that of intact cells. Nucleoside pyrophosphates of bases other than adenine must have been nearly absent, hydrolyzed by apyrase, or converted to ATP by the action of cell transphosphorylases.

If the pump activity had shown any correlation with cell ATP, the Na^+-gradient hypothesis would have been greatly weakened; but the lack of correlation observed does not strengthen it correspondingly. A pump with ATP as energy source might show no such correlation if the ATP were generated in a small compartment of the cell which neither lost much ATP nor admitted apyrase during lysis. Also, the pump might be saturated at very low concentrations of ATP. For such reasons, experiments of this type cannot rule out the action of ATP.

One test of the Na^+ gradient hypothesis had been applied earlier (Vidaver, 1964b). It was shown that glycine pumping required a Na^+ gradient, and that the direction of the Na^+ gradient determined the direction in which glycine was pumped. Including this earlier test, a total of four independent tests have been applied. The results of each conform to the predictions from the hypothesis. Each test was as capable of weakening the hypothesis as strengthening it. Two tests, the induced-pump test and the test reported in the earlier paper, were capable of flatly disproving it. These four therefore were all fair tests. Although no one test by itself is conclusive, taken together they appear to establish the hypothesis as correct.

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REFERENCES

Christensen, H. N., Riggs, T. R., Fischer, H., and Palatine, I. M. (1952), *J. Biol. Chem. 198*, 1.
Crane, R. K., and Lipman, F. (1953), *J. Biol. Chem. 201*, 235.
Lee, K-H., and Eiler, J. J. (1951), *Science 114*, 393.
Martin, J. B., and Doty, D. M. (1949), *Anal. Chem. 21*, 965.
Osawa, S., Allfrey, V. G., and Mirsky, A. E. (1957), *J. Gen. Physiol. 40*, 491.
Riggs, T. R., Walker, L. M., and Christensen, H. N. (1958), *J. Biol. Chem. 233*, 1479.
Vidaver, G. A. (1964a), *Biochemistry 3*, 662.
Vidaver, G. A. (1964b), *Biochemistry 3*, 795 (first of series of three papers in this issue).
Vidaver, G. A. (1964c), *Biochemistry 3*, 799 (preceding paper, this issue).

Differences in the Metabolism of *N*-Hydroxy-*N*-2-fluorenylacetamide in Male and Female Rats*

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Studies on metabolism of carbon-14-labeled *N*-hydroxy-*N*-2-fluorenylacetamide (N-OH-2-FAA) were performed in Fischer rats to elucidate the mechanism of action of this carcinogenic chemical. Adult male rats excreted less of a dose in the urine than female rats. The urine of male rats contained more sulfate conjugates and less of the glucuronic acid conjugates than that of female rats. The ether-soluble metabolites after hydrolysis of the glucosiduronic acids contained 7-, 5-, and 3-hydroxy-FAA in addition to N-OH-FAA. In the urine of female rats there was considerably more N-OH-FAA whereas in males there was more 5- and 7-hydroxy-FAA. The sulfuric acid ester fraction contained mostly 7-hydroxy-FAA and 7-amino-2-fluorenol. More radioactivity was bound to liver proteins in male than in female rats but the kidney proteins of males and females contained approximately equal amounts of activity. Thus there was a sex difference in the metabolism of the carcinogen *N*-hydroxy-FAA in Fischer-strain rats.

There is a considerable body of evidence that in addition to species and strain effects, there may be sex-linked differences in the metabolism of various drugs (Axelrod, 1956; Quinn *et al.*, 1958). Thus male rats acetylate sulfanilamide more extensively than do females (Franz and Lata, 1957). On the other hand, homogenates or liver-cell fractions from female rats metabolized steroid hormones at a faster rate than did those from males (Yates *et al.*, 1958; Leybold and Staudinger, 1959).

In the case of the carcinogenic aromatic amine derivative *N*-2-fluorenylacetamide (FAA),[1] we have shown that there is only a limited influence of sex on the metabolism of a single dose of this compound. There was a tendency for greater urinary levels of hydroxylated metabolites conjugated with sulfuric acid in the mature male rats while mature females excreted more glucuronides (Weisburger and Weisburger, 1963a). In this connection it should be mentioned that there is a significant difference in the response of male and female rats to the carcinogenic action of FAA, the livers of males being appreciably more susceptible. Moreover, hypophysectomy, adrenalectomy, gonadectomy, and similar modification of the hormonal milieu

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[1] Abbreviations used in this work: FAA or 2-FAA, *N*-2-fluorenylacetamide; N-OH-FAA, *N*-hydroxy-*N*-2-fluorenylacetamide (*N*-2-fluorenylacetohydroxamic acid); 3-OH-FAA, the 3-hydroxy derivative, etc.; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2-(5-phenyloxazolyl)benzene; DEAE, diethylaminoethyl-; 7-OH-2-FA, 7-hydroxy-2-fluorenamine.

have an inhibiting effect on the carcinogenicity of FAA to the liver. Excess of pituitary hormones, on the other hand, promotes liver tumor development (cf. Weisburger and Weisburger, 1958, 1963b; Weisburger *et al.*, 1964).

The comprehensive studies of J. A. and E. C. Miller *et al.* (1960, 1961) have provided a considerable body of evidence that *N*-hydroxylation is an important reaction leading to the active intermediate in the case of the carcinogenic aromatic amines. Ring hydroxylation, followed by conjugation, is the other chief metabolic reaction undergone by such compounds. With FAA less active or inactive products results, suggesting that this latter pathway is a detoxification route (Weisburger and Weisburger, 1963b).

It seemed of interest, therefore, to determine whether there might be a difference in the metabolism of *N*-hydroxy-*N*-2-fluorenylacetamide as a function of sex since the biological effect of this compound is also influenced strongly by the hormonal environment (Weisburger *et al.*, 1964). In the present paper we are reporting on the urinary metabolites of N-OH-FAA in male and female adult Fischer-strain rats. The salient findings consist in a sex-linked difference in (1) the glucuronic acid and sulfuric acid conjugates, (2) the amount of N-OH-FAA excreted as glucosiduronic acid, and (3) the amount of radioactivity bound to the liver proteins.

Experimental

9-Carbon-14-labeled N-OH-FAA was purchased from Tracerlab, Inc., Boston, Mass. The material gave a single spot on chromatography in cyclohexane–*tert*-butanol–acetic acid-water (16:4:2:1), a specific chromatographic system. The commercial material was diluted with nonisotopic N-OH-FAA, mp 148°, to yield a compound with a specific activity of 3.8×10^6 cpm/mg.

Reference compounds N-OH-FAA (E. C. Miller *et al.*, 1961), *N*-(1-, 3-, 5-, and 7-hydroxy-2-fluorenyl)-acetamide, and 2-amino-7-fluorenol were prepared by published methods (cf. Weisburger and Weisburger, 1958).

Mature male and female Fischer rats were obtained from the Animal Production Section, NIH. They were injected intraperitoneally with the labeled N-OH-FAA as a suspension prepared in an all-glass Potter-Elvejhem homogenizer in 7% acacia-isotonic saline solution at three dose levels, 50, 40, and 10 mg/kg body weight. The animals were housed in stainless steel metabolism cages (Acme Metal Products Co., Chicago) permitting the separate collection of urine and feces. Purina laboratory chow and water were available freely. At the end of 24, 48, 72, and 96 hours the animals were necropsied by withdrawal of blood from the abdominal aorta under ether anesthesia, followed immediately by perfusion with isotonic saline through the vena cava. This procedure removed the blood from most organs owing to the pumping action of the heart. Liver and kidney were excised immediately, cooled, and homogenized (4 ml/g of 0.2 M sodium acetate buffer, *p*H 6) in a Potter-Elvejhem apparatus. Aliquots of the homogenate were counted to yield total tissue activity. The protein-bound activity was determined on the total protein which was obtained by precipitation with 10% trichloroacetic acid, followed by exhaustive washing of the pellets in the centrifuge tube with acetone (4 times), ethanol (twice), ether-ethanol (twice) and ether (once) (Gutmann *et al.*, 1960). Alternatively, the liver and kidney homogenates were centrifuged, the pellet was resuspended in an equal volume of buffer, and the supernatants were combined. Upon separate precipitation with trichloroacetic acid and workup, the insoluble and soluble proteins were obtained.

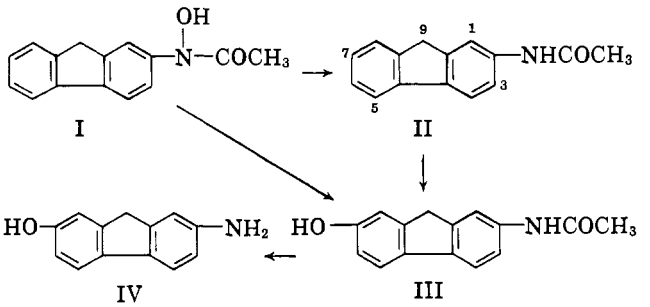

FIG. 1.—Some metabolites of N-OH-FAA (I). In addition to ring-hydroxylated metabolites at 7- position, III and IV, excreted as glucosiduronic acids and sulfuric acid esters, metabolites with a hydroxy group at 3- and 5- position were also found, chiefly as glucuronic acid conjugates. Owing to similar mobilities of I and 1-HO-FAA, this compound was not seen, but Miller and Miller (1960) conclusively showed it to be produced from I.

The metabolites in the urine were determined by previously published methods (Weisburger *et al.*, 1956). Briefly, the free compounds were removed from the urine by ether extraction at *p*H 7. The glucosiduronic acids were hydrolyzed by incubation with bacterial β-glucuronidase (Sigma Chemical Co., St. Louis) followed by ether extraction. The aqueous phase was adjusted to *p*H 1 (*p*H meter) and boiled 15 minutes. The mixture was adjusted to *p*H 7 with sodium bicarbonate and extracted with ether, thereby removing the metabolites previously conjugated with sulfuric acid. Completeness of hydrolysis and reliability of the identification was checked by paper chromatography. The feces were dried in a vacuum desiccator.

The quantity of metabolites conjugated with sulfuric acid was estimated by chromatography of urine on DEAE-cellulose (Grantham *et al.*, 1964). The ion exchanger, treated according to Sober and Peterson (1956), was last converted to the chloride form with hydrochloric acid (Ishidate *et al.*, 1963) and used as a 1.3 × 5–cm column. Typically, 5 ml of rat urine containing N-OH-FAA metabolites was diluted with an equal volume of water and applied to the column. Free metabolites and the glucuronide of N-OH-FAA were eluted with 100 ml of water. Other glucosiduronic acids appeared in the effluent with 100 ml of 0.02 N HCl, and sulfates were removed with 100 ml of 0.5 N HCl. For identifying purposes, each fraction was (1) chromatographed on paper in *sec*-butanol–3% ammonium hydroxide in comparison to urine, (2) extracted with ether, and (3) hydrolyzed by β-glucuronidase or dilute acid (see above) and the ether-soluble metabolites liberated were removed. (4) For each fraction the ether extracts were chromatographed in the cyclohexane solvent system.

Urine and aqueous phases were chromatographed in *sec*-butanol–3% ammonia (3:1). Ether extracts were resolved with the cyclohexane solvent system. The chromatograms were exposed to Royal Blue X-ray film (Eastman Kodak Co.) for 2–4 weeks in order to localize the radioactive areas. Quantitative data on individual metabolites were secured by cutting out the appropriate radioactive spots and counting the radioactivity on the paper strip in the scintillation counter. All chromatograms were run with appropriate synthetic reference compounds. In addition, ultraviolet

TABLE I

EXCRETION OF RADIOACTIVITY AFTER INTRAPERITONEAL INJECTION OF [9-^{14}C]-N-OH-FAA INTO RATS[a]

Sex	Urine				Feces	Urinary Metabolites[b]		
	0–24 hr	24–48 hr	48–72 hr	72–96 hr		Free	Glucuronides[c]	Sulfates
	Per Cent of Dose[d]					Per Cent of Urine[d]		
Male	32 (8)	2.8 (6)	0.5 (5)	0.16 (3)	22 (9)	3.4 (8)	23 (8)	35 (8)
Female	45 (8)	1.9 (7)	0.6 (5)	0.2 (3)	21 (9)	5.6 (8)	47 (7)	19 (6)

[a] Males weighing 275–325 and females 165–190 g were injected with 10 mg/kg of compound. For other details see text. [b] Usually determined on the 24-hour urine samples. [c] In one series of experiments, the aqueous phase was diluted with an equal volume of water prior to incubation with β-glucuronidase in order to assess the possible presence of an enzyme inhibitor in the urine. Identical data were obtained. [d] The figure in parenthesis is the number of observations for which the average is given.

TABLE II

URINARY METABOLITES OF N-HYDROXY-2-FLUORENYLACETAMIDE IN MALE AND FEMALE RATS[a]

Spot No.	Identity[c]	Mobility[d]	Per Cent of Fraction[b]					
			Free		Glucuronides		Sulfates[e]	
			Male	Female	Male	Female	Male	Female
1			26	21	11	5.9	40	38
2	7-OH-2-FA	3–8	20	21	14	10		
3	7-OH-FAA	9–14	15	14	19	9.5	26	23
4	5-OH-FAA	16–24	9.4	9.8	17	12		
5		27–34	6.7	6.6	5	4.7		
6	3-OH-FAA	40–47	8.3	12	6	3.7		
7	FAA, 1-OH-FAA	54–66	13	10				
8	N-OH-FAA	58–72			25	50		

[a] See experimental details for procedure. [b] About 5–20 × 10^3 cpm were applied to each strip. The amounts in each fraction are given in Table I. The absolute quantities of metabolites can be readily obtained by multiplying the percentages in Table II by those in Table I and relating this value to the dose. [c] FAA is N-2-fluorenylacetamide, 7-OH etc., the 7-hydroxy derivative, etc. [d] Mobility is measured from front to back of spot and is listed × 100. [e] After ether extraction or column separation and hydrolysis, followed by paper chromatography, there was a general darkening of the radioautographs, somewhat more intense in the region of the 5-, 3-, and N-OH-FAA. Whether this represents actual metabolites or may be an artifact (due to the acid-catalyzed decomposition and rearrangement of N-OH-FAA sulfate) remains uncertain. In any case it was not seen in similar fractions during studies of the metabolism of FAA.

spectra and specific color tests were used to confirm unambiguously the identity of some of the metabolites.

Determination of Radioactivity.—Carbon-14 was measured on a liquid scintillation counter set for optimal efficiency, utilizing a scintillation mixture of toluene (700 ml/liter), methanol (300 ml/liter), PPO (3 g/liter), and POPOP (100 mg/liter). Solids such as feces, proteins, or homogenates were digested completely in 3 ml of Hyamine at 60° for 0.5 hour on a shaking incubator, followed by addition of 12 ml of a toluene scintillation mixture (5 g PPO, 100 mg POPOP, 1 liter toluene). Corrections were made for background, and also for quenching by addition of internal standards.

RESULTS

Intraperitoneal injection of N-OH-FAA at a level of 50 mg/kg was fatal to adult male and female Fischer-strain rats within 18 hours. At a dosage of 40 mg/kg the male rats died within 36 hours but the female rats survived. Although 30 mg/kg appeared to be a tolerable level for both male and female rats, the remainder of our experiments were performed at a dosage of 10 mg/kg since it was found earlier with FAA that any sex-linked difference in metabolism became more apparent at a dosage lower than the maximally tolerated one (Weisburger and Weisburger, 1963a).

Excretion of Radioactivity.—Most of the radioactivity from the injected N-OH-FAA was excreted during the first day in the urine and in the first two days in the feces by male and female rats (Table I). The urine of females contained more of the isotope than that of the males. Only small amounts of the dose were found in the urine after the first day.

Urinary Metabolites.—As is the case with 2-FAA, a relatively small amount of the urinary metabolites was observed as unconjugated material (Table I). Chromatography of these free materials showed them to consist of 5- and 7-OH-FAA, N-OH-FAA, and FAA. In addition, a fair amount of isotope in this fraction was found in materials with low mobility (Table II).

The glucosiduronic acids were the major components in the metabolites from female rats (47% of urine). Noteworthy is the fact, however, that in male rats this fraction accounted for only 23%. On the other hand, conjugation with sulfuric acid of the phenolic metabolites was larger in male rats (35%) as compared to female rats (19%). Column chromatography on DEAE-cellulose gave similar values for sulfuric acid esters, namely, 34 and 20%, in males and females, respectively.

Examination of the individual metabolites in the glucosiduronic acid fraction showed that one-fourth to one-half was in the form of apparently unchanged N-OH-FAA. Substantial amounts of 5- and 7-OH-FAA and a small quantity of 3-OH-FAA were identified. In male rats the *N*-hydroxy derivative accounted for less of this fraction than in females. There also appeared a material with a R_F value of 27–34, a mobility somewhat faster than the 5-hydroxy derivative of 2-FAA but slower than that of the 3-hydroxy derivative. A material with such a mobility has been noted earlier in connection with studies of the metabolism of FAA where it was found both in urine and in

fractions derived from the hydrolysis of liver proteins containing metabolites from FAA (Miller *et al.*, 1960; Weisburger *et al.*, 1961).

The major component in the metabolites conjugated with sulfuric acid were the 7-hydroxy derivatives of 2-FAA and of 2-fluorenamine. They were present in larger amounts in urine of male rats.

Tissue Metabolites.—In a typical experiment the serum of male rats had an activity of 6200 and 3400 counts/ml after 2 and 4 days, respectively. The corresponding figures for female rats were 3870 and 2670 counts/ml. Thus in males the serum carried more radioactivity than in females. Likewise in males the liver had a somewhat larger total activity and the portion of this activity bound to protein also was higher. On the other hand, the isotope content of the kidneys was rather similar for both sexes (Table III). In one series the soluble and insoluble proteins were separated for liver and kidney. As had been found earlier with FAA, the soluble liver proteins generally showed higher specific activity than the insoluble proteins.

TABLE III

BINDING OF RADIOACTIVITY FROM [9-^{14}C]-N-OH-2-FAA TO TISSUE PROTEINS

Organ	Time (hr)	Sex	Dose in Organ (%)	Radioactivity of Proteins (mμmoles/g) Soluble	Radioactivity of Proteins (mμmoles/g) Insoluble	Dose Bound to Protein (%)
Liver	48	M	1.9	150	84	1.1
	96		1.5	←57→		0.9
	48	F	1.5	102	63	0.75
	96		0.9	←47→		0.7
Kidney	48	M	0.26	63	35	0.09
	96		0.2	←32→		0.08
	48	F	0.25	62	51	0.09
	96		0.2	←35→		0.1

DISCUSSION

N-OH-FAA (I), which appears to be a proximate agent crucial in the carcinogenicity of FAA, is in turn metabolized further as already demonstrated by Miller and Miller (1960) and Miller *et al.* (1960) using male rats, oral intake, and techniques not involving isotopes. One reaction undergone by N-OH-FAA is dehydroxylation to FAA (II). This conclusion is based on the finding not only of FAA, but also of the products expected by the further metabolism of this compound by hydroxylation at various positions of the fluorene ring system. Thus, the occurrence of 3-, 5-, and 7-hydroxylated metabolites in the urine of animals fed N-OH-FAA can be explained by the known transformation undergone by FAA. It is possible, however, that the *N*-hydroxy derivative of FAA might undergo ring-hydroxylation on its own prior to *N*-dehydroxylation. In this instance one could find dihydroxylated compounds such as have been demonstrated in the case of the further metabolism of 1-OH- and 3-OH-FAA (Weisburger *et al.*, 1959a). Certain of the metabolites of low mobility during chromatography in the cyclohexane solvent system may consist of these materials. The directive effect of the *N*-hydroxy group on further substitution is not established. Conceivably it could affect the *para* carbon, which in the case of the fluorene ring is at the extended 7- position. The data (Table II) speak in favor of such a possibility, for the total amount of 7-OH-FAA (III) found as free compound, glucuronic acid, and sulfuric acid conjugate is larger after giving N-OH-FAA than after FAA itself. FAA usually gives more of the 5-hydroxy derivative, excreted free and as glucosiduronic acid.

The data presented clearly show that female Fischer-strain rats appear to excrete more N-OH-FAA than male rats as the glucuronic acid conjugate. Possibly *N*-dehydroxylation of the compound or ring-hydroxylation followed by the dehydroxylation occurs to a lesser extent in female rats than in male rats. The type of hydroxylated metabolite may have a bearing on the relative quantities of glucuronic and sulfuric acid conjugates excreted. Thus the larger amounts of the glucosiduronic acid fraction may be ascribed to the larger ratio of N-OH-FAA in females. The increased level of sulfuric acid conjugates in males may be related to the higher production of the 7- (or 2-) hydroxy-derivatives which are characteristically the only hydroxylated metabolites of FAA (Weisburger *et al.*, 1959b) and of fluorene (Grantham, 1963) so conjugated.

This explanation would satisfy our finding of higher glucosiduronic acid levels excreted by female rats and higher amounts of sulfuric acid esters by male rats, which appears to be novel and related to N-OH-FAA. The specific enzyme system forming glucuronides of *N*-hydroxy compounds has not yet been investigated. With phenolic hydroxy derivatives uridinediphosphoglucuronic acid, itself formed by oxidation of the corresponding glucose derivative in the soluble fraction of the cell, is reacted with phenols at the microsome level by means of glucuronyl transferase. In this connection, Inscoe and Axelrod (1960) reported that the ability of liver microsomes from male rats to make *o*-aminophenol glucuronide was about four times as great as the ability of those from female rats due to a greater glucuronyl transferase activity in male rat liver. Other investigators have discussed specific instances of sex-linked differences in drug metabolism (cf. Knox *et al.*, 1956; Popper and Schaffner, 1957; Brodie, 1962; Remmer, 1962; Williams, 1962). Most of the reports deal with observations on the sleeping time after administration of anesthetics, as related to detoxification of these drugs. In these instances the rat seems to be the only species so far exhibiting a sex-related effect. Ours seems to be the first observation of a quantitative difference in the excretion of glucuronic and sulfuric acid conjugates by rats as a function of sex.

There is less conjugation with glucuronic acid in both male and female animals and somewhat higher conjugation with sulfuric acid when compared to our earlier studies with FAA in which glucosiduronic acids usually amounted to 50–70% of the urinary radioactivity and sulfuric acid esters accounted for 10–20% (Weisburger and Weisburger, 1963a). These figures are to be compared with 20–50% approximately of the glucosiduronic acid and 20–35% of the sulfate found with N-OH-FAA.

Even though the liver and other tissues of rats are endowed with deacetylase activity, the major portion of the urinary metabolites was in the form of the acetyl derivatives. As with FAA, there is apparently an equilibrium between deacylation and reacetylation which in rats lies on the side of the latter reaction (Peters and Gutmann, 1955). However, deacetylation may be an important reaction from the viewpoint of the carcinogenicity of the material. In other studies we have noted that the deacetylated product, namely, *N*-2-fluorenylhydroxylamine, was a very reactive compound which combines readily with proteins and nucleic acids. Production of this metabolite in the course of the biochemical transformations of N-OH-FAA might logically explain the attachment of label

to a variety of tissue components, as was also found in the course of the present studies. However, the significance of such reactions and the role they play in the carcinogenic process requires to be elucidated since similar combinations of radioactivity were observed with aminofluorenols (Weisburger *et al.*, 1961) which apparently are noncarcinogenic. Nevertheless, there was more radioactivity bound to the liver of male rats which is also the more susceptible organ with N-OH-FAA. Thus, despite the higher reactivity of the intermediate hydroxylamine, a certain selectivity was exhibited. None of this selectivity was apparent in the case of the kidneys, an organ usually not a target of the carcinogenic effect with this compound. In any case, the metabolic behavior of N-OH-FAA noted in these studies bears a closer relationship to its observed biological properties than does that of the precursor FAA. However, it is still somewhat premature to draw cause-and-effect conclusions in terms of carcinogenicity.

References

Axelrod, J. (1956), *J. Pharmacol. Exptl. Therap. 117*, 322.

Brodie, B. B. (1962), *Ciba Found. Symp. Enzymes Drug Action, 1961*, 317.

Franz, J. M., and Lata, G. F. (1957), *Endocrinology 60*, 602.

Grantham, P. H. (1963), *Biochemistry 2*, 610.

Grantham, P. H., Weisburger, E. K., and Weisburger, J. H. (1964), *Proc. Intern. Congr. Biochem. 6th, New York, 1964*, (in press).

Gutmann, H. R., Seal, U. S., and Irving, C. C. (1960), *Cancer Res. 20*, 1072.

Inscoe, J. K., and Axelrod, J. (1960), *J. Pharmacol. Exptl. Therap. 129*, 128.

Ishidate, M., Tamura, Z., and Samejima, K. (1963), *Chem. Pharm. Bull (Tokyo) 11*, 1014.

Knox, W. E., Auerbach, V. H., and Lin, E. C. C. (1956), *Physiol. Rev. 36*, 164.

Leybold, K., and Staudinger, H. (1959), *Biochem. Z. 331*, 389.

Miller, E. C., and Miller, J. A. (1960), *Biochim. Biophys. Acta 40*, 380.

Miller, E. C., Miller, J. A., and Hartmann, H. A. (1961), *Cancer Res. 21*, 815.

Miller, J. A., Cramer, J. W., and Miller, E. C. (1960), *Cancer Res., 20*, 950.

Miller, J. A., Wyatt, C. S., Miller, E. C., and Hartmann, H. A. (1961), *Cancer Res. 21*, 1465.

Peters, J. H., and Gutmann, H. R. (1955), *J. Biol. Chem. 216*, 713.

Popper, H., and Schaffner, F. (1957), Liver: Structure and Function, New York, McGraw-Hill, p. 645.

Quinn, G. P., Axelrod, J., and Brodie, B. B. (1958), *Biochem. Pharmacol. 1*, 152.

Remmer, H. (1962), *Ciba Found. Symp. Enzymes Drug Action, 1961*, 276.

Sober, H. A., and Peterson, E. A. (1956), *J. Am. Chem. Soc. 78*, 751.

Weisburger, E. K., and Weisburger, J. H. (1958), *Advan. Cancer Res. 5*, 331.

Weisburger, J. H., Grantham, P. H., Morris, H. P., and Weisburger, E. K. (1961), *J. Natl. Cancer Inst. 27*, 153.

Weisburger, J. H., Pai, S. R., and Yamamoto, R. S. (1964), *J. Natl. Cancer Inst. 32*, 881.

Weisburger, J. H., and Weisburger, E. K. (1963a), *Acta Unio Intern. Contra Cancrum 19*, 513.

Weisburger, J. H., and Weisburger, E. K. (1963b), *Clin. Pharmacol. Therap. 4*, 110.

Weisburger, J. H., Weisburger, E. K., Grantham, P. H., and Morris, H. P. (1959a), *J. Natl. Cancer Inst. 22*, 825.

Weisburger, J. H., Weisburger, E. K., and Morris, H. P. (1959b), *Arch. Biochem. Biophys. 80*, 187.

Weisburger, J. H., Weisburger, E. K., Morris, H. P., and Sober, H. A. (1956), *J. Natl. Cancer Inst. 17*, 363.

Williams, R. T. (1962), *Ciba Found. Symp. Enzymes Drug Action, 1961*, 239.

Yates, F. E., Herbst, A. L., and Urquhart, J. (1958), *Endocrinology 63*, 887.

Effect of Antimycin A on Oxidative Phosphorylation with Ferricyanide as Electron Acceptor*

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Antimycin A inhibits respiration of rat liver mitochondria more than 90% when measured in a Warburg-flask-assay system with potassium ferricyanide as electron acceptor, confirming results obtained by continuous spectrophotometric assays. Previous failures to observe inhibition in a Warburg-flask-assay system are due to the destruction and inactivation of antimycin A by potassium ferricyanide in the absence of mitochondria. Antimycin A and potassium ferricyanide undergo a chemical reaction at neutral *p*H to yield products that absorb at longer wavelengths. The reaction rate is dependent on the *p*H of the reaction media and on the concentration of potassium ferricyanide. Antimycin A is bound by rat liver mitochondria in a manner which protects it from destruction by potassium ferricyanide.

Potassium ferricyanide has been used as a terminal electron acceptor in place of oxygen in studies of oxidative phosphorylation by mitochondria and two principally different methods have been employed for the measurement of ferricyanide reduction. In this laboratory (Copenhaver and Lardy, 1952; Maley and Lardy, 1955) and in work by Ernster (1961) the reaction was carried out in Warburg vessels and the ferricyanide reduction was determined by measuring its concentration at the beginning and at the end of the reaction, whereas Pressman (1955) and Estabrook (1961) followed the reduction directly by spectrophotometric methods. With the former method, P:$2e^-$ values between 0.8 and 1.5 for DPN-linked substrates and of about 0.6 for succinate (Copenhaver and Lardy, 1952; Maley and Lardy, 1955; Ernster, 1961) were found. Somewhat higher values were observed

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