present in the spectra of both the normal and the "lethal" forms.

Our results demonstrate that the "lethal" variant of *Candida albicans* and the "petite colonie" mutant of *Saccharomyces cerevisiae* described by Ephrussi (*3*) have the same physiological impairment. This fact suggests that the hereditary loss of the respiratory function may be a frequent phenomenon among microorganisms endowed with both aerobic and anaerobic metabolism.

ANIBAL ALVAREZ
JUAN E. MACKINNON
Instituto de Higiene,
Facultad de Medicina,
Montevideo, Uruguay

References

1. J. E. Mackinnon, *J. Infectious Diseases* **66**, 59 (1940).
2. W. J. Nickerson and G. Falcone, *Science* **124**, 722 (1956).
3. B. Ephrussi, *Nucleo-Cytoplasmic Relations in Micro-Organisms* (Clarendon, Oxford, 1953).

27 May 1957

O-Methylation of Epinephrine and Other Catechols in vitro and in vivo

For the past 20 years the catechol amines, epinephrine and norepinephrine, have been considered to be transformed in the body, mainly by monoamine oxidase. However, recent investigations have shown that these amines may be inactivated by enzymes other than monoamine oxidase (*1*). The important observation of Armstrong and McMillan (*2*), that a major metabolic product of epinephrine and norepinephrine is 3-methoxy-4-hydroxy mandelic acid, suggested that methoxy derivatives of the catechol amines may be the precursors of the deaminated compound. I wish to report the isolation of an enzyme from rat liver which catalyzes the O-methylation of epinephrine and other catechols. In addition, the presence of methoxy epinephrine and methoxy norepinephrine in the urine was demonstrated.

Table 1. Enzymatic O-methylation of epinephrine. The soluble supernatant fraction from 10 mg of rat liver was incubated at 37°C with 0.1 μmole of l-epinephrine bitartrate, 50 μmole of phosphate buffer (*p*H 7.4), 20 μmole of $MgCl_2$, and water to make a final volume of 0.5 ml. After 30 minutes of incubation, the reaction mixture was assayed for epinephrine (*13*).

Additions	l-Epinephrine metabolized (μmole)
None	0.000
S-Adenosylmethionine, 0.05 μmole (*8*)	0.041
S-Adenosylmethionine, 0.1 μmole	0.065

Enzymes have been found which transfer the methyl group of S-adenosylmethionine to the nitrogen of nicotinamide (*3*) and guanidoacetic acid (*4*). These findings suggested the possibility that phenols also could accept methyl groups by a similar mechanism. Incubation of the soluble supernatant fraction of rat liver with epinephrine, adenosine triphosphate, and methionine resulted in the disappearance of the cathechol amine. In the absence of either cofactor, no metabolism took place. Cantoni has shown that an enzyme in the liver can form S-adenosylmethionine from adenosine triphosphate and methionine (*5*). When S-adenosylmethionine was substituted for adenosine triphosphate and methionine, a more rapid disappearance of epinephrine was observed (Table 1). The metabolic product was extracted into an ether-isoamyl alcohol mixture at *p*H 9.0 and was then returned to dilute hydrochloric acid. The acid extract contained a phenolic compound which did not give a reaction for catechols (*6*). These observations were taken as evidence that an enzyme present in rat liver can transfer the methyl group of S-adenosylmethionine to one of the phenolic groups of epinephrine.

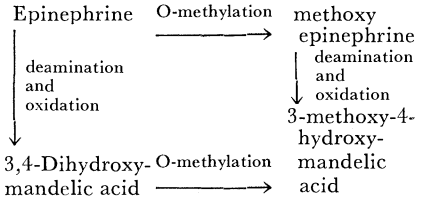
Several other catechols (norepinephrine, 3,4-dihydroxyphenylethylamine, and 3,4-dihydroxybenzoic acid) also served as substrates for the enzyme. The enzyme has been purified 20-fold and is stimulated considerably by magnesium ions. Supernatant fractions of brain, kidney, and spleen can also O-methylate epinephrine, but to a lesser extent than the liver.

Evidence for the identity of methoxy epinephrine formed by the enzyme was obtained by deaminating and oxidizing the metabolite with a monoamine oxidase preparation to yield 3-methoxy-4 hydroxymandelic acid as follows. Methoxy epinephrine was incubated with rat-liver mitochondria, and the metabolic product was extracted with ethyl acetate at *p*H 1, returned to a dilute sodium bicarbonate solution, and reextracted into ethyl acetate at *p*H 1. The resulting compound, when subjected to paper chromatography with several solvent systems (*7*), had the same R_f values and color reaction as an authentic sample of 3-methoxy-4-hydroxymandelic acid (*8*). A marked depression in the formation of the deaminated derivative was observed when the mitochondrial preparation was obtained from a rat that had been pretreated with iproniazid, an inhibitor of monoamine oxidase.

Urine of rats was examined for methoxy epinephrine after incubation with β-glucuronidase. Phenolic amines were extracted from a sodium chloride-saturated urine at *p*H 9.0 into an ether-isoamyl alcohol mixture and returned to dilute hydrochloric acid. The acid extract was saturated with sodium chloride, adjusted to *p*H 9.0, and shaken with *n*-butanol. After the butanol extract was evaporated to dryness, the residue was dissolved in a small volume of an ether-methanol mixture and subjected to two-dimensional chromatography (solvent systems I and III). A compound was found that had the same R_f values (0.70 and 0.52) and color reaction as the enzymatically formed methoxy epinephrine. The amount of methoxy epinephrine excreted in the urine of rats was markedly elevated after the intraperitoneal administration of iproniazid and l-epinephrine bitartrate (*9*).

Rat urine was also examined for methoxy norepinephrine after the intraperitoneal administration of l-norepinephrine bitartrate. Urine was incubated with β-glucuronidase and extracted as described in the preceding paragraph. After the extract had been subjected to two-dimensional chromatography (solvent systems I and III), a compound was found that had the same R_f values (0.54 and 0.43) and color reaction as enzymatically formed methoxy norepinephrine (*10*).

On the basis of the experiment described, the following scheme for the metabolism of epinephrine and its congeners is suggested:



Barger and Dale (*11*), as well as other investigators, have shown that numerous derivatives of epinephrine have sympathomimetic action. It is conceivable that some of the activity of the catechol amines could be mediated through their methoxy transformation products (*12*).

JULIUS AXELROD
National Institute of Mental Health,
National Institutes of Health,
Bethesda, Maryland

References and Notes

1. E. C. Greisemeyer *et al.*, *Proc. Soc. Exptl. Biol. Med.* **84**, 699 (1953); U. S. von Euler and S. Hellner-Björkman, *Acta Physiol. Scand.* **33**, Suppl. 118, 21 (1955); U. S. von Euler and B. Zetterstrom, *ibid.* **33**, Suppl. 118, 26 (1955); S. J. Carne and J. D. Graham, *J. Physiol. (London)* **135**, 339 (1957).
2. M. D. Armstrong and A. McMillan, *Federation Proc.* **16**, 146 (1957).
3. G. L. Cantoni, *J. Biol. Chem.* **189**, 203 (1951).
4. G. L. Cantoni and P. J. Vignos, *ibid.* **209**, 647 (1954).
5. G. L. Cantoni, *ibid.* **204**, 403 (1953).
6. H. Weil-Malherbe and A. D. Bone, *Biochem. J. (London)* **51**, 311 (1952).
7. The following solvent systems [described by M. D. Armstrong, N. F. Shaw, P. E. Wall,

J. Biol. Chem. 218, 293 (1956)] were used: I, isopropyl alcohol-ammonia (5 percent), 8/2; II, benzene-propionic acid-H_2O, 2/2/1; III, *n*-butanol-acetic acid-H_2O, 8/2/2. Phenolic compounds were visualized by spraying the chromatograms with diazotized *p*-nitroaniline [H. G. Bray, W. V. Thorpe, K. White, *Biochem. J.* (*London*) 46, 271 (1950)]

8. S-Adenosylmethionine and 3-methoxy-4-hydroxymandelic acid were kindly supplied by G. L. Cantoni and M. S. Fish, respectively, National Institutes of Health.
9. Enzymatically formed methoxy epinephrine and the metabolite isolated from the urine after the administration of l-epinephrine bitartrate had the same R_f, fluorescent spectrum, and partition coefficients as a synthetic sample of 3 methoxy-4-hydroxyphenyl-2-methylaminoethanol (synthesized by S. Senoh and B. Witkop, National Institutes of Health).
10. The solvent systems sharply separated methoxy epinephrine and methoxy norepinephrine.
11. G. Barger and H. H. Dale, *J. Physiol.* (*London*) 41, 19 (1910).
12. A detailed report of this work is in preparation.
13. U. S. von Euler and I. Floding, *Acta Physiol. Scand.* 33, Suppl. 118, 45 (1955).

26 July 1957

Isolation of Cytopathogenic Viral Agent from Feces of Cattle

In the early part of September 1956, 19 beef calves, 3 to 4 months of age, were purchased and brought into an experimental barn unit. Approximately 10 days later the calves developed temperatures varying from 104° to 106°F, and several also had a slight cough and some nasal exudate. The calves were treated with broad-spectrum antibiotics and recovered uneventfully.

Two pregnant cows were brought into the same barn unit on 7 October. These cows developed fever and nasal exudate within a week. One cow recovered in a few days but aborted approximately 1 month later. The other cow developed pulmonary complications and was treated with broad-spectrum antibiotics over a period of 21 days. When it was examined 2 to 3 months later, this cow was not pregnant. The aborted 7-month-old fetus and both cows were found to be negative for evidence of infection caused by *Vibrio fetus, Leptospira pomona,* and *Brucella abortus* (*1*).

On 5 January 1957, nine beef calves, 3 to 6 months old, were purchased and housed in a separate pen in the aforementioned experimental barn unit. Within a week several of these calves developed temperatures varying from 104° to 106°F, slight cough, moderate nasal exudate, and considerable mucus in the stools. These animals maintained normal food intake and recovered without treatment in approximately 1 week.

A viral agent, cytopathic for bovine kidney cells grown *in vitro*, was recovered from (i) fecal samples collected from four of the latter calves during the late stages of the illness and also 3 months after recovery; (ii) a pool of fecal samples collected 15 February 1957 from the two cows, one of which aborted; (iii) samples of ascites fluid, stomach fluid, and placental fluid collected from the aborted fetus; and (iv) a pool of fecal samples from calves in another experimental unit which had fever and slight nasal exudate.

Ten pooled fecal samples collected from calves and cows with no history of illness were found free from this virus as judged by the absence of cytopathic changes in inoculated tissue cultures.

Tissue cultures of bovine kidney (*2*) were prepared in tubes of the trypsin digest method described by Youngner (*3*). The cells were grown in a nutrient medium consisting of Hank's solution with 0.5 percent lactalbumin hydrolyzate and 10 percent bovine serum. When cell sheets were formed on the glass surface, this nutrient medium was withdrawn and replaced with a maintenance medium consisting of Hank's solution containing 0.5 percent lactalbumin hydrolyzate but no serum. To both media were added 100 units of penicillin, 200 μg of streptomycin, and 100 units of mycostatin (*4*) per milliliter.

Fecal samples were suspended in phosphate-buffered saline containing 500 units of penicillin and 500 μg of streptomycin per milliliter. These suspensions were strained through a four-layer pad of cheesecloth into stainless-steel tubes which were centrifuged at 10,000 rev/min for 30 minutes in a refrigerated centrifuge. Each supernatant was subsequently diluted 1/2 with maintenance medium and dispensed in quantities of 1 ml to each of five tissue-culture tubes. After 2 to 3 hours, this inoculum was withdrawn and replaced with fresh maintenance medium. The fetal fluids which were clear and free of bacteria were diluted 1/5 with maintenance medium and dispensed in quantities of 1 ml to each of five tissue-culture tubes.

Cytopathic changes characterized by focal rounding and shrinking of cells were observed in the bovine kidney cell cultures within 12 to 24 hours. These changes progressed until all cells were affected and fell off the glass surface after 24 to 96 hours (Fig. 1). The fluid of culture tubes in which the majority of cells were affected also was considerably more acid than the fluid of the unaffected control tubes.

Ten serial passages of infected cell culture fluids carried out have regularly resulted in cytopathic cell changes as well as in increased acidity of the culture fluids. The viral agents recovered from the different sources have behaved identically in these aspects.

Replicate titrations of various tissue culture passage levels showed a $TCID_{50}$ of 10^7 to 10^{12} per milliliter. Cell cultures inoculated with the lowest dilution of infected fluids showed typical cytopathic changes as early as 4 hours after inoculation, while those inoculated with the highest dilution revealed such changes 36 to 42 hours after inoculation.

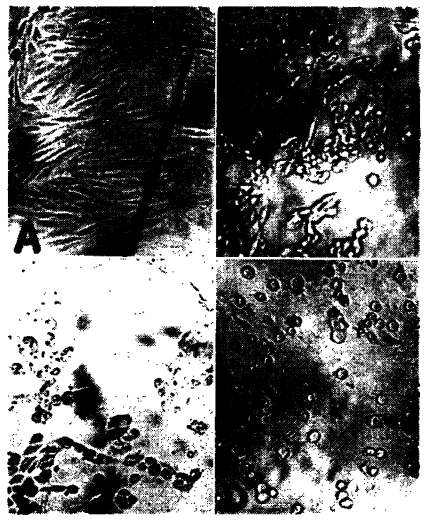


Fig. 1. Bovine kidney cells (about × 15). (*A*) Normal cells growing on the glass wall of a test tube. (*B*) Early cytopathic effect (1+) of virus growth; a number of cells have become shrunken or rounded, but most of the cell sheet appears normal. (*C*) Progression of virus-induced cytopathic changes (2 to 3+); a large number of cells are rounded or shrunken. (*D*) Final stage of cytopathic changes (4+); no normal cells remain, and most cells have fallen off the glass surface.

The increase in acidity at the time of severe or complete cell destruction varied from 0.2 to 0.4 *p*H units as compared with normal cell cultures. The viral agent replicates and produces cytopathic changes in bovine kidney cell cultures at *p*H 6.4 to 8.4. The cytopathic changes develop more slowly and seem less extensive at *p*H 6.4 to 6.6 than at *p*H 7.4 to 8.4. Considerable rounding and degeneration were observed in noninfected control cultures at *p*H 8.4.

The virus passes through a fine sintered glass filter without appreciable loss of titer. It will also pass an ultrafilter membrane (*5*) with average pore size of 0.05 to 0.08 μ, but not through a membrane with average pore size of 0.01 to 0.05 μ.

The virus, which is ether-resistant, survives storage in tissue-culture maintenance fluid (*p*H 7.6 to 7.8) at −10° to +22°C for at least 2 months, at 37°C for at least 120 hours, and at 56°C for at least 30 minutes.

Pooled preinfection and convalescent serums from the four calves, from whose feces virus was isolated, were tested for specific neutralizing antibodies against the virus. Tissue-culture neutralization tests were carried out in accordance with the method described by Paul and Melnick for paired poliomyelitis serums (*6*).