both supplemental arginine and lysine had similar growth depressing effects, only lysine had an effect on plasma cholesterol levels which became significant at the level of 4%. In addition to hypercholesterolemia, 5% lysine supplements also altered the relative ratios of high density to low density lipoproteins; a change which was due mainly to increased levels of LDL. The results of stepwise 1% supplemental increments of both arginine and lysine up to a maximum of 5% total on chick plasma cholesterol levels are presented in the figure. It can be seen that the effects of lysine on plasma cholesterol levels are only slight from 1–3% but that 4% and 5% supplementation with lysine resulted in significant hypercholesterolemia.

Discussion. Arginine-lysine antagonism in the chick is a well known phenomenon[10]. This antagonism affects the rate of growth, and the metabolic site of the antagonism is the kidney where a high concentration of lysine is believed to induce kidney arginase activity. However, since in both mammals and birds the liver is the major site of lipoprotein metabolism and since compared to mammals only trace amounts of arginase are present in the chicken liver, it is not clear whether lipoprotein metabolism would be affected by arginine-lysine antagonism in the chick. Our results from chicks fed supplemental crystalline lysine and arginine show that, as in ureotelic mammals, excess dietary lysine does stimulate hypercholesterolemia. It can be concluded, therefore, that the cholesterolemic effects of arginine-lysine imbalance are not mediated by urea cycle anaplerosis.

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2 Reprint requests to: F.K. Burnsides Research Laboratory, 1208 W. Pennsylvania Avenue, Department of Food Science, University of Illinois, Urbana (IL 61801, USA).
3 P. Hevia and W.J. Visek, J. Nutr. *109*, 32 (1979).
4 K.K. Carroll, Nutr. Rev. *36*, 1 (1978).
5 N.R. Yadav and I.E. Liener, Nutr. Rep. Int. *16*, 385 (1977).
6 D. Kritchevsky, S.A. Tepper and J.A. Story, Fedn Proc. *37*, 747 (1978), abstract.
7 B. Schepartz, Regulation of Amino Acid Metabolism in Mammals. W.B. Saunders Co., London 1973.
8 T. Toda, D. Leszczynski and F. Kummerow, J. Path. *134*, 219 (1981).
9 D.E. Leszczynski, J.C. Cleveland and F.A. Kummerow, Clin. Cardiol. *3*, 252 (1980).
10 A.J. Leslie, J.D. Summers, R. Grandhi and S. Leeson, Poultry Sci. *55*, 631 (1976).

Somatic cell hybrids producing inhibitors of melanotic melanoma tyrosine hydroxylase

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Summary. Splenic lymphocytes from BALB/c mice pre-immunized with purified tyrosine hydroxylase (TH) were fused with murine myeloma N/1 cells. Supernatants of only 2 from large number of cloned cell hybrids contained an inhibitor of TH.

Human malignant melanoma cells have 2 characteristics that vary in magnitude from a cell line to another: Melanin synthesis is a specialized function of pigment cells and differentiates melanotic melanoma from other tumor cells. It arises from the oxidation of tyrosine by tyrosine hydroxylase. In human melanomas, tyrosine hydroxylase activity varies with the degree of melanization. The magnitude of response and binding of estrogens also vary from one melanoma cell line to another. Depending on the culture medium, an estradiol-non-responsive melanotic cell line (E^-Mel^+) could be derived from an estradiol-responsive melanotic line (E^+Mel^+) by cultivation of the latter cells in the absence of estradiol[5,6], and melanotic cells (Mel^+) could be converted into amelanotic by deprivation from tyrosine. In general, melanotic melanoma cells bind and respond to estradiol, whereas amelanotic cells do not[7].

In melanocytes tyrosine hydroxylase exists in multimolecular forms, a soluble cytoplasmic and an insoluble membrane form[8]. In mouse and human tumors the T_1 and T_2 tyrosine hydroxylases are soluble, while T_3 is a particulate membrane associated enzyme[9–12].

The present studies examined the characteristics of a tyrosine hydroxylase inhibitor (M-THI) found in spent culture media of cloned cell hybrids obtained from fusion of murine myeloma cells with splenic lymphocytes of BALB/c mice pre-immunized with highly purified tyrosine hydroxylase.

Materials and methods. Purified tyrosine hydroxylase (polyphenol oxidase) was obtained from Sigma Company, and was purified by gel filtration to yield 20,000 U/mg[13].

Human malignant melanotic melanoma HMMC-ShA, and HMMC-WJP were established using tumor biopsies as explants from 2 male adults, whereas HMMC-ZBJ was from a female adult. The amelanotic melanoma cell lines HMMC-Sr and HMMC-KM were also established from tumor biopsies from 2 male adults[14].

A group of 10 BALB/c (10–20 g) female mice were immunized with purified tyrosine hydroxylase (TH). Spleenocyte suspensions were prepared and splenic lymphocytes were separated on Ficol Hypaque columns.

An equal number of 10^6 of spleen lymphocytes and murine myelome ($NS_1/1$) cells were mixed in DMEM and centrifuged at $600 \times g$ for 10 min. The supernatant was removed, and the cell pellets were resuspended in 1.5 ml of 50% polyethylene glycol 1500 in DMEM (pH 7.5–7.8). The cells were washed, and suspended in DMEM containing 20% fetal calf serum, hypoxanthine (0.1 mM), amethopterin (0.4 µM) and thymidine (3 µM) (DMEM-HAT). Aliquots of each suspension were dispensed into 96 well Coster plates with 50 µl of DMEM per well and incubated in a humidified 5% CO_2 air atmosphere. The wells were fed every 72 h with 100 µl of HAT-free DMEM supplemented with 20% FCS. Wells that became acidic 7–14 days after fusion and containing cell clusters were assayed for anti-tyrosine hydroxylase (M-THI). 48 out of the 96 wells showed active cellular proliferation. Supernatant of only 1 (Hy-7) out of the 48 wells contained the TH-inhibitor. The cells of this well were once more cloned and 3 times subcultured in vitro, then injected s.c. into athymic Nu/Nu mice. 4 out of 5 inoculated animals developed tumors.

When reached 1.5 × 0.5 cm in size, the tumors were dissected and reexplanted for in vitro culture. The cells were recloned to size and shape, and supernatants were assayed for TH-inhibitor. This in vivo-in-vitro cycle was repeated 3 times. Finally, supernatants from the 4th in vitro subculture were pooled, concentrated by gel filtration and used as the M-THI.

Inhibitors endigeneous to the melanoma cells were obtained as follows: Aliquots of 5 ml of melanoma cell suspensions (10^7 cells/ml) were centrifuged. The cell pellets were homogenized in 0.1 M phosphate buffer of pH 6.8 for 2 min at 0–4 °C, then centrifuged at 700 × g for 10 min. The supernatant contained the soluble cellular tyrosine hydroxylase inhibitor (SC-THI). The residue was treated with lipase (10 units/mg protein) to solubilize the particulate tyrosine hydroxylase inhibitor (P-THI).

The tyrosine hydroxylase (EC-1-10-3-1) activity of the

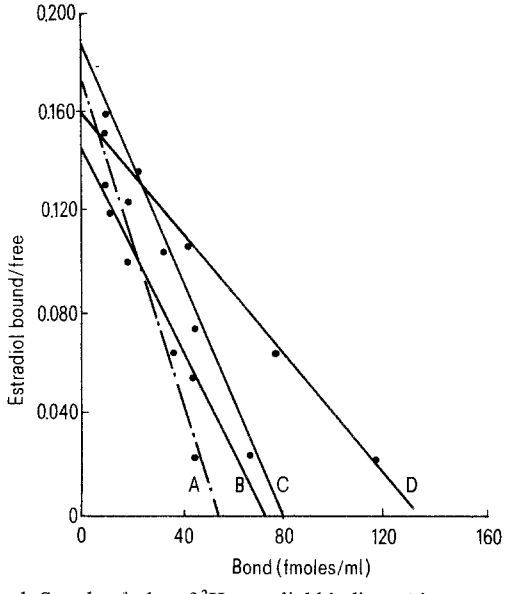

Figure 1. Scatchard plot of ^{3}H-estradiol binding: *A* in a system with 10 µg of purified tyrosine hydroxylase, and in cytosol of human malignant melanoma cell lines *B* HMMC-ShA, *C* HMMC-WJP and *D* HMMC-ZBJ.

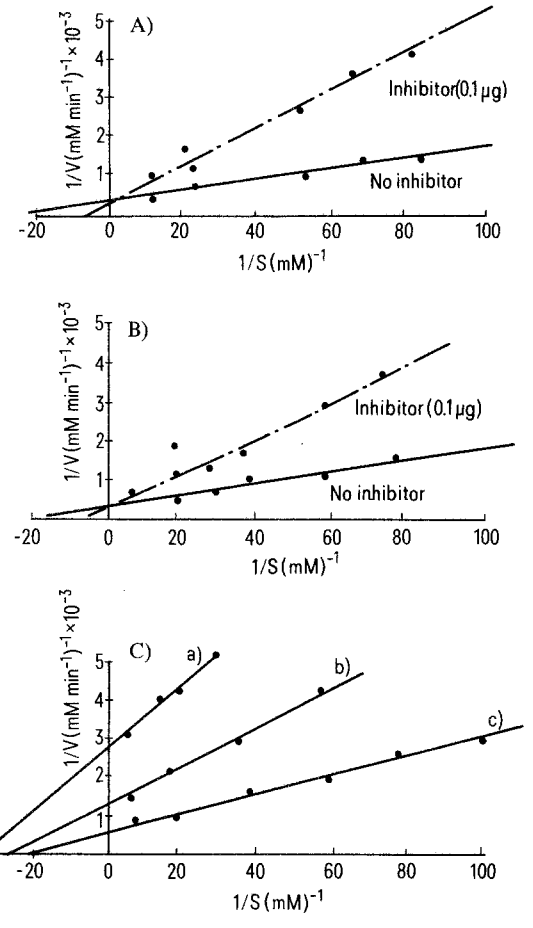

Figure 2. Lineweaver-Burk plots for 1 (ring 3′,5′-^{3}H) tyrosine in presence and absence of the tyrosine hydroxylase inhibitor (M-THI). *A* purified tyrosine hydroxylase (40 µg): ^{3}H-tyrosine without the inhibitor (——) and with 0.1 µg inhibitor (- · - · -). *B* Tyrosine hydroxylase from cytosol of melanotic melanoma (40 µg protein): ^{3}H-tyrosine without the inhibitor (——), and with 0.1 µg inhibitor (- · - · -). *C* Lineweaver-Burk plots for the enzyme activity of 3 human melanoma cell lines a) HMMC-ShA, b) HMM-WJP and c) HMMC-ZBJ.

Table 1. Effect of tyrosine hydroxylase inhibitor and L-DOPA on melanotic melanoma cell lines

Cell lines	Cultivation period (h)	^{3}H-Estradiol binding (fmole/mg protein) Standard	+M-THI	+L-DOPA	Tyrosine hydroxylase ^3H_2O formed cpm/h/10^6 cells Standard	+M-THI	+L-DOPA	Melanin biosynthesis A_{400}nm/10^6 cells Standard	+M-THI	+L-DOPA
HMMC-ShA	72	595±35	487±29	372±19	445±21	327±21	245±17	1.30±0.01	0.92±0.01	0.78±0.01
HMMC-WJP	72	817±45	658±40	524±32	807±68	727±52	645±45	1.66±0.04	1.32±0.03	1.05±0.01
HMMC-ZBJ	72	548±29	442±25	327±17	425±78	325±27	217±14	0.93±0.05	0.72±0.02	0.59±0.01
HMMC-ShA	144	373±22	264±19	173±15	298±19	185±15	113±12	1.04±0.01	0.84±0.02	0.65±0.02
HMMC-WJP	144	587±34	458±31	347±24	625±39	517±31	327±19	1.37±0.05	1.04±0.03	0.75±0.02
HMMC-ZBJ	144	357±20	239±18	122±14	275±22	198±19	135±11	0.69±0.02	0.41±0.02	0.15±0.01
HMMC-ShA	216	147±14	78±6	37±5	142±14	79±9	31±4	0.76±0.03	0.54±0.02	0.42±0.01
HMMC-WJP	216	238±18	114±9	58±4	375±34	187±17	98±8	0.85±0.04	0.62±0.03	0.53±0.03
HMMC-ZBJ	216	114±11	65±7	31±7	117±11	84±9	42±4	0.32±0.03	0.15±0.02	0.10±0.01
HMMC-ShA	576	417±19	9±0.8	4±0.6	376±41	3.2±0.2	1.7±0.1	0.98±0.03	0.04±0.01	0.02±0.01
HMMC-WJP	576	532±27	11±0.7	6±0.8	421±37	3.8±0.3	1.4±0.1	1.32±0.09	0.03±0.01	0.03±0.01
HMMC-ZBJ	576	478±36	4±0.2	3±0.02	319±25	2.7±0.2	1.3±0.1	1.17±0.07	0.02±0.01	0.04±0.01
HMMC-ShA	1152	613±38	8±0.6	3±0.04	462±25	1.8±0.04	0.8±0.09	1.28±0.03	0.01±0.01	0.01±0.01
HMMC-WJP	1152	788±39	5±0.02	2±0.01	811±62	2.1±0.05	0.9±0.07	1.71±0.04	0.01±0.01	0.01±0.01
HMMC-ZBJ	1152	539±27	3±0.01	4±0.02	437±73	1.0±0.02	0.8±0.06	1.01±0.05	0.01±0.01	0.01±0.01

M-THI represent the inhibitor isolated from the culture media of the cloned hybrid cells (Hy-7) which was produced from the fusion of myeloma cells with murine spleen lymphocytes preimmunized with highly purified tyrosine hydroxylase.

purified enzyme and the cytosol fraction was assayed in absence and in presence of the M-THI by monitoring the amount of ^3H_2O released into the medium during conversion of 1(ring-3′,5′-^{3}H) tyrosine to dihydroxyphenylalanine[15,16].

^{3}H-estradiol-binding to the purified enzyme and to the tumor cell cytosol fraction in presence and absence of the M-THI was monitored by well established methods[17,18]. Melanin content was measured by the colorimetric method described by Whittaker[19].

Conversion of melanotic to amelanotic melanoma cells was examined as follows: Aliquots (10^7 cells/ml) of melanotic melanoma HMMC-ShA, HMMV-WJP and HMMC-ZBJ, each was seeded into 3 groups (6 dishes/group). The cells were allowed to proliferate in A) DMEM supplemented with 20% FCS, B) DMEM supplemented with 20% FCS and M-THI (1.5 µg/ml), and C) DMEM supplemented with 20% FCS and L-DOPA (10 µg/ml). All the 3 groups were incubated in the same incubator with 5% CO_2 humidified air. At 72 h intervals, they were fed with the same volume (2 ml) of their fresh nutrients. They were split at the same time with the same proportion (1:5). All the 3 types of cell culture maintained the same doubling time.

Results and discussion. Scatchard analysis of ^{3}H-estradiol-binding to cytosol from 3 melanoma cell lines, and to a system with 10 µg of the purified tyrosine hydroxylase is presented in figure 1. These analyses were completed by means of multiconcentration saturation analysis using dextran-charcoal analysis (DCCA). Apparent estrogen binding of more than 5 fm/mg protein was observed. Cytosols of 5 amelanotic cell lines did not bind ^{3}H-estradiol.

The curves in figure 2A show double reciprocal Lineweaver-Burk plots for cleavage of 1 (ring 3′,5′-^{3}H) tyrosine by purified tyrosine hydroxylase in presence and absence of the M-THI. The enzyme assayed without the inhibitor was found to have a K_m(app.)-value of 0.05 nM and a V_{max} of 2.18 µM min^{-1} at 23 °C and pH 7.5 (0.53 µg enzyme/ml). When the inhibitor was present, the double reciprocal plot gave the same V_{max} but different K_m(app.) suggesting a competitive inhibition.

The curves in figure 2B represent a double reciprocal plot for the cleavage of ^{3}H-tyrosine by tyrosine hydroxylase of melanotic melanoma cytosol in presence and absence of the M-THI. The double reciprocal plots suggest M-THI as a competitive inhibitor of ^{3}H-tyrosine hydrolysis by the cytosol enzyme.

The curves in figure 2C show that cytosol tyrosine hydroxylase from 3 melanotic melanoma cell lines hydrolyze ^{3}H-tyrosine with different K_m, suggesting different rates of melanogenesis.

The data depicted by curve A in figure 3 indicate that the inhibitor M-THI blocks ^{3}H-estradiol-binding to the purified enzyme at dilutions greater than 1:250. Inhibition of ^{3}H-estradiol-binding to cytosols of melanotic melanoma cells varied from cell line to another (curves B, C, and D). M-THI had statistically non-significant effect on ^{3}H-estradiol binding to cytosols of amelanotic melanoma (curve E), or to cytosols of mammary carcinoma cells (not shown in figure 3).

The data summarized in table 1 indicate that cultivation of melanotic melanoma cells in culture media supplemented with either M-THI or L-DOPA decreased ^{3}H-estradiol binding to the cytosols. Parallel decrease occurred in cyto-

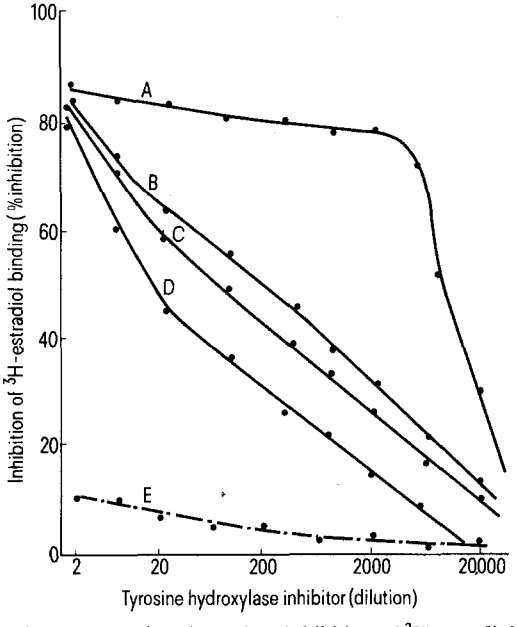

Figure 3. Concentration-dependent inhibition of ^{3}H-estradiol binding to purified enzyme and to cytosols of human tumors cells. Inhibition of ^{3}H-estradiol-binding to purified tyrosine hydroxylase (curve A), and to cytosols of melanotic melanoma HMMC-ShA, HMMC-WJP and HMMC-ZBJ cell lines (curves B, C, and D) respectively. Curve E inhibition of ^{3}H-estradiol-binding to cytosols of amelanotic melanoma HMMC-KM cell line.

Table. 2. Characteristics of tyrosine hydroxylase inhibitors from melanoma cells and from culture medium of clone somatic cell hybrid 'Hy-7'

	Melanoma cell inhibitors Soluble	Particulate	Cloned cell hybrid inhibitor M-THI from 'Hy-7'
Protein reaction	(+)	(+)	(+)
Filtrate from amicon membrane:			
Initial solution	98±8.3	96±8.1	98±8.7
XM-200	75±5.2	91±7.6	8±0.7
XM-100	87±6.3	12±0.9	0.2±0.01
XM- 50	97±6.8	4.5±0.2	0.3±0.01
UM-20	5.5±0.2	0.9±0.01	0.2±0.01
PM-30	2.3±0.1	0.2±0.01	0.2±0.01
PM-10	1.5±0.03	0.1±0.01	0.1±0.01
After treatment with:			
Trypsin	0.9±0.08	1.3±0.09	0.5±0.01
Lipase	87±6.7	98±6.3	97±7.8
Precipitin formation with purified tyrosine hydroxylase	(-)*	(-)*	1:512

The above results are presented as percent inhibition of a standard tyrosine hydroxylase preparation. They represent the average of 3 separate assays ±SD. * No precipitin was formed at 1:4, 1:8 and 1:16 ratios enzyme to inhibitor.

sol tyrosine hydroxylase activities and melanin biosynthesis. These changes did not occur in control cultures carried on in parallel in the standard medium.

In soft agar, melanotic and amelanotic melanoma cells produce 2 major morphologically distinct types of colonies. The 2 major colony variants were: a) groups of 50–250 dark (melanotic) small (5–20 µm diameter) cells, and b) groups of 5–50 light, large (20–40 µm diameter) cells. Control cultures in standard DMEM maintained their dark small morphology for over 30 subcultures. On the other hand, on the 3rd subculture, each of the experimental cultures, i.e. cultures grown in media supplemented with either M-THI inhibitor or L-DOPA, gave a mixture of the 2 types of colonies, and on the 7th subculture they gave mostly the light, large colonies.

Human melanoma cells contain 2 types of endogeneous tyrosine hydroxylase (TH) inhibitors. By filtration through Amicon membranes, the data in table 2 indicate that the inhibitors differed in molecular size. The M-THI inhibitor from supernatants of the cloned hybrids had the highest molecular weight. All the 3 inhibitors are protein in nature, but only M-THI in presence of the enzyme produced a precipitin band in agar suggesting that it is an antibody to tyrosine hydroxylase.

Whilst the method in somatic hybridization, introduced by Köhler and Milstein[20] has proved entirely successful for the production of antibodies against particulate antigens such as those on the cell surface[21,22] and viruses[23], it has only recently been successfully applied to soluble tumor markers such as CEA[2], hCG[25] and alpha-fetoprotein[26].

Inhibition of tyrosinase activity produces regression of abnormal cell growth in human and mouse melanoma[27–29]. The described experiments showed the binding of estrogen to tyrosine hydroxylase present in cytosols of melanotic melanoma cells, thus confirming reports by other investigators[17,18]. They also demonstrated the production of an enzyme inhibitor by somatic cell hybrids. The role of these inhibitors in the modulation of melanin biosynthesis, differentiation, morphology and oncogenicity of human malignant melanoma are under current studies.

1 Mailing Address: 180 Longwood Drive, Kankakee (Illinois 60901, USA).
2 M. Romsdahl and I.S. Cox, in: Proceedings of the 20th annual clinical conference on cancer, p.251. University of Texas System Cancer Center, M.D. Anderson Hospital and Tumors Institute, Chicago. Year Book Medical Publishers, Chicago 1976.
3 Y.M. Chen and W. Chavin, Proc. Soc. exp. Biol. Med. *121,* 497 (1966).
4 Y.M. Chen and W. Chavin, Cancer Res. *35,* 606 (1975).
5 A.A. Hakim, Cancer Biochem. Biophys. *4,* 173 (1980).
6 A.A. Hakim, Annals Inst. Pasteur, Paris *131C,* 155 (1980).
7 K.S. McCarty, J. Wortman, S. Stowers, D.B. Lubahn, K.S. McCarty, and H.F. Seigler, Cancer *46,* 1463 (1980).
8 V. Ulrich and W. Duppel, in: The Enzymes, vol.12, p.253. Ed. P.D. Boyer. Academic Press, New York 1974.
9 J.B. Burnett, J. biol. Chem. *246,* 3079 (1971).
10 J.B. Burnett and H. Seiber, J. Invest. Dermat. *52,* 1999 (1969).
11 Y.M. Chen and W. Chovin, Oncology *31,* 147 (1975).
12 Y.M. Chen and W. Chovin, Cancer Res. *35,* 606 (1975).
13 A.A. Hakim, Proc. Soc. exp. Biol. Med. *168,* 82 (1981).
14 A.A. Hakim, Neoplasma *24,* 81 (1977).
15 A.O. Kawa, M. Nakayama, M. Nohara and T.T. Tehen, Archs Biochem. Biophys. *148,* 548 (1972).
16 S.H. Pomerantz, J. biol. Chem. *241,* 161 (1968).
17 K.S. McCarty, T.K. Barton, C.H. Peete and W.T. Creesman, Cancer *42,* 512 (1978).
18 K.S. McCarty, Jr, T.K. Barton, B.I. Fetter and W.T. Creesman, Am. J. Path. *96,* 147 (1979).
19 J.R. Whittaker, Devl. Biol. *8,* 99 (1963).
20 G. Kohler and C. Milstein, Nature *256,* 41 (1975).
21 M.M. Trucco, J.W. Stocker and R. Cappellinin, Nature *273,* 666 (1978).
22 K. Imai, A.-K. Ng and S. Ferrone, J. natl Cancer Inst. *66,* 489 (1981).
23 H. Koprowski, W. Gerhard and C.M. Croce, Proc. natl Acad. Sci. USA *74,* 2985 (1977).
24 R.S. Accolla, S. Carnell, M. Phan, D. Hemann and J.P. Mach, Peptides Biol. Fluids *27,* 31 (1979).
25 C. Stahli, T. Staehelin, V. Miggiano, K. Schmidt, Jr, and P. Haring, J. Immun. Meth. *32,* 297 (1980).
26 Y.K. Tsung, A. Milursky and E. Alpert, N. Engl. J. Med. *302,* 180 (1980).
27 H.B. Demopoulos, Cancer *19,* 657 (1966).
28 B.T. Hourani and H.B. Demopoulos, Lab. Invest. *21,* 434 (1969).
29 W.L. Ryan and J.A. Elliot, Archs Biochem. Biophys. *125,* 797 (1968).

Close relationship of mitochondria with intercellular junctions in the adrenaline cells of the mouse adrenal gland

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Summary. In adrenaline cells, junctional complexes formed by alternating gap junctions and attachment plaques were identified in close proximity to bilateral clusters of mitochondria. It is suggested that this proximity is related to a role of gap junctions in metabolic coupling.

Gap junctions have been structurally identified between contacting cells of most tissues especially glandular epithelia. Only recently, however, freeze-fracture studies have revealed the presence of gap junctions in the adrenal medulla[1]. Gap junctional particle aggregates were shown to be relatively scarce, with many linear and loop-like configurations. The possibility existed therefore that adrenomedullary cells might possess small and non-macular gap junctions previously missed in studies of thin sections, revealing only attachment plaques. In contrast to freeze-fracture preparations, where cell types could not be identified, adrenaline and noradrenaline cells are easily distinguished in thin sections by the differential electron density of their storage granules after glutaraldehyde fixation[2]. Thus intercellular junctions were carefully reinvestigated in thin sections of mouse adrenal medulla. The mouse adrenal medulla was chosen because its cellular composition is well known[3]. Moreover, freeze-fracture studies showed that no tight junctional fibrils were present in this species. 3 mice were perfused through the heart with buffered 2.5% glutaraldehyde for 10 min. Small pieces of tissue were immersed in the same fixative for 1–3 h, post-fixed in osmium tetroxyde and 'en bloc' treated by uranyl acetate.

Small or linear gap junctions are expected to appear as