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Influence of 2α-Methyl-17β-Hydroxy-5α-Androstan-3-one on Incorporation of Glycine-2-C^{14} into Proteins of a Rat Mammary Fibroadenoma. A Possible Bioassay Method.* (27897)

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Huggins(1,2) and Glenn(3,4,5) and their coworkers suggested a test involving the action of steroids on growth of a transplanted rat mammary fibroadenoma as a means of finding possible useful therapeutic agents for human mammary tumors. Huggins(2) suggested the use of the effective steroid 2α-methyl-17β-hydroxy-5α-androstan-3-one on the basis of this rat test and Glenn *et al.*(5) were able to suggest the active anti-tumor steroid 9α-fluoro-11β, 17α-acetoxy-6α-methylpregna-1, 4-diene-3, 20-dione for human trial at least partly on the basis of its activity against the rat mammary fibroadenoma. This communication reports an assay procedure both more rapid and more efficient than those previously available. This new test uses the end-point of glycine-2-C^{14} incorporation into the tumor proteins.

Method. Aged Sprague-Dawley rats bearing spontaneous mammary fibroadenomas obtained from the Sprague-Dawley Co. (Madison, Wisc.) and a transplanted tumor kindly furnished by Dr. E. Myles Glenn, Upjohn Co., Kalamazoo, Mich., served as the starting tumors.

The transplantation technic of Glenn *et al.* (3,4) was followed with few modifications. Female Sprague-Dawley rats, 51 ± 2 days old and weighing 140 ± 15 g were anesthetized with ether and through a dorsal incision, channels were made in the subcutaneous connective tissue with surgical forceps about ½ inch caudal to each front leg. Seeds weighing 70 ± 20 mg were prepared with a nasal cutting forceps from a parent tumor, which would afford enough seed tumors for a complete experiment. Transplants soaked in either saline or Eagle's Medium were quickly inserted into previously made channels and the incision closed with surgical wound clips. The animals were kept at constant temperature and fed Purina Lab Chow *ad libitum* and tap water.

Rats with suitable sized tumors, usually 30 or 35 days after transplantation, were randomized and divided into experimental groups. The test compounds were suspended in an aqueous suspending medium consisting of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethyl cellulose (0.5%), and benzyl alcohol (0.9%) and injected subcutaneously once daily usually for 14 days. The daily dose was contained in 0.5 ml of medium. Control animals received only the vehicle and were run in each experiment in parallel with the test compound. On the final day of treatment, glycine-2-C^{14} in 0.5 ml of saline per rat was injected intraperitoneally and food was removed. Twenty-four hours later tumors were removed, weighed, and processed for the isolation and counting of the protein.

Tumors were frozen in liquid nitrogen and pulverized in a stainless steel mortar. The crushed tissue was washed successively with 5% trichloracetic acid at 90°C for 15 minutes, 5% trichloracetic acid at room temperature, 80% ethanol, 95% ethanol, 95% etha-

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TABLE I. Influence of Graded Doses of 2α-Methyl-17β-Hydroxy-5α-Androstan-3-One Injected Daily for 14 Days on Tumor Weight and Glycine-2-C^{14} Uptake in Tumor Protein.

Exp	Total dose of 2α-methyl-17β-hydroxy-5α-androstan-3-one (mg)	No. of tumors	Mean tumor wt (mg)	Mean specific activity of tumor protein (cpm/mg)
A	0	6	292 ± 40*	62.0 ±6.3*
	7	7	169 ± 27	45.3 ±4.6
	14	6	215 ± 38	50.2 ±2.9
	28	7	214 ± 31	39.9 ±2.4
	56	8	157 ± 14	40.3 ±1.5
B	0	6	205 ± 25	66.4 ±3.3
	7	8	170 ± 13	45.2 ±2.2
	14	8	150 ± 18	60.7 ±2.6
	28	9	173 ± 21	49.8 ±3.0
	56	11	145 ± 10	53.3 ±3.0
C	0	7	441 ±145	64.9 ±5.9
	3.5	7	258 ± 46	38.6 ±3.8
	7	7	321 ± 85	36.4 ±6.2
	14	6	153 ± 62	42.4 ±4.7
	28	8	404 ±175	37.1 ±3.0
D	0	10	355 ± 44	52.9 ±1.8
	1.75	9	221.8± 23	48.3 ±5.0
	3.5	8	229.1± 24	34.3 ±4.8
	7.0	7	225.0± 27	39.6 ±2.2

* ± standard error.

nol-ether (3:1) at 80°C twice, 95% ethanol, and anhydrous ether. Treatment of the protein residue with 1.4 M mercaptoacetic acid resulted in no loss of radioactivity.

All determinations of radioactivity were made on solid samples plated on tared aluminum planchets and counted in a gas flow proportional counter for a sufficient period to obtain 5% statistical accuracy. Specific activity is expressed in terms of counts per minute per mg of protein and corrected for both self-absorption and counting efficiency.

Results. Preliminary studies indicated that when 2α-methyl-17β-hydroxy-5α-androstan-3-one injections were started 35 days after tumor transplantation 7- and 10-day treatment periods resulted in highly variable responses using both the tumor weight and glycine-2-C^{14} uptake end-points. When the treatment period was extended to 14 days quite reasonable data were obtained. Four experiments described in Table I illustrate that weight changes in tumors as a result of steroid treatment are variable and unreliable as compared to the index of specific activity of tumor protein. The specific activity of the tissue protein from solvent treated control animals was remarkably constant for the 4 studies, varying from 52.9 ± 1.8 to 66.4 ± 3.3. Total doses of 3.5 mg inhibited glycine-2-C^{14} uptake into the tumor proteins. Increasing the dose from 2 to 16 times the minimal effective dose produced only a small additional response. Maximum inhibition was less than 50%.

The data in Table II indicate the relative potencies of 2α-methyl-17β-hydroxy-5α-androstan-3-one and testosterone propionate. It is recognized that these experiments are not ideal since for a comparative study both compounds should be studied either as the free compound or as the propionate. Nonetheless, the propionate of testosterone was consistently less active than free 2α-methyl-17β-hydroxy-5α-androstan-3-one although the propionate is expected to be significantly more active than the free compound.

The mean specific activities of the tumor proteins were reasonably constant although the source and age of tumor varied (Table III). Maximum variation in mean specific activity for vehicle treated animals was from 46.8 ± 5.0 to 66.4 ± 3.3. For the Upjohn tumors derived from transplanted tissues ranging from 240 to 332 days the mean specific activity variation was from 48.5 ± 5.0 to 66.4 ± 3.3. The 3 groups of tumors derived from Sprague-Dawley rats showed a

TABLE II. Influence of Graded Doses of 2α-Methyl-17β-Hydroxy-5α-Androstan-3-One (2α) and Testosterone Propionate (TP) Injected Daily for 14 Days on Tumor Weight and Glycine-2-C^{14} Uptake in Tumor Protein.*

Exp	Compound administered (total dose, mg)	No. of tumors	Mean specific activity of tumor protein (cpm/mg)
E	0	9	106.2 ± 8.1†
	TP (3.5)	11	96.0 ± 7.6
	TP (7.0)	13	85.3 ± 5.2
	2α (3.5)	12	65.5 ± 6.2
	2α (7.0)	12	72.5 ± 4.8
F	0	15	76.8 ± 6.1
	TP (3.5)	7	64.0 ± 4.0
	TP (7.0)	19	57.5 ± 4.2
	2α (3.5)	9	55.7 ± 3.7
	2α (7.0)	14	43.5 ± 2.0

* Inj started 35 days after transplantation. Each animal received 5 μc of glycine-1-C^{14} in 10 μmoles 24 hr before autopsy.

† ± standard error.

TABLE III. Influence of Source and Age of Tumor or Mean Specific Activity of Tumor Protein (5 μc Glycine-2-C^{14} in 10 μmoles of Glycine Injected 24 Hours before Autopsy).

Source of tumor	Age of tumor transplant (days)	No. of tumors	Mean specific activity of tumor protein (cpm/mg)
Sprague-Dawley	Spont.*	7	65.7 ± 4.8†
Upjohn	247	6	59.5 ± 3.7
"	240	5	48.5 ± 5.0
Sprague-Dawley	174	7	46.8 ± 5.0
Upjohn	290	6	62.0 ± 6.3
"	296	6	66.4 ± 3.3
"	332	10	52.9 ± 1.8
Sprague-Dawley	56	7	64.9 ± 5.9

* Spontaneous.
† ± standard error.

maximum variation from 46.8 ± 5.0 to 65.7 ± 4.8.

The results reported in all 3 Tables are on the basis of autopsies being performed 24 hours after administration of the glycine-2-C^{14}. When this interval was decreased to 6 hours similar data in magnitude and variation were obtained.

Conclusions. A preliminary method has been described for assessing the inhibitory action of compounds on a transplantable rat mammary fibroadenoma. Sufficient data have been obtained to indicate that this can be a useful procedure if the end-point of tumor growth is the glycine-2-C^{14} uptake in the tumor proteins. Crude tumor weight was inadequate on the basis of high variation within groups and between groups.

Reproducible inhibition was obtained with both 2α-methyl-17β-hydroxy-5α-androstan-3-one and testosterone propionate and the former compound appears to be the more active.

Summary. The uptake of glycine-2-C^{14} into the proteins of a rat mammary fibroadenoma has been studied as a possible bioassay procedure for detecting useful antitumor steroids and other substances for further studies in humans. A preliminary method has been evolved consisting in transplantation of the tumor to 140 ± 15 g female rats and after 35 days injecting the rats once daily for 14 days with the test compounds. On the last day of treatment the rats received one injection of glycine-2-C^{14}. Twenty-four hours after the last injection of the test compound and the glycine-2-C^{14} injection, the tumors are removed, weighed and C^{14} specific activity of the tumor protein determined. The tumor was inhibited by testosterone propionate but more regularly and more intensely by equal weights of 2α-methyl-17β-hydroxy-5α-androstan-3-one, a steroid.

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Influence of Age and N, N′-Diethylene-N″-Phenethylphosphoramide (PEDP) Upon Nucleic Acid and Ribonuclease Activity in Tumor Tissue.* (27898)

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A recent report by Allison *et al.*(1) indicates that the development of the Walker 256 carcinosarcoma transplanted into the rat is maximum when ribonuclease activity in the

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