tive deserts of the world, the Australian species produce urine whose concentrations are equivalent to or exceed those of other forms that are not dependent on exogenous water (Table 1). In fact, to our knowledge, the values for *N. alexis* and *L. hermannsburgensis* are the highest and second highest, respectively, measured for mammals. The highest single value for urine osmotic pressure (urine, 6340 mosmole/liter; urine : blood ratio, 17 : 1) previously measured was obtained from a Saharan sand rat (*Psammomys obesus*) on a diet of halophytic plants (*1*).

Certain North American desert rodents have unusually low pulmocutaneous and fecal losses of water compared to nondesert forms (*1*). The comparative data, including ours for Australian forms, indicate that water loss may be reduced by the excretion of unusually dry feces in desert rodents deprived of water (Table 2). However, the data available indicate a wide range of pulmocutaneous water loss regardless of habitat and degrees of water independence (Table 2). Expressed as a function of oxygen consumption, which is directly related to the production of metabolic water, the rate of pulmocutaneous water loss was lowest among our animals in the form most dependent on water (*N. cervinus*); the higher rates of *N. alexis* and *L. hermannsburgensis* were comparable to that of the laboratory rat.

While the Australian desert rodents discussed herein will drink water greedily in the laboratory, they are in general not dependent on drinking water under conditions of low relative humidity, moderate temperatures, and a diet of dry, carbohydrate-rich seeds. They appear to owe this independence to extreme renal capacities for concentrating urine and to reduced output of water in the feces. Under natural conditions this efficiency in water conservation, together with a nocturnally active and a diurnally fossorial existence, yield a combination of physiological and behavioral adaptations which ensures survival under the potentially stressful conditions of extreme aridity, diurnal heat, and very low, periodic rainfall characteristic of the Australian desert.

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Sulfhydryl Groups and Estradiol-Receptor Interaction

Abstract. *The characteristic ability of rat uteri to take up tritiated estradiol in vitro or to retain estradiol previously incorporated either in vivo or in vitro is destroyed by treating the tissue with various sulfhydryl-blocking reagents. The two radioactive estradiol-receptor complexes, observed in uterine homogenates in the supernatant fraction and in an extract of the nuclear fraction, respectively, are disrupted by brief exposure to organic mercurials in the cold. Sulfhydryl groups of uterine receptor substances apparently play a vital role in estradiol binding, perhaps indirectly through contribution to receptor conformation.*

It is now well established that estrogen-responsive tissues, such as uterus and vagina, contain minute amounts of apparently unique components called "estrogen receptors" which show a striking affinity for estradiol and certain other estrogens, both in vivo (*1*) and in vitro (*2, 3*). Strong but reversible interaction of hormone with receptor, without chemical transformation of the steroid molecule, appears to be an early step—if not the initial one—in the uterotrophic process (*3, 4*). Our study demonstrates that sulfhydryl groups of the receptor substance are essential to its ability to associate with estradiol (*5*).

On exposure to dilute ($10^{-10}M$) solutions of tritiated estradiol in Krebs-Ringer-Henseleit (KRH) glucose buffer (*3*), the uterine horns from immature rats rapidly accumulate radioactive steroid until the concentration in the tissue is several hundred times that of the medium (Fig. 1). Slit uterine horns of 24-day-old rats were stirred for 1 hour at 38°C in 500 ml KRH glucose buffer (*p*H 7.3) alone or containing $0.001M$ iodoacetamide (*IA*), *N*-ethylmaleimide (*NEM*), or *p*-hydroxymercuribenzoate (*PHMB*), after which they were stirred for various periods at 38°C in 500 ml of the buffer containing $10^{-10}M$ estradiol-6,7-H^3 (specific activity, 57 mc per micromole; solution contains 12,500 disintegrations per minute per milliliter). Radioactivity is determined by combustion of freeze-dried tissues by modified Schöniger technique to produce tritiated water counted in liquid-scintillation spectrometer.

As shown in Fig. 1, incubation of the uteri with iodoacetamide markedly decreases their ability to take up estradiol, whereas similar treatment with *N*-ethylmaleimide or *p*-hydroxymercuribenzoate, even for as little as 15 minutes, completely eliminates the characteristic affinity of the tissue for estrogen. The latter agents reduce the amount of radioactivity entering the tissue to that observed with rat diaphragmatic muscle, a nontarget tissue in which the small uptake of radioactivity is not affected by treatment with sulfhydryl reagents. Similar abolition of estradiol uptake is effected when $0.001M$ *p*-hydroxymercuribenzoate or methylmercurihydroxide are present during exposure of the uteri to the hormone solution.

Tritiated estradiol, previously incorporated into rat uteri, either after subcutaneous injection of the hormone in the animal or by exposure of the iso-

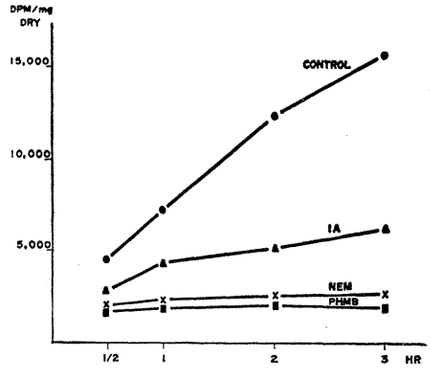

Fig. 1. Effect of sulfhydryl reagents on uptake of estradiol by uterine tissue in vitro. Each point represents the median value of five horns.

lated uterine horns to estradiol solutions, remains bound to the uterine tissue on repeated or continuous washing with buffer alone (*2*, *3*). If sulfhydryl-blocking reagents are added to the washing medium, the radioactive steroid is readily eluted (Fig. 2). The rate of estradiol release is more rapid with *p*-hydroxymercuribenzoate than with *N*-ethylmaleimide and slowest with iodoacetamide. These differences in activity among the various blocking agents are in the same order as those observed in the uptake experiments (Fig. 1) and probably reflect the relative rates of reaction with sulfhydryl groups in the tissue. The small amount of radioactivity present in uteri treated with sulfhydryl reagents prior to estradiol uptake is not firmly bound and is eluted readily on washing with buffer (Fig. 2B).

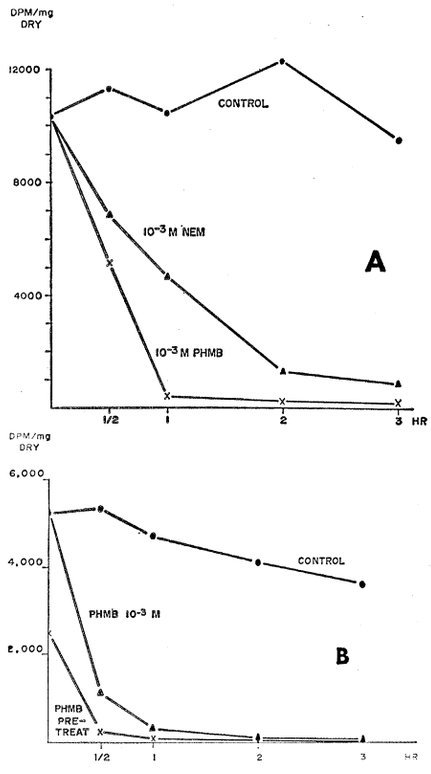

Fig. 2. Effect of sulfhydryl reagents on uterine retention of estradiol previously incorporated in vivo or in vitro. (A) Slit uterine horns of 24-day-old rats were excised 2 hours after single subcutaneous injection of 0.11 μg (21.9 μc) of estradiol-6,7-H^3 in 0.5 ml of saline; then they were stirred at 38°C for various periods in 500 ml of KRH glucose buffer, *p*H 7.3, with or without 0.001*M* sulfhydryl reagent, with the eluting medium changed at each time point. (B) Uterine horns from untreated rats were stirred for 1 hour at 38°C in KRH glucose buffer or in "PHMB pretreat" buffer containing 0.001*M* PHMB. The uteri were then stirred for 30 minutes at 38°C in buffer containing $10^{-10}M$ estradiol-6,7-H^3, rinsed briefly, and eluted with buffer, with or without 0.001*M* PHMB as described under (A). Radioactivity was determined by combustion; each point represents the median value of five horns.

The ability of sulfhydryl reagents to inhibit uterine uptake and retention of estradiol does not, in itself, prove that sulfhydryl groups actually are involved in the estrogen-receptor interaction. Their effect could result from the blocking of sulfhydryl-dependent enzymatic processes which furnish energy required to put the hormone on the receptor and keep it there. Evidence that sulfhydryl groups of the receptor substance actually participate, directly or indirectly, in the estrogen-binding phenomenon is provided by observations that sulfhydryl-blocking reagents disrupt the radioactive estradiol-receptor complexes which can be detected in uterine extracts.

When homogenates of rat uteri, previously exposed to physiological amounts of tritiated estradiol either in vivo or in vitro, are fractionated by differential ultracentrifugation, most of the radioactive steroid is bound in the heavy or nuclear fraction, but, depending on homogenization conditions, a variable portion of the radioactivity appears in the high-speed supernatant fraction (*6*). Using homogenates prepared in hypotonic medium, Toft and Gorski (*7*) have shown that the estradiol in the supernatant fraction is bound to a macromolecule which can be recognized conveniently by ultracentrifugation in sucrose density gradients in which the radioactivity sediments at about 9.5*S*. We observed (*5*, *8*) that repeated extraction of the nuclear sediment with cold 0.3*M* potassium chloride solubilizes the major portion of the uterine radioactivity as a different complex sedimenting at about 5*S* (*9*). Both receptor substances appear to be at least partly protein in nature, in that both complexes are destroyed by incubation at 10°C with pronase, but not by ribonuclease or deoxyribonuclease.

The integrity of both hormone-receptor complexes was found to depend on sulfhydryl groups. When a solution containing either of the complexes is exposed to 0.001*M* *p*-hydroxymercuribenzoate or methylmercurihydroxide for 30 minutes at 2°C, the association is disrupted, and the radioactive estradiol is released (Fig. 3). Fifty slit uterine horns from 24-day-old rats were stirred at 38°C for 2 hours with $10^{-10}M$ estradiol-6,7-H^3 in KRH-glucose buffer, rinsed, and then homogenized (12 strokes with a glass pestle) at 2°C in 2.5 ml of 0.01*M* tris buffer, *p*H 7.4, containing 0.0015*M* ethylenediaminetetraacetic acid. The homogenate was centrifuged at 204,000*g* for 1 hour at 2°C, and the supernatant was separated. The sediment was again suspended at 2°C in 2.5 ml of tris buffer containing 0.3*M* KCl, homogenized gently, and centrifuged at 204,000*g* for 1 hour at 2°C to give the nuclear extract. Fifty μl of tris buffer alone or containing 0.007*M* PHMB was added to 300-μl portions of either the supernatant fraction or the nuclear extract. These buffered fractions were kept (with occasional stirring) at 0°C for 30 minutes, and then portions (200 μl) were layered on 4.8 ml of a 5 to 20 percent sucrose gradient containing tris buffer. After centrifuging at 204,000*g* for 7.3 hours at 6°C, 50 fractions (100 μl) were drawn off from the bottom and counted directly in a dioxane-xylene scintillation mixture.

A similar disruption of the estradiol-receptor complexes is observed with *N*-ethylmaleimide, although this compound reacts rather slowly in the cold, so that only partial liberation of the hormone occurs during 1 hour of treatment. Along with the previously described effects of sulfhydryl reagents on estradiol uptake and retention by uter-

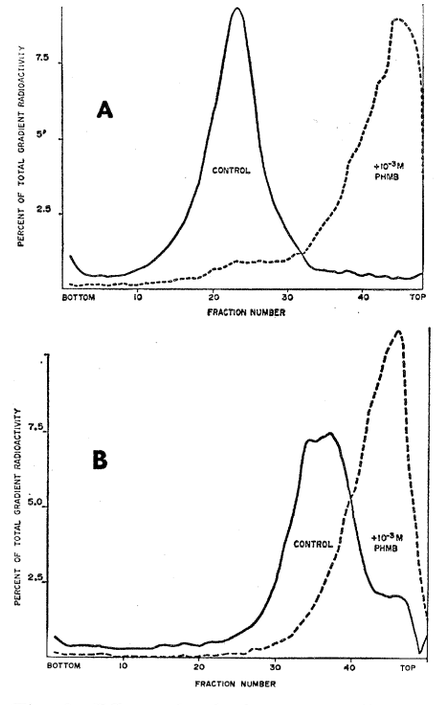

Fig. 3. Effect of *p*-hydroxymercuribenzoate on radioactive estradiol-receptor complexes present in: (A) supernatant fraction; (B) nuclear extract. Total count/min in the gradient: (A) 4430; (B) 3460.

ine tissue, these observations indicate that, in both the 9.5*S* and 5*S* complexes, sulfhydryl groups of the receptor substances actually participate in the estrogen-binding phenomenon, perhaps indirectly by contributing to receptor structure or conformation.

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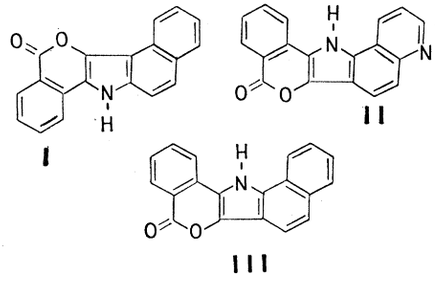
9. Tritiated estradiol also binds to a component of rat serum (probably albumin), giving rise to a peak of radioactivity likewise sedimenting in the region of 5*S*. This association, which is much weaker than the binding to uterine receptors, has been found by T. Suzuki in our laboratory to be unaffected by sulfhydryl-blocking reagents. In that homogenates of uteri contain appreciable amounts of serum proteins, comparison of the sedimentation pattern in the presence and absence of organic mercurials provides a convenient test to determine whether a 5*S* peak represents estradiol associated with the receptor from uterine nuclei or an artifact resulting from the interaction of free estradiol with a serum contaminant.
10. Supported by PHS research grant CA-02897 from the National Cancer Institute and a Ford Foundation fellowship grant (D.J.H.).

3 August 1967

5-Oxo-5H-benzo[e]isochromeno-[4,3-b] indole, a New Type of Highly Sarcomagenic Lactone

Abstract. *The title compound, which belongs to a new synthetic group of polycyclic lactones derived from isocoumarin, exhibits a remarkably high degree of carcinogenicity* in situ *when injected in mice. Cancer-inducing activity has also been found in similar isocoumarins, a family known to include many naturally occurring substances.*

In recent years much attention has been paid to the local carcinogenic effects of natural (*1*) or synthetic (*2*) compounds bearing lactone groups, the most widely investigated being the polycyclic furanoid lactones of the aflatoxin family (*3*). We wish to record here the outstanding activity of 5-oxo-5*H*-benzo[*e*]isochromeno [4,3-*b*] indole (I), an entirely new type of polycyclic carcinogen in whose molecular structure a lactone function is present in the form of an isocoumarin system.



I II III

This substance, recently synthesized in our laboratory (*4*), was tested in two strains of mice, strain C3H and strain Swiss (Carshalton, Surrey, United Kingdom). It was administered by subcutaneous injection in the flank of 3- to 4-month-old male and female mice (three injections of 0.6 mg in 0.2 ml of neutral, sterile olive oil, 1 month elapsing between each injection). The results, recorded in Table 1, evidence the remarkable speed and magnitude of the action of compound I, which rank it among the most potent carcinogenic agents known.

Carcinogenicity was also found, although to a lesser degree, in another nitrogenous coumarin similar to I, namely 8-oxo-8*H*-isochromeno[4′,3′:4,5] pyrrolo[2,3-*f*]quinoline (II); tested in mice of C3H strain (14 male and 14 female) in the same conditions as for I, this substance gave one sarcoma in two males after 309 days, and one sarcoma in two females after 282 and 296 days respectively. Further, carcinogenicity in this family of lactones is highly dependent on chemical structure, as witnessed by the total inactivity of 5-oxo-5*H*-benzo[*g*]isochromeno[4,3-*b*] indole (III), which although isomeric with I, had not elicited any tumors in 14 male and 14 female mice (C3H strain) after 450 days.

These findings have a twofold significance. First, compounds such as I and II represent an entirely new class of carcinogens, which can be considered as structurally hybrid between the current polycyclic carcinogens (hydrocarbons, benzacridines, benzocarbazoles) and the lactones. Second, that such a highly carcinogenic substance as I should be found in the chemical family of isocoumarins is of particular importance in view of the presence of the isocoumarin ring in the molecule

Table 1. Sarcomagenic effect of compound I.

Strain C3H				Strain Swiss			
Male		Female		Male		Female	
Survival (days)	Sarcoma on day:	Survival (days)	Sarcoma on day:	Survival (days)	Sarcoma on day:	Survival (days)	Sarcoma on day:
140	90	115	82	103	78	150	102
140	90	115	82	103	78	150	102
140	90	117	89	110	82	150	102
140	101	117	89	112	82	150	102
140	107	117	89	149	102	153	112
140	107	117	89	149	102	153	112
194	152	117	89	149	109	153	112
201	152	117	92	149	109	153	112
241	186	117	92	176	136	153	122
256	186	117	92	176	136	157	122
363	302	167	129	183	142	176	142
373	302	174	132	183	142	176	142
450*		194	140			182	149
450		197	140			182	149

* Animals showing no tumor after 450 days were killed. Tested in over 600 mice of each sex and belonging to the same strains, the solvent used alone has never shown any sarcomagenic activity. In the present experiment the controls consisted of 28 mice (14 male and 14 female) of each strain.