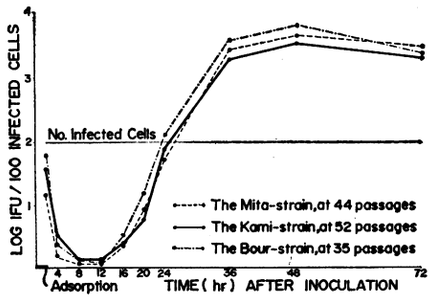

Fig. 1. One-step growth curve of the TRIC agents in HeLa cell monolayers grown on coverslips in Leighton tubes. Cells were infected with 10 inclusion forming units (IFU) per cell. For virus assay, subcultures were made at intervals in new monolayers grown on coverslips with a dilution capable of infecting 1 to 5 percent of the cells. After incubation for 72 hours, the coverslips were stained with Giemsa and the inclusions counted. From the number of the cells containing inclusions in original- and sub-cultures, the IFU titer of the culture was calculated (*6*).

The tissue culture was kept at 35°C after inoculation with the TRIC-agents.

To inoculate the tissue culture, an emulsion of infected yolk sacs prepared in YLH-medium (*5*) containing 10 percent bovine serum was centrifuged at a low speed. Three milliliters of the supernatant, with an ELD_{50} (50 percent egg lethal dose) of about 10^4 per milliliter, was added to each bottle containing the HeLa cells which had been washed twice with YLH-medium (*5*). After 4 hours the cells were again washed with YLH- medium and 10 ml of the same medium containing 2 percent bovine serum was added. For inoculation back into yolk sacs, the cells were suspended in the culture medium by the aid of a rubber spatula 72 hours after they had been inoculated, and 0.4 ml of the suspension was injected into each yolk sac of eggs which had been incubated for 6 days. The embryos then died only 8 to 12 days after inoculation. Before proceeding to the next inoculation into tissue culture, therefore, passages through yolk sacs had to be repeated until the embroys died within 5 days.

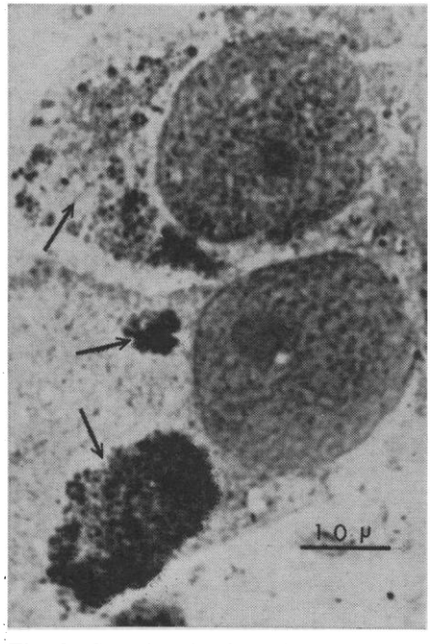

Fig. 2. Cytoplasmic inclusions (arrows) in a smear from a HeLa cell monolayer grown in a 250-ml bottle, 72 hours after inoculation with the Mita-strain in 23rd passage in cell culture. Giemsa stain.

After two (Kami-strain) or three (Mita- and Bour-strains) such alternating passages, serial passage in tissue culture was attempted. Cells were suspended in the culture medium by means of a rubber spatula, 72 hours after inoculation. The suspension was homogenized in a motor-driven Pyrex homogenizer, and was then centrifuged. Three milliliters of the supernatant was added to each bottle containing the HeLa cells which had been washed twice as before. After 4 hours, 7 ml of YLH-medium containing 2 percent bovine serum was added to each bottle without withdrawing the inoculum.

In experiments with the Kami- and the Bour-strains, 10 percent or less of the cells were infected before 10 passages in tissue culture had been completed, as shown by the examination of smears stained for cytoplasmic inclusions. The proportion of infected cells increased thereafter and, after 20 passages, 80 percent of the cells became infected. At the same time, a cytopathic effect became evident. Infected cells assumed spherical shape with granularity of the cytoplasm as observed in unstained preparations.

The Mita-strain became adapted to tissue culture more rapidly than the other strains. After five passages, the proportion of infected cells increased up to 50 percent and, after eight passages, there was an apparent inclusion formation in up to 80 percent of the cells.

Figure 1 shows one-step growth curves of the three strains in tissue culture as determined by the method described by Furness *et al.* (*6*). Figure 2 shows typical cytoplasmic inclusions as observed in a smear from a HeLa cell monolayer infected with the Mita-strain. The Kami-, Mita-, and Bour-strains have now survived more than 80, 50, and 40 passages in tissue culture, respectively. In our laboratory we have never received the TE-55 and the LB-1 strains. The present results are, therefore, not due to a contamination with known strains adapted to tissue culture.

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5. The YLH medium consisted of 1 g of yeast extract, 5 g of lactalbumin hydrolysate, and 25 mg of streptomycin, dissolved in 1 liter of Hanks balanced salt solution.
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Hormone Excretion Patterns in Breast and Prostate Cancer Are Abnormal

Abstract. *In patients with breast and prostate cancer hormone excretion patterns differ in a similar way from patterns in persons without cancer.*

Patients with advanced cancer of the breast or prostate are given palliative hormone therapy ranging from administration of estrogens and androgens to ablation of endocrine glands. Remission occurs in some cases. Similar effects have not been observed with such regimens in cancers at other sites. The initial observation by Beatson that inoperable cancer of the breast in premenopause women responded to castration (*1*) has been extended, and the findings suggest that carcinoma of both breast and prostate may be hormone-dependent (*2*). In studies of the urinary excretion of hormone metabolites in women with cancer of the breast the concentration of androgens was abnormally low, and this difference was useful in discriminating between nonresponders

and responders to pituitary or adrenal ablation in patients with advanced disease (*3*).

The purpose of this study was to search for abnormalities of excretion of hormone metabolites in patients with relatively early cancers, that is, patients first entering the hospital for diagnosis, from whom urine was collected before diagnostic biopsy, surgical intervention, or specific therapy. The intricate relationships within the endocrine system and the diversity of response to hormone therapy in palliative treatment of breast and prostate cancer also suggested the possibility of a more complex endocrine abnormality than has been previously reported.

This report is concerned with these endocrine relations in patients with primary cancer of the breast and the prostate. Patients and normal controls matched with respect to age and sex were selected on negative evidence of myocardial infarction, hypertension, previous or coexisting cancer, renal or hepatic dysfunction, and previous hormone therapy. All cancers were adenocarcinoma, histologically verified. In the patients with breast cancer, all females, the disease was limited to the breast or extension to regional lymph nodes. Patients in the prostate group had cancers with invasion of adjacent tissues and distant metastases.

The hormone metabolites determined were estrogen fractions, 17-ketosteroid fractions, corticoids (Porter-Silber chromogens, 17-ketogenic steroids), pregnanediol, and the biologically active gonadotropin residue (*4*). In addition, the total protein and the protein-bound carbohydrate in the gonadotropin residue were determined; the endocrine activity of these fractions was not studied. The baseline measurements in this analysis are age, height and weight, and the specific gravity and creatinine content of the urine.

The measurements are given in Table 1 as the means and standard deviations of the logarithms of the observed values in each of the three cancer groups. Examination of these comparisons (Table 1) would suggest that androsterone, etiocholanolone, 11-oxygenated ketosteroids, beta fraction, estrone, estradiol, and gonadotropin protein are different in the premenopause cancer subjects than in the normal controls. The postmenopause cancer subjects do not differ so noticeably from the controls in these respects, only estriol, gonadotropin activity and gonadotropin protein appearing to be significantly different. In prostate cancer the amounts of androsterone, estradiol, gonadotropin protein, and gonadotropin carbohydrate in the urine are different, but there is also a difference in the baseline matching variates age, weight, and specific gravity. This appearance of poor baseline matching of the normal controls for prostate cancer presents some difficulty in interpretation of the univariate excretion differences. Reasons for these differences are not apparent, but they are taken into account in the subsequent multivariate analysis.

From the data of Table 1 it is evident, as a first approximation, that in all three comparisons the cancer subjects exhibit similar deviations from their respective controls: (i) high concentration of protein in the gonadotropin residue, (ii) low androgen concentration, especially of androsterone (not quite significant in the postmenopause women), and (iii) a disturbance of the excretion of estrogens.

While the univariate method of examining the data yields indications of abnormality in individual variates, these are not readily interpretable because of the intricate system of inter-correlations of the variates. To study the 18-variate system as a whole we applied the linear discriminant function method (*5*), which makes use of the multivariate interactions as well as the single-variate

Table 1. Mean excretion levels in patients (upper figures) with cancer of the breast and prostate and in normal control subjects (lower figures). Mean logarithm and standard deviation.

Variate	Premenopause (7 cancer, 24 normal)		Postmenopause (21 cancer, 20 normal)		Men (21 cancer, 39 normal)	
	Mean	S.D.	Mean	S.D.	Mean	S.D.
			Baseline measurements			
Age, log years	1.56	0.09	1.83	0.08	1.85	0.05
	1.48	0.15	1.81	0.06	1 80	0.06*
Weight, log *g*	4.82	2.71	4.80	2.77	4.81	2.73
	4.76	2.72	4.79	2.74	4.86	2.72*
Height, log cm	2.21	0.42	2.19	0.42	2.23	0.43
	2.22	0.42	2.20	0.42	2.24	0.42
Specific gravity, log ratio	0.01	0.00	0.01	0.00	0.01	0.00
	0.01	0.00	0.01	0.00	0.01	0.00†
Creatinine, log *g*/24 hours	0.13	0.10	−0.02	0.14	0.04	0.12
	0.08	0.08	−0.05	0.10	0.06	0.15
			Androgen complex (log mg/24 hours)			
Androsterone	−0.58	0.24	−0.64	0.45	−0.23	0.33
(DHEA-eq)	0.05	0.32†	−0.48	0.31	0.10	0.26*
Etiocholanolone	−0.32	0.22	−0.27	0.39	0.11	0.19
(DHEA-eq)	0.23	0.31†	−0.22	0.24	0.22	0.21
11-oxygenated ketosteroids	−0.27	0.18	−0.11	0.32	0.01	0.21
(DHEA-eq)	0.04	0.22*	−0.11	0.20	0.06	0.19
Beta fraction	−1.09	0.42	−1.24	0.11	−1.12	0.38
(DHEA-eq)	−0.53	0.48*	−1.14	0.29	−1.11	0.40
Pregnanediol	0.25	0.43	0.04	0.24	−0.33	0.27
(Pregnanediol-eq)	0.34	0.29	0.11	0.38	−0.28	0.19
			Estrogen complex (log μg/24 hours)			
Estrone	0.30	0.41	−0.18	0.37	0.19	0.35
(Estrone-eq)	0.70	0.37*	−0.33	0.28	0.36	0.26
Estradiol	−0.27	0.32	−0.78	0.29	−0.39	0.35
(Estradiol-eq)	0.08	0.40*	−0.82	0.22	−0.14	0.24*
Estriol	0.95	0.17	0.64	0.35	0.62	0.21
(Estriol-eq)	0.84	0.28	0.32	0.09†	0.56	0.21
			Corticoid complex (log mg/24 hours)			
Porter-Silber corticoids	0.67	0.08	0.60	0.20	0.61	0.13
(Hydrocortisone-eq)	0.61	0.12	0.52	0.13	0.60	0.17
17-ketogenic steroids	0.95	0.14	0.76	0.22	0.92	0.23
(DHEA-eq)	0.81	0.16	0.67	0.17	0.88	0.18
			Gonadotropin residue complex (log mg/24 hours)			
Biological activity	−0.51	0.17	0.56	0.42	−0.27	0.37
(Estrone-eq)	−0.55	0.18	0.34	0.37*	−0.30	0.25
Protein	1.69	0.27	1.65	0.36	1.90	0.37
(Albumin-eq)	1.42	0.13*	1.30	0.39†	1.40	0.20†
Carbohydrate	0.93	0.13	0.92	0.22	0.81	0.18
(Glucose-eq)	0.79	0.08	0.81	0.26	0.66	0.16*

* The *t* ratio for difference between cancer and normal > 2.00, considered individually significant at the 5 percent level for a single comparison. † The *t* ratio for difference between cancer and normal > 3.75, a set all of which may be considered simultaneously significant in view of the multiple comparisons made.

means. The discriminant function provides a pattern of coefficients, or weights, (Table 2A) for the 18 variates which provide an index score on which the cancer and control groups are maximally separated, or discriminated. The baseline physiologic measurements are included in the discriminant, any initial inequality in the matching of the samples thus being adjusted.

Significant discrimination was found in comparison of both pre- and postmenopause breast cancer patients with normal controls and of prostate cancer patients with normal men. The coefficient of multiple determination, R^2, is used to evaluate the success of the discrimination. The determination (R^2 = 0.91) for premenopause breast cancer indicates that 91 percent of the variance in clinical state (cancer or normal) is "explained" by or associated with concentrations observed in the 18 predicting variates. The discrimination with these 18 variates is less good in postmenopause women ($R^2 = 0.69$) and in the men ($R^2 = 0.75$), but all three discriminants are highly significant.

The relative size of the individual coefficients of the discriminant function indicates the relative importance of each variate in determining the clinical state, given that the other variates are held constant (mathematically). Inspection of the 18 coefficients suggests that in each of the three comparisons the androgens, the estrogens, and the gonadotropin residue are important after adjustment for the baseline values, while the corticoids are not important. The endocrine disturbance associated with cancer might therefore be adequately attributed to variations in androgen, estrogen, and gonadotropin residue complexes alone.

Discrimination by a single complex alone is possible, but much power is lost, the R^2's dropping to 0.47, 0.21, and 0.35 for discrimination with only the androgen complex; to 0.42, 0.30, and 0.29 for the estrogens alone; and to 0.36, 0.24, and 0.54 for the gonadotropin residue complex, in the three comparisons of cancer patients with normal controls respectively. The corticoids alone give no significant discrimination. Thus no endocrine complex by itself adequately describes the endocrine abnormality in these cancers.

Across-complex discrimination, with a single representative of androgens, of estrogens and of gonadotropin residue, improves the situation, giving with only three endocrine variates about three fourths of the determination possible with all 13 of the endocrine variates. Many three-variate across-complex combinations could be selected, with nearly equal discriminant power, and choice of a preferred combination is somewhat arbitrary.

For discriminating premenopause breast cancer, the combination of gonadotropin residue carbohydrate, estrone, and etiocholanolone is most powerful; in the postmenopause, the most powerful combination is gonadotropin residue protein, estriol, and androsterone; and in prostate cancer, it is gonadotropin residue protein, estradiol, and estriol. The discriminant power does not, however, differ greatly in these alternative combinations, so that if one were to choose a single combination applicable to all three comparisons, a generally effective one would be gonadotropin residue protein, estriol, androsterone. We therefore chose this reduced set of variates as a means of providing an adequate description of the endocrine abnormality observed in these three cancer groups. Table 2B gives the linear discriminant functions for these three variates alone, which are highly significant in all three comparisons.

The three-variate discriminant index score has been evaluated for each subject, and the distributions of the scores are illustrated in Fig. 1. The discriminant scores give good separation of

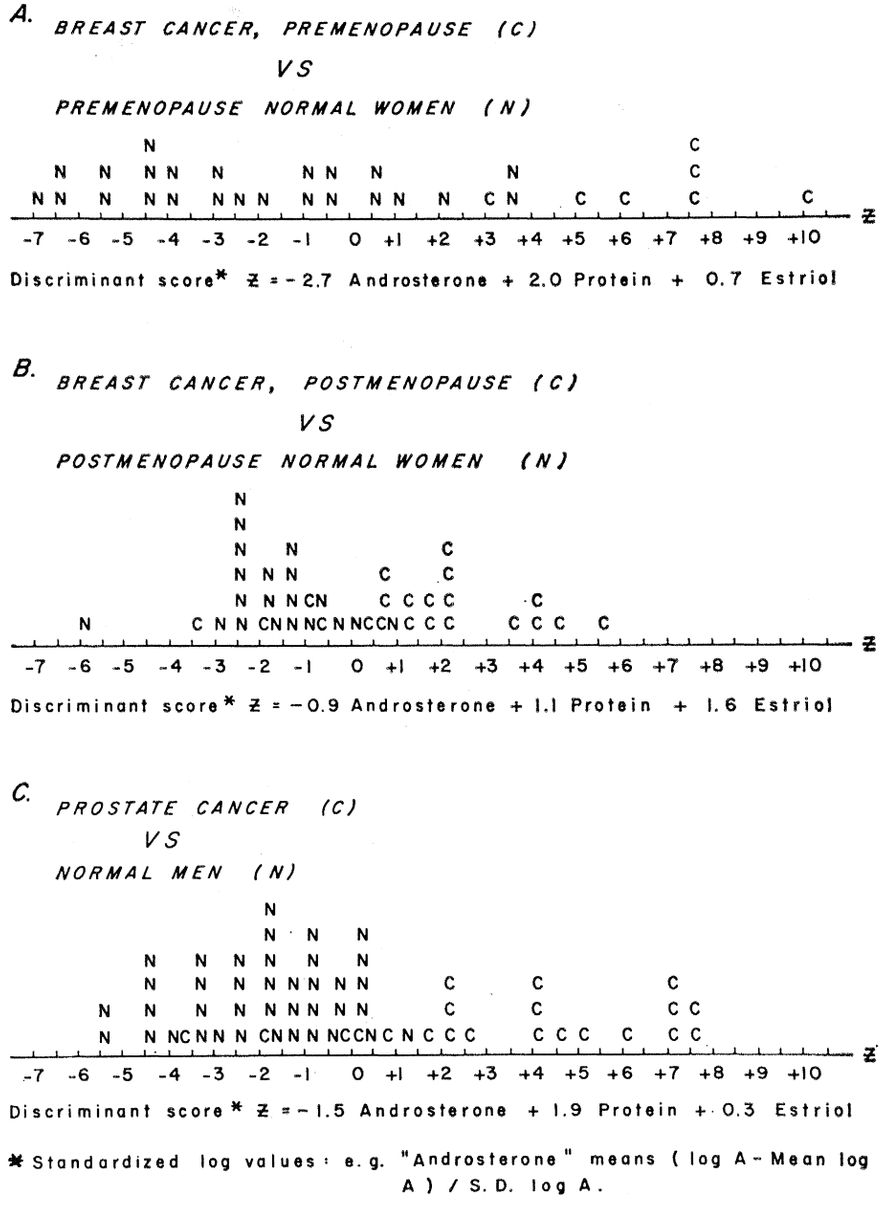

Fig. 1. Distribution of 3-variate discriminant scores of subjects; premenopause women (*A*), postmenopause women (*B*), and men (*C*). Each *N* represents a normal subject's discriminant score, each *C* a cancer patient's score.

Table 2. Discriminant function coefficients for comparison of excretion patterns in cancer with those in matched normal controls. Coefficients are in standard units, hence comparable within each comparison column.

Variate	Breast cancer premenopause (N = 7) compared with premenopause normal (N = 24)	Breast cancer postmenopause (N = 21) compared with postmenopause normal (N = 20)	Prostate cancer (N = 21) compared with normal men (N = 39)
A. 18-variate discriminant		*Baseline measurements*	
Age	3.8	0.3	0.2
Weight	−6.5	−1.4	−1.3
Height	−1.3	1.0	−0.0
Specific gravity	5.2	−0.2	−0.8
Creatinine	6.6	1.1	−0.1
		Androgen complex	
Androsterone	−9.5	−3.8	−1.5
Etiocholanolone	0.3	1.6	−0.7
11-oxygenated ketosteroids	−5.1	−0.7	0.9
Beta fraction	4.5	−1.0	1.2
Pregnanediol	−5.7	2.3	1.3
		Estrogen complex	
Estrone	−7.1	−0.1	−2.4
Estradiol	3.0	−2.1	−1.0
Estriol	5.6	2.9	2.1
		Corticoid complex	
Porter-Silber corticoids	1.6	2.4	0.3
17-Ketogenic steroids	0.6	−0.2	0.1
		Gonadotropin residue complex	
Gonadotropin residue:			
Biological activity	0.0	0.4	−1.8
Chemical composition			
Protein	5.4	2.6	4.7
Carbohydrate (protein-bound)	8.0	−1.5	−1.4
Determination (R^2)	0.91	0.69	0.75
P	<0.010	<0.025	<0.001
B. 3-variate discriminant			
Androsterone	−2.7	−0.9	−1.5
Gonadotropin residue protein	2.0	1.1	1.9
Estriol	0.7	1.6	0.3
Determination (R^2)	0.63	0.47	0.56
P	<0.001	<0.001	<0.001

cancer and normal subjects. This analysis, however, is not primarily aimed at possible use of the discriminant in diagnosis, but at description of the pattern of endocrine disturbance related to the presence of cancer. Indeed, although this same endocrine disturbance is not found in sick (nontumor) controls, there is indication of a complex endocrine disturbance associated with benign tumors of the breast and of the prostate.

Although the origin and endocrine activity, if any, of the protein fraction of the gonadotropin residue is not known, increased protein was associated with a multiple steroid disturbance in the cancer patients. The physiological significance of this combination of abnormalities in the excretion pattern must await further investigation, but the result suggests a more extensive disturbance of hormone relationships in cancer than hitherto reported. This multiple disturbance in both breast and prostate cancer may be related, among other possibilities, to their similar cell type as adenocarcinomas, that is, originating in columnar epithelium, or to the analogous response of breast and prostate tissue as target organs of the endocrine glands.

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6. The laboratory work on which this report is based was done under the direction of S. M. Myers, University of Southern California School of Medicine. We thank L. G. Crowley and L. J. Lombardo, Jr., for collaboration in selection of patients and collection of urines, G. F. Baier III and volunteers of California Men's Colony, Western Data Processing Center, and the Computing Facility, University of California, Los Angeles. Supported by the National Cancer Institute, USPHS, and the Bella and André Meyer Foundation.

26 May 1964

Atrophy of Skeletal Muscle in Chick Embryos Treated with Botulinum Toxin

Abstract. *Botulinum toxin was given in large intravenous doses to 7- and 12-day chick embryos. Atrophy of skeletal muscle resulted without significant atrophy of other organs. The histological appearance of muscle was consistent with denervation. The results suggest that neural acetylcholine release may play a significant role in "trophic transmission" from nerve to muscle.*

Botulinum toxin is known to prevent the release of acetylcholine from motor nerve terminals (*1*). Local injection of the toxin reproduces certain physiological effects of muscle denervation (motor paralysis, spread of the receptor zone, and spontaneous fibrillations) (*2*). This suggests that acetylcholine may serve a dual function, both as an impulse transmitter and as a "trophic transmitter" to muscle. Experimental interruption of axonal conduction by local anesthetics (*3*) and by nerve compression (*4*) produces motor paralysis, but fails to eliminate the nerve's trophic effect. The possibility remains that the spontaneous quantal release of acetylcholine by the nerve terminals (*5*) is adequate to prevent the atrophic consequences of denervation.

A structural change has been sought in the motor end plates of frog and cat muscles injected locally with botulinum toxin, but none was found with electron microscopic techniques (*2*). Feng *et al.* (*6*) observed hypertrophy of "slow" adult fowl muscle, with shrinkage of "fast" muscle, up to 8 weeks after local injection of botulinum toxin.

I have administered comparatively enormous amounts of botulinum toxin intravenously to chick embryos, with resulting atrophy and degeneration of the skeletal muscle fibers. The nature of the atrophy suggests true denervation, a point which will be defined by further studies. Systemic administration of botulinum toxin in minute amounts produces death by respiratory paralysis in most animals; since respiratory gas exchange in the chick embryo is accomplished by passive diffusion across the chorioallantoic membrane (*7*), the embryo readily survives doses which would be lethal to 20,000 or more hatched chickens (*8*).

The method of intravenous chorioallantoic injection was described previously (*9*). Type A crystalline botu-