induced supernatants which caused stimulation on day 7 and inhibition on day 42 (figure 2). Supernatants of peritoneal washing cell cultures gave the following pattern of migration. Supernatants from animals exposed to bovine IgG_2 caused stimulation on day 28 and 49 and inhibition on day 14 while supernatants from animals exposed to bovine IgG_2 + detergent caused stimulation on day 7 and day 49 and inhibition on day 14 (figure 2).

Discussion. SDBS exerted an adjuvant effect on the immune response to aerosolized bovine IgG_2. In animals exposed to IgG + detergent titers of specific antibodies in BAW supernatants were significantly higher shortly after primary immunization and in serum, antibodies persisted at higher levels for several weeks and remained higher after secondary immunization. Observed alterations in macrophage migration did not seem to be influenced by exposure to detergent at time of immunization. Taken with a previous report of adjuvanticity of SDBS in solution with *Bacillus subtilis* protease[7] the present results may indicate a general adjuvanicity of SDBS given with aerosolized antigen. This may suggest a rather universal utility of SDBS as aerosol adjuvant although it is likely that its function should be confirmed with specific antigens.

The mechanisms of adjuvant activity of detergent are speculative but may include alteration of membrane permeability, interaction and alteration of antigen, or stimulation of cells responding to antigen. Dioctyl sodium sulphosuccinate increases absorption of poorly absorbable drugs across gut epithelium[11] and a similar phenomenon may occur in the respiratory tract. Detergents bind avidly to protein molecule which could effect antigenicity[12] and conjugation of lipids to protein antigens has been shown to alter the immune response to those materials[13]. In each case, detergent may enhance immune response. Surface active materials can stimulate cellular metabolism and differentiation[14] and release of lysolecithin-like materials from macrophages has been implicated in the adjuvant activity of a variety of materials[15]. SDBS, therefore acting upon lymphocytes or macrophages may enhance antibody synthesis.

It is thought that live, replicating bacterial vaccines stimulate a better immune response at mucosal sites than do antigenically identical killed vaccines[2]. This may be due to the ability of live vaccines to provide both a critical antigen mass and penetration of innate mucosal barriers. However the convenience of using killed organisms or their components may be better exploited of adjuvants of the type described here improve the immune response to the level seen with live agents. In that SDBS enhanced both the serum and BAW antibody response it may be useful when aerosol immunization is more practical or associated with fewer side effects than is parenteral administration[16,17].

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2 R.H. Waldman and R. Ganguly, J. infect Dis. *130,* 419 (1974).
3 H.B. Kaltreider, Am. Rev. resp. Dis. *113,* 347 (1976).
4 W.H.O. Technical Report, No. 595, Geneva 1976.
5 M. Masa, I. Yano, G. Yanagida, Y. Tanaka, S. Kuwajima and S. Yamamoto, FEBS Lett. *83,* 237 (1977).
6 H. Snippe, M. Beller and J.M.N. Willers, Immunology *33,* 931 (1977).
7 R.J.F. Markham and B.N. Wilkie, Int. Archs Allergy appl. Immun. *51,* 529 (1976).
8 R. Duncan, B.N. Wilkie, F. Hiestand and A.J. Winter, J. Immun. *108,* 965 (1972).
9 B.N. Wilkie, B. Pauli and M. Gygax, Path. Microbiol. *39,* 393 (1973).
10 R.J.F. Markham, Thesis, University of Guelph, 1977.
11 S.H. Malik, D.H. Canaham and M.W. Gouda, J. Pharm. Sci. *64,* 987 (1975).
12 C. Tanford, in: Advances in Protein Chemistry, vol. 23, p. 122. Ed. C.B. Anfinsen, M.L. Anson, J.T. Edsall and F.M. Richards. Academic Press, New York 1968.
13 M.O. Dailey, W. Post and R.L. Hunter, J. Immun. *118,* 963 (1977).
14 K. Kakinuma, T. Hatae and S. Minokani, J. Biochem. *79,* 795 (1976).
15 P.G. Munder, M. Modelell, E. Ferber and H. Fischer, in: Mononuclear Phagocytes. Ed. R. van Furth, Blackwell, Oxford 1970.
16 R. Ganguly, B. Durrer and R.H. Waldman, Am. J. Dis. Child. *128,* 821 (1974).
17 R.W. Scott and A.F. Glauberg, J. Am. med. Ass. *166,* 147 (1975).

Circadian variation in urinary melatonin in clinically healthy women in Japan and the United States of America[1]

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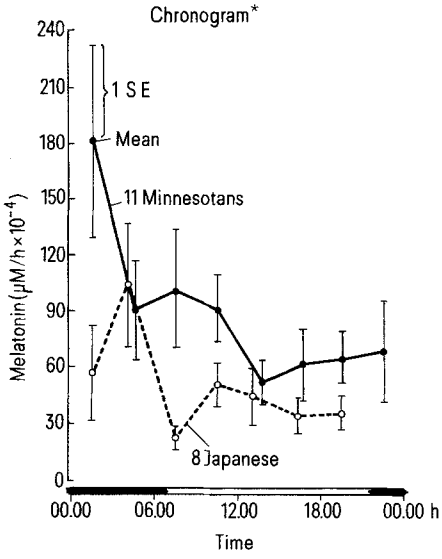
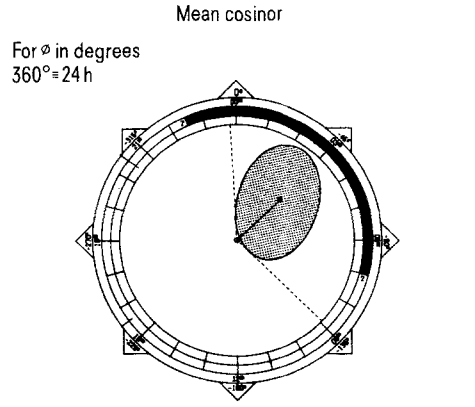
Summary. Urinary melatonin excretion is lower in East-Asian (Japanese) than in North-American (whites of mixed ethnic origin) women. Moreover, a statistically significant circadian rhythm is demonstrated by population-mean cosinor in the data pool from both groups of women. Furthermore, statistical significance characterizes interactions of effects from geographic differences (between ethnic groups) with temporal factors. Such spatio-temporal interactions await further scrutiny with a view inter alia of carcinogenesis as it is influenced by a spectrum of intermodulating rhythms.

Circadian rhythms have been reported for urinary excretion and blood levels of melatonin in human beings, rats and calves[2-6], along with changes in serum melatonin during the menstrual cycle[7]. Apart from basic physiologic and pharmacologic aspects beyond our scope, interest in this variable stems from the question whether melatonin excretion may vary in populations with different breast cancer risk. To study this possibility, adult female subjects, some menstrually cycling, others post-menopausal (table 1) were admitted to the Clinical Research Center at the University of Minnesota in Minneapolis between 08.00 h and 09.00 h, for a stay of about 28 h between mid-July and





No. of subjects	P	Amplitude (95% C.L.+) as % of mesor	Acrophase (95% C.L.+)
19	0.034	35 (2,71)	-47°(-357°,-134°)

Chronogram at left shows urinary melatonin excretion by Japanese and Minnesotan women at several timepoints during 1-day span; amplitude and acrophase estimates from all 19 subjects were combined in mean cosinor displayed at right. * Each woman contributed 6–8 samples collected over 1 single 24-h span; data for each population averaged across all subjects for 3-h intervals (no Japanese samples between 21.00 and 24.00 h).

early September 1977. Each subject was instructed to note at home the time of her 1st morning urinary voiding on the day of admission. Thereafter urine specimens were collected in the hospital approximately every 2 h while the subject was awake and whenever a subject awoke from sleep, until the time of discharge (about 28 h later). The time and total volume of each voiding was noted by nurses for each urine specimen, and aliquots were distributed into plastic bottles and frozen at −20 °C. Each subject collected 10–12 timed voidings during hospitalization, with times differing among subjects. As part of a larger study, blood samples were also drawn every 20 min during the span of hospitalization, for analyses of hormones, including prolactin.

For each subject, an analysis for melatonin was performed on 6 of the timed voidings, usually including the one collected just before sleep, the one just after arising, and the others spaced as evenly as possible throughout the span of wakefulness. Similar sampling procedures were also carried out in Kyushu, Japan, and the samples were sent from Kyushu to Minnesota in dry ice. All samples arrived in Minnesota completely frozen and were immediately stored at −20 °C. Both Minnesotan and Japanese samples were then transported to Sweden, again in dry ice, and again stored at −20 °C at St Göran's Hospital until melatonin could be determined by radioimmunoassay[8].

Table 1. Subjects investigated

Geographic site (breast cancer risk)	Number of Subjects Pre-menopausal (age, years)	Post-menopausal (age, years)	Subtotals
Minnesota (low)	1 (35½)	3 (55½–59)	11
Minnesota (high)	3 (34½–35)	4 (46–67½)	
Japanese (low)*	4 (30–35½)	4 (52–59)	8
Subtotals	8	11	

* Japanese volunteers all were selected according to criteria indicating a low breast cancer risk.

On the data thus obtained, several analyses were carried out to examine the possibility of a difference between the 2 populations, and also to search for a time effect. Unequal numbers of samples were available from Japan and Minnesota in given time intervals for consideration in an idealized day (table 2). For an analysis of variance (requiring equal numbers) samples were picked from among the totals available, using from the larger number of Minnesota samples primarily samples of subjects belonging to the high breast cancer risk group, since breast cancer risk was one of the points of interest. At the outset only the data between 06.00 h and 21.00 h (shown in table 2) were used. Thereafter, the analysis was repeated with the addition of data between 00.00 h and 06.00 h from each of 8 subjects. The mean value (±SE) for the latter span (not shown in table 2) from the samples collected in Minnesota was 176.60±53.32 as compared to 86.49±22.92 for samples collected in Kyushu.

Prior to carrying out an analysis of variance on the original data, the assumption of homogeneity of variances was tested by the F_{max} statistic proposed by Hartley, which yielded $F_{max} = 86.26$ with (12.7) degrees of freedom. Consequently, this assumption had to be rejected. When logarithmic transformation was applied to the original data, the desired homogeneity was achieved. The analysis of variance summary in table 3 showed statistically significant differences between groups. Moreover, the time-group interaction is statistically significant, thereby attesting to the fact that the intergroup difference depends upon time. When all data, i.e., including those collected between 00.00 h and 06.00 h are considered, time also constitutes a statistically significant source of variation, as seen in the analysis of variance summary in table 4. Again the interaction effect nearly achieved statistical significance at the 5% level ($0.05 < p < 0.06$).

These results should be re-examined for the same effects by a graphic presentation of all data and, hence, the figure presents the means of the transformed data and shows that

Table 2. Urinary melatonin excretion by clinically healthy women in USA and Japan, Mean values for consecutive 3-h spans*

Geographic site	Time span 6.00–9.00	9.00–12.00	12.00–15.00	15.00–18.00	18.00–21.00
Minnesota	101.50	87.89	45.55	65.44	65.70
Kyushu	18.22	50.66	45.39	35.02	36.62

* Data from 8 subjects in each location covering only wakefulness span to balance both N of subjects and N of samples.

a very low value occurred in the timespan from 06.00 h to 09.00 h in the Japanese sample tested. Moreover, when lines are drawn to connect the values for each group, they barely intersect at one of the timespans here considered, i.e., the Japanese have consistently lower values of melatonin excretion compared to the North Americans. Thus, statistically significant differences among timespans and between groups were attained, the latter difference demonstrable even with a simple non-parametric sign test.

An interesting separate question in the present study is whether there is a significant difference in mean melatonin values for the night span, from 00.00 h to 04.00 h, when compared to the combined mean of the other spans. A simple contrast test was applied to the (log-transformed) data and confirmed the above hypothesis, yielding a p-value < 0.05. Hence, these data on 19 subjects extend the scope of earlier studies showing high melatonin excretion during the time of darkness (and habitual rest).

The results summarized with ANOVA must be viewed with the realization that the process of choosing 96 observations from a larger total is somewhat arbitrary. The selection of data from the larger set was originally carried out to permit a balanced table of data for computational purposes. Nonetheless, the results here summarized have important potential for exploiting rhythms which may lead to an early detection or warning of impending breast cancer.

The data from both groups were subjected to a mean-cosinor analysis[9,10], to yield the results shown in the figure. The question may be raised whether it is permissible to pool all data from Minnesota and Japan for such cosinor analysis after a significant population difference has been documented. As noted in connection with cosinor techniques[9], one can discuss populations, groups within populations and individuals in a given group and consider then concomitantly, provided that one makes some allowance for any repeated tests on the same data. With the realization that only the mean cosinor is done, it can be seen that the cosinor's 95% confidence region does not overlap the pole, a finding documenting a statistically significant rhythm. Table 5 shows an agreement between the current results and other studies on urine. It can be seen that the difference between the acrophase found earlier in data for Lynch and that in this study is only 2°; i.e., 8 min, since 360° are equated to 24 h.

The question may also be raised how differences in melatonin excretion may be related to differences in risk of breast cancer between Minnesotan and Fukuokan (Japanese) women. Since pinealectomy was reportedly associated with accelerated tumor growth[11,12] and since pineal extracts reportedly counteracted malignancy[13], pineal function may be involved in inhibition of carcinogenesis, acting conceivably via intermediary metabolism. However, if the pineal hormone constitutes a brake to carcinogenesis, one might expect melatonin excretion to be greater in Japanese women because they are presumably at lower risk. Conceivably, the hypothesis may be reconciled with the results if the reason the particular women from the high-risk Minnesotan group do not as yet have demonstrable cancer, is that they currently have a pineal brake to carcinogenesis. Much more work on much larger samples will be needed to scrutinize this possibility in longitudinal studies.

Although plasma prolactins from the same women have not yet been analyzed, these same women were part of an earlier study revealing higher peak values of prolactin in the Japanese compared to white Americans of mixed ethnic background. An inverse relationship of melatonin excretion to plasma prolactin levels is consistent with the inverse relationship seen in the circannual rhythm of these hormones in rodents[14]. The latter studies showed a decrease in the circannual fluctuations of prolactin when pinealectomy was performed. The circadian rhythm here described also complements a report on changes in melatonin with the human menstrual cycle[7]. A circaseptan rhythm in the mammalian pineal gland has also been reported[15].

Elsewhere, we have reviewed and partly documented the facts that a spectrum of rhythms with different frequencies - with 1 cycle in about 24 h (circadian), in about 7 days (circaseptan), in about 1 month (circatrigintan) and about 1 year (circannual) characterizes the urinary excretion of 17-ketosteroids[16]. These frequencies also may be reflected in urinary melatonin excretion. Among other effects, these spectra may influence carcinogenesis.

Table 3. Analysis of variance summary*

Source of variation	Degrees of freedom	Sum of squares	F-value	p-value
Group (G)	1	13.45	13.41	< 0.01
Time (T)	4	6.47	1.61	> 0.10
Interaction (T × G)	4	10.77	2.68	< 0.05
Error	70	70.20		
Total	79	100.88		

* On transformed data. Homogeneity of variances had to be rejected ($F_{max} = 31.46$). Hence, natural logarithmic transformation was used before ANOVA. After transformation the homogeneity of variances was accepted ($F_{max} = 9.11$).

Table 4. Analysis of variance summary*

Source of variation	Degrees of freedom	Sum of squares	F-value	p-value
Group (G)	1	15.05	10.10	0.01
Time (T)	5	19.55	4.18	0.01
Interaction (G × T)	5	10.86	2.32	0.05
Error	84	78.50		
Total	95	123.96		

* Logarithmically-transformed data.

Table 5. Apparently similar timing of circadian melatonin rhythm in urine and plasma of several species

Species	Source	Diurnally (D) or nocturnally (N) active	Circadian acrophase (95% confidence limits)	Reference
Human beings	Urine	D	− 6° (+44°, −93°)	This study
Human beings	Urine	D	− 4° (+12°, −26°)	Lynch[2]
Human beings	Plasma	D	−25° (− 2°, −55°)	Vaughan[3]
Sheep	Serum	D	−27° (−11°, −43°)	Rollag[4]
Cattle	Plasma	D	− 9° (+35°, −56°)	Hedlund[5]
Rats	Urine	N	− 0°	Lynch[2]

Acrophase: Timing of high values in relation to middle of daily dark span, with 360°≡24 h, 15° = 1 h; based on fitting 24 h cosine curve in the case of human beings, sheep and cattle; in the case of rats a rough macroscopic approximation is listed, the excretion during a 12-h dark span being compared with that during a 12-h light span.

When approaches based on homeostatic single samples fail to discern the mechanism underlying variability both in health and disease (in the latter case chronopathology quantifiable possibly as a rhythm alteration), a spectral analysis completed on a reference sample of test individuals from populations at different risk for a given cancer (such as that of the breast) may yield information of interest to both students of oncology and of chronobiology.

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2 H.J. Lynch, Y. Ozaki, D. Shakal and R.J. Wurtman, Int. J. Biometeorol. *19*, 267 (1975).
3 G.M. Vaughan, R.W. Pelham, S.F. Pang, L.L. Loughlin, K.M. Wilson, K.L. Sandoch, M.K. Vaughan, S.H. Koslow and R.J. Reiter, J. clin. Endocr. Metab. *42*, 752 (1976).
4 M.D. Rollag and G.D. Niswender, Endocrinology *98*, 482 (1976).
5 L. Hedlund, M.M. Lischko, M.D. Rollag and G.D. Niswender, Science *195*, 686 (1977).
6 L. Wetterberg, J. neural Transm., suppl. XIII, 289 (1978).
7 L. Wetterberg, J. Arendt, L. Paunier, I.C. Sizonenko, W. van Donselaar and T. Heyden, J. clin. Endocr. Metab. *42*, 185 (1976).
8 L. Wetterberg, O. Eriksson, Y. Friberg and B. Vangbo, Clin. chim. Acta *86*, 169 (1978).
9 F. Halberg, F. Carandente, G. Cornelissen and G.C. Katinas, Chronobiologia *4*, suppl. 1 (1977).
10 F. Halberg, Y.L. Tong and E.A. Johnson, in: The Cellular Aspects of Biorhythms, p.20. Symposium on Biorhythms. Springer 1967.
11 E. Katagiri, Osaka Igakki Zasshi *43*, 315 (1944).
12 T.K. Das Gupta and J. Terz, Cancer Res. *27*, 1306 (1967).
13 R. Hofstätter, Krebsarzt *14*, 307 (1959).
14 R.J. Reiter, J. exp. Zool. *191*, 111 (1975).
15 L. Vollrath, A. Kantazjian and C. Howe, Experientia *31*, 458 (1975).
16 F. Halberg, M. Engeli, C. Hamburger and D. Hillmann, Acta endocr., suppl. *103*, 5 (1965).

A study of the enzyme activity in the seminal vesicles of castrated and hormone-replaced castrated mice

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Summary. Castration provokes a time-related decrease in weight, protein, β-glucuronidase and glucose-6-phosphate dehydrogenase activity of seminal vesicles. A dose-dependent stimulation of these parameters is obtained with 5α-DHT. Cyproterone acetate counteracts the stimulatory effects due to androgen. Acid and alkaline phosphatases remain largely unaffected by these treatments.

It is well-recognized that the function of the seminal vesicles is completely androgen-dependent, and that castration and administration of the antiandrogenic steroid cyproterone acetate results in an atrophy of these glands (for bibliographical details see Neumann[2]). Information, however, is lacking upon the androgen-dependency of several biochemical parameters. A more detailed study of these might be of use for a better comprehension of the physiology of this accessory sex gland.

This work was undertaken to study the time-related effects of castration, effects of androgen-replacement therapy and those of the antiandrogen cyproterone acetate on the enzyme activity in mice seminal vesicles.

Adult male mice (Swiss albino race) weighing 25–30 g were used. Castration was done via the scrotal route. Groups of 5 animals were sacrificed at 7, 14, 21, 28 and 42 days after castration. At the 6-week postcastration term, groups of remaining mice received daily s.c. injections respectively of 5 µg 5α-dihydrotestosterone (DHT), 50 µg DHT, 50 µg DHT + 0.5 mg cyproterone acetate (CPA) and maize oil vehicle. The hormone treatment was continued for 15 days before the mice were sacrificed. All animals in experimen-

Influence of castration, 5α-dihydrotestosterone and cyproterone acetate on weight, protein content and enzyme activity in seminal vesicles of mouse[a] (values are means ± SEM for 5 determinations)

Experimental groups	Organ-somatic index	Protein (µg/mg tissue)	β-glucuronidase	Acid phosphatase	Alkaline phosphatase	Glucose-6-phosphate dehydrogenase
Intact control	9.82 ± 0.32	12.1 ± 6.28	4.68 ± 1.01	1.73 ± 0.67	2.79 ± 1.76	543 ± 222
Castrated						
7 days	5.10 ± 0.57[c]	12.9 ± 3.01	2.18 ± 0.57[c]	1.00 ± 0.08[b]	2.51 ± 0.80	126 ± 79[c]
14 days	2.10 ± 0.34[c]	11.5 ± 7.60	3.33 ± 1.68	1.12 ± 0.22	2.28 ± 0.52	209 ± 31[b]
21 days	1.47 ± 0.21[c]	5.8 ± 3.01	3.41 ± 0.34[b]	1.67 ± 0.09	3.34 ± 0.87	135 ± 13[c]
28 days	1.07 ± 0.27[c]	4.4 ± 1.90[b]	1.74 ± 0.84[c]	1.44 ± 0.17	2.83 ± 0.30	66 ± 19[c]
42 days	0.33 ± 0.09[c]	1.1 ± 0.29[c]	0.90 ± 0.04[c]	2.14 ± 0.51	4.23 ± 1.07	5.1 ± 1.1[c]
Castrated + maize oil	0.46 ± 0.11	1.9 ± 0.50	0.70 ± 0.28	2.90 ± 0.20	4.22 ± 0.74	7.1 ± 0.4
Castrated + 5 µg DHT	1.75 ± 0.23[e]	11.1 ± 2.60[e]	6.00 ± 1.35[e]	2.76 ± 0.81	4.72 ± 1.67	134 ± 35[e]
Castrated + 50 µg DHT	5.81 ± 0.44[e]	63.6 ± 9.11[e]	6.28 ± 0.66[e]	3.36 ± 0.56	5.23 ± 2.02	711 ± 114[e]
Castrated + 50 µg DHT + CPA	0.80 ± 0.10[d]	4.06 ± 1.4[d]	1.05 ± 0.19	3.00 ± 0.41	3.74 ± 1.44	7.5 ± 0.7

[a] Enzyme activities expressed as nmoles paranitrophenol liberated/µg protein min for acid and alkaline phosphatases, as nmoles phenolphthalein liberated/µg protein min for β-glucuronidase and as pmoles NADP reduced/µg protein min for glucose-6-phosphate dehydrogenase. [b,d] Significantly different respectively from intact and castrated + oil controls ($0.02 < p < 0.05$). [c,e] Significantly different respectively from intact and castrated + oil controls ($p < 0.01$).