Brain Research Bulletin, Vol. 19, pp. 129–133. © Pergamon Journals Ltd., 1987. Printed in the U.S.A. 0361-9230/87 $3.00 + .00

Stimulation of the Paraventricular Nucleus Area of the Hypothalamus Elevates Urinary 6-Hydroxymelatonin During Daytime

J. YANOVSKI,*[1] J. WITCHER,* N. ADLER,*
S. P. MARKEY† AND D. C. KLEIN‡[2]

**Department of Psychology, University of Pennsylvania*
3813-15 Walnut Street, Philadelphia, PA 19104
†Section on Analytical Biochemistry, Laboratory of Clinical Science
National Institute of Mental Health, National Institutes of Health, Bethesda, MD 20892
‡Section on Neuroendocrinology, Laboratory of Developmental Neurobiology
National Institute of Child Health and Human Development, National Institutes of Health
Bethesda, MD 20892

Received 25 August 1986

YANOVSKI, J., J. WITCHER, N. ADLER, S. P. MARKEY AND D. C. KLEIN. *Stimulation of the paraventricular nucleus area of the hypothalamus elevates urinary 6-hydroxymelatonin during daytime.* BRAIN RES BULL **19**(1) 129–133, 1987.—The paraventricular nucleus of the hypothalamus (PVN) is thought to be a part of the neural circuit comprising the melatonin rhythm generating system (MRGS). Electrical stimulation of the PVN during the early lights-on period significantly elevated urinary 6-hydroxymelatonin content to nearly 50% of night levels; stimulation during the lights-off period did not produce significant changes. In contrast to the effects of PVN stimulation, stimulation of surrounding brain areas was without effect at either time. This observation confirms the participation of the PVN in the MRGS.

Paraventricular nucleus Hypothalamus 6-Hydroxymelatonin Urine Circadian rhythms
Melatonin rhythm generating system

THE anatomical structures and neural circuits that participate in the control of melatonin secretion from the pineal gland appear to include the retina, retinohypothalamic tract, suprachiasmatic nucleus (SCN), paraventricular nucleus (PVN) of the hypothalamus, intermediolateral cell column of the spinal cord, superior cervical ganglion (SCG), inferior carotid nerve and nervi conarii [2, 7, 8, 11, 16]. Together with the pineal gland these structures constitute a melatonin rhythm generating system (MRGS). The SCN appears to contain a circadian oscillator which determines the timing of pineal gland activity. Light acts through the retina to adjust the phase of the oscillatory system, ensuring that it is entrained to the environmental lighting cycle [10]. Light also acts to override transmission to the pineal gland, and thereby block stimulation of the pineal gland at night [4,6].

The latest addition to the MRGS is the PVN. Histochemical studies first demonstrated SCN projections to the PVN [1, 20, 21], as well as PVN projections to the intermediolateral cell column of the spinal cord, the region containing the preganglionic cell bodies that project to the SCG [22–24]. Later it was found that lesions of the PVN abolish the light/dark differences in pineal N-acetyltransferase activity, pineal melatonin and urinary 6-hydroxymelatonin [9]. PVN lesions also mimic pinealectomy in their ability to prevent short-day-induced testicular regression [12,15].

Recently, short-term electrical stimulation of the PVN in acutely blinded, urethane anesthetized animals has been reported by Reuss *et al.* [17] to decrease late dark period rat pineal N-acetyltransferase activity without significantly affecting pineal melatonin content. In the guinea pig similar stimulation also alters the electrical activity of single pinealocytes [18,19]. Such data provide physiological evidence of the importance of the PVN in the regulation of the pineal gland, but are in conflict with one proposed operation of the MRGS, that at night excitatory signals from the SCN pass through the PVN to stimulate the pineal gland [6]. The data from Reuss *et al.* [17] suggest that excitation of the PVN by the SCN might inhibit stimulation of the pineal gland.

[1]Trainee, Medical Scientist Training Program, NIH Grant 5-T3GM07170.
[2]Requests for reprints should be addressed to D. C. Klein, National Institutes of Health, Building 10 Room 8D-42C, Bethesda, MD 20892.

The purpose of the current study was to further define the neural circuit mediating the transmission of photic and circadian information from the hypothalamus to the pineal gland. We examined the effects of electrical stimulation of the PVN on pineal melatonin production in awake, freely moving animals during the day and night. Melatonin production was estimated by measuring the urinary content of 6-hydroxymelatonin conjugates. Urinary 6-hydroxymelatonin conjugates are the major urinary metabolites of melatonin [7], and serve as a valid approximation of melatonin released by the pineal gland [13].

METHOD

Animals

Female Sprague-Dawley rats (275 to 350 gram, Charles River Co., Wilmington, DE) were maintained in a windowless facility under controlled lighting conditions (LD 14:10, lights on at 1800). Food and water were provided ad lib.

Stereotaxic Surgery

Rats were anesthesized with intraperitoneal ketamine hydrochloride (120 mg/kg) and acepromazine (2.4 mg/kg). Each rat was implanted with a 0.25 mm monopolar stainless steel stimulating electrode (MS303; Plastic Products Co., Roanoke, VA), aimed stereotaxically at either the junction of the parvo- and magnocellular parts of the PVN or the lateral preoptic area (LPO), which served as a control stimulation site. Coordinates were derived from the stereotaxic atlas of Paxinos and Watson [14] and were confirmed by histological evaluation in a pilot study. The PVN coordinates were 1.8 mm posterior to bregma, 0.5 mm lateral to the midline, and 8.0 mm below the surface of the skull, with the skull horizontal between bregma and lambda. The LPO coordinates were 0.1 mm rostral to bregma, 1.5 mm lateral to the midline, and 8.0 mm below the surface of the skull.

A stainless steel wire loop, placed subcutaneously over the frontal periosteum and wrapped around the stainless steel screws used to support the electrode assembly, served as an indifferent electrode. All subjects were allowed at least three weeks to recover from surgery before electrical stimulation.

Sample Collection

Animals were placed in Nalgene metabolic cages (Thomas Co.) within a light-tight, anechoic, temperature regulated chamber and provided food and water ad lib. During the 14-hour lights-on period, the room was illuminated by a single 25 watt bulb (approximately 75 lux at cage level). The chamber was lit by a Kalt giant red safelight during the 10-hour lights-off period. Animals were housed for two days within the metabolic cages prior to the commencement of the urine collection.

Samples were collected in tubes on dry ice and stored at −30°C until analysis. Urine samples were obtained in two 12-hour collections, from 0700 to 1900 and from 1900 to 0700. The lighting schedule was LD 14:10, with lights on at 0600. Therefore, the dark period collection included an hour of light before the beginning of the dark time and after its conclusion. The anechoic chamber was never entered during the dark period.

Urine was collected during the light and dark periods on consecutive days, first without and then with electrical stimulation.

TABLE 1

URINARY 6-HYDROXYMELATONIN OF PVN-IMPLANTED RATS DURING CONTROL AND STIMULATION PERIODS

Location of Electrode	Urinary 6-Hydroxymelatonin (ng/collection period) Light Control	Light Stimulated	Dark Control	Dark Stimulated
PVN (N=5)	5.7 ± 2.5	27.0 ± 7.9*	42.5 ± 11.8†	77.1 ± 25.3
Caudal and Rostral (N=4)	12.4 ± 4.8	15.2 ± 7.2	58.5 ± 20.5†	79.8 ± 21.5

Animals were classified according to the results of histological examination as PVN (electrode within the PVN), CAUDAL (electrode far caudal to the PVN) and ROSTRAL (electrode far rostral to the PVN) placements. Three-way analysis of variance revealed significant effects of PVN stimulation (*$p<0.05$) and light/dark condition (†$p<0.01$). Pre-planned t-tests for dependent measures were subsequently performed and are reported in the text.

TABLE 2

URINARY 6-HYDROXYMELATONIN OF LPO OR VENTRAL PALLIDUM IMPLANTED RATS DURING CONTROL AND STIMULATION PERIODS

Location of Electrode	Urinary 6-Hydroxymelatonin (ng/collection period) Light Control	Light Stimulated	Dark Control
LPO (N=5)	22.6 ± 2.9	20.6 ± 2.8	110.3 ± 16.3
VP (N=3)	36.9 ± 11.4	15.6 ± 8.9	99.3 ± 39.3

Animals were classified according to results of histological examination as LPO (electrode in LPO) or VP (electrode in ventral pallidum). There were no significant differences between control and stimulated rats following early stimulation.

Electrical Stimulation of the PVN

Rats were electrically stimulated for 3 hours at either of two times: one hour after lights-off or one hour after lights-on. Stimulation characteristics were modeled after those used by Bowers and Zigmond [2] to stimulate the SCG and elevate pineal N-acetyltransferase activity. Stimulations were 310 A (constant current source) of 10 Hz bursts (pulse duration 1.0 msec), lasting for 2 seconds, initiated every 20 seconds. Half the animals were first stimulated during the day and the other half during the night. A two week period separated the two stimulation periods.

Biochemical Analysis

Quantitative analysis of urine samples for 6-hydroxymelatonin content was performed by gas chromatography-negative chemical ionization mass spectrometry [25].

Data are presented as the mean±SE of urinary 6-hydroxymelatonin. Statistical analysis was performed

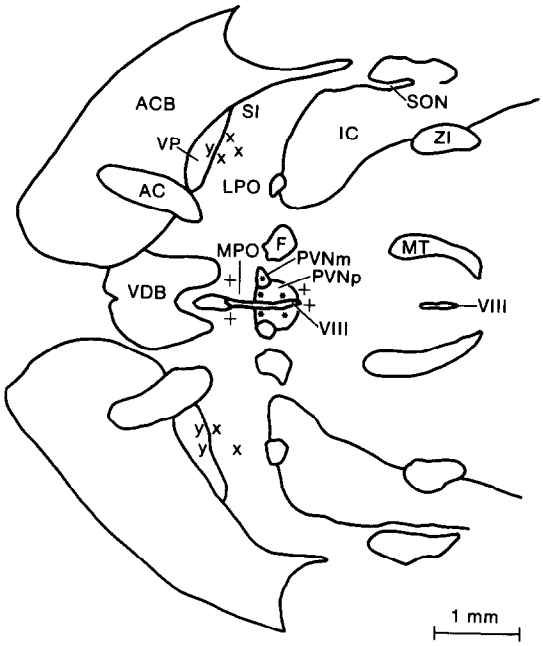

FIG. 1. Diagram of stimulating electrode placements using a horizontal brain section. AC, anterior commissure; ACB, nucleus accumbens; F, fornix; IC, internal capsule; LPO, lateral preoptic nucleus; ME, mamillothalamic tract; PVNm, paraventricular hypothalamic nucleus pars magnocellularis; PVNp, paraventricular hypothalamic nucleus pars parvocellularis; SI, substantia innominata; SON, supraoptic nucleus; VDB, nucleus of the vertical limb diagonal band; VIII, third ventricle; VP, ventral pallidum; ZI, zona incerta; *, electrode aimed at PVN lying within the PVN; +, electrode aimed at PVN lying outside the PVN; x, electrode aimed at LPO lying within LPO; y, electrode aimed at LPO lying outside the LPO.

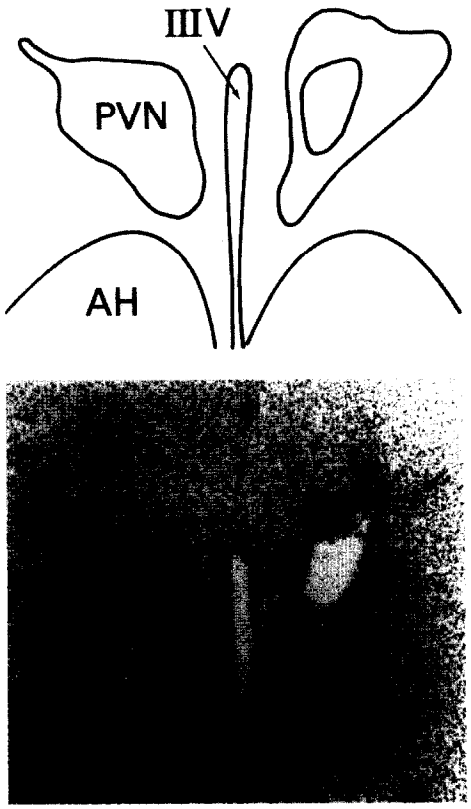

FIG. 2. Photomicrograph of typical PVN stimulation site. Top: Line drawing of a coronal section through the PVN from a rat which had an electrode implanted within the right PVN. Abbreviations PVN, hypothalamic paraventricular nucleus; III V, third ventricle; AH, anterior hypothalamic nucleus. Right: Photomicrograph of same coronal section (20 m, 1% thionin stain ×10).

using 3-way analysis of variance with repeated measures, and dependent t-tests [26].

Histological Analysis

Frozen brains were placed on specimen plates and sectioned in a cryostat. Coronal sections (20 μm) were cut through the area of the implant, and stained with 1% thionin for verification of the electrode site. None of the electrode placements obliterated the PVNs, although occasionally there was a unilateral loss of cellular content (see Fig. 1). Two evaluators, blind to the outcome of the experiment, examined brain sections to determine the location of the electrodes and the extent of PVN damage.

RESULTS

Histology

Histological verification of implantation sites was obtained for the seventeen rats used to obtain the data presented in Tables 1 and 2. Nine rats were implanted wih electrodes aimed at the PVN. In five animals the electrode placement was within the PVN; in four it was far outside the bounds of the PVN (Fig. 1). These four inaccurate placements contained two overly caudal and two excessively rostral implantations. None of the electrodes produced bilateral PVN damage, although as much as 40 to 50% of the implanted PVN was damaged in two of the animals with accurate implantation sites (Fig. 2). All eight rats whose electrodes were aimed at the lateral preoptic area (LPO) were found to have electrode placements within the LPO or ventral pallidum (Fig. 1).

Day/Night 6-Hydroxymelatonin

The average urinary 6-hydroxymelatonin values of the two groups of "PVN implanted" animals (Table 1) were about 9 ng during the light period, and 50 ng during the dark period prior to stimulation. There was a significant difference between unstimulated dark and light period values independent of whether the electrode had been accurately implanted in the PVN ($t=3.9$, $p>0.005$); on an individual basis it was found that each animal produced more 6-hydroxymelatonin during the dark period ($p>0.005$, sign test for matched pairs; [3]).

Similar results were obtained for animals with LPO implants (Table 2); the dark period 6-hydroxymelatonin content, 106.2±17.3 ng was significantly elevated when compared to the light period content, 28.0±5.2 ng ($t=5.6$, $p<0.001$).

Effects of Stimulation in Animals With Accurate PVN Implants

For 5 animals with electrode placements within the PVN,

the mean light urinary 6-hydroxymelatonin content after stimulation during the early light stimulation was significantly greater than the pre-stimulation value ($t=3.57$, $p<0.02$). However, after stimulation in the early portion of the dark period, the 6-hydroxymelatonin content for the dark collection period was not significantly greater than prestimulation nocturnal values.

Effects of Stimulation at Implant Sites Outside the PVN

For animals in which electrode placement was outside of the PVN, stimulation during the day or night did not alter urinary 6-hydroxymelatonin (Table 1). Similarly, stimulation of LPO during early light period did not produce a significant increase in urinary 6-hydroxymelatonin (Table 2).

DISCUSSION

These results demonstrate that electrical stimulation of the PVN during the light period can significantly increase daytime urinary 6-hydroxymelatonin conjugates. The effects of electrical stimulation of the PVN on melatonin appear to be specific: stimulation of brain regions outside of the PVN but within the hypothalamus does not change urinary 6-hydroxymelatonin. Destruction of the PVN has previously been shown to mimic pinealectomy and eliminate the circadian rhythms of the enzymes involved in melatonin production, in pineal melatonin content, and in urinary 6-hydroxymelatonin [9, 12, 15]. Taken together, these findings support the hypothesis that the PVN is a functional part of the MRGS, and that the PVN is driven by the SCN.

All animals whose electrodes were accurately implanted in the PVN also showed some increase in their urinary 6-hydroxymelatonin levels following dark-time stimulation. However, the magnitude of the change was variable. The failure to detect a significant increase in dark-time urinary 6-hydroxymelatonin following stimulation above normal nocturnal levels may be due to the fact that the stimulation parameters were not optimal. Alternatively, it is possible that dark-time pineal melatonin output may be nearly maximal, making it impossible for electrical stimulation to increase the production of more melatonin. If the latter explanation is the case, experiments examining the pineal response to electrical stimulation of the PVN after nighttime exposure to light, which decreases nocturnal melatonin production [4,5], might unmask a larger effect of dark-time PVN stimulation.

The results also support the view that SCN neurons generate the rhythm in melatonin production by stimulating the neural activity of the PVN at night in the dark, thus, increasing the firing rate of PVN neurons [6]. Alternatively, SCN neurons may not make synaptic contact within the PVN which are required for the control of melatonin production. Rather, there might be a monosynaptic pathway from the SCN to the intermediolateral cell column that passes through the PVN. Stimulation of the PVN with neuroactive chemicals could help distinguish between these two possibilities, i.e., synaptic communication versus fibers of passage at the level of the PVN.

It is interesting to note that the 3-hour daytime stimulation period increased 6-hydroxymelatonin production to more than half that produced normally during an entire night period. Apparently the stimulation parameters used mimic physiological stimulation of the downstream system. The stimulation parameters employed were based on a determination of the minimal intensity of stimulation of the superior cervical ganglia necessary to maximally stimulate N-acetyltransferase activity [2].

The results presented in this study are not in complete agreement with those of Reuss *et al.* [17]. Although both studies indicate that PVN stimulation influences some parameters of pineal function, the effects of stimulation are opposite. In the present study, the effect of stimulation was to elevate melatonin production during the day; the effect of stimulation in the previous study was to inhibit pineal melatonin and N-acetyltransferase activity to a small degree during the day; the inhibitory effects of stimulation at night were larger. These results may not be incompatible because of a number of fundamental differences in experimental design. For example, in the previous study the investigators used an acute surgical preparation, blinding and anesthesia during the stimulation period [17]; none of these were part of the present study. Also, the time of stimulation relative to the light-dark cycle was also different; a 24-hour rhythm in sensitivity to stimulation might produce different responses to PVN stimulation at different times of day in the two studies. Finally, results in the present study reflect total melatonin release during a 12-hour collection period following a 3-hour stimulation period. The previous study used a short stimulation period (0.25 to 1 hour stimulation) and measured pineal melatonin and N-acetyltransferase activity only at a single point in time, at the end of stimulation. This choice of a single time point for analysis, in contrast to the integrated response measured in the present study over a period of 12 hours, probably precluded detection of an increase in melatonin production. The reason for this conclusion is that stimulation of melatonin production by administration of adrenergic agonists or electrical pulses is characterized by a marked lag period followed by a rapid increase in N-acetyltransferase activity and melatonin [2, 5, 6]. It is probable that large increases in pineal function will not be detected in a single point-in-time analysis after a 0.25 to 1 hour stimulation period, and that a longer period is required.

The successful use of the electrical stimulation technique in this study indicates it may also help identify neural structures involved in the MRGS which connect the PVN and the SCG. In addition, the use of the ^{14}C-2-deoxyglucose autoradiographic method in conjunction with PVN electrical stimulation may enhance the description of this circuit.

REFERENCES

1. Berk, M. L. and J. A. Finkelstein. An autoradiographic determination of the efferent projections of the suprachiasmatic nucleus of the hypothalamus. *Brain Res* **226:** 1–13, 1981.
2. Bowers, C. W. and R. E. Zigmond. The influence of the frequency and pattern of sympathetic nerve activity on serotonin N-acetyltransferase in the rat pineal gland. *J Physiol (Lond)* **330:** 279–296, 1982.
3. Hays, W. L. *Statistics for the Social Sciences*. New York: Holt, Rinehart and Winston, 1973.
4. Illnerova, H., M. Backstrom, J. Saaf, L. Wetterberg and B. Vangbo. Melatonin in rat pineal gland and serum: rapid parallel decline after light exposure at night. *Neurosci Lett* **9:** 189–193, 1978.

5. Illnerova, H. and J. Vanecek. Complex control of the circadian rhythm in N-acetyltransferase activity in the rat pineal gland. In: *Vertebrate Circadian Systems*, edited by J. Aschoff, S. Daan and G. Groos. Berlin: Springer-Verlag, 1982.
6. Klein, D. C. Photoneural regulation of the mammalian pineal gland. In: *Photoperiodism, Melatonin and the Pineal*. London: Pitman (Ciba Foundation Symposium 117), 1985, pp. 38–56.
7. Klein, D. C., D. Auerbach, M. A. A. Namboodiri and G. H. T. Wheler. Indole metabolism in the pineal gland. In: *Pineal Gland: I. Anatomy and Biochemistry*, edited by R. Reiter. Boca Raton: CRC Press, 1981, pp. 199–227.
8. Klein, D. C. and R. Y. Moore. Pineal N-acetyltransferase and hydroxyindole-O-methyltransferase: Control by the retinohypothalamic tract and the suprachiasmatic nucleus. *Brain Res* **174:** 245–262, 1979.
9. Klein, D. C., R. Smoot, J. L. Weller, S. Higa, S. P. Markey, G. J. Creed and D. M. Jacobowitz. Lesions of the paraventricular nucleus area of the hypothalamus disrupt the suprachiasmatic spinal cord circuit in the melatonin rhythm generating system. *Brain Res* **10:** 647–652, 1983.
10. Klein, D. C. and J. L. Weller. Indole metabolism in the pineal gland: a circadian rhythm in N-acetyltransferase. *Science* **169:** 1093–1095, 1970.
11. Klein, D. C., J. L. Weller and R. Y. Moore. Melatonin metabolism: Neural regulation of pineal serotonin: acetyl coenzyme A N-acetyltransferase activity. *Proc Natl Acad Sci USA* **68:** 3107–3110, 1971.
12. Lehamn, M. N., E. L. Bittman and S. W. Newman. Role of the hypothalamic paraventricular nucleus in neuroendocrine responses to daylength in the golden hamster. *Brain Res Bull* **308:** 25–32, 1984.
13. Markey, S. P. and P. E. Buell. Pinealectomy abolishes 6-hydroxymelatonin excretion by male rats. *Endocrinology* **111:** 425–426, 1982.
14. Paxinos, G. and C. Watson. *The Rat Brain in Stereotaxic Coordinates*. New York: Academic Press, 1982.
15. Pickard, G. E. and F. W. Turek. The hypothalamic paraventricular nucleus mediates the photoperiodic control of reproduction but not the effects of light on the circadian rhythm of activity. *Neurosci Lett* **43:** 67–72, 1983.
16. Reppert, S. M. and D. C. Klein. Mammalian pineal gland: Basic and clinical aspects. In: *The Endocrine Functions of the Brain*, edited by M. Motta. New York: Raven Press, 1980, pp. 327–372.
17. Reuss, S., J. Olcese and L. Vollrath. Electrical stimulation of the hypothalamic paraventricular nuclei inhibits pineal melatonin synthesis in male rats. *Neuroendocrinology* **41:** 192–196, 1985.
18. Reuss, S. and P. Semm. Electrophysiological investigations on the sympathetic and central innervation of the mammalian pineal gland. *Neurosci Lett [Suppl]* **10:** 406, 1982.
19. Reuss, S., P. Semm and L. Vollrath. Electrophysiological investigations on the central innervation of the rat and guinea-pig pineal gland. *J Neural Transm* **60:** 31–43, 1984.
20. Stephan, F. K., K. J. Berkley and R. L. Moss. Efferent connections of the rat suprachiasmatic nucleus. *Neuroscience* **6:** 2625–2641, 1981.
21. Swanson, L. W. and W. M. Cowan. The efferent connections of the suprachiasmatic nucleus of the hypothalamus. *J Comp Neurol* **160:** 1–12, 1975.
22. Swanson, L. W. and H. G. J. M. Kuypers. The paraventricular nucleus of the hypothalamus: cytoarchitectonic subdivisions and organization of projections to the pituitary, dorsal vagal complex, and spinal cord as demonstrated by retrograde fluorescence double-labeling methods. *J Comp Neurol* **194:** 555–570, 1980.
23. Swanson, L. W. and H. G. J. M. Kuypers. A direct projection from the ventromedial nucleus and retrochiasmatic area of the hypothalamus to the medulla and spinal cord of the rat. *Neurosci Lett* **17:** 307–312, 1980.
24. Swanson, L. W. and P. E. Sawchenko. Paraventricular nucleus: A site for the integration of neuroendocrine and autonomic mechanisms. *Neuroendocrinology* **31:** 410–417, 1980.
25. Tetsuo, M., S. P. Markey, R. W. Colburn and I. J. Kopin. Quantitative analysis of 6-hydroxymelatonin in human urine by gas chromatography-negative chemical ionization mass spectrometry. *Anal Biochem* **110:** 208–215, 1981.
26. Winer, B. J. *Statistical Principles in Experimental Design*. New York: McGraw Hill, 1971.