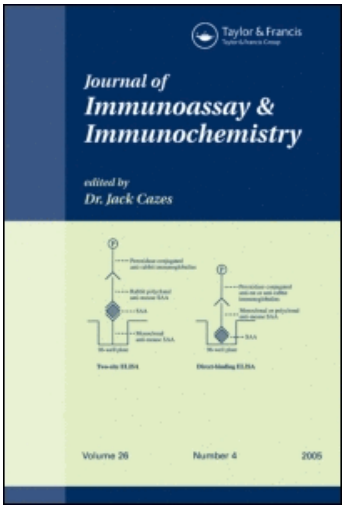

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

A Radioimmunoassay for N-Acetylserotonin in Biological Tissues

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To cite this Article Pang, S. F. , Brown, G. M. , Campbell, S. L. , Snieckus, V. , De Silva, S. O. , Young, S. N. and Grota, L. J.(1981) 'A Radioimmunoassay for N-Acetylserotonin in Biological Tissues', Journal of Immunoassay and Immunochemistry, 2: 3, 263 — 276
To link to this Article: DOI: 10.1080/15321818108056981
URL: http://dx.doi.org/10.1080/15321818108056981

JOURNAL OF IMMUNOASSAY, 2(3&4), 263-276 (1981)

A RADIOIMMUNOASSAY FOR N-ACETYLSEROTONIN IN BIOLOGICAL TISSUES

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(Key Words: Radioimmunoassay, N-acetylserotonin, serum, pineal, brain, retina)

ABSTRACT

We have developed a specific radioimmunoassay for N-acetylserotonin. Cross-reactivity of 23 related or selected compounds is less than 1 percent; sensitivity is 10 to 25 picograms per tube; within assay coefficient of variation is 7.5 to 8.4%; and between assay coefficient of variation is from 7.1 to 11.1%. Satisfactory parallelism has been demonstrated for rat, hamster and rabbit serum, rat and hamster serum extract and rat brain retina and pineal extract. An extracted sample of rat serum gave one peak on HPLC which corresponded to authentic N-acetylserotonin. Daytime levels of N-acetylserotonin have been established for each of the tissues studied. This radioimmunoassay provides a sensitive and specific method for determination of N-acetylserotonin levels in biological tissues.

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INTRODUCTION

N-acetylserotonin, a derivative of serotonin and the precursor of melatonin, was first demonstrated in the pineal (1). Treatment with N-acetylserotonin inhibited compensatory ovarian and adrenal hypertrophy in mice (2, 3) and lowered TSH levels in rat serum (Tang and Pang, unpublished results). Immunization with a N-acetylserotonin-bovine serum albumin conjugate, produced antiserum against melatonin and N-acetylserotonin (4), decreased serum testosterone, prolactin and corticosterone levels but elevated serum TSH levels in rats (5, 6, 7). These findings, together with the fact that the circadian rhythm of serum N-acetylserotonin in rats (8) was significantly lowered by pinealectomy (9), suggested that N-acetylserotonin may be a pineal hormone. N-acetylserotonin was also demonstrated in the retina and brain of rats (10 - 19) and its concentrations were not lowered by pinealectomy (13, 17). Thus, it has been proposed that N-acetylserotonin may be a neurotransmitter, a neuromodulator, and/or a hormone.

In the past, N-acetylserotonin was quantified by fluorometric assay (18, 20), radioenzymatic assay (21, 22) and gas chromatography-mass spectrometry (19, 23, 24) which had one or a number of drawbacks, namely: low sensitivity, non-specificity and inefficiency. Previously, our laboratory developed a N-acetylserotonin radioimmunoassay (12). However, the antiserum used bound equally well with melatonin and N-acetylserotonin (4), which is not totally satisfactory. In this report, a more sensitive and more specific radioimmunoassay for N-acetylserotonin is documented and used for the determination of N-acetylserotonin concentrations in pineal, retina, brain and serum samples.

MATERIALS AND METHODS

Preparation of the Hapten and Conjugate

The hapten, 1-(p-carboxy)benzyl-N-acetylserotonin (NAS-PCB), was synthesized by the phase-transfer catalytic alkylation method (25) and conjugated to give 1-(p-carboxy) benzyl-N-acetylserotonin-bovine serum albumin (NAS-PCB-BSA) using ethylcarbodiimide (ECDI; 1-ethyl-3(3-dimethyl-aminopropyl) carbodiimide HCl; Sigma). Briefly, 16.3 mg BSA and 20 mg ECDI were dissolved in 16 ml of deionized water and the solution added slowly to 4 ml of methanol containing 5.1 mg of NAS-PCB. The mixture was adjusted to pH 6.5 with dilute NaOH, rotated by Roto-Torque (Model 7637, Cole Parmer) for 4 h at room temperature (23 ± 1°C) and dialysed in

20/32 of dialysis tube (union Carbide) overnight in running tap water. The resulting conjugate was evaluated by UV spectrometry (Spectrophotometer model 25, Beckman) as having a molar ratio of 21 to 1 of NAS-PCB to BSA.

Production of Antiserum

The conjugate NAS-PCB-BSA (50 µg) was mixed with complete Freund's adjuvant (Difco) and injected subcutaneously into two rabbits at 2 sites over the hips. Then, subcutaneous booster injections of 25 µg NAS-pcb-BSA in saline were given monthly. Antisera were harvested 10-14 days after each booster injection. The antiserum used in these studies was harvested 6 months after the first injection.

Preparation of Samples

Blood samples were collected in the light period after decapitation of rats and hamsters or by venepuncture of rabbits. The samples were kept at 0-4°C for 1-16 h. Serum samples were obtained after centrifugation at 4000 x g for 20 min and stored in a freezer at -20°C until assay or extraction.

Extraction of serum samples was achieved by addition of one volume of serum to five volumes of petroleum ether. The mixture was swirled and centrifuged at 4000 g for 20 min, the organic phase discarded and the purified serum collected. Then, one volume of the purified serum was mixed with ten volumes of methanol, swirled again and centrifuged (4000 g for 10 min.). The supernatant was transferred to another centrifuge tube, dried under nitrogen and stored at -20°C.

Other tissue samples from rats killed in the light period were dissected out immediately after decapitation, frozen on dry ice and stored at -20°C. On the day of extraction, the tissues were weighed and homogenized in 3 to 10 ml of methanol. The homogenate was centrifuged at 40,000 g for 30 min, the supernatant collected, dried under nitrogen and stored at -20°C.

Before assay, the appropriate amount (0.5-2 ml) of gelatin-phosphate buffer was added, the tube vortexed and centrifuged at 40,000 g for 20 min, the supernatant collected and 10-500 µl used for radioimmunoassay (RIA).

Gelatin Phosphate Buffer pH 6.5

In about 800 ml deionized water was dissolved sodium phosphate dibasic, anhydrous (Fisher) (0.01M); sodium chloride (Baker Analysed) (0.15 M); EDTA, disodium salt (Baker Analysed) (0.025M) and Thimerosal (Sigma) (0.1g). The pH

was adjusted to 6.5 with 10N NaOH and the volume to 1 litre, and the buffer was stored at 4°C for a maximum of one month. Immediately prior to use, it was heated slightly to dissolve gelatin powder, USP (Baker Analysed) (1g/l).

Radioactive N-Acetyl-Serotonin

(2, 4, 6-^{3}H) acetyl serotonin, 35 Ci/mmol, was obtained from Amersham (stable for 9 months at -20°C in ethanol: water solution (98:2 v/v)) and used to make a working solution of 2000 cpm/50 µl in gelatin phosphate buffer, prepared freshly immediately prior to use.

Standard N-Acetyl-Serotonin: (Sigma)

A stock solution of 10µg/50µl NAS in absolute ethyl alcohol (Consolidated Alcohols Ltd.), was stable at -20°C for 4-6 weeks and 100µl was diluted to 1ml with buffer (i.e. 1µg/50µl) immediately prior to use. It was serially diluted with buffer to give 500, 250, 100, 50, 20 and 10 pg/50µl.

Anti-NAS:

Rabbit antiserum (R228 9/6/80) raised against NAS-PCB-BSA, as described above, diluted 1:200 in buffer containing carrier normal rabbit serum (1µl/ml), was stable for at least 1 month at -20°C. Attempted removal (stripping) of endogenous antigen from this antiserum by three different procedures (26 - 28) made little difference to binding when compared to the untreated antiserum. The ethanol and NaI procedures did not remove NAS added to the antiserum while urea treatment removed NAS but did not enhance binding.

Ammonium Sulfate Solution (Saturated):

Sufficient ammonium sulfate (Baker Analysed) was added, with constant stirring, to deionized water to assure 100% saturation and the pH adjusted to 7.0 with NaOH. After filtration through a millipore type SM 5.0 µm filter, the solution could be stored indefinitely at room temperature in a polyethylene bottle.

Scintillation Cocktail:

With constant shaking 12.0g PPO (Fisher) and 0.25g Bis MSB (New England Nuclear) were dissolved in 2 ℓ Toluene (Fisher) in a 4 ℓ amber bottle and 1 ℓ Triton X100 (Rohm & Haas Canada Inc.) added with shaking.

Scintillation Vials:

Plastic Minivials 6ml (Wheaton).

Procedure for Radioimmunoassay:

Appropriate amounts of 0.1% gelatin phosphate buffer, pH 6.5 and sample or standard (50 to 500 µl) to give a final volume of 650 µl were placed in 12 x 75 borosilicate tubes and 50 µl ^{3}H NAS and 100 µl anti-NAS (diluted 1:1300) were added. The tubes were shaken and incubated 40 h at 4°C. Saturated ammonium sulfate (pH 7.0), 650 µl, was added with an Oxford pipettor (Model R), the tubes were vortexed gently and incubated at 4°C for a further 1 h. They were then centrifuged at 2800 g for 20 minutes at 4°C (Beckman J6 Model, Bucket rotor JS 5.2). The supernatant was decanted, and 550 µl deionized water added to the pellet. The tubes were vortexed vigorously and 500 µl volumes of the mixture were transferred to plastic minivials, together with 5ml of scintillation cocktail. Each vial was counted for 10 min (Beckman LS 7000). Total count tubes (500 µl deionized water and 50 µl ^{3}H label), non-specific binding tubes (600 µl buffer plus 50 µl label), standard curve, controls and unknown samples were run in duplicate.

Cross-reactivity of Anti-N-acetylserotonin Serum:

Twenty-three indoles related to N-acetylserotonin or selected biogenic amines were evaluated (Table I). The potencies of those compounds with significant inhibition of binding in the assay were calculated as the concentration which displaced 50% of specific binding in the zero standard tubes.

Recovery:

20,000 cpm of ^{3}H NAS was added to tissue or serum prior to extraction. Recovery of radioactivity determined for four samples of each was rat pineal 89.2 ± 0.8%, rat retina 103.5 ± 1.3%, rat brain 90.1 ± 1.1%, rat serum 96.4 ± 0.3% and hamster serum 97.5 ± 3%.

HPLC:

One ml of rat serum was extracted as for the radioimmunoassay procedure. The frozen extract was dissolved in 100 µl of buffer and separated by reverse-phase HPLC using a procedure for separation of indoles (29). This involves separation of the compounds with a Waters C_{18} µBondapak column (300 mm x 3.9 mm I.D., 10 µm average particle size) (Waters Assoc. Milford, MA, USA) using a buffer which was 65% 0.01 M sodium acetate, pH 4.25, and 35% methanol. The

TABLE I

Cross-reactivity of Related and Selected Compounds in the N-acetylserotonin Radioimmunoassay.

Compound	Binding Equivalence to NAS
N-acetylserotonin (Sigma)	100
*N-acetylserotonin-PCB (V. Snieckus)	100
*Melatonin-PCB (V. Snieckus)	0.5
6-hydroxymelatonin (Sigma)	0.5
O-acetyl-5-methoxytryptophol (Dr. Silman)	0.3
*N-acetyltryptamine (V. Snieckus)	0.2
Melatonin (Sigma)	0.12
γ-amino-n-butyric acid (Sigma)	0.02
5-methoxytryptamine (Sigma)	0**
*N-propionic acid melatonin (V. Snieckus)	0
5-methoxy-NN -DMT (Sigma)	0
Serotonin creatinine sulphate (Sigma)	0
N-acetyltryptophan	0
5-methoxytryptophan	0
5-methoxytryptophan (Sigma)	0
5-methoxytryptophol	0
N-methyltryptamine (Sigma)	0
Tyramine (Sigma)	0
L-tryptophan (Sigma)	0
5-methoxy - 3 - indoleacetic acid (Aldrich)	0
Tryptamine (Sigma)	0
Epinephrine (Sigma)	0
Metanephrine (Sigma)	0
Tyrosine (Sigma)	0

* these compounds are not known to exist in nature

** 0 cross-reactivity is less than 0.001%

eluate was collected in 0.5 ml fractions. Fifty µl of each fraction was assayed directly in the radioimmunoassay. Fractions from the chromatography of a water blank were assayed and contained undetectable N-acetylserotonin. The elution profile of authenic N-acetylserotonin was determined separately using fluorometric detection.

RESULTS

Binding Characteristics of the Assay:

A typical standard curve for N-acetylserotonin is shown in Figure 1. The values generated in a Scatchard plot of the data were 1) equilibrium constant (k): 0.6×10^{-9} L.mol $^{-1}$ and binding capacity: 4.8×10^{-7} mol. L^{-1}.

Precision and Sensitivity:

The within and between assay variability were assessed by inclusion of quality control samples (unextracted rat serum and/or rabbit serum) in routine assays (30). The results are as shown (Table II). The sensitivity of the assay, based on the dose of N-acetylserotonin producing 12% (twice the standard deviation of the binding at zero concentration) inhibition of binding was 10-25 pg/tube.

Specificity:

There were significant cross-reactivities for NAS-PCB (100%), melatonin-PCB (0.5%), 6-hydroxymelatonin (0.5%), 0-acetyl-5-methoxytryptophol (0.3%), N-acetyltryptamine (0.2%), melatonin (0.12%) and γ-amino-n-butyric acid (0.02%) while 16 other compounds had no observable cross-reactivity (Table I).

Validation:

A. Parallelism

Parallel inhibition curves were demonstrated for rat, hamster and rabbit serum, for rat and hamster serum extract and for rat brain, retina and pineal extract with the standard in the radioimmunoassay. Typical results are shown in Fig. 2. In rats, hamsters and rabbits 20 µl of serum are readily assayed.

B. High Pressure Liquid Chromatography (HPLC)

An extracted sample of rat serum was chromatographed on HPLC, and radioimmunoassay of the eluates gave only one peak corresponding to authentic N-acetylserotonin.

Concentrations of N-acetylserotonin in Biological Tissues:

The concentrations of N-acetylserotonin in the rat tissues obtained in the day time is shown in Table III. There is a ten fold variation in the serum concentration of NAS among the species tested.

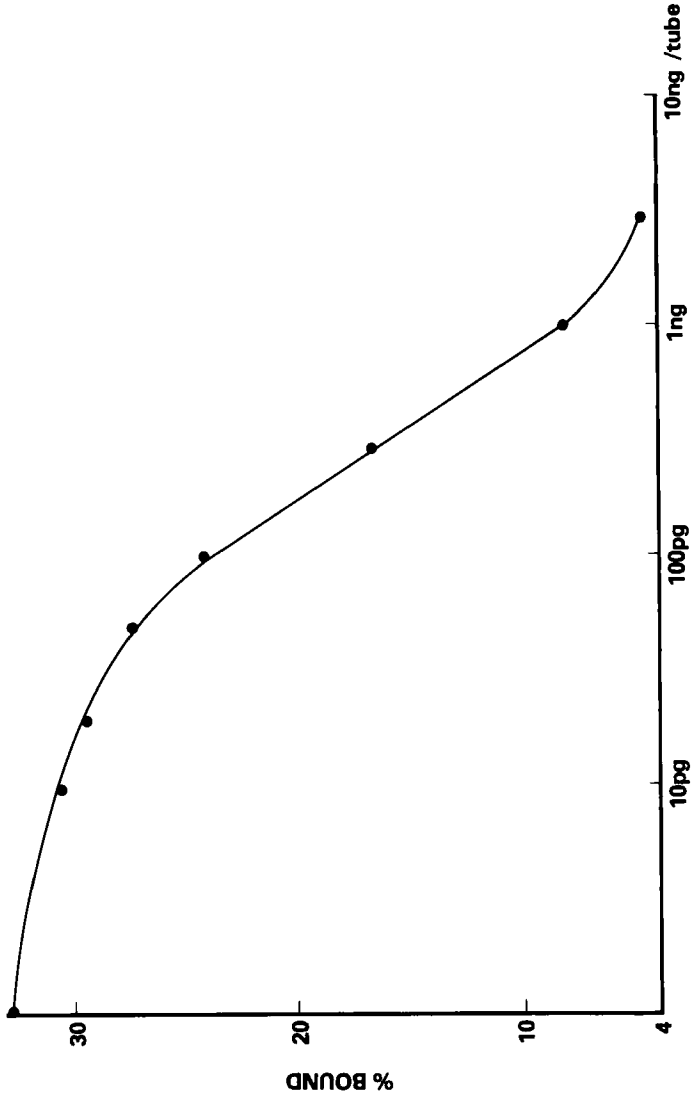

Figure 1

Typical Inhibition Curve for the N-acetylserotonin assay.

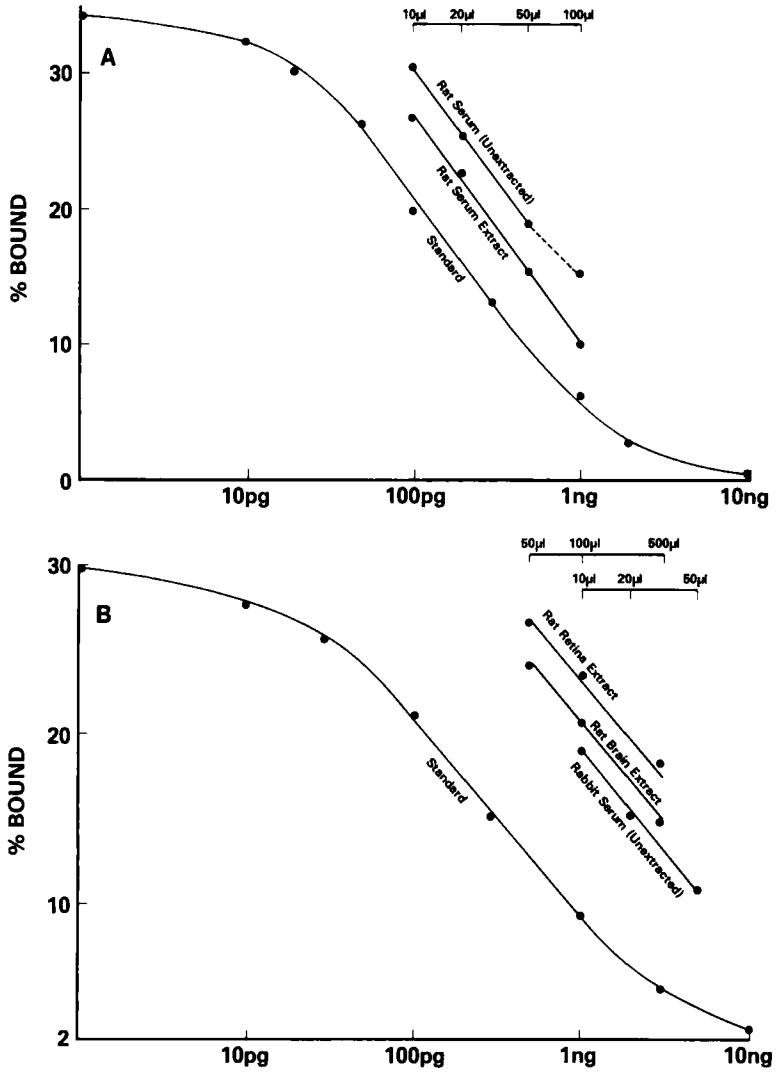

Figure 2

Parallel Inhibition Curves of A) Rat Serum and Rat Serum Extract and B) Rat retina extract; rat brain and rabbit serum with the standard in the N-acetylserotonin radioimmunoassay.

TABLE II

Precision of the N-acetylserotonin Assay

	pg/tube	Within Assay CV%	Between Assay CV%	Number of Assays
Control A	285.6	7.5	11.1	17
Control B	142.0	8.4	7.7	15
Control C	160.6	7.7	7.1	12

TABLE III

Levels of N-acetylserotonin in Biological Tissues
mean ± standard error (Number of samples)

Unextracted samples		
Rat serum	2.59 ± 0.45 ng/ml	(8)
Hamster serum	0.95 ± 0.15 ng/ml	(8)
Rabbit serum	9.69 ± 1.06 ng/ml	(6)
Extracted samples		
Rat serum	3.58 ± 0.55 ng/ml	(6)
Rat pineal	371 ± 62 pg/pineal	(6)
Rat retina	203 ± 62 pg/pair of retina	(5)
Rat brain	340 ± 55 pg/g	(6)
Hamster serum	0.92 ± 0.1 ng/ml	(8)

DISCUSSION

In 1970, Miller and Maickel (20) used the reaction of o-phthaladehyde with N-acetylserotonin which yielded a highly fluorescent compound to study the level of N-acetylserotonin in the rat pineal, hypothalamus, medulla and mid-brain and in the dog pineal and hypothalamus. N-acetylserotonin could be detected only in the rat and dog pineal. N-acetylserotonin was later found in the rat pineal by radioenzymatic assay (21, 22) or gas chromatography-mass spectrometry (GCMS) (23, 24). However, using an immunohistochemical technique, N-acetylserotonin was demonstrated in the cerebellum and brain stem of rats (10, 11) and Narasimhachari and co-workers (18) have recently confirmed its

presence in the rat hypothalamus using o-phthalaldehyde. N-acetylserotonin has also been identified and quantified by GCMS in tissues pooled from several rats (19) but quantification of the amount of N-acetylserotonin in brain tissues remains a problem, requiring more sensitive techniques.

The presence of N-acetylserotonin in the pineal (12, 20 - 23) suggests that N-acetylserotonin is not metabolized completely into melatonin and may be a pineal hormone (12). This is supported by the findings of a circadian rhythm of serum N-acetylserotonin in rats (8, 32) which is significantly lowered by pinealectomy (9). Additionally, the demonstration of N-acetylserotonin in the cerebellum, trigeminal roots and the reticular formation suggests a neuromodulator or neurotransmitter role (10, 11, 13).

A sensitive and specific method for the determination of N-acetylserotonin levels in biological tissue would be of value and was partially achieved by a previous radioimmunoassay for N-acetylserotonin (12) which enabled the quantitation of N-acetylserotonin in the pineal, retina, brain and serum samples of rats and chickens (8, 12, 13, 14). However, the assay required prior removal of melatonin from the sample (8, 13, 14) or a mathematical deduction of its level. Furthermore, the assay lacked sensitivity.

The present radioimmunoassay of N-acetylserotonin overcomes some of the problems. Cross-reactivity studies with 23 related and selected compounds indicated that the antiserum is very specific for N-acetylserotonin and only four potentially important compounds (6-hydroxymelatonin, O-acetyl-5-methoxytryptophol, melatonin and γ-amino-n-butyric acid) cross-react significantly in our assay system. However, they would have to be present at concentrations 100 - 100,000 times higher than the level of N-acetylserotonin to interfere.

The within and between assay CV were satisfactory and the parallelisms demonstrated between the inhibition curves for the standard and extracted or unextracted samples suggest that an unbiased estimation of N-acetylserotonin levels in biological tissues can be obtained. Additional validation was provided by HPLC with the similar elution profile of authentic N-acetylserotonin and the extracted sample of rat serum suggesting that N-acetylserotonin is being measured and the assay system is specific for N-acetylserotonin.

The levels of N-acetylserotonin in rat, hamster and rabbit tissues were determined by this radioimmunoassay with no extraction or following relatively simple methods of extraction by petroleum ether and methanol. The higher level of N-acetylserotonin determined in rat serum by this assay as compared with earlier reports (8, 9), may partially reflect a low recovery for the extraction method employed. The levels of N-acetylserotonin in other rat tissues are comparable to those reported previously (12 - 14, 21 - 24).

While the role of N-acetylserotonin as a potential hormone, neurotransmitter and neuromodulator (31) remains to be elucidated, the development of the present radioimmunoassay should facilitate further investigations (32).

ACKNOWLEDGMENT

The investigators would like to acknowledge financial support from the Medical Research Council (MA 5981 and MA 7327), and the expert secretarial assistance of Mrs. L. Koutalos.

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