COMET ASSAY REPORT

A Comet assay in exfoliated cells from the buccal mucosa after administration of an electronic microbicide *in situ*

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STUDY TITLE

A Comet assay in exfoliated cells from the buccal mucosa after administration of an electronic microbicide *in situ*

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TESTING FACILITY

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LABORATORY PROJECT IDENTITY

CONTENTS

GOOD LABORATORY PRACTICE

Acceptance of Report

I hereby declare that the study described in this report was conducted under my supervision and that to the best of my knowledge and belief the study was conducted in compliance with accepted international standards of Good Laboratory Practice. However, the critical phases of the study and the final report have not been subject to QA inspection or audit.

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This report is approved by BIBRA Management.

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CRITICAL DATES

Start date (first experiment)	:	5 February 1997
Preliminary study	:	30 January 1997
Second experiment	:	7 February 1997
Report date	:	14 February 1997

Responsible staff

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Archives

All raw data, documentation, any relevant specimens and a copy of the protocol and final report will be retained, for a period of 10 years, in the Department of Genetic and Reproductive Toxicology at BIBRA International under the appropriate reference. Specimens will be retained as long as they afford evaluation.

1. SUMMARY

The study reported was conducted to assess the ability of an electronic microbicide to induce damage in exfoliated cells from the buccal mucosa of a volunteer using the Comet assay. Cells were collected from the buccal mucosa by scraping with a wooden spatula from four areas in the mouth (cheek areas adjacent to the gum line), the first being used as the source of untreated, control cells (negative and positive) and the other three providing cells after exposure *in situ* to the DENTRON Biogun for 2, 4 and 8 minutes. This device emitted a stream of electrically-charged air particles including the superoxide radical anion (O_2^-). Cells used as the positive control were treated with 20 μM hydrogen peroxide for 30 minutes.

The cell samples were tested using the Comet assay described by Singh *et al.* (1988) and Anderson *et al.* (1994), with the incorporation of proteinase K (0.05 mg/ml) into the lysing solution to digest the structural proteins surrounding the cells and enabling the release of the DNA. A computerized image analysis system (KOMET 3.0 - Kinetic Imaging Ltd.) was used to analyse the tail moments and tail lengths of the comets produced. The tail moment is considered the most sensitive measurement. Two experiments were conducted.

No biologically significant increases were observed at any of the exposure times by comparison with the negative control values under the experimental conditions used.

2. OBJECTIVE AND INTRODUCTION

2.1. Objective

The objective of the study was to assess the ability of an electronic microbicide to induce damage in exfoliated cells from the buccal mucosa using the Comet assay.

2.2. The Comet assay - Principle of the assay

The alkaline single cell gel electrophoresis assay or Comet assay is a sensitive and rapid method for detection of DNA strand breaks, alkali-labile sites or intermediates in base and nucleotide-excision repair. It provides information about damage in individual cells. Isolated cells are embedded in between agarose layers, lysed *in situ* in the presence of detergent and alkali, subjected to electrophoresis, and stained with ethidium bromide to visualize their DNA. Small DNA fragments migrate out of the nucleus forming a 'comet' tail and the breakage can be estimated from the extent of the migration by comparison of untreated with treated cells.

This assay has been used to demonstrate DNA strand breaks in e.g. human and mouse sperm (Singh *et al.*, 1989), human lymphocytes (Anderson *et al.*, 1994) and buccal cells (Rojas *et al.*, 1996). There are two comprehensive review articles (McKelvey-Martin *et al.*, 1993 and Fairbairn *et al.*, 1995).

2.3. The DENTRON Biogun

The DENTRON Biogun is an electronic microbicide which kills a wide variety of micro-organisms on electrically conductive surfaces using a concentrated stream of electrically charged gaseous particles derived from the surrounding air including the superoxide radical anion (O_2^-). This is thought to act as a nucleophile on the phospholipid bilayer of microbial cells, causing a de-esterification of fatty acids and leading to an increase in surface charge and a weakening of the membrane. Under hypotonic conditions this results in cell lysis and death. The anions are generated by charging a sharp metallic point to a high negative voltage relative to the surface being treated, voltages of -10kV to -20kV being typical.

Details of the structure of the device and its function (as supplied by DENTRON Ltd.) can be found in Appendix 1.

2.4. Basis of the study

Cells from the buccal mucosa of a volunteer from 4 areas in the mouth (cheek areas adjacent to the gum lines) were sampled. The first area provided cells for the negative and positive controls, and the remaining areas used for 2, 4 and 8 minute *in situ* exposure periods to the Biogun.

The Comet assay was performed using the mucosal cells to assess the extent of any damage caused by the Biogun. The assay was performed twice.

3. TEST AND CONTROL ARTICLES

3.1. Test article

3.1.1. Definition

This was an electronic microbicide called the Biogun which delivered a concentrated stream of gaseous anions derived from the atmosphere.

3.1.2. Supply

The equipment required to generate the anions - the Biogun - was provided by the sponsor, DENTRON Ltd. The sponsor was responsible for the quality assurance of the Biogun.

3.2. Control articles

3.2.1. Negative control

RPMI 1640 medium plus L-glutamine (Gibco Ltd., Paisley, Scotland, UK) was used as the negative control article.

3.2.2. Positive control article

Hydrogen peroxide (Sigma, Cat No. H1009) was used as the positive control article at doses of 400 and 20 μM *in vitro*. This was chosen because superoxide radical anions are unstable in an aqueous medium and can spontaneously dismutase to form hydrogen peroxide. Hydrogen peroxide, in turn, in cellular systems forms reactive hydroxyl radicals (Anderson, 1996).

4. PROCEDURES

4.1. Sampling of cells

The cells from the buccal mucosa were collected by scraping with a clean wooden spatula. Before cell removal, the mouth was washed with physiological saline to remove debris. The inside of the mouth was scraped at the appropriate place agreed by the sponsor. Cells were removed from the spatula with RPMI medium into a cell culture dish, washing the spatula several times to ensure all cells were collected in a total of 500 μl medium. The suspension was transferred to an eppendorf tube and centrifuged at 700 x g for 3 minutes. The supernatant was discarded and the untreated cells used as a negative control.

4.1.1. Cell supply

The Managing Director of DENTRON Ltd. (Mr Jonathan Copus) was the volunteer for the exposures and provided the cell samples. A document was signed by Mr Copus taking full responsibility for providing the samples and being exposed to the Biogun.

4.2. Experimental procedure

4.2.1. Number of assays

The assay was carried out on two separate occasions at a time interval of 48 hrs to allow for cell recolonisation after scraping.

4.2.2. Mouth sites

Four mouth sites were used:

a) Top right region - control area, cells for both positive and negative controls

b) Lower right region - low dose area, 2 min exposure

c) Lower left region - middle dose area, 4 min exposure

d) Top left region - high dose area, 8 min exposure

4.2.3. Treatment of cells

4.2.3.1. With electronic microbicide

Treatment of the cells *in situ* was with a concentrated stream of gaseous anions, electronically generated, and administered for 2, 4 and 8 minutes. Doses were decided in consultation with the sponsor.

4.2.3.2. With hydrogen peroxide

Cells were treated for 30 minutes with 400 μM (first assay) and 20 μM (second assay) hydrogen peroxide at 37°C to provide a positive response.

Duplicate slides were prepared at each dose level, including the no treatment, negative control group.

4.2.4. Slide preparation

Fully frosted microscope slides (Surgipath, Winnipeg Manitoba) were covered with 110 μl of 0.5% normal melting agarose (NMA) at about 50°C in Ca^{2+} and Mg^{2+} free phosphate buffered saline (PBS(A)). They were immediately covered with a 24 x 60 mm coverslip and then kept at room temperature for about 5 min to allow the agarose to solidify. This layer was to promote the attachment of the second layer of 0.5% low melting point agarose (LMA). Treated or control cells were mixed with 75 μl LMA to form a cell suspension. After gently removing the coverslip, the cell suspension was rapidly pipetted onto the first agarose layer, spread using a coverslip, and maintained on an ice-cold flat tray for 5 min to solidify. After removal of the coverslip, the third layer of LMA (75 μl) at 37°C was added, spread using a coverslip and again allowed to solidify on ice for 5 min. After removal of the coverslip, the slides were immersed in freshly prepared lysing solution (2.5 M sodium chloride (NaCl), 100 mM ethylenediamine tetra-acetic acid, sodium salt (Na_2 EDTA), 10 mM Tris, 1% sodium sarcosinate, pH 10), with 1% Triton X-100, 10% dimethyl sulphoxide (DMSO) and 0.05 mg/ml proteinase K (Boehringer Mannheim) added just before use, and incubated for ½ hr at 37°C.

4.2.5. Electrophoresis

The slides were removed from the lysing solution, drained and placed in a horizontal gel electrophoresis tank side by side, avoiding spaces and with the agarose ends facing each other, nearest the anode. The tank was filled with fresh electrophoresis solution prepared using water at 4°C (1 mM Na_2 EDTA and 300 mM sodium hydroxide (NaOH)) to a level approximately 0.25 cm above the slides.

The slides were left in the solution for 20 min to allow the unwinding of the DNA and expression of alkali labile damage before electrophoresis. Electrophoresis was conducted for 20 min using 24 volts, and the current adjusted to 300 milliamperes by raising or lowering the buffer level. All of these steps were conducted under dimmed light (the apparatus was covered with a black cloth) to prevent light-induced DNA damage. After electrophoresis, the slides were placed horizontally on a rack and Tris buffer (0.4 M Tris, pH 7.5) was added gently drop wise to neutralize the excess alkali. The slides were left for 5 min. This neutralizing procedure was repeated 3 times.

4.2.6. Staining

60 μl Ethidium bromide (20 μg/ml) was added to each slide and covered with a coverslip. The slides were placed in a humidified air-tight container to prevent drying of the gel and analyzed within 3-4 hr.

4.3. Observations

4.3.1. Slide scoring

Slides were examined at a 50-fold magnification resulting from a 40 x objective and a 1.25 camera projection lens. They were visualised with a 12.5 x eye piece on a fluorescence microscope (Zeiss, R.G. equipped with an excitation filter of BP 546/10 nm and a barrier filter of 590 nm). A camera was attached to the microscope and the images of cells received by the camera through the projection lens appeared on the computer monitor screen at a magnification of x 1000. The system was calibrated to measure tail length using a stage micrometer. 25 cells were scored from each duplicate slide (i.e. 50 cells in total).

4.3.2. Analysis by computer

A computerized image analysis system (KOMET 3.0 - Kinetic Imaging Ltd, Liverpool, UK) was used to measure various comet parameters. The ones chosen for presentation were comet tail moment and tail length. The tail moment is equivalent to the integrated value of density multiplied by migration distance and is considered to be the most sensitive measurement but the tail length is also widely used. The system set up used for these experiments was as follows: head threshold 8%, tail threshold 1%, smoothing value 1; background height 20; tail break length 10. A minimum of 25 cells was read from each slide.

5. RESULTS

5.1. Meter readings

During the exposure period the readings on the Biogun meter (microamperes) showed constant small fluctuations. Recordings were made of these values as changes occurred (rather than at a set time interval) and the results are shown in Appendix 2 and Table 1. Mean values ranged between 95.0 and 101.7.

5.2. Experimental data - tail moments and tail lengths

The data obtained from experiments 1 and 2 are shown in Table 2 and Appendix 3. In experiment 1 the concentration of the positive control, hydrogen peroxide, was found to be too high and resulted in severe toxicity to the cells. In experiment 2, at a lower dose, the positive control gave the anticipated response when compared with its respective negative control value.

Although a formal statistical analysis was not carried out there was no biologically significant increase in the amount of comet damage following treatment.

6. DISCUSSION

The evaluation of DNA in buccal mucosal epithelial cells is considered to be a good biomarker of early damage in the target tissue of the mouth (Rojas *et al.*, 1996). This site was thought to be the most relevant for treatment with the superoxide radical anion stream because of the application of the Biogun in dentistry. In addition, it has been possible to expose cells in the buccal cavity directly *in situ*.

Although the meter readings from the Biogun fluctuated during the exposure period, the fluctuations were not considered to have affected the treatment periods in the study. Saliva which collected in the mouth during exposure very occasionally resulted in the ion emitter being briefly withdrawn beyond the 10 mm from the surface being treated. Since the Biogun maintains a substantially constant current at emitter-to-surface distance of between 2 and 10 mm, this resulted in a drop in current to values in the range of 20-30 microamperes. This is reflected in the raised standard error values for the 8 minute exposure in experiment 1 and 4 minute exposure in experiment 2. The one observed increase to a value of 211 μamps was due to a momentary arcing of the Biogun. It is believed that these deviations made no difference to the overall doses delivered and the μamps for all readings were satisfactory.

Due to the high background (negative control) level of damage in the buccal mucosal cell as found in this study, the data are inappropriate for formal statistical analysis, because the statistical power attained would be too low i.e. the assay has low sensitivity. However, this level of background damage has been reported by other authors (Rojas *et al.*, 1996) and is due to the nature of the cells in the buccal mucosa which are constantly being recolonised and sloughing-off. This occurs because of the continuous division of cells in the basal membrane. Those cells which are attainable for analysis by scraping with a wooden spatula are those which are easily removable, but are in the final stages of cellular life.

In conclusion, no biologically significant increases were observed at any of the exposure times by comparison with the negative control values under the experimental conditions used.

7. REFERENCES

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Fairbairn, D.W., Olive, P.L. and O'Neill, K.L. (1995). The Comet assay: a comprehensive review. *Mutation Research* **339**, 37-59.

Table 1. Summary of meter readings from the DENTRON Biogun during exposure periods

Experiment No.	Time of exposure (min)	Meter reading (microamperes) Mean ± SE
1	2	101.7 ± 1.504
	4	99.4 ± 4.230
	8	96.4 ± 16.055
2	2	100.4 ± 6.991
	4	95.0 ± 15.932
	8	99.5 ± 9.185

Table 2. Summary of data from Experiments 1 and 2

Treatment	n	Experiment 1 Mean ± SE		Experiment 2 Mean ± SE	
		Tail moments	Tail lengths	Tail moments	Tail lengths
Negative control	50	78.82 ± 6.46	107.16 ± 4.24	43.12 ± 6.32	89.84 ± 4.46
2 min*	50	44.40 ± 4.54	68.54 ± 3.10	54.42 ± 5.14	100.61 ± 3.41
4 min*	50	54.19 ± 5.11	86.45 ± 3.69	61.54 ± 5.04	100.28 ± 2.64
8 min*	50	50.66 ± 4.94	78.43 ± 3.92	48.18 ± 4.93	87.11 ± 2.60
Positive control					
(Expt 1 400 μM H_2O_2)	7	107.2 ± 24.5†	116.2 ± 23.1†		
(Expt 2 20 μM H_2O_2)	50			79.11 ± 5.76	111.54 ± 3.03

* = treatment with electronic microbicide (DENTRON Biogun).
n = number of cells read.
† = This result was considered invalid due to toxicity
H_2O_2 = hydrogen peroxide.

THE DENTRON BIOGUN

The Dentron Biogun is an electronic microbicide which kills a wide variety of micro-organisms on electrically-conductive surfaces using a concentrated stream of electrically charged gaseous particles derived from the surrounding air. The technique has a large number of potential applications from instrument sterilisation to preserving food, but the main area of initial concern is dentistry and medicine, including dermatology and chiropody. The technique is non-invasive and practically free from adverse side-effects.

The principal biologically-active species of particle has been identified as the superoxide radical anion (O_2-·); this is thought to act as a nucleophile on the phospholipid bilayer of microbial cells, causing a de-esterification of fatty acids and leading to an increase in surface charge and a weakening of the membrane, and therefore under hypotonic conditions to cell lysis and death[1]. The anions are generated by charging a sharp metallic point to a high negative voltage relative to the surface being treated, voltages of -10kV to -20kV being typical.

The gradient of the electric field formed at the point is increased by the proximity of a surface with a relatively positive charge. The number of ionisations occurring per second at the point can be found by measuring the current flowing in the conductor connecting the point to the negative pole of the high-voltage generator, according to the formula

$$N = I/e$$

where N = the number of ionisations per second,
I = the current in amperes, and
$e = 1.602 \times 10^{-19}$ coulombs.

Of more practical interest is the number of ions impinging on the treated surface, and so measurements are taken of the current flowing in the conductor connecting this surface to the positive pole of the high-voltage generator, which is normally within a few volts of earth potential. When the system is used for medical and dental applications the sharp point takes the form of a short length of PTFE-insulated, silver-plated copper wire mounted in a small plug with its free end cut at an oblique angle to form what is known as an *emitter assembly*, the flexibility of the emitter wire eliminating the possibility of cross-infection through so-called "needle-stick injury". The emitter assembly is mounted in an insulated handpiece connected by flexible cable to a base unit containing the high-voltage generator, while both the patient and the practitioner are connected to the positive pole of the high-voltage generator via a wristband and a "curly cord" earth-type conductor. For medical and dental uses the recommended distance between the end of the emitter and the surface being treated is between 2mm and 10mm with a mean of 6mm, and under these conditions internal regulatory circuitry in the base unit maintains a constant output current of 100µA ± 3µA.

The Biogun has been shown to kill a large number of micro-organisms *in vitro,* including the following:

Acinetobacter sp., *Actinomyces actinomycetemcomitans, Actinomyces naeslundii* genospecies 1, *Actinomyces naeslundii* genospecies 2, *Actinomyces georgiae, Actinomyces gerensceriae, Actinomyces isrealii, bacillus* sp., *Candida albicans, Clostridium perfringens, Enterobacter agglomerans, Escherichia coli, Klebsiella pneumoniae, Lactobacillus* sp., *Proteus* sp., *Pseudomonas aeruginosa, Pseudomonas putida, Psychrobacter immobilis, Salmonella, Staphylococcus aureus, Staphylococcus sanguis, Streptococcus mutans, Tinea*[2,3,4].

When primary root caries lesions were treated *in vivo* with a prototype Biogun, 99.8% of the total microflora were found to be killed within 4 minutes. The micro-organisms identified included mutans streptococci, lactobacilli, yeasts and Gram-positive pleomorphic rods[5]. Production models of the device promise even quicker results. Clinical success is also well established against the conditions *Tinea pedis* (athlete's foot), *verruca pedis,* hand warts, onychomycosis, exudative venous ulcers,

acne, thrush, and cold sores caused by the *Herpes simplex* virus. Other potentially susceptible conditions include impetigo, candidiasis (including angular cheilitis), gingivitis and periodontitis.

Safety

The Biogun conforms with the requirements of the European Medical Devices Directive 93/42/EEC (Class IIa) and to the relevant particular standards for electrical safety[6]. EMC compatibility is independently certified[7]. The device is inherently safe in electrical terms for the following reasons:

1. Although the maximum output voltage is approximately 20kV, the output current (the parameter of concerned with safety) is regulated at 100μA, 300 times lower than the lowest safety limit for direct electrical current through the human body set by the British Standards Institution, 30mA for 10,000 mS giving no harmful effects 8. The maximum output current under arcing conditions (i.e. maintaining a spark between the tip of the ion emitter and the surface undergoing treatment) is limited to a maximum of 220μA.
2. Even without the regulatory feedback circuitry the theoretical maximum output current is given by the formula $I = (D + A/2)/R$, where I = maximum instantaneous output current in amperes, D = direct voltage between the output socket and earth in volts, A = peak-to-peak voltage of the ripple component of the output and R = total series resistance composed of R74-79 in ohms. This formula gives a "worst case" result since it ignores the effect of the further series resistance incorporated in the grounding cords. Substituting real values in the above equation gives $I = (19800 + 990)/(19.8 \times 106) = 1.05mA$.
3. The circuitry of the Biogun incorporates several layers of safety devices, both active and passive, to protect users in the event of a fault.
4. Experimental multiple fault conditions were simulated by disabling the regulatory feedback circuitry, replacing the fuses with solid conductors, and shorting out the resistor chain in series with the output. Over a series of five experiments, the average maximum output current obtained was 2.03 mA and the absolute maximum output current 4.17mA for <1 second before thermal runaway in the drive transistor loaded the oscillator to a point where oscillation ceased.

Electrical therapies are normally used only with caution on epileptics, pregnant women and people fitted with a cardiac pacemaker – although the normal output current of the Biogun is regulated at a level far below that at which adverse effects might be expected, and the device has in fact been safely used on members of both the last two groups.

The electrical generation of gaseous anions also inevitably produces a small quantity of ozone, a gas which in sufficient concentration has been shown to have deleterious physiological effects. However, the European Medical Devices Directive 93/42/EEC specifies that "any risks which may be associated with their use constitute acceptable risks when weighed against the benefit to the patient and are compatible with a high level of protection of health and safety"[9]. There are thus no ozone exposure limits directly applicable to the Biogun, although *occupational* exposure limits are embodied in COSHH Document EH 40/96, which prescribes a limit of 0.2 ppm over 15 minutes, exposure limits over other times being derived from the formula

$$m = 15/t \times 0.2$$

where m = the exposure limit in parts per million, and
t = exposure time in minutes.

Applying this formula to the Biogun over the prescribed treatment duration of 4 minutes gives an exposure limit of 0.75 ppm. The Ozone emissions from the Biogun have been measured in two independent series of tests: St Bartholomew's and the Royal London Hospital Medical College (LHMC) tested the unit in its quiescent state (i.e. operating in free air), Southern Counties Scientific Services Ltd (SCSS) conducted trials simulating dental use of the device (i.e. with the emitter tip approximated to an earthed conductive surface). As indicated in the following tables, the LHMC tests give a "worst case" result which is 7.5 times lower than this limit, while corresponding figure for the SSCS results is 1.5 times lower than the limit. When operated in accordance with the dental instructions (i.e. at an emitter/surface distance of 6mm and using an aspirator) the result was Over 9 times lower than the occupational safety limit.

Ozone tests conducted by St Bartholomew's and the Royal London Hospital Medical College

CONDITIONS:
The increase in levels of ozone above ambient generated by the Biogun was measured using a Gas Sampling Pump (Detectawl, Milton Keynes) fitted with the ozone 18L detector tube (detects 0.025 to 3ppm ozone). It was held at a distance of 20 mm from the Biogun emitter tip while it was activated for up to 65 minutes continuously *in vitro* on three occasions. On the first occasion, Test 1, there was some ventilation as the window of the laboratory was open some two meters away. Over 65 minutes no ozone was detected. On the other two occasions there was no ventilation and the values found are given in Table 1.

AFTER X MINUTES	TEST 1 (PPM)	TEST 2 (PPM)	TEST3 (PPM)
0	0.0	0	0
5	0.0	0	0.05
10	0.0	0	0.05
15	0.0	0.05	0.05
20	0.0	0	0.05
25	0.0	0.05	0.05
30	0.0	0.1	0
35	0.0	0.1	0
40	0.0	0.05	0
45	0.0	0.05	0.05
50	0.0	0.1	0
55	0.0	0.05	0
60	0.0	0.1	0
65	0.0	0.0	0

Table 1

Ozone tests conducted by Southern Counties Scientific Services, Southampton

CONDITIONS:
Emitter/probe distance: 20mm
Emitter/aspirator inlet distance (where used): 10mm
Samples taken after 15 minutes of continuous operation

EMITTER/SURFACE DISTANCE (mm)	PPM WITHOUT ASPIRATOR	PPM WITH ASPIRATOR
3.0	0.50	0.45
6.0	0.22	0.08
9.0	0.06	<0.01

Table 2

Time for which the Biogun could be used before it reached the occupational safety limit (using the Southern Counties figures)

EMITTER/SURFACE DISTANCE (mm)	WITHOUT ASPIRATOR (minutes)	WITH ASPIRATOR (minutes)
3.0	6.00	6.67
6.0	13.64	37.50
9.0	50.00	300.00

Table 3

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[6] EN 60601, EN 60601-1/1A and EN 60601-1-2

[7] SGS United Kingdom Ltd Test Reprt No. 1464, 3rd October, 1996.

[8] BSPD 6519.

[9] 93/42/EEC Annex i.

Appendix 2. Meter readings from the DENTRON Biogun during exposure periods - first assay

Time of exposure	Meter readings (current in microamperes)							
	No.	Reading	No.	Reading	No.	Reading	No.	Reading
2 minutes	1	98.9						
	2	101.6						
	3	102.0						
	4	102.2						
	5	102.3						
	6	103.1						
	7	103.2						
	8	102.3						
	9	102.3						
	10	101.9						
	11	103.1						
	12	100.5						
	13	98.5						
4 minutes	1	102.0	19	102.5	37	100.2	55	100.7
	2	102.1	20	102.2	38	99.8	56	102.2
	3	102.3	21	102.3	39	100.4	57	96.0
	4	93.4	22	103.4	40	98.3	58	78.6
	5	102.2	23	93.5	41	99.7	59	95.6
	6	101.7	24	102.4	42	101.5	60	101.4
	7	102.3	25	93.7	43	94.5	61	96.5
	8	102.4	26	102.5	44	97.2	62	91.1
	9	103.0	27	96.8	45	102.1	63	100.7
	10	102.2	28	101.2	46	102.6	64	91.0
	11	94.5	29	100.3	47	102.7	65	101.2
	12	102.0	30	94.9	48	101.0	66	101.9
	13	101.7	31	97.2	49	99.3		
	14	102.4	32	100.6	50	94.8		
	15	103.0	33	98.0	51	102.5		
	16	102.4	34	102.4	52	101.4		
	17	101.0	35	101.7	53	94.3		
	18	103.1	36	94.0	54	99.5		

continued....

Appendix 2. continued.

Time of exposure	Meter readings (current in microamperes)							
	No.	Reading	No.	Reading	No.	Reading	No.	Reading
8 minutes	1	79.9	34	100.9	67	103.2	100	99.7
	2	87.2	35	102.9	68	104.1	101	103.6
	3	100.9	36	103.4	69	103.1	102	85.1
	4	99.1	37	103.1	70	102.7	103	102.1
	5	89.1	38	102.5	71	102.1	104	103.3
	6	94.6	39	84.6	72	103.1	105	103.8
	7	99.7	40	102.6	73	100.6	106	101.7
	8	99.1	41	103.8	74	99.8	107	103.5
	9	91.5	42	102.6	75	102.2	108	101.7
	10	103.3	43	102.9	76	103.0	109	99.0
	11	103.4	44	101.8	77	102.4	110	89.9
	12	103.5	45	99.8	78	103.0	111	99.2
	13	103.5	46	102.5	79	102.8	112	103.2
	14	103.2	47	98.5	80	103.3	113	103.5
	15	102.1	48	103.1	81	103.2	114	103.5
	16	102.9	49	91.5	82	101.6	115	99.1
	17	103.1	50	96.9	83	99.6	116	103.4
	18	102.7	51	103.6	84	92.7	117	98.3
	19	102.7	52	102.9	85	90.6	118	98.6
	20	103.2	53	103.1	86	95.6	119	100.1
	21	102.9	54	102.6	87	87.0	120	103.1
	22	102.6	55	102.4	88	82.9	121	99.5
	23	103.5	56	103.3	89	74.6	122	69.5
	24	102.9	57	102.5	90	81.3	123	100.1
	25	101.6	58	103.1	91	73.4	124	98.9
	26	102.0	59	102.9	92	70.0	125	91.4
	27	102.7	60	91.9	93	101.7	126	96.1
	28	102.9	61	103.0	94	96.8	127	95.0
	29	103.7	62	102.9	95	97.6	128	87.1
	30	98.9	63	99.1	96	83.0	129	102.2
	31	102.7	64	103.1	97	54.7	130	99.7
	32	211.4	65	102.5	98	18.8	131	91.0
	33	91.8	66	103.3	99	71.9	132	103.4

continued....

Appendix 2. continued.

Time of exposure	Meter readings (current in microamperes)							
	No.	Reading	No.	Reading	No.	Reading	No.	Reading
8 minutes (cont'd)	133	101.2	143	59.0	153	103.7	163	91.6
	134	103.6	144	76.1	154	102.1	164	87.4
	135	97.5	145	102.3	155	103.5	165	96.7
	136	56.5	146	97.2	156	103.2	166	71.4
	137	97.3	147	88.6	157	103.1	167	94.5
	138	103.1	148	99.3	158	102.6	168	103.4
	139	81.6	149	103.4	159	103.3	169	37.2
	140	61.0	150	101.8	160	101.4	170	87.5
	141	45.0	151	102.5	161	89.7	171	103.4
	142	54.9	152	103.7	162	79.6		

continued....

Appendix 2 Meter readings from the DENTRON Biogun during exposure periods - second assay

Time of exposure	Meter readings (current in microamperes)							
	No	Reading	No	Reading	No	Reading	No	Reading
2 minutes	1	101.2	12	102.1	23	98.7	34	102.5
	2	102.1	13	102.5	24	102.1	35	59.3
	3	101.9	14	101.9	25	102.1	36	101.9
	4	90.9	15	101.2	26	101.2	37	102.3
	5	102.9	16	102.0	27	107.3	38	102.2
	6	99.8	17	101.9	28	102.4	39	102.5
	7	97.1	18	101.6	29	102.4	40	102.1
	8	102.5	19	102.1	30	101.9	41	101.4
	9	89.3	20	102.8	31	102.2	42	99.4
	10	102.1	21	101.8	32	102.4	43	102.3
	11	102.1	22	100.1	33	102.4	44	106.2
4 minutes	1	98.2	17	102.5	33	54.2	49	102.7
	2	101.32	18	103.1	34	91.3	50	77.9
	3	90.8	19	102.9	35	96.7	51	101.3
	4	82.4	20	102.5	36	89.5	52	93.4
	5	88.1	21	102.8	37	92.7	53	28.1
	6	96.7	22	101.9	38	102.4	54	89.6
	7	102.5	23	101.2	393	102.6	55	102.9
	8	98.4	24	97.3	40	102.9	56	102.5
	9	85.6	25	37.2	41	89.6	57	103.4
	10	87.9	26	76.8	42	92.7	58	97.9
	11	99.9	27	80.9	43	102.6	59	102.9
	12	102.7	28	97.4	44	97.7	60	103.6
	13	102.8	29	102.1	45	102.5	61	103.6
	14	102.6	30	102.9	46	102.9	62	101.9
	15	102.2	31	103.0	47	101.9	63	98.1
	16	102.8	32	88.1	48	102.6	64	94.1

continued....

Appendix 2. continued.

Time of exposure	Meter readings (current in microamperes)							
	No	Reading	No	Reading	No	Reading	No	Reading
4 minutes (cont'd)	65	103.6	75	102.9	85	102.6	95	102.9
	66	102.7	76	102.9	86	103.0	96	96.3
	67	102.9	77	102.7	87	102.9	97	101.3
	68	88.9	78	103.0	88	101.5	98	98.7
	69	99.6	79	102.4	89	101.6	99	103.6
	70	80.9	80	99.4	90	99.4	100	95.8
	71	96.9	81	102.8	91	91.0	101	99.8
	72	102.6	82	103.2	92	81.5	102	96.9
	73	102.6	83	95.8	93	99.5	103	83.1
	74	33.6	84	102.6	94	102.8	104	97.2
8 minutes	1	102.2	22	102.4	43	101.9	64	102.3
	2	102.1	23	102.0	44	101.7	65	102.3
	3	99.5	24	101.8	45	101.6	66	102.1
	4	101.8	25	101.6	46	102.7	67	102.6
	5	104.8	26	101.2	47	101.2	68	102.4
	6	67.1	27	102.0	48	102.1	69	102.0
	7	99.2	28	101.7	49	102.1	70	101.6
	8	102.5	29	101.8	50	102.6	71	102.6
	9	92.7	30	101.5	51	102.7	72	103.6
	10	89.1	31	102.6	52	102.5	73	102.4
	11	90.9	32	102.6	53	101.9	74	102.4
	12	100.3	33	102.5	54	101.8	75	101.9
	13	97.2	34	102.3	55	102.5	76	99.9
	14	96.2	35	102.6	56	102.3	77	105.6
	15	102.2	36	102.7	57	103.4	78	102.4
	16	87.2	37	102.3	58	101.7	79	101.3
	17	101.4	38	102.2	59	102.5	80	101.2
	18	101.9	39	102.5	60	102.6	81	101.2
	19	102.8	40	101.9	61	102.7	82	100.2
	20	99.6	41	102.6	62	103.1	83	100.3
	21	101.2	42	102.0	63	102.4	84	101.9

continued....

Appendix 2. continued.

Time of exposure	Meter readings (current in microamperes)							
	No	Reading	No	Reading	No	Reading	No	Reading
8 minutes (cont'd)	85	100.6	121	102.9	157	101.2	193	102.6
	86	99.6	122	102.6	158	102.9	194	102.4
	87	99.3	123	102.6	159	102.9	195	102.4
	88	100.3	124	101.7	160	101.6	196	96.5
	89	102.1	125	102.5	161	103.4	197	101.7
	90	101.9	126	102.3	162	102.5	198	102.9
	91	99.2	127	103.9	163	102.9	199	102.4
	92	100.2	128	99.7	164	102.9	200	102.1
	93	99.3	129	101.1	165	99.6	201	101.8
	94	98.5	130	101.6	166	102.4	202	102.4
	95	100.3	131	102.3	167	102.5	203	102.7
	96	76.8	132	101.2	168	102.5	204	99.8
	97	72.6	133	105.5	169	101.6	205	99.3
	98	101.6	134	102.7	170	102.0	206	105.2
	99	101.3	135	103.9	171	96.9	207	68.6
	100	102.9	136	100.4	172	102.5	208	101.7
	101	49.9	137	37.2	173	101.2	209	95.7
	102	102.1	138	100.4	174	102.6	210	99.2
	103	99.4	139	102.1	175	99.5	211	101.2
	104	102.7	140	101.2	176	102.8	212	97.3
	105	102.5	141	101.6	177	101.3	213	101.2
	106	102.8	142	101.4	178	29.3	214	93.6
	107	102.8	143	102.1	179	101.2	215	97.6
	108	102.4	144	100.8	180	97.9	216	98.6
	109	102.4	145	100.8	181	64.1	217	97.1
	110	103.1	146	70.1	182	102.2	218	102.4
	111	103.9	147	97.2	183	100.1	219	102.4
	112	101.9	148	98.9	184	102.1	220	99.8
	113	100.9	149	102.2	185	102.4	221	98.6
	114	100.5	150	102.3	186	99.7	222	94.2
	115	102.7	151	102.1	187	101.5	223	97.0
	116	103.1	152	101.3	188	102.8		
	117	102.5	153	98.7	189	101.8		
	118	101.4	154	84.7	190	101.9		
	119	102.7	155	102.9	191	102.7		
	120	102.4	156	102.5	192	102.7		

Appendix 3 Tail Moment and Tail Length Data for Experiments 1 and 2

Comet Tail Moment Values

Experiment 1

-ve ctrl	2-min	4-min	8-min	+ve ctrl
116.44	59.457	69.765	0.47529	64.378
70.249	64.508	2.2530	14.508	67.039
99.05	3.0379	23.90	58.025	249.03
111.67	90.069	77.808	114.70	108.14
126.26	58.637	82.982	4.6657	102.4
49.762	41.90	107.30	61.194	73.024
129.97	84.991	43.595	69.458	86.131
249.03	69.886	79.656	35.553	
104.63	60.605	68.367	68.059	
2.1497	73.479	123.61	64.332	
50.811	11.480	9.5068	25.469	
39.829	35.168	1.2193	45.931	
2.2634	67.452	109.55	1.7968	
80.515	3.3237	68.186	64.669	
116.44	79.303	69.960	7.203	
110.62	0.244	112.88	33.315	
103.12	0.43126	119.79	2.9888	
102.96	61.415	11.558	110.54	
106.40	17.549	20.079	46.07	
81.32	32.454	25.537	30.168	
38.105	15.057	6.97	8.014	
42.300	8.3246	71.027	50.839	
93.620	8.6581	9.7171	22.381	
76.263	0.1764	55.198	27.170	
76.563	70.918	37.567	5.8699	
137.44	69.91	97.331	89.365	
58.95	5.0432	108.06	1.3700	
61.908	75.999	0.13673	70.044	
76.100	11.252	26.790	55.619	
56.39	72.876	83.317	91.523	
21.612	18.744	18.915	66.76	
122.34	24.780	67.87	124.58	
121.60	9.7276	2.1078	64.059	
10.179	46.309	85.396	51.901	
99.515	81.666	91.680	39.29	
16.268	74.849	30.471	69.460	
49.276	82.414	77.001	75.520	
100.12	55.401	11.569	0.60289	
11.99	0.75092	5.2390	110.47	
102.21	1.0930	73.551	93.464	
94.244	84.868	88.61	89.151	
67.549	15.498	35.451	5.5983	
102.39	72.316	60.130	93.181	
94.582	51.093	34.594	86.417	
86.592	24.59	33.04	79.068	
21.0	73.344	22.129	44.918	
0.81572	106.52	53.291	56.028	
29.553	0.6374	54.297	71.029	
119.3	76.558	76.617	24.803	
98.392	65.01	64.084	5.3368	

Experiment 2

-ve ctrl	2-min	4-min	8-min	+ve ctrl
118.83	59.517	96.10	78.457	9.0305
1.6198	64.410	32.136	88.789	91.228
0.43365	63.344	78.190	42.392	72.400
120.26	86.713	92.597	115.24	84.781
1.8510	37.311	9.4515	106.60	70.048
112.82	71.502	78.439	41.47	111.24
107.27	101.32	39.555	92.006	109.27
6.7690	16.350	53.124	2.7724	116.37
1.6172	3.0820	87.087	22.078	88.364
1.6249	70.084	73.698	74.065	111.5
63.705	5.6993	97.732	34.814	71.095
0.31812	53.4	101.84	34.410	6.7839
5.0296	52.840	60.150	58.798	6.9847
5.1325	70.295	40.809	23.116	81.013
114.43	108.81	78.591	37.188	82.357
3.9305	90.093	75.65	35.250	109.05
3.2893	6.3759	24.772	76.863	7.6702
5.4747	12.134	76.088	21.972	111.8
48.012	103.96	100.51	80.066	130.69
2.8671	21.327	7.4564	3.7988	10.714
0.04792	1.5056	15.918	67.032	115.31
76.26	4.0083	60.314	33.995	99.965
6.2619	47.037	12.14	6.204	92.376
29.572	13.866	4.2821	88.267	69.979
93.958	3.5712	7.0487	23.303	114.51
74.216	19.636	119.02	92.142	2.9938
69.716	37.775	55.103	70.215	118.06
1.1284	91.351	6.463	18.729	101.26
1.1284	106.19	82.158	6.7662	5.3210
54.654	96.395	17.208	5.895	74.914
114.75	31.121	44.726	7.8747	103.91
6.3484	37.12	47.152	36.476	91.309
5.5595	98.896	79.626	75.928	87.356
19.058	80.910	36.656	74.21	117.00
62.325	33.779	43.232	121.39	115.44
71.34	60.709	116.7	69.680	79.616
43.499	116.82	20.247	12.098	115.2
86.937	68.34	110.13	19.083	73.180
6.7623	20.293	87.751	94.296	83.597
90.792	23.092	109.47	41.647	5.6360
107.43	45.291	8.6957	19.523	3.9544
112.86	92.106	105.71	93.238	50.265
5.009	85.57	127.37	14.878	108.08
83.474	38.649	94.635	95.908	138.83
76.605	62.461	67.603	28.646	16.059
17.569	116.86	32.52	77.35	112.75
1.6130	105.63	104.39	2.6784	105.94
107.47	5.4590	62.98	9.7006	111.04
0.98462	75.182	43.142	10.346	112.33
3.0771	2.8817	50.441	21.439	46.865

continued...

Appendix 3 Tail Moment and Tail Length Data for Experiments 1 and 2

Comet Tail Length Values

Experiment 1

-ve ctrl	2-min	4-min	8-min	+ve ctrl
132.25	66.451	83.225	29.677	72.258
106.45	80.645	30.967	56.774	94.193
106.45	23.87	92.258	74.193	249.03
127.09	98.064	118.06	138.06	125.16
132.25	67.741	94.193	61.290	105.16
96.12	67.096	123.22	74.193	78.709
134.83	88.387	108.38	83.225	89.032
249.03	73.548	116.77	56.12	
117.41	69.677	87.741	74.838	
61.935	83.225	132.25	99.354	
83.87	63.225	64.516	98.064	
82.580	64.516	29.677	65.161	
47.096	76.774	119.35	42.580	
92.903	45.806	91.612	72.903	
123.22	83.87	90.967	47.096	
112.25	24.516	129.67	63.87	
113.54	30.322	133.54	42.580	
107.74	72.903	83.87	122.58	
12	56.774	85.806	71.612	
113.54	65.161	67.096	99.354	
90.967	50.322	45.161	50.967	
89.677	50.967	92.258	89.677	
116.12	56.774	54.838	54.838	
87.096	18.709	95.483	68.387	
95.483	79.354	96.12	41.290	
146.45	75.483	110.32	100.64	
94.838	42.580	118.06	36.774	
12	78.709	27.096	102.58	
81.290	52.258	61.935	72.903	
96.12	87.741	12	114.19	
87.096	62.580	78.709	76.774	
132.25	70.322	78.709	141.93	
129.67	74.838	40.645	78.709	
76.12	84.516	101.2	80.645	
130.32	94.838	102.58	71.612	
91.612	87.741	77.419	10	
85.806	87.741	83.225	101.93	
111.61	75.483	62.580	2	
112.90	32.903	63.225	130.96	
125.16	41.290	85.161	101.93	
98.709	10	105.80	101.2	
93.548	69.032	66.451	47.096	
116.12	83.225	89.032	118.7	
110.32	69.032	87.096	92.903	
94.838	75.483	74.193	85.161	
91.612	87.741	69.677	81.935	
43.225	132.25	81.935	82.580	
94.838	4	87.096	8	
125.80	79.354	87.741	71.612	
127.74	83.225	95.483	49.677	

Experiment 2

-ve ctrl	2-min	4-min	8-min	+ve ctrl
125.16	101.93	98.709	114.19	4
29.677	129.67	90.322	98.709	105.80
45.161	109.67	89.677	84.516	87.741
133.54	116.77	10	130.32	10
38.709	110.32	67.096	116.12	111.61
125.16	123.87	110.96	78.709	124.51
118.7	118.7	107.09	102.58	12
6	65.161	107.09	67.096	129.03
68.387	54.193	122.58	68.387	98.064
69.677	94.193	92.258	92.903	12
90.322	75.483	110.32	78.064	125.16
30.322	107.74	116.77	83.225	112.90
8	116.77	10	97.419	124.51
90.967	101.2	78.709	91.612	119.35
128.38	131.61	83.225	82.580	129.03
117.41	118.06	126.45	77.419	135.48
89.677	100.64	81.935	91.612	98.709
57.419	85.161	8	69.032	117.41
91.612	125.80	116.12	98.709	143.22
74.193	109.03	74.193	70.967	62.580
17.419	32.258	88.387	83.87	127.09
116.12	62.580	112.25	75.483	123.22
112.90	102.58	77.419	95.483	117.41
123.22	82.580	74.193	98.064	108.38
125.80	63.225	72.258	70.322	125.80
83.225	109.67	132.90	110.96	98.064
89.032	95.483	92.258	94.838	129.03
36.12	108.38	72.258	78.709	105.16
36.12	121.93	100.64	68.387	103.22
105.80	110.32	93.548	69.032	106.45
131.61	90.967	98.709	71.612	118.7
103.87	98.064	76.12	76.12	10
84.516	114.83	102.58	10	113.54
90.322	118.7	85.806	98.709	126.45
85.161	116.12	93.548	124.51	120.64
83.87	97.419	132.90	91.612	109.03
73.548	132.90	85.806	72.903	129.03
110.32	109.03	120.64	67.096	123.87
103.22	81.935	121.93	114.19	96.774
108.38	64.516	128.38	105.16	10
129.03	123.87	90.322	56.774	39.354
123.22	105.80	120.64	114.83	87.741
100.64	118.06	137.41	79.354	130.96
107.74	112.90	12	109.03	140.64
130.32	105.16	100.64	87.096	98.064
90.322	130.96	101.93	95.483	121.93
43.225	127.74	127.74	58.064	127.74
12	50.322	104.51	60.645	127.74
69.032	91.612	112.25	52.258	123.22
88.387	54.193	82.580	80.645	92.258