THE DEVELOPMENT AND APPLICATIONS OF A NEW LIQUID HANDLING DEVICE

By

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ABSTRACT

This thesis describes the development of a new device for carrying out liquid chemical reactions on a very small scale, and its application to a number of problems in the biological sciences including clinical biochemistry, cell manipulation and crystallography. The device operates by containing all reactants as droplets in an inert immiscible liquid, all within a system of conduits. By moving the immiscible liquid, droplets can be dispersed, subdivided, stored or coalesced. The thesis includes a description of the device, details of the evolution of the design, and reports of experiments to test its performance.

The requirements for two types of detector, bioluminescent and photometric are discussed, and preliminary experiments were performed to demonstrate their feasibility. There follows an investigation of the application of the device to clinical chemistry, especially paediatric testing. Two examples of chemical procedures to measure glucose were executed, dispensing reactants both with the device, and by other means.

In order to investigate the utility of the device for handling live cells, it was demonstrated that the device could be sterilised, and operated aseptically. This opens up applications in cell fusion, and transformation in molecular biology. Preliminary experiments showed that plant cells were clearly visible within the device, and droplets containing single cells could be coalesced. It was also shown that the activity of two restriction enzymes was not significantly reduced in droplets in silicone carrier liquid. Experiments were also included to show the degree of contamination of the walls when droplets containing bacteria are moved and stored in the conduits. Moderate contamination was observed.

Finally, a pilot study was conducted to investigate the utility of the device for identifying the appropriate conditions for producing crystals of newly purified proteins. This showed great promise and photographs of two proteins are shown, for which the appropriate conditions were already known.

Further development of the device is also discussed in each of these areas.

CONTENTS

LIST OF TABLES

LIST OF ILLUSTRATIONS AND GRAPHS.

List of Plates

Glossary and Abbreviations.

ABTS (r)	2,2´-Azinobis(3-ethylbenzthiazoline) sulphonate
FMN	Flavin mononucleotide
NAD	Nicotinamide-adenine dinucleotide
NADH	Nicotinamide-adenine dinucleotide, reduced
"The device"	The Droplet Reactor

ACKNOWLEDGEMENTS

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I must firstly express my deepest gratitude to my supervisors Professor Brian S.Hartley and Dr. David Leak. I would like to thank Professor Hartley for making it possible for me to work here, for his advice and many welcome suggestions for applications, and for his energy in arranging funding. I would like to thank Dr. Leak for his knowledgeable advice on biochemical issues, his valuable suggestions for experiments, his constant assistance in administrative matters, and for excusing me from the laboratory washing-up rota.

Mrs. Sarah Jones and Mr. David Manuel provided the reagents and the protocols on which the luminescent assays were based. I am also grateful to Dr. Tony Cass for his advice on questions of physical chemistry and other issues. Special thanks are also due to Professor D. Blow and Dr. Dennis Maeder for making the application to crystallography a reality.

Mr. Maurice Pimm of the Physics Students' Workshop slowly taught me the fundamentals of metal-work, in which Mr. Bernard Keene of the Biochemistry Workshop also provided much needed advice.

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I would also like to thank my collaborators outside the college; Dr. Jan White of the University of Sheffield for coming to the Centre to collaborate in performing an initial investigation of applications of the device to Crystallography, and Dr. Mike Jones of Rothamstead Experimental Station for helping to investigate plant cell fusion with the device.

I would also like to thank Mahmoud Mahmoudian for being such a good-natured and tolerant bench-mate.

Finally I would like to thank Dr. Ernest Foulkes and all at the Foulkes Foundation for making this project feasible financially.

CHAPTER 1. INTRODUCTION.

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1.1. HISTORY OF THE PROJECT.

While working in the molecular Biology Department at Edinburgh University in 1981 I realised that there was a need for a device that could accurately and conveniently handle small volumes of chemical reactants; in many experiments one uses larger volumes than are necessary in order to have enough to place in a test-tube or to fill a pipette. As a solution to this problem I invented the idea of dispensing and coalescing droplets of reactant in an immiscible liquid, within a conduit system. Besides operating on a smaller scale than had previously been convenient, this system also had the advantages of ease of automation and efficient containment.

I tried to licence the idea to instrument manufacturers and visited a number of companies in Britain and the USA, but received no more than verbal encouragement. After this I looked for a laboratory where I could develop the device myself, which I eventually found when Professor Hartley offered a place at the Centre For Biotechnology. This thesis represents two years work investigating applications of the device in clinical chemistry, crystallography, microbiology and cell manipulation.

1.2. SUMMARY OF THE METHOD.

Figure 1 a-d and plates 1 a-d on page 19 illustrate the principle of the invention. If aqueous reactants are to be used, hydrocarbon, organic fluorocarbon or silicone oil can be used as a carrier liquid. Fluorocarbon liquid has previously been used in certain continuous-flow analysers as a separating and encapsulating fluid (Cassaday 1985).

The manipulation of reactant droplets takes place in a branched conduit, the "reaction conduit". This is initially filled with carrier liquid. This reaction conduit is connected via constrictions, such as narrow steel tubing, to reservoirs for reactants. One of the reservoirs is usually loaded with a sample, while one or more others contain the appropriate reagents.

First a predetermined volume of sample is dispensed from its reservoir (figure 1a.) This volume is now separated from the reservoir by moving the immiscible liquid, to form a droplet (figure 1b) which is moved to a position near a coalescing station (where the conduit branches.) A droplet of reagent from another reservoir is produced by a similar sequence, and moved into the coalescing station (figure 1c), where it is constrained by a narrowing of the conduit. The sample droplet is now moved by a flow of the immiscible liquid into the coalescing station. This causes the droplets to coalesce, forming one larger droplet (figure 1d), referred to in this thesis as the "resultant"

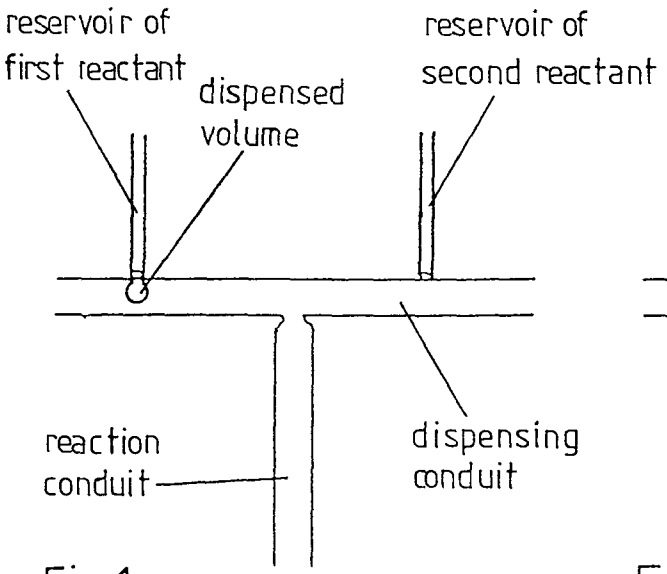


Fig.1a

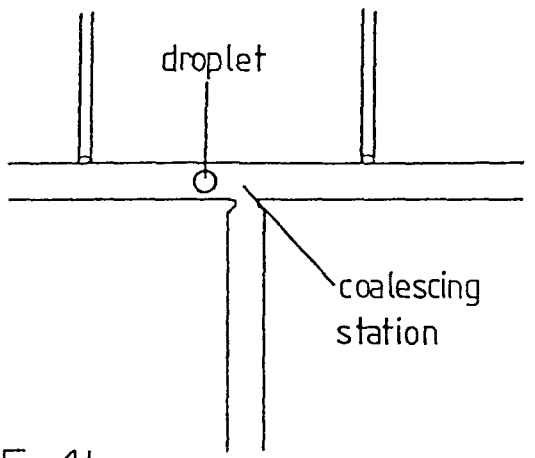


Fig.1b

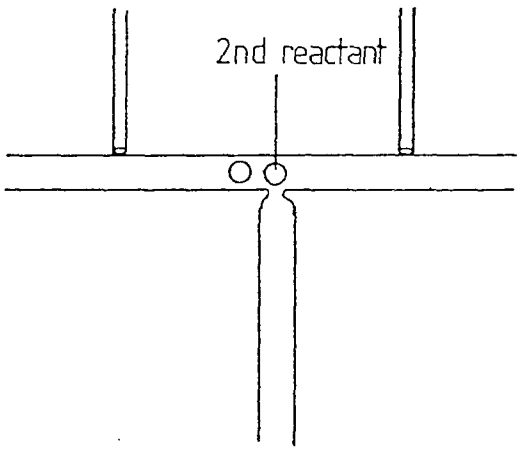


Fig.1c

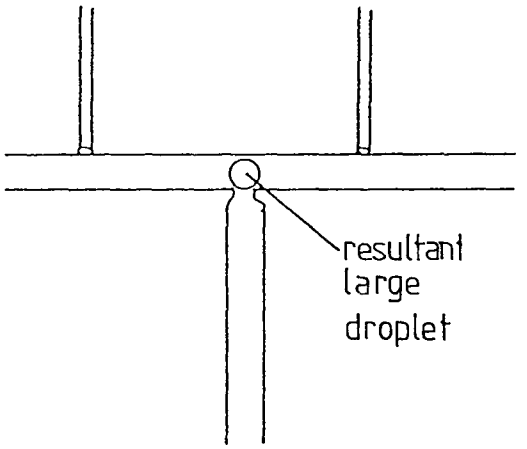


Fig.1d

Figure 1A-D: The method of the invention.

droplet. The resultant droplet may now be analysed by some device attached to the reaction conduit, or it may be passed out of the block assembly to be utilised or analysed by some other means.

Plates 2a-d show the same sequence of events.

In order to made the droplets visible, a dye was incorporated in the reactants and samples. The dye dextran blue was used for this purpose in all of the experiments reported, except for those involving bioluminescence, in which the appropriate computer routine was devised using solutions containing dextran blue. After this the dye solutions were replaced by clear reactant solutions, and the same routine repeated. In the final version of the device, Embodiment III, a dye, Azulene, was incorporated into the carrier solution, and clear reactant solutions were used in all cases, as described in chapter 3.

A British patent for the invention was granted during the course of the project and it is given in appendix 1 (Shaw Stewart 1982).

1.3. ADVANTAGES.

This method has the following advantages:

1. Small scale of operation.

No minimum has yet been found for the size of droplets that can be coalesced in this way. If a droplet is visible or can be

detected then it can be coalesced and its contents can be reacted with another droplet. No difficulty was found in coalescing droplets of 10 nl., and the chief limitation for small-scale applications lies in the detection or use of the reaction products.

2. Ease of automation.

The method uses only components for moving liquids, and can be automated using pistons, pumps and valves, with a suitable means of control such as a computer. Thus a minimum of mechanical components and conventional robotics are needed.

3. Efficient containment.

The reactants are all contained in a sealed system before and after combination. This has the effects of (1) reducing the risk of contamination of the laboratory and worker by noxious samples, and (2) reducing the risk of contamination of the sample by the laboratory environment. (1) would be useful in analysing pathogens such as HILV and hepatitis B, or handling radioisotopes, while (2) may be useful for handling very sensitive or valuable samples, such as RNA.

4. Gravity Independence.

This technique is unusual in that it is as easy to perform in a microgravity environment as it is on Earth. This is useful at a

time when there is increasing emphasis on unmanned automatic systems for analysis in space. It should be noted that one widely-used class of clinical analysers, the centrifugal analysers (figure 7), was invented for space operation.

1.4. DESCRIPTION OF THE APPARATUS.

1.4.a. Block Assembly.

A novel method of construction was utilised to build the conduits for use in the method described in the previous section. This involved forming indentations of the appropriate configuration in the surface of a PTFE block, which was clamped against a transparent plate. The conduit formed in this way was connected to reservoirs of reactant and carrier, and to an outlet, using PTFE and steel tubing. This construction is illustrated in figure 2, which shows an exploded view of Embodiment I of the invention. Plate 2 also shows Embodiment I.

This system allows any reasonable number of connections to be made, and allows the configuration and cross section of the conduits to be easily varied by manufacturing new blocks. (Some examples of blocks with different configurations are shown in plate 4, page 151.) Three different embodiments were used in this project, all of which used a motorised syringe unit to move both the carrier liquid and the reactants. All of the engineering was designed and built in house, except for the version of the motorised syringe used in Embodiment III, which was designed in house and manufactured by a commercial engineer using engineering drawings provided.

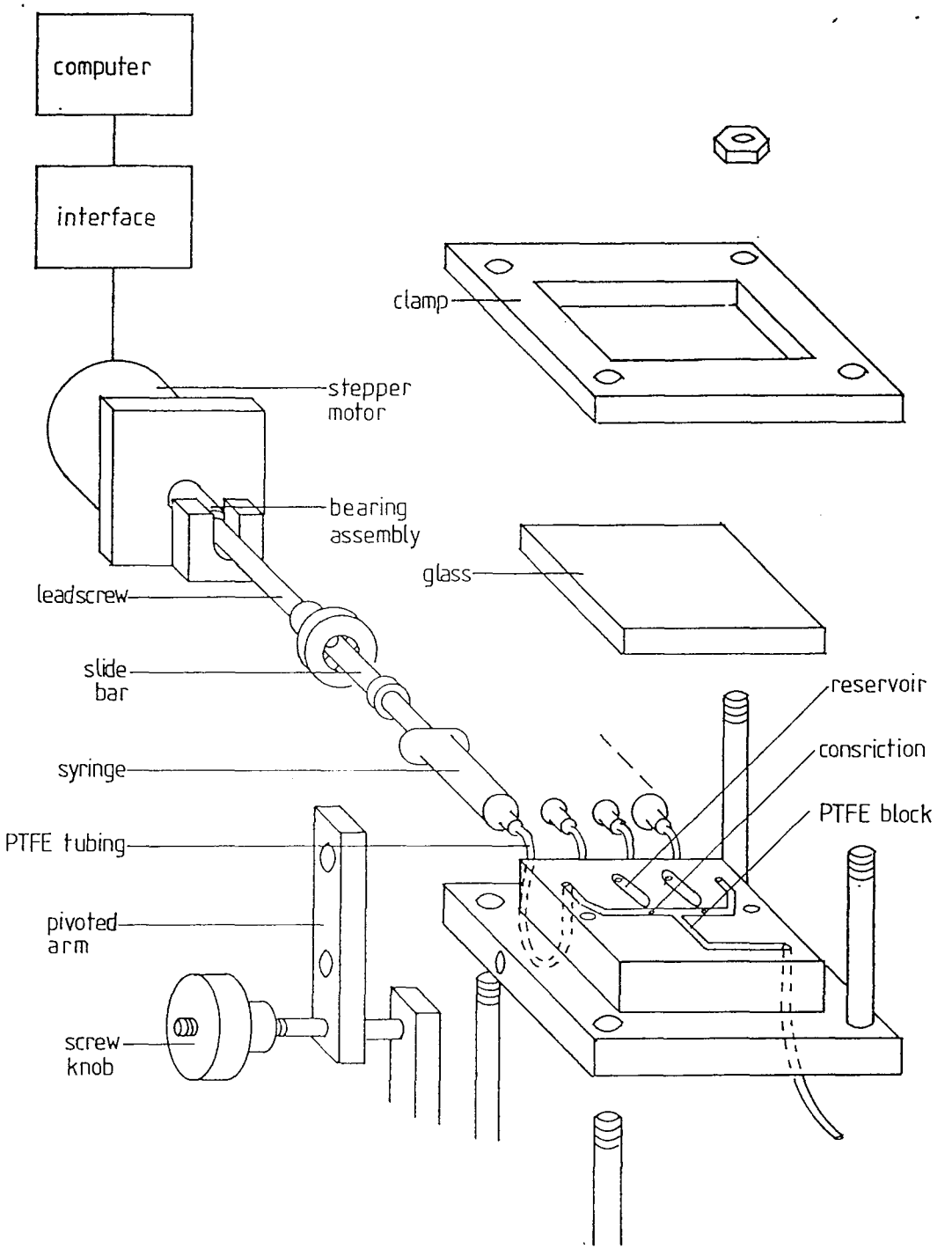


Figure 2: Exploded view of Embodiment I.

The apparatus will now be described in more detail with reference to figure 2, which shows an exploded view of the device as it was at the start of the project, referred to as Embodiment I. Subsequent embodiments are described in chapter 3. Indentations are shown on the surface of a PTFE block, which was clamped against a sheet of glass by tightening bolts. The conduits thus formed were connected by PTFE tubing to two of the motorised syringes, which were used to move the carrier liquid. Larger indentations were machined to form a pair of reservoirs to contain reactants within the block prior to dispensation. These reservoirs were each connected to the main conduit via a constriction, and to motorised syringes via PTFE tubing. The syringes were moved automatically by leadscrews which were turned by stepper motors. These stepper motors were controlled by a computer, using a laboratory-made electronic interface.

Carrier liquid could be passed through the PTFE tubing into the reservoirs to displace a predetermined volume of each reactant into the main conduits. The reactants were loaded into the reservoirs by means of a syringe needle (not shown in fig 2) which also acts as a valve as described in the next chapter. The outlet end of the main conduit was connected to a piece of PTFE tubing (which I refer to as the "outlet tubing" in this thesis) which could be connected to a container for waste. It could also be used to introduce samples manually. In any event the end of the outlet tubing was open to atmospheric pressure.

In Embodiment I the PTFE tubing connections were made by flanging the end of the tubing and pressing the flanged end against a hole in the block with a screw connector. The whole assembly was pivoted on an arm, the position of which was adjustable using a screw knob. This allowed loading of samples under gravity and removal of air bubbles.

Figure 2 shows a device with two reagent reservoirs, and this is the configuration used in the prototypes described. However it should be appreciated that this number of reservoirs was used for ease of construction, and that it was envisaged that the device would eventually have anything from two to thirty channels to allow a number of tests to be performed on the same sample. The two syringes which contain carrier liquid and are connected directly to the main conduits are referred to in this thesis as the "carrier" syringes, while the syringes which are used to dispense reactants at the dispensing stations are referred to as the "reactant" syringes.

1.4.b. Motorised Syringes.

The reactants and carrier liquid are both moved using motorised syringes. Computer-controlled motorised syringes have been used by other workers where the maximum accuracy is required together with automation (e.g. Stieg and Nieman 1980). Figure 3 and plate 3 (page 150) shows the design of these motorised syringes. They work on the following principle: the plunger of a gas-tight

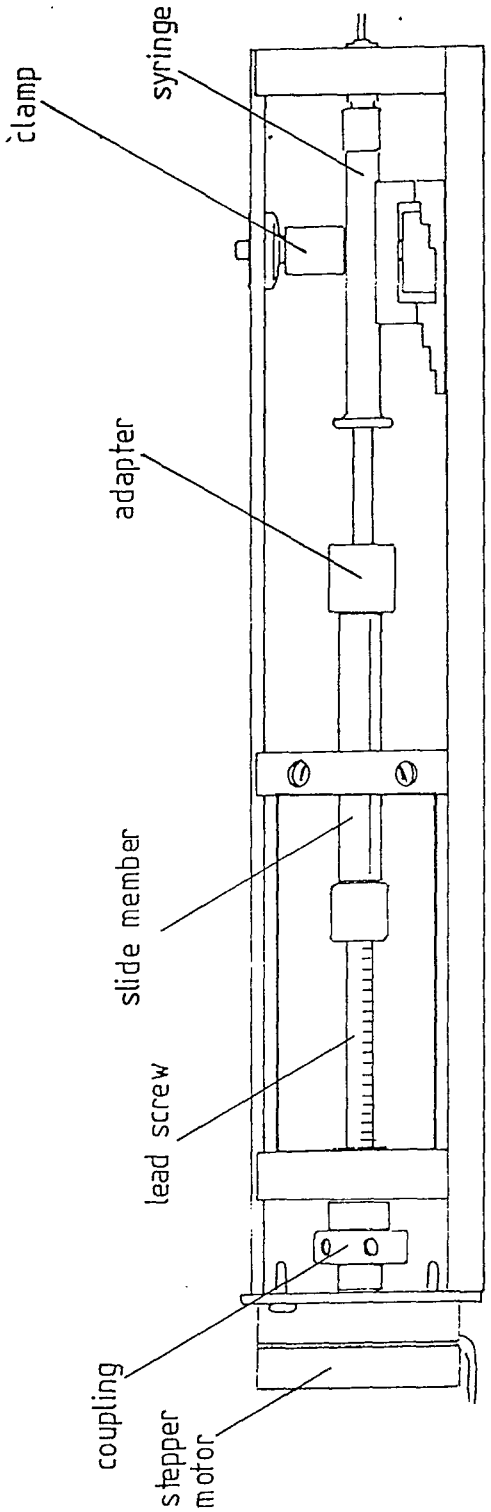


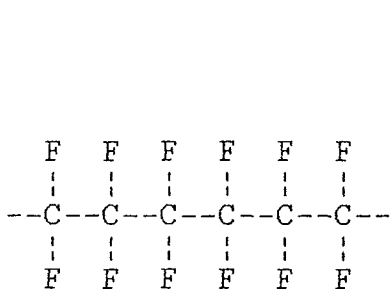
Figure 3: Motorised Syringes.

syringe is moved in by a lead-screw, the shaft of which is turned by a stepper motor. The female thread is attached to a slide member which is hollow to allow the leadsrew to "telescope" up inside it when the syringe plunger is withdrawn. This slide member also prevents the female thread and the plunger from rotating. The stepper motor is activated and controlled by the CBM computer, via an interface device, which uses four SAA1027 stepper motor driver integrated circuits.

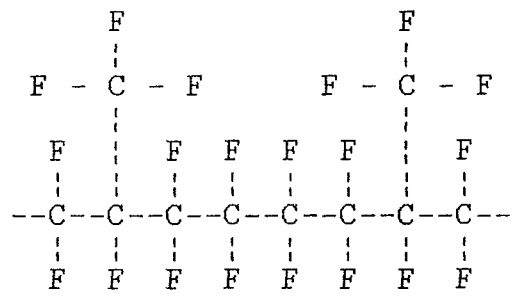
1.4.c. Materials.

It is essential that all the components that are exposed to the liquids used in the device should have strongly hydrophobic surfaces, to encourage wetting by the carrier, and to prevent the aqueous droplets from sticking. Although other materials such as polypropylene were tried, the only materials that were found that prevented droplets from breaking up under some circumstances were the solid fluorocarbons, including PTFE and FEP which were finally used. PTFE is highly hydrophobic, having a contact angle with water of 108 (Fox 1950). PTFE was used to form the block on which the reaction channels were formed. It is also highly resistant to chemical attack (Du Pont, data sheet - "Teflon in chemical services"). FEP has broadly similar properties (Du Pont, data sheet - "FEP"), and this material was used was used to form the top window of the device since it is transparent. A problem with both these materials is that they are very good electrical insulators, which in this application means that the conduits and

tubing tend to become charged by "static" electrical charge (Imperial Chemical Industries 1978). Because the liquids are not equally charged they tend to become attracted or repelled from particular areas of the conduits, sometimes to the point were it is impossible to move a droplet past a particular position. To avoid this effect it is necessary to carefully discharge the components by immersing in water before assembly. PTFE and FEP have the following chemical structures:



PTFE



FEP

1.5. DETECTORS.

Two types of detectors were developed for use with the device: those based on the absorbtion (colorimetry) and the emission (bioluminescence) of light. Absorbtion detectors are relatively simple and inexpensive to build, but are insensitive in comparison to other systems. They also present special problems for the droplet reactor system, because the light has to pass both into and out of the droplet in a regular and repeatable way. In particular, the immiscible liquid must not refract or scatter

the light. However, absorbance methods have the advantage that the large number of chemical methods are already available for this technology. In the past these have mainly been relatively insensitive analyses, measuring blood components and other analytes with concentrations in the milli-molar region. However, enzyme linked immunoassays and enzyme amplification methods have recently been developed which are able to detect micro-molar or lower concentrations of hormones, tumour markers and drugs (Stanley et al. 1985).

1.6. COMPETITIVE TECHNOLOGY.

There are many types of automatic laboratory analysers but the main principles are illustrated by the four main types in use in clinical laboratories; segmented flow, flow injection, centrifugal and discrete analysers. The segmented flow method (Kessler (patent) 1962) was widely adopted in the 1960s and 1970s for performing clinical and other analyses. This allowed a far greater number of clinical tests to be performed. However, the method had several disadvantages (Stockwell 1978): it required sample volumes of at least a 1 ml. for each test to be performed. It sometimes suffered from "carry over", that is, samples were sometimes contaminated by other samples that had just passed through the device. And, most seriously, the method used relatively large amounts of reagents. This feature, combined with a lack of flexibility in the tests performed for each sample, led researchers to investigate other automatic and continuous flow methods.

The first important replacement of this method was Flow Injection Analysis (Hansen (patent) 1977), (Ranger 1981). The most important advantages of this method are its high sampling rate, lower sample volumes and a nearly instant analytical read-out (Ruzicka 1980). It is not, however, easily adapted to perform many different tests simultaneously. This is also a problem for centrifugal analysers, (Stockwell 1978) which unfortunately require a complex dispensing unit to fill the individual rotors.

Discrete methods retain the sample as an entity (e.g. Uffenheimer (patent) 1981), and the appropriate tests can be chosen individually for each sample, but these systems are complex and expensive, and more difficult to maintain than continuous flow methods (Stockwell 1978.)

1. Segmented flow (Auto Analysers.) (figure 4)

This technology, illustrated in figure 4, was pioneered by Technicon in the early 1960s (Kessler, 1962). The sample 1 is converted into a flowing stream 2, and air segments are introduced into this stream. This segmenting prevents the mixing of sequential samples. The segmented stream is then merged with a stream of reagent 4, mixed, and incubated by passing through a long coiled conduit 5, and pumped through a flow-through measuring cell 6 and hence to waste. An immiscible liquid is sometimes used as the segmenting fluid.

2. Flow Injection. (figure 5)

Flow injection is similar to segmented flow analysis, except that fine bore tubing is used, so that segmenting fluid is not needed (Hansen and Ruzicka (patent), 1977; Ruzicka and Hansen, 1979). The sample 1 is injected into a stream of reagent 2, carried through a mixing coil 3, and through a flow-through detector cell 4 to waste.

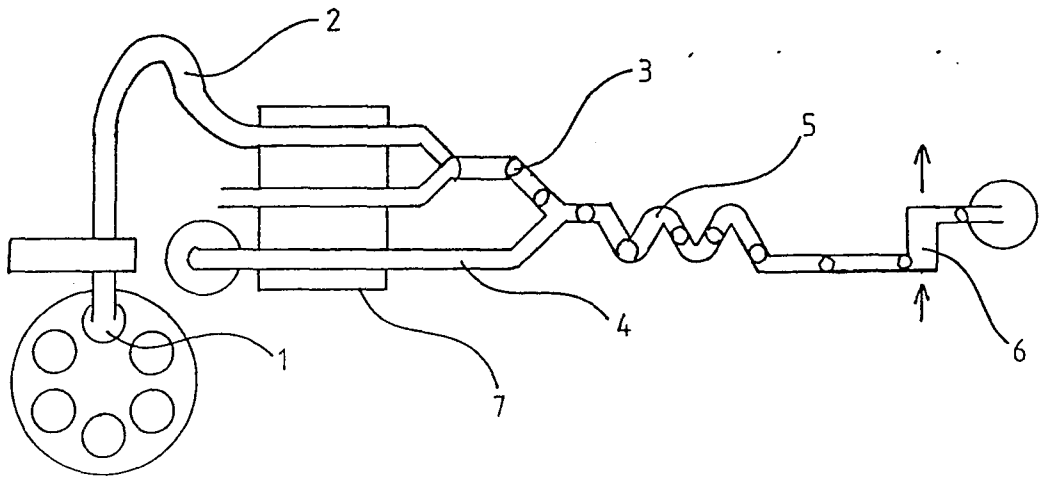


Fig 4

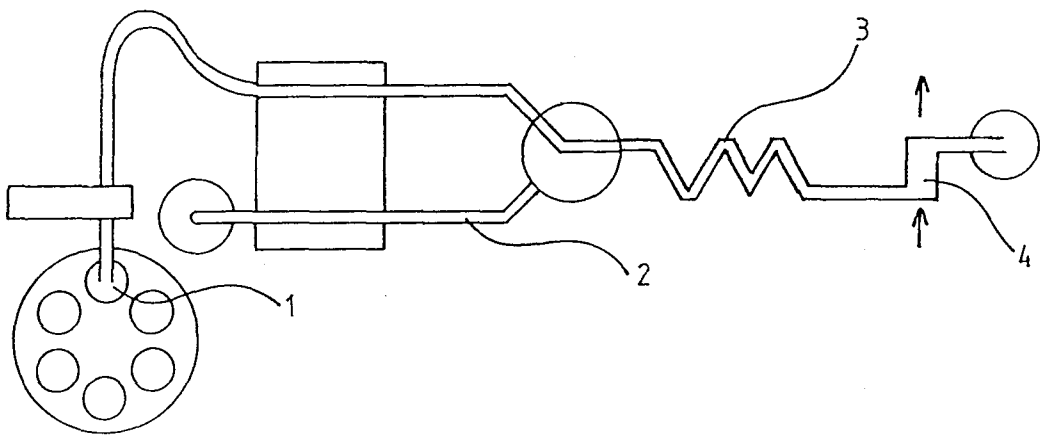


Fig. 5

Figures 4 and 5: Segmented Flow (Auto Analyser), and Flow Injection.

3. Discrete Analysers. (figure 6.)

In discrete analysers each sample 1 is placed in a separate receptacle 2, where dilution, reagent addition and mixing are performed using mechanical means 3 (e.g. Uffenheimer, patent, 1982). Finally each sample is presented to the detector 4. Discrete Analysers are the equivalent of traditional manual methods, and can be extremely flexible, but are often mechanically complex.

4. Centrifugal Analysers. (fig 7.)

It is interesting to note that these analysers (Stockwell 1978) were first developed for space applications. The sample and reagents are dispensed into wells 1a, 1b on a centrifuge rotor 2. When the centrifuge is started, the samples are mixed by centrifugal force and moved into cuvettes 3 on the rotor, which intercept the light beam of a photometer.

In addition to these well-known automatic analysis methods, there are a number of procedures which are competitive with the Droplet Reactor in that they allow the analysis of very small quantities of material. Examples of these procedures are the microfluorometric method used by Outlaw (1980) to measure the starch in individual plant guard cells in droplets in oil drops, using a microscope fluorometer, the high sensitivity automatic protein sequencer developed by Hunkapiller (1984), which performs gas-phase Edman degradation, and can determine the sequence of up

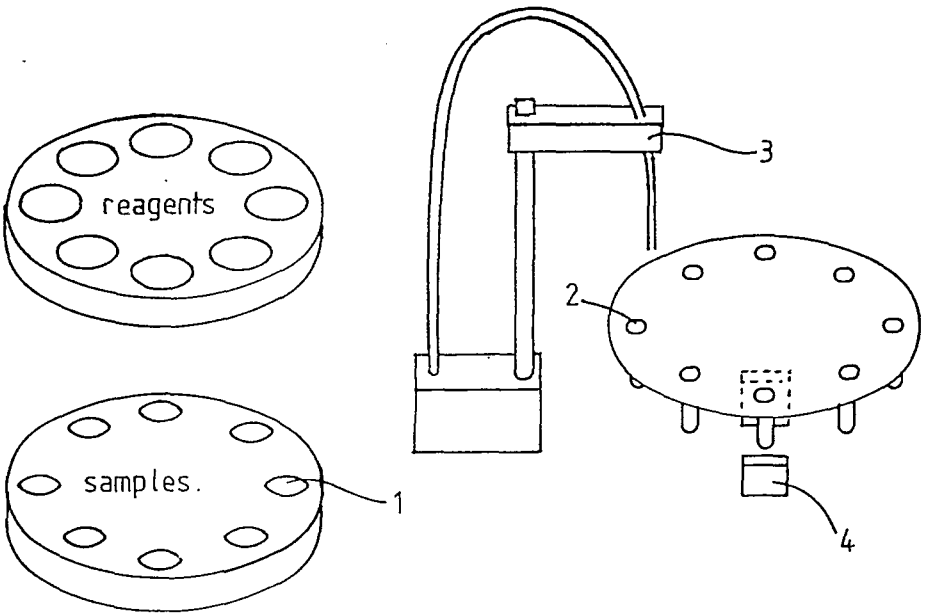


Fig.6

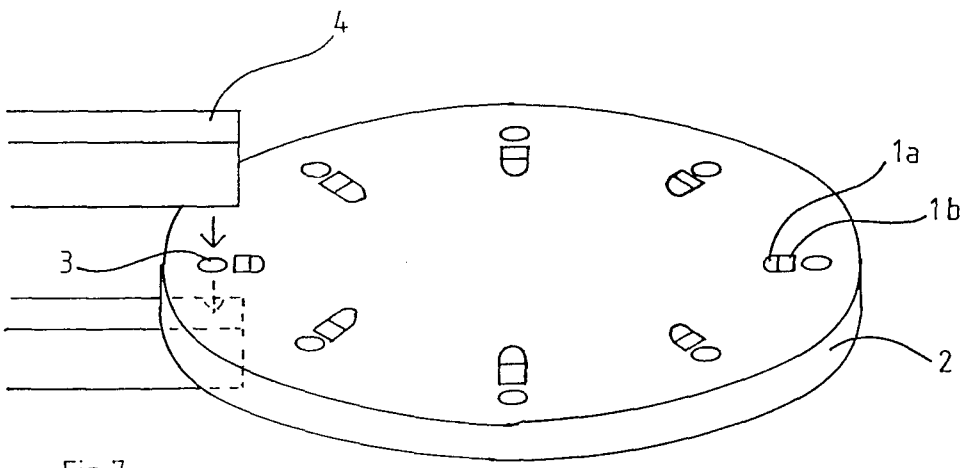


Fig.7

Figures 6 and 7: Discrete Analysers and Centrifugal Analysers.

to 40 residues from a few hundred pmoles of peptides. Bowman (1984) has also developed a system for measuring the electrolytes in nanolitre samples from individual kidney tubules, which uses a flow-through colorimeter with a working volume of 0.3 ul and a path length of 1.1 cm.

1.7. APPLICATIONS INVESTIGATED.

A large number of potential applications for the Droplet Analysers have been suggested including forensic science, process control, low cost cell sorting, toxicity and mutagenicity testing, molecular biology, microbial screening, space research and enzymology. During the work at Imperial College, however, it has been necessary to restrict the project to a few applications, which are clinical biochemistry, cell manipulation, molecular biology and crystallography.

1. Clinical Biochemistry.

The market for automatic biochemical analysers is vast, with larger hospitals performing over a million tests a year, and with world-wide sales of successful products. These devices can cost £50 000 to £250 000 each (Fyffe, 1985), and a device which could perform clinical tests more cheaply (including interest on the capital investment), than those on the market would have enormous commercial potential.

In addition, there is an unsatisfied market for a device which can analyse sample volumes of less than 10 microlitres for paediatric testing. The present device can potentially analyse volumes of less than one microlitre, and is easily automated. The analysis could be based on colorimetry, fluorescence, chemiluminescence or bioluminescence.

2. Cell Manipulation.

It may prove possible to handle a single bacterial cell or bacterial clone in each droplet, without serious contamination of the walls or other droplets. If cells were included in the droplet medium at a low enough concentration, so that on average each droplet contained only one or a few cells, it would be possible to screen for mutants which synthesised or degraded specific compounds. Eventually it may be advantageous, for major automated molecular biology projects, to carry out transformations of "competent" cells, where the restriction digestion and ligation of DNA, and the transformation is carried out in one Droplet Reactor.

Using eukaryotic cells, a device equipped with a pair of electrodes could be used for cell fusion e.g. of green plant protoplasts (Sender, 1979). This apparatus might also be used for the transfer of DNA across the cell walls of plant cells (electroporation).

Each of these applications has potential problems associated with it; bacterial manipulation proved difficult because of contamination of the walls of the conduits by bacteria from the droplets (this problem may not be insuperable). In the case of cell fusion it proved difficult to construct and position electrodes in such a way that they make good electrical contact with a droplet without breaking it up.

3.Crystallography.

One of the most time-consuming procedures in protein crystal preparation for x-ray crystallography is identifying a set of conditions which cause a particular protein to crystallise. This is done by mixing small volumes of the protein with a range of precipitating agents at different concentrations, and with a range of concentrations of certain other additives (Ward, 1986). Such a survey could initially be carried out in the device, with the automatic incrementation of additive concentrations. After dispensing the protein and additives in the device, it is envisaged that some method of scanning droplets rapidly to detect the presence of crystals can be devised.

4. Other Applications.

a. Space research.

As mentioned in section 1.3 , one of the advantages of the droplet reactor is its ability to operate in micro-gravity

conditions. The droplet reactor could thus be applied to automatic analysis in space. This might be useful for physiological testing, for example to determine the effect of space flight on organisms, or for automatic process control such as the maintenance of constant environmental conditions during the culture of cells in space.

b. Fraction collecting.

A stream of liquid from a separating device (such as a chromatographic column) could be converted into a series of droplets, which could optionally be reacted by coalescing with other droplets, and tested or stored.

c. Cell sorting.

It would be possible to convert the device into a low-cost cell sorter. Droplets, (in carrier liquid instead of in air as in conventional cell-sorters) each containing one (or a few) cells, could be passed through a laser detector assembly. This would measure absorbance, scattering, fluorescence, conductivity etc., and valves would be activated to move the droplet into one or more storage conduits according to the result of the analysis. Such a device would probably have a lower throughput than conventional electrostatic cell sorters (because the viscosity of the carrier would prevent such rapid droplet formation), but it would have the advantage that it could easily and cheaply produce small batches of sorted cells.

CHAPTER 2. MATERIALS, EQUIPMENT AND METHODS.

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2.1. SOURCES OF MATERIALS.

Table 1. The Suppliers of the Chemicals Used.

Chemical	Supplier
2,2-Azino-bis(3-ethylbenzthiazoline) sulphonate (ABTS)	Boeringer Mannhiem GmbH.
Agar, Nutrient Agar CM3	Oxoid Ltd., Wade Rd., Basingstoke, Hants RG24 OPW
ATP, disodium salt, grade 2	Sigma Chemical Company Ltd., Fancy Rd., Poole, Dorset, BH17 7NH.
Azulene	Sigma Chemical Company Ltd.
Brilliant Blue R, 65%	Sigma Chemical Company Ltd.
DNA, plasmid pFK::Tn7 5s (c.c.c.)	This laboratory, see below.
DTT	Sigma Chemical Company Ltd.
Decanal, grade 1.	Sigma Chemical Company Ltd.
Ethanol, Absolute Alcohol A.R.	James Burrough plc., Beefeater Ho., Kennington La., London. SE11.
Fluorocarbon liquid, Fluorinert FC-40	Sigma Chemical Company Ltd.
FMN, commercial grade	Sigma Chemical Company Ltd.
Glucose oxidase, Fermcozyme 653AM	Hughes and Hughes Ltd., Elms Industrial Estate, Church Rd. Haroldwood, Romford, RM3 OHR
Glucose-6-phosphate dehydrogenase from bakers'yeast, type IX	Sigma Chemical Company Ltd.
Hexokinase, from yeast, type VI	Sigma Chemical Company Ltd.
Hind III, restriction enzyme	Amersham International plc., West Lion Road, Little Chalfont, Amersham, Bucks, HP7 9NA.

Hydrogen Peroxide, A.R. grade	Fisons Scientific Apparatus, Bishop Mead Road, Loughborough, Liecs, LE11 ORG.
Kanamycin sulphate	Sigma Chemical Company Ltd.
KH PO , SLR grade	Fisons plc.
K HPO , SLR grade	Fisons plc.
Luciferase	This laboratory, see below
Magnesium chloride A.R. grade	Fisons plc.
-NAD, grade II, disodium salt	Boeringer Mannheim GmbH.
-NADH, grade II, disodium salt	Boeringer Mannheim GmbH.
Nutrient Broth CM1	Oxoid Ltd.
Peroxidase RZ 1.0 (for glucose oxidase method)	Hughes and Hughes Ltd.
Peroxidase, grade 1 (for ABTS method)	Boeringer Mannheim GmbH.
Polyoxyethylene sorbitan monolaurate	Sigma Chemical Company.
Propan-2-ol, A.R. grade	Fisons plc.
Reductase (to use with Luciferase)	This laboratory, see below.
Restriction Enzyme Buffer, E8	Amersham International plc.
Silicone liquid, Dow Corning (r) 200/1 cs	Hopkin and Williams., Chadwell Heath, Essex, England
Tetracycline Hydrochloride	Sigma Chemical Company Ltd.
Turpentine substititute	J. H. Woodmans Ltd.

Luciferase and reductase were prepared in this laboratory, by Mrs. Sarah Jones, essentially according to the method of Hastings (1978.)

The plasmid DNA was prepared in this laboratory by Carlos Flores, essentially according to the method of Blair (Blair 1972.)

The bacterial strain J53RP4 of E.Coli was used. This strain has resistance to Ampicillin, Kanamycin and Tetracycline and it was obtained from Datta (1972).

2.2. EQUIPMENT USED.

Table 2. Equipment Used, and the Model and Manufacturer.

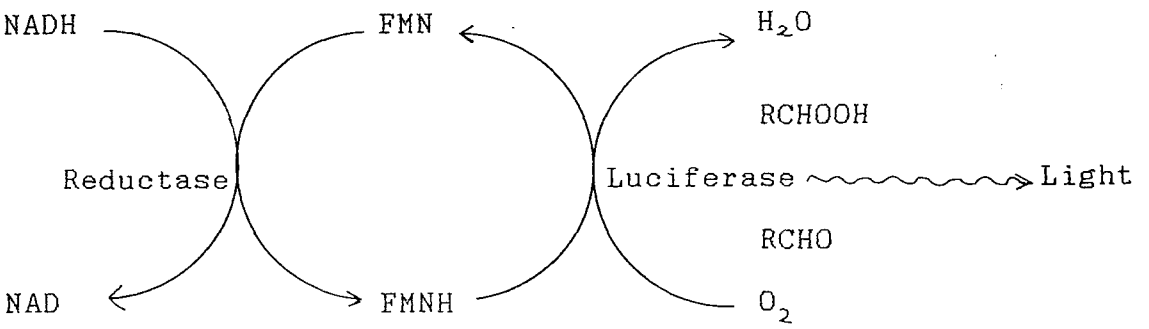
Equipment	Model	Manufacturer
Computer	CBM 3032, cassettes	Commodore.
Cuvettes, disposable, acrylic	4ml.	B.C.L.
Electronic interface	SCO32 IEEE488 - 32 bit	Thorn-EMI.
Light-emitting diode, "sweet spot" visible emitter, (red, approx	625 nm.)	Radio Spares.
Luminometer	1250, with display	LKB Wallac.
Micropipettes	Pipetman, P 20 P 100 P 200 P 1000 F 10	Gilson.
Optic Fibres	1mm polymer core	Radio Spares.
Power Supply	20 A	Henry's Radio.
Stepper motors Control I.C.	ID 35) SAA 1027)	McLennan Servo Supplies Ltd.
Spectrophotometer	Lambda 3	Perkin-Elmer.
Syringes, Gas Tight	5 ml. 1005 TLL 50 ul. 1750 TLL 500 ul. 1705 TLL	Hamilton.
Teflon block	10 mm.	Fluorocarbon Ltd.

2.3. METHODS.

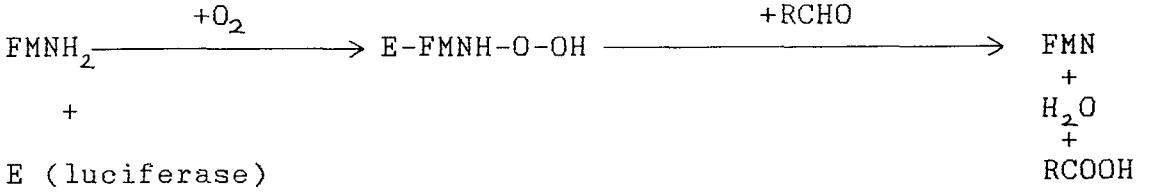
Four distinct assay systems were used in this project. These were assays for NADH, hydrogen peroxide, and two assays for glucose. These four methods used bacterial luciferase, peroxidase and an indicator dye, glucose oxidase and peroxidase, and a bioluminescence method, respectively. In many cases the concentration of the analytes was kept constant and the performance of the of the assays was measured in different conditions likely to be encountered in the Droplet Reactor.

2.3.a. Bioluminescence.

A long-chain aldehyde is oxidised by luciferase in the presence of $FMNH_2$ and O_2 to the corresponding carboxylic acid. This reaction produces light which is measured in a luminometer. The FMN produced is recycled to $FMNH_2$ by a reductase which oxidises NADH.



Via;



Method.

For the standard method these solutions were used;

50 mM. phosphate buffer pH 7 (using the potassium salts.)

0.5 mM. FMN.

0.1 mg/ml. decanal in propan-2-ol.

5 mg/ml luciferase and 2.2 mg/ml reductase mixture.

1 mM NADH.

The FMN, NADH and enzyme mix were made up or diluted in phosphate buffer.

To perform an assay the following ingredients were placed in a cuvette;

870 ul. assay buffer

10 ul. FMN

10 ul. decanal

100 ul. NADH

10 ul. enzyme mix

The enzyme mix was the last ingredient to be added. The ingredients were mixed by inversion and the luminescence measured in the luminometer.

This method was adapted in various ways to make it appropriate for the experiments that were performed. To test the luminometer that was constructed in the laboratory, a range of concentrations of NADH were used (300, 100 and 30 uM.)

To test the sensitivity of the components of this system to silicone carrier liquid, 1/10th strength enzyme mixture and 10 uM NADH were used (see page 93). Full-strength ingredients were used in the experiment to test the solubility of the components in silicone fluid (page 94), as they were in the experiment to test the stability of the luciferase and reductase in three different carriers (see page 97).

2.2.b.Bioluminescence assay for glucose.

The luminescent method was also adapted to measure glucose, by omitting NADH and generating it by adapting the protocol of Lowry (1972). This protocol was designed to assay glucose, with NADH normally being measured spectrophotometrically. The assay works on the principle that the glucose is phosphorylated using hexokinase to produce glucose-6-phosphate, which is converted to glucose-6-P-gluconolactone and NADH by glucose-6-P dehydrogenase:

$$\text{Glucose} + \text{ATP} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP}$$

$$\text{Glucose-6-P} + \text{NAD}^{+} \xrightarrow[\text{dehydrogenase}]{\text{glucose-6-P}} \text{6-P-gluconolactone} + \text{NADH} + \text{H}^{+}$$

These steps were followed:

1. The following solutions were prepared:

Reagent I:

Concentration		Mass in 1 ml.
1 M	$MgCl_2$	95 mg
10 mM	NAD+	6.4 mg
10 mM	ATP	5 mg
0.4 U/ml	glucose-6-phosphate	1.5 ug
0.44 U/ml	hexokinase	4.6 ug

in 50 mM phosphate buffer pH 7.6.

Reagent II:

Concentration
1 M $MgCl_2$
5 uM FMN
0.01 mg/ml decanal
8.8 ug/ml luciferase
7.2 ug/ml reductase

in 50 mM phosphate buffer pH 7.6

2. 50 microlitres each of reagent I and reagent II were placed in a cylindrical cuvette and the luminescence peak (after about 3 minutes) was measured in the luminometer.

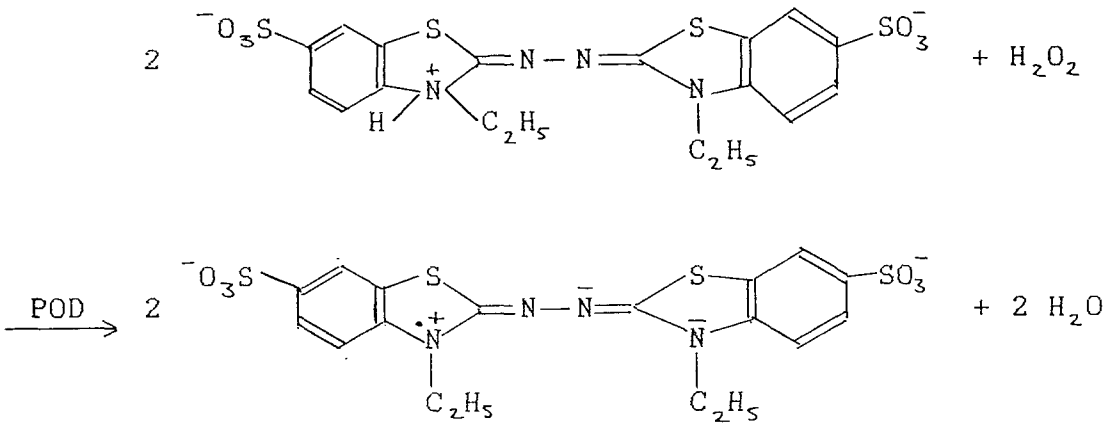
3. To a cuvette were added 50 microlitres of reagent I and 10 microlitres of 5 mM glucose. This mixture was incubated for two minutes at room temperature.

4. 50 microlitres of reagent II were added and the luminescence was measured in the luminometer.

2.2.c. Peroxidase/ABTS assay for hydrogen peroxide.

Principle.

This assay is based on the method of Putter (1983.) Peroxidase can be detected by its ability to dehydrogenate ABTS in the presence of hydrogen peroxide.



A coloured compound is produced, and the absorbance of the solution is measured at 405 nm.

Method.

Reagent; 20 mM ABTS
10 mM hydrogen peroxide

Procedure;

1. 0.5 ml of ABTS/hydrogen peroxide reagent was placed in a clean 1ml disposable cuvette.

2. A 2 ul sample to be tested for the presence of peroxidase was added.

3. The reactants were mixed and incubated at room temperature for 10 minutes.

4. The absorbance at 405 nm was measured against a blank containing only ABTS/hydrogen peroxide solution.

2.2.d. Colorimetric Assay for Glucose.

A standard clinical colorimetric method was also used to assay glucose. This was the method of Trinder (1969), as modified by Pennock (1973). The method was designed for segmented flow analysis. The following reactions occur:

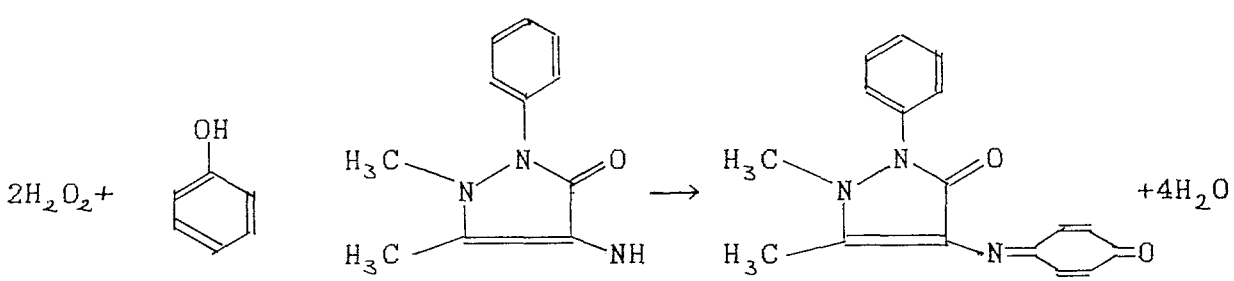
1. Glucose + Oxygen $\xrightarrow[\text{(glucose oxidase)}]{}$ gluconolactone + hydrogen peroxide

$\downarrow$

gluconic acid

2. Hydrogen Peroxide + phenol + 4-aminophenazone $\xrightarrow[\text{(peroxidase)}]{}$

phenazone quinonemine + water



The quinone imine dye produced is purple and is measured photometrically at 505 nm.

Method.

The following solutions were made up:

1. Buffer-enzyme-dye reagent:

Fermcozyme 653AM	15 ml
Peroxidase RZ 1.0	15 mg
4-aminophenazone	300 mg
0.1M phosphate buffer pH7.0	to 1 litre

2. Saline phenol solution:

18% sodium chloride solution	50 ml
1.0M phenol solution (shaken)	9.3 ml
Distilled water	to 1 litre

The following steps comprised the standard method:

1. To 0.1 ml. of glucose standard or sample was added to 3 ml of saline phenol reagent and well mixed.

2. To 1 ml of this mixture 2 mls of buffer-enzyme-dye were added and mixed well.

3. The resulting mixture was incubated at 37°C for 10 minutes, and the absorbance read at 515 nm.

CHAPTER 3. THE EVOLUTION OF THE DESIGN OF THE APPARATUS.

CHAPTER 3. THE EVOLUTION OF THE DESIGN OF THE APPARATUS.

3.1. INTRODUCTION.

A number of improvements were made to the design of the apparatus as the project progressed. The experiments described were performed in three distinct embodiments of the apparatus which are described below, together with the reasons for making each alteration.

3.2. THREE EMBODIMENTS.

Embodiment I.

The first embodiment is shown in figure 8 (and plate 2, page 150). In addition to the indentation that formed the reaction conduit, large reservoirs for the reactants (about 2 ml) were machined into the block. Reactant was dispensed into the reaction conduit, by displacement, by moving carrier into the reservoir through a thin steel tubing inlet. The reactant reservoirs could be reloaded by passing reactant through separate thin steel tubing inlets. The tubing was a tight fit in the block and the ends of these inlets were blocked with epoxy resin with reactant being introduced through a hole in the side of the tubing. The inlet could therefore be sealed by pulling the tubing partially out of the block. Note also that the reservoirs were connected to the reaction conduit by a short length of steel tubing. This prevented carrier from seeping into or out of the reservoir during dispensation.

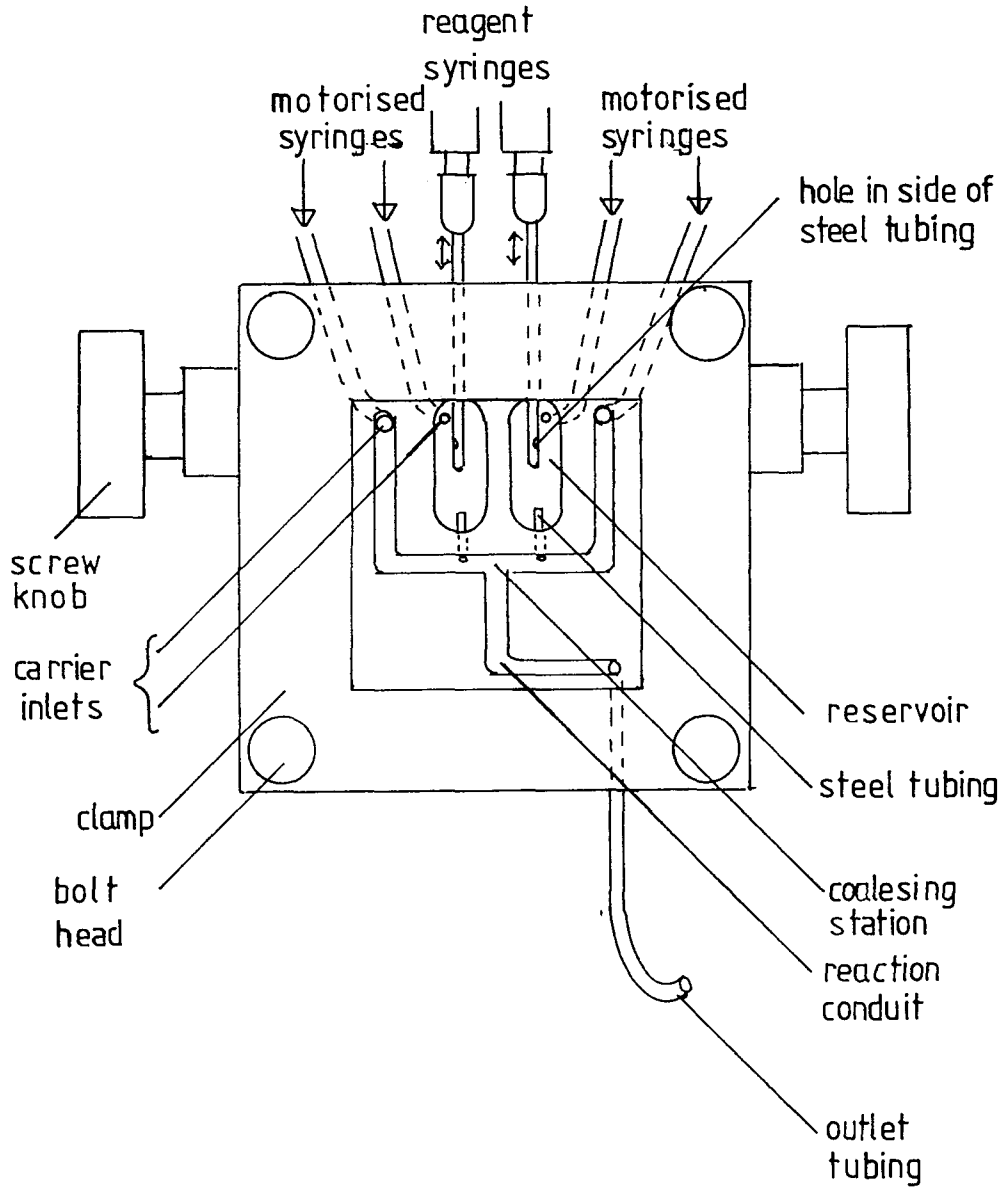


Figure 8: Plan view of Embodiment I.

The advantage of this embodiment was that all of the reactants loaded into the device can be inspected visually. To make it possible to remove air bubbles the whole block assembly was mounted on a pivoting arm so that the angle of the reservoir to the horizontal could be adjusted. During use it was found that the design suffered from the following disadvantages:

1. It was difficult to remove all of the reactant from a reservoir, and difficult to flush and clean the device without opening up the block.

2. Air bubbles became entrapped in the reservoirs.

3. Loading the reservoirs without introducing air bubbles was slow and difficult.

4. The need to adjust the angle of the device during loading and dispensation added unnecessary engineering complexity.

5. There was the possibility that hidden air bubbles were trapped at the connections of the tubing.

6. It was difficult to sterilise the reservoirs.

The arrows indicate the position of the motorised syringes.

Embodiment II.

Figure 9 (and plate 5) illustrates an improved version of the device, which avoided these difficulties. In this embodiment, the inlet needles of the motorised syringes passed directly into the reaction conduit. Thus the reactant syringes and the connecting tubing formed the reservoirs for the reactants. The problems involved in flushing the system and adjusting the angle of the block were thus avoided. However there was a danger of contamination or carry-over due to the dead volumes of the needle connections at the syringe, especially if luer fittings were used. The needles used had rounded tips.

The main problem encountered with this device was in loading the reactant syringes. This could be done either by disconnecting the syringes, filling them, and reconnecting; or by sucking reactant up the outlet tubing into the syringes. The second method was much quicker, but produced a danger of contamination.

Embodiment III.

Figure 10 (and plate 6) shows the device in its final form, Embodiment III. This is the same as Embodiment II, except that more accurate motorised syringes were used, designed in house and made by a commercial engineer. Three way valves were also added, which were designed and built to allow reloading of the reactant syringes.

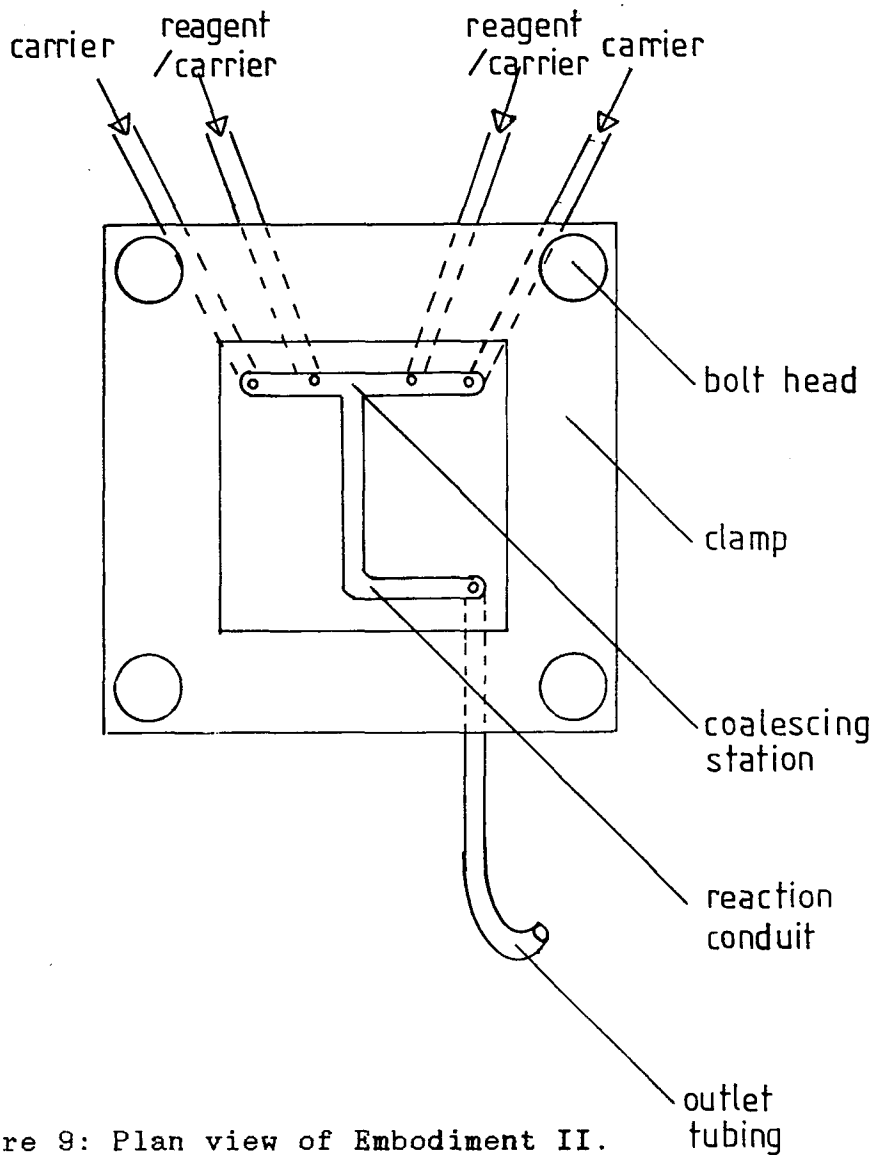


Figure 9: Plan view of Embodiment II.

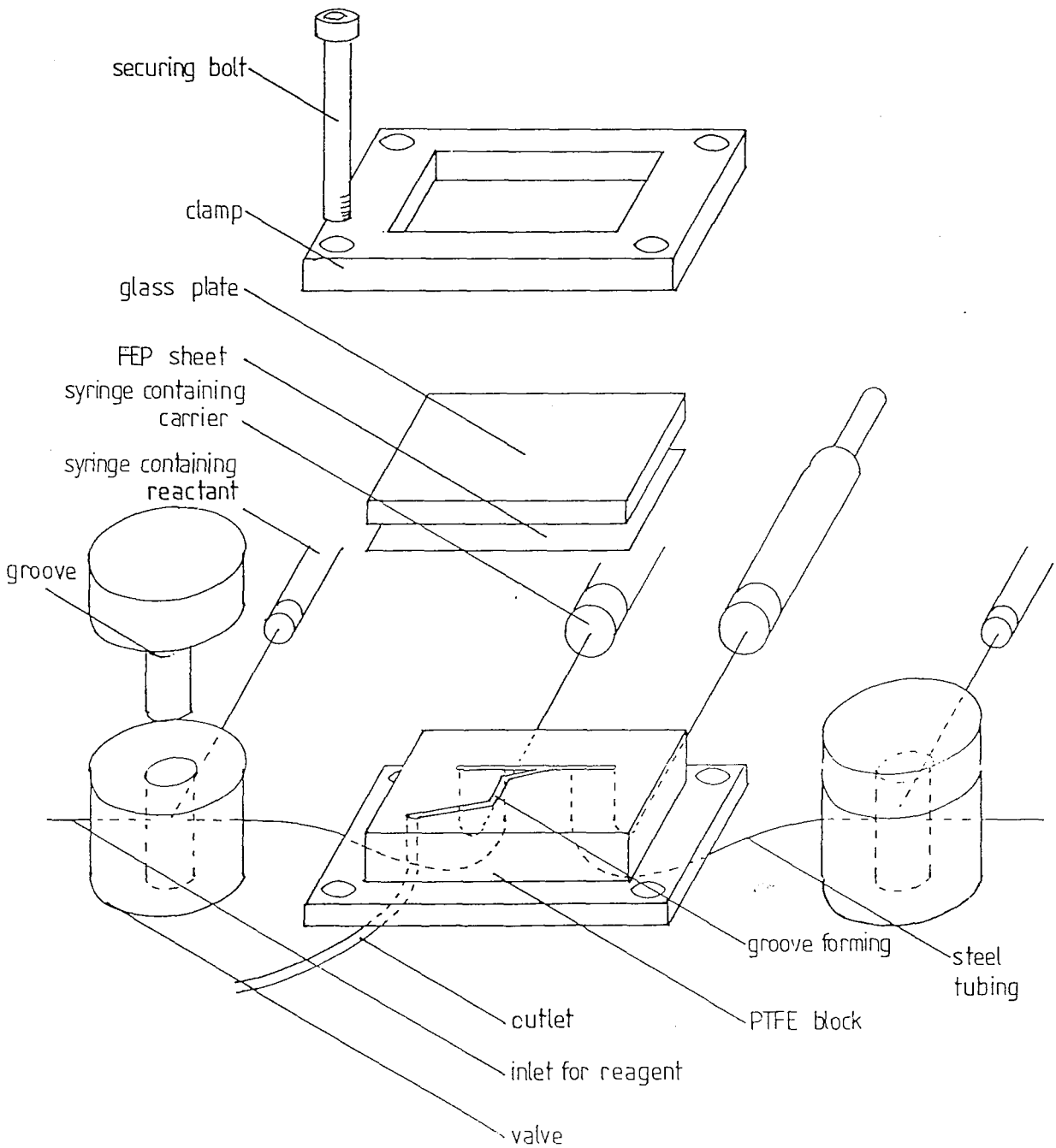


Figure 10: Exploded View of Embodiment III.

Another innovation which was introduced at the same time was to incorporate a dye in the carrier liquid rather than the aqueous reactants. As described in chapter 1, a dye was usually used to make the droplets visible. Usually this was incorporated in the droplets until a computer routine could be devised, after which it was left out. In embodiment III, the droplets were made visible by incorporating the blue dye Azulene in the carrier. The droplets were then less distinct because the outline of the droplets was blurred, but this method has the advantage that the computer routines could be developed without reloading the reactant reservoirs. The dye Azulene was selected in consultation with Dr. Maeder, on the basis of its molecular structure, as a compound that would be soluble in silicone liquid.

Embodiment III was constructed too late to be used in any of the experiments described in this thesis, but it was used to demonstrate that the Droplet Reactor could be easily flushed and loaded with a new set of reagents. It also showed that droplets could be dispensed with greater accuracy. This accuracy of dispensation has not yet been rigorously determined, but preliminary experiments show that the variation in volume of droplets dispensed in this way is less than 5% for 1 ul droplets.

Embodiments II and III have the disadvantage, however, that small volumes of reactants contained in the syringe connections cannot be visually inspected. The potential contamination problems can in general be overcome by using high quality gas-tight syringes with a low dead volume in the connection. Moreover, for small

volumes of reactant the syringes can be partially filled with carrier liquid.

However for applications where a very valuable or noxious sample is to be loaded once only, the design with the reservoir within the block (Embodiment I) is still probably the best.

3.3. TUBING CONNECTIONS.

The design was further improved by changing the method of connecting the PTFE tubing to the block, as shown in figures 11a and 11b. In Embodiment I the tubing was flanged, and held in position with a screw connector to form a standard low-pressure connection, as shown in figure 11a. This had the disadvantages that it sometimes leaked, that it formed an uneven surface where contamination could occur, and that the connection could not be inspected visually when assembled. The junctions were therefore modified in Embodiments II and III so that the tubing was held by a friction connection. Holes were drilled in the block of a slightly smaller diameter than the tubing, and the tubing was forced in, as shown in figure 11b.

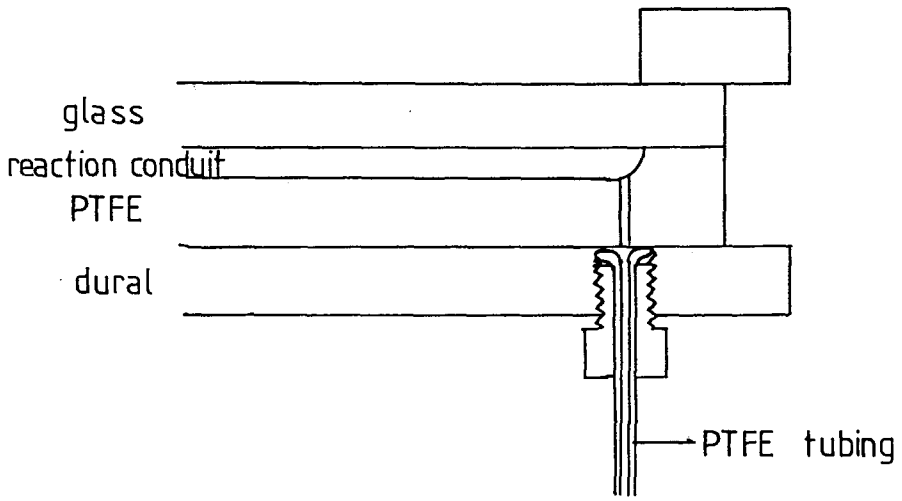


Figure11a Flanged tubing connection.

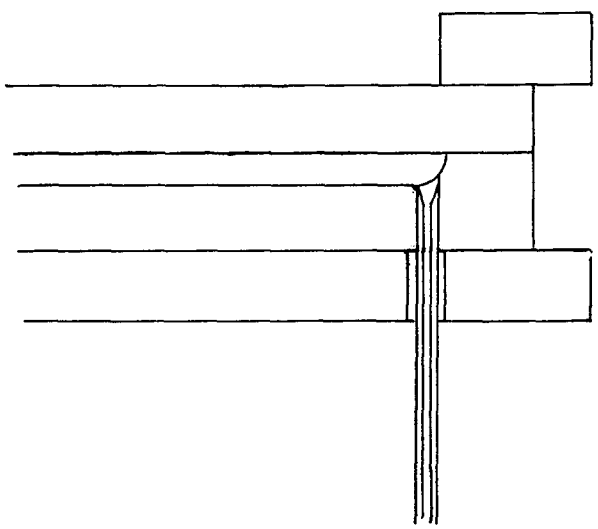


Figure 11b Friction tubing connection

Figures 11A and B: Flanged tubing connector and friction tubing connection.

3.4. DETECTORS.

Two types of detectors were used and tested with the device; a flow cell for photometric analysis, and a photomultiplier for bioluminescence,

3.4.a. Photometric Flow cell.

An absorbtion flow cell was designed and built which would be suitable for measuring the absorbance of droplets. Carrier liquid and aqueous droplets can be passed through this flow cell, and the absorbance of the droplet is measured as it passes through a length of PTFE tubing. The internal diameter of the tubing is small enough to elongate the droplet, so that it spans the distance between the ends of two optic fibres. This arrangement is illustrated in figure 12. One optic fibre was connected to a fluorescent light (later to a light emitting diode) and the end of the other was directed at a segment of paper in the chamber of the LKB luminometer used in the luminescence experiments. The length of the light-path was seven millimetres and the internal volume (ie. minimum sample volume) about five microlitres. The internal diameter of the PTFE tubing was 1 mm.

3.4.b. Method and Results.

This flow cell was tested by passing through solutions of Brilliant Blue dye the absorbance of which had been determined spectrophotometrically at 515nm. The absorbance of the dye solution was first adjusted to 3.20 by adding small volumes of

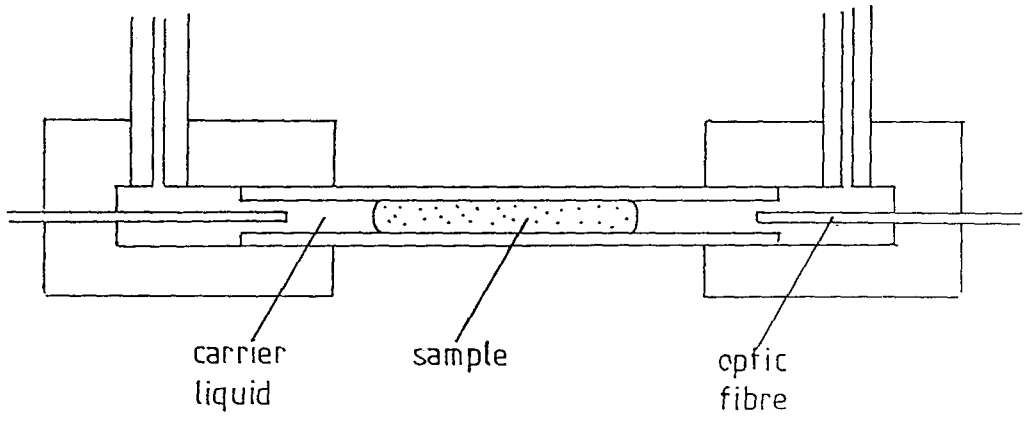


Figure 12: A laboratory-constructed photometric flow cell.

water. This solution was injected into the flow cell by syringe and a reading on the luminometer was recorded. This solution was then diluted to give a series of solutions, the absorbances of which were determined in the spectrophotometer. These solutions were injected in turn into the flow cell and readings were recorded on the luminometer.

Table 3 gives the readings obtained. and log10 (the reading for water / the reading obtained) is plotted in figure 13 against the absorbance measured in the spectrophotometer.

Absorbance	Reading	Log10(reading for water / reading)
3.20	66	0.811
3.00	99	0.635
2.70	143	0.475
2.30	152	0.449
2.00	178	0.380
1.70	196	0.339
1.30	219	0.288
1.00	257	0.220
0.70	280	0.183
0.30	338	0.102
0 water	427	0

Table 3. Comparison of absorbtion readings obtained in a laboratory-made flow-through microphotometer with a commercial spectrophotometer. Light was emitted by a red LED, passed through the sample chamber, and transmitted by another optic fibre to a commercial luminometer which was used as a light meter. Log10 (the reading for distilled water / the reading for the sample) was calculated to give a value corresponding to absorbance.

3.4.c. Interpretation.

The curve deviated from a linear response because (1) some extraneous light was entering the microphotometer without passing through the flow cell, and (2) the luminometer was measuring

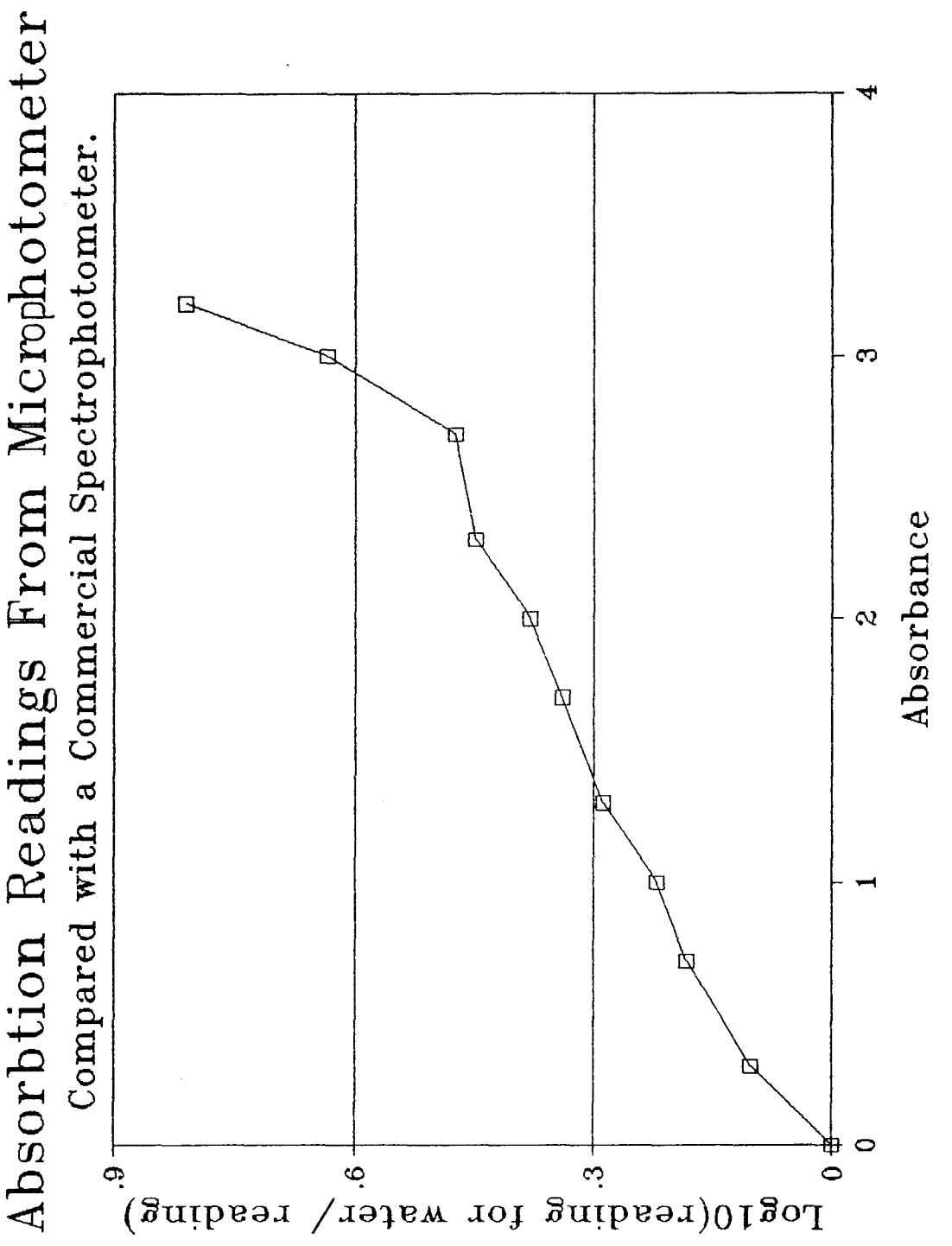


Figure 13. Absorbtion readings from a microphotometer compared with a commercial photometer.

light over a wider band-width than the spectrophotometer. The response was not a logarithmic or hyperbolic type of response, and the central part of the curve was approximately linear. It is not possible at this stage to say how much light was transmitted through the flow-cell after reflecting off its walls. The flow-cell clearly worked, but it needed to be improved, particularly a more constant light source was needed (the L.E.D. and its relatively cheap power supply showed small fluctuations in output.) Also, experiments should be performed to ensure that air bubbles or droplets of oil trapped in the light path or in contact with the optic fibres were not affecting the light transmitted. It is possible that the ends of the optic fibres had small droplets of carrier or sample adhering to them when the liquid in the cell was changed, and these may cause refraction of light.

However, this simple flow cell shows great potential for measuring the absorbtion of droplets. It is easily constructed and could be incorporated directly into the block assembly of the device. By elongating the droplets, a light-path of conventional dimensions is obtained, so that standard chemical procedures can be followed, except that smaller volumes are used.

For very small volume droplets, below 1 microlitre, it will be necessary to elongate droplets still further by using smaller diameter tubing. It is interesting to note that in some instances the tubing can form a highly effective light guide. In particular, silicone liquid, which has a high refractive index,

can be used to carry light to and from a droplet contained in PTFE tubing as shown in figure 14. In this configuration, the droplet would be positioned in the middle of a length of tubing, with carrier liquid on each side of it. It would be important that the volume of droplets can be kept constant, so that the light-path is constant. A number of other materials were considered and Table 4 gives the refractive indices of some common materials. To act as an efficient light-guide the fluid inside the tubing must have a different (preferably higher) refractive index than the tubing. Silicone oil (about 1.45) works well in PTFE tubing (1.3), but unfortunately the refractive index of water (1.33) is close to that of PTFE, so that water in PTFE does not work efficiently. This also suggests that there is a danger that variations in metabolites other than that being measured may influence the light transmitted by altering the degree of internal reflection.

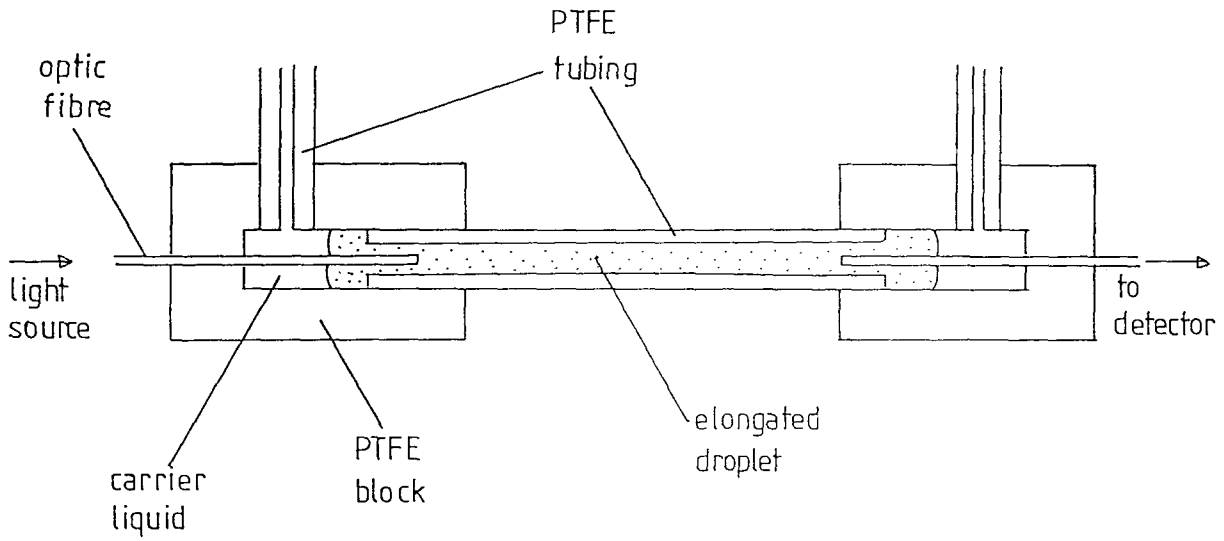


Figure 14, Photometric flow cell where light is conducted to and from sample by carrier acting as a light-pipe.

Material	Refractive index at 589nm.
Fluids;	
Silicone Oil	1.45 Approx
Water	1.33
Saturated sucrose soln.	1.50
Air	1.00
NaCl soln.(density 1.189)	1.37
Solids;	
Polycarbonate	1.60
Cellulose	1.46-1.50
Nylon	1.53
Low density polyethylene	1.50-1.54
Polystyrene	1.59
PTFE	1.30-1.40
PTFCE	1.43
Soda glass	1.51
Flint glass	1.57

Source; Handbook of Chemistry and Physics.

Table 4. The refractive indices of fluids and solids materials which could be used in constructing a flow-through photometer to measure the absorbance of droplets. Combinations of materials that have widely different refractive indices will give rise to reflections at the interface, particularly if the light is passed into a material with lower refractive index. Note that silicone oil has a higher refractive index, and that this fluid forms an effective light-guide in a PTFE (low refractive index) tube.

3.5. Photomultiplier Detector for Bioluminescence.

3.5.a. Introduction.

It was felt that bioluminescence is in many respects the ideal analysis system for this application. It is versatile and highly sensitive. Moreover an accurate reading can in theory be obtained that is almost independent of the exact geometry of the droplet being tested. This independence of geometry is illustrated in figure 15, which shows a cross-section through the block assembly with a photomultiplier mounted over it. As shown, a large proportion of the light emitted is captured by an end-viewing photomultiplier, including light which is reflected off the PTFE block. By contrast, measurements of the absorbance of a droplet by photometry would be affected by light scattering and diffraction as light enters and leaved the droplet. It was therefore decided to build a bioluminescence system which could be used with the device.

This system took over four months to design and construct. A photomultiplier (side-viewed, Hitachi IP21) was mounted directly over the block assembly in a light-tight box. Note that it was also necessary to mask the outlet tubing for about 80 mm of its length to prevent light from travelling up the tubing into the block assembly. The system also comprised a 1000 V power supply, a screened amplifier, and pulse-counting circuitry, which allowed data collection with the same Commodore CBM microcomputer which

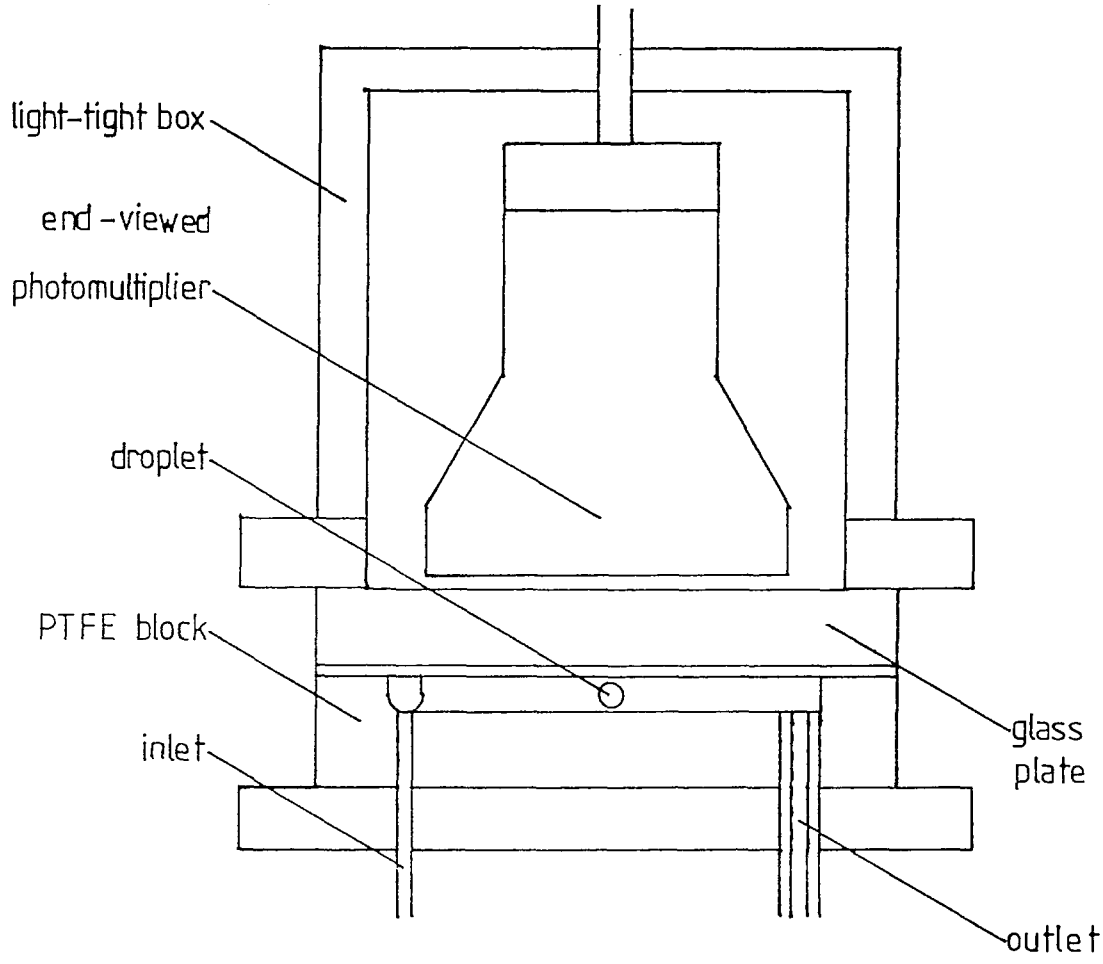


Figure 15: Detection of bioluminescence by a photomultiplier mounted over the block.

was used to control the liquid-handling components. The pulse counting circuitry was provided with a rotary switch so that pulses (which correspond to individual photons) could be integrated over 3 seconds, 300 milliseconds, 30 milliseconds or 3 milliseconds.

3.5.b. Method and Results.

This detector was tested using a small commercial radio-active light source (a "beta-light".) The beta-light was placed on top of the glass plate of the block assembly, just below the opening of the photomultiplier tube, which was contained in a light-tight box. It was found that in order to achieve useful sensitivity, the voltage had to be turned up to a point where a background of 500 counts per second were detected. At this level, there were fluctuations in the count of about 100%, which meant that the photomultiplier could not be used for quantitative work. These fluctuations were probably due to an unstable power supply. At higher voltage levels, (giving around 30,000 counts per second) the signal was more stable. These readings from the beta-light now showed a coefficient of variation of about 30%.

The device was used to measure the light generated by bioluminescence to illustrate its utility. Details of the biochemistry of the luminescence system are given on page 43. A reagent containing FMN (5 mM.), decanal (1 ug/ml), bacterial luciferase (5 ug/ml) and bacterial reductase (2.2 ug/ml) in 50mM phosphate buffer (pH 7) was prepared. Three solutions of different strengths of NADH in buffer were also prepared, 300uM, 100uM and 30uM. 100 microlitres of NADH solution were added to 900 microlitres of the reagent in a cuvette, and it was placed 1 cm below the photomultiplier window. After 30 seconds five three-second readings were taken. Table 5 lists the results, which are plotted in figure 16.

NADH concentration	300 uM	100 uM	30uM
Counts in 30ms	2503	0938	0911
	1523	1428	0766
	1299	1132	0727
	2776	0857	0819
	1667	1283	0913
Mean counts	1954	1128	0777
Coefficient of variation %	30	19	10

Table 5. Photon counts from a bioluminescence system for three concentrations of NADH measured in a laboratory-made photometer. The system is based on bacterial luciferase in which FMN is cycled between its reduced and oxidised forms, and NADH is oxidised. The coefficient of variation is the standard deviation divided by the mean, expressed as a percentage.

3.5.c. Interpretation.

These experiments were performed to show that NADH could in practice be measured with this type of equipment. The three concentrations of NADH were distinguished, but the detector was too imprecise in its present form to perform useful laboratory analysis. It is unclear at present why the coefficient of variation should be less for the lower concentrations of NADH.

There were two changes that could be made to this system to improve its performance. Firstly a more stable power supply could be used as the photomultiplier was highly sensitive to changes in potential. Secondly an electronic mechanism could be

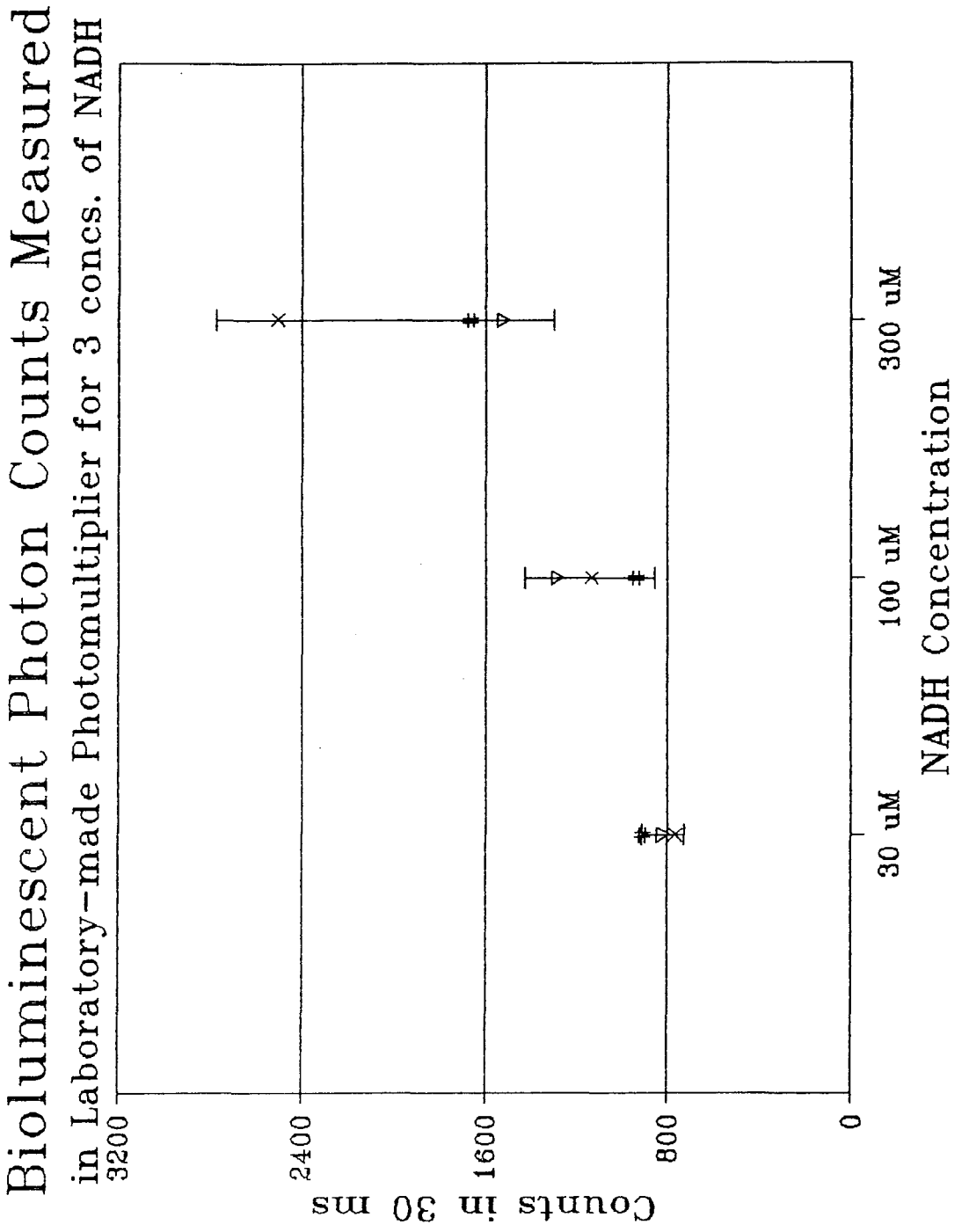


Figure 16: Bioluminescent photon counts measured in a laboratory-made photomultiplier for 3 concentrations of NADH.

installed that would accept only those pulses from the Photomultiplier that were above a certain threshold level and below a certain maximum level. This would help to screen out thermal emissions and cosmic ray pulses.

However, as access to a commercial photomultiplier-based luminometer (made by LKB) became available it was decided not to persist in developing the photomultiplier system. It was not possible to place the photomultiplier tube of this commercial instrument closely over the block assembly, but as it took around 30 secs for the luciferase system to develop its full light emission it was found that it was possible to pass the droplet out of the device into a cuvette containing carrier liquid, and to place this cuvette into the measuring chamber of the luminometer. Experiments of this design are described in the next chapter. Obviously, this system is manual and requires automation. The best system would be the arrangement where the photomultiplier is mounted directly above the block, so that bioluminescence can be detected as soon as the reactants are combined. The earlier developments showed that it was possible to enclose the block and the photomultiplier in an effective light-tight box. The only reason that this arrangement was not used in the experiments that follow is that the laboratory-made photometer system gave unstable readings.

CHAPTER 4. TECHNICAL PERFORMANCE.

CHAPTER 4. TECHNICAL PERFORMANCE.

4.1. INTRODUCTION.

Before proceeding to detailed applications of the apparatus, it was necessary to test the physical performance of the device. The accuracy with which droplets could be dispensed was tested, and the prevalence of contamination of one droplet with another, after which the chemical and physical interaction between certain reactants and carriers was determined by various methods.

4.2. ACCURACY OF DISPENSATION.

4.2.a Introduction.

First the performance was tested of the motorised syringes used to move the liquids in the device, and the accuracy with which the droplets could be dispensed using the block assembly was also tested. The accuracy of movement was tested by two methods; moving liquid down a glass capillary, and colorimetry. The accuracy of dispensation was then tested by the colorimetric method.

4.2.b. Method and Results.

To test the consistency of the movement of the motorised syringes each of the four syringes was in turn connected to a piece of PTFE tubing which was connected to a siliconised 5 microlitre glass capillary by a short length of silicone rubber tubing. The capillary was held against a 15 cm rule. The syringe was loaded

with a solution of the dye Brilliant Blue R, and the syringe plunger was moved until the blue dye reached the start of the glass capillary. The plunger was then moved further a certain number of steps (under the control of the computer) and the distance moved by the front of the blue dye solution was measured and recorded. All of the syringes proved to have a small irregularity which caused up to 15% extra to be dispensed per step at one position in every rotation of the leadscrew (ie. every 48 steps.) This problem was traced to oversized holes in the couplings which joined the motors to the leadscrews. This caused the tips of the leadscrews to rotate off axis. These couplings were therefore replaced with couplings with reamed holes.

After this modification a more regular rotation of the motorised syringes was obtained. The motorised syringes were again tested with dye solution and siliconised glass micropipettes. Using a 500 microlitre syringe, 3.1 microlitre volumes were displaced, using 48 steps for each measurement. This gave readings which were accurate to the limits of resolution of this method ie. about 3-5%. This is a significant achievement for a syringe drive with over 5700 steps per syringe travel. Equivalent commercial devices have 1000 steps per syringe travel (e.g. Hamilton's "Microlab M.", from V.A. Howe and Co. Ltd., 12-14 St. Ann's Crescent, London SW1 2LS, also Gilson's "Diluter 401", from Anachem, Charles St., Luton, Beds. LU2 OEB., and the "T40 Sampler" from Hook and Tucker Instruments Ltd., Vulcan Way, New

Addington, Croydon, CRO 9UG.)

One syringe was now tested using a more accurate method. The syringe was loaded with Brilliant Blue R dye solution, which was dispensed with a length of PTFE tubing, with a disposable micropipette tip on the end, into a cuvette containing exactly 3 ml. of water. This water was dispensed with a syringe and needle to give the greatest repeatability. The dye was mixed with the water in the cuvettes using a small spatula and the absorbtion was measured in the spectrophotometer at 585 nm. Table 6 summarises the readings that were obtained when three series of volumes were dispensed, corresponding to 1 step, 6 steps and 48 steps (a different solution of dye was used in each series.) These motions were expected to dispense volumes of 0.17, 1 and 7.8 ul. respectively. The coefficient of variation is the standard deviation divided by the mean of the results.

Number of steps	1	6	48
Expected Vol. Dispensed, ul	0.17	1	7.8
Total number of readings	20	20	20
Mean Reading, Absorbance at 585nm.	0.849	0.981	1.039
Standard Deviation	0.073	0.019	0.006
Coefficient of Variation	8.6%	1.9%	0.71%

Table 6. The absorbances of solutions of dye dispensed by a motorised syringe, without using the block assembly. Exactly 3 ml of deionised water was dispensed into each plastic cuvette manually and concentrated dye solution (brilliant blue) was added to it with a motorised syringe from a steel needle. (The concentration of the dye solution was different for each column and was not known.) After careful mixing the absorbance was measured at 500 nm.

A similar procedure was also used to test the accuracy of dispensation of droplets within the block assembly, using Embodiment I of the device, in which reservoirs were machined into the block. One of these reservoirs was filled with dye solution, which was dispensed as individual droplets. A precise number of steps was moved by the dye dispensing syringe to dispense the droplet, followed by ten steps by both syringes containing carrier to separate the droplet from the reservoir and move it past the coalescing station. This procedure was repeated automatically, producing a series of droplets in the outlet tubing. These were ejected one at a time with the accompanying carrier, into 2 mls. of water in a cuvette. The mixture was

carefully stirred, without allowing the carrier (which is less dense than water) to come into contact with the optic surfaces of the cuvette. Droplets were dispensed by moving the dispensing syringe 3, 6 and 9 steps. Table 7 summarises the results.

	3 steps	6 steps	9 steps
Mean Reading	0.486	0.932	1.467
Number of readings	20	20	20
Standard deviation	0.035	0.029	0.034
Coefficient of variation	7.2%	3.1%	2.4%

Table 7. The absorbances measured when aqueous droplets of dye, which were dispensed within the block assembly, were mixed with exactly 2 ml. of deionised water. Droplets accompanied by about 20 ul of carrier liquid were added to the water in plastic cuvettes, and stirred together allowing the carrier liquid to remain on the surface of the mixture. The absorbances were measured at 500 nm.

4.2.c. Interpretation.

This degree of accuracy is not sufficient for many important applications such as clinical testing. Although not tested, it is believed that Embodiment II of the device in which hollow needles form the reservoirs for the reactants, together with improvements in the accuracy of motorised syringes, will provide greater accuracy. The planned improvements in the design of the motorised syringes are described in chapter 8.

4.3. CONTAMINATION.

4.3.a. Introduction.

One potential problem envisaged was the transfer of biological and non-biological material from one droplet to another via the walls of the conduits. An experiment was therefore designed to detect the transfer of peroxidase between droplets. The peroxidase was detected by the oxidation of ABTS in the presence of hydrogen peroxide. The method is based on that described by Putter (Putter 1983) and is described in chapter 2.

4.3.b. Method and results.

In this experiment a droplet (6 ul) of peroxidase (2500 U / ml in phosphate buffer pH7) was moved through the main conduits of the device, followed by a droplet (also 6 ul) of phosphate buffer (50mM pH7.0). For this experiment, Embodiment II was of the device was used.

First the device was primed by the following steps;

1. One of the syringes was filled with 50 mM phosphate buffer (pH 7.0), and all air bubbles were removed from the system.

2. The PTFE outlet tubing was placed in strong peroxidase solution (2500 U / ml) in phosphate buffer pH7 (containing Brilliant Blue R dye to make the droplet visible) and 2 ul were aspirated into the outlet tubing.

3. The PTFE outlet was then placed in carrier liquid, and carrier liquid (silicone oil) was aspirated until the enzyme droplet reached the inner end of the main conduit. This site is referred to hereafter as the peroxidase droplet's "resting place".

4. Three droplets of 5 ul each of phosphate buffer were dispensed and passed out of the PTFE outlet to wash out the device.

This priming procedure was performed once only at the start of the experiment.

Four different procedures were each performed three times. For the first three tests the following steps were followed;

1. The enzyme droplet was moved along approximately five centimetres of the conduit, then returned, without stopping, to its resting place. A 2 ul buffer droplet was then dispensed from the reservoir containing buffer, moved to the outlet end of the conduit, and out of the PTFE outlet tubing.

2. This buffer droplet, which may have picked up peroxidase, was dispensed out of the PTFE outlet tubing into a 1 ml. cuvette containing 0.5 ml. of a solution of ABTS (20mM.) and hydrogen peroxide (10mM.,) and the droplet was carefully mixed in with a spatula to prevent silicone carrier from adhering to the optic surfaces of the cuvette, and the mixture was incubated at room

temperature for 6 minutes.

3. The absorbtion at 405 nm. was determined against a blank containing only ABTS and hydrogen peroxide.

Steps 2 and 3 were performed a total of twelve times. For buffer droplets 1 to 3 step 1 above was followed. The following procedures replaced step 1 for subsequent droplets:

Droplets 4 to 6; the peroxidase droplet was moved up and down the main conduit without stopping, as before, but this time the buffer droplet was brought to rest for 1 minute in a part of the main conduit that had been swept by the peroxidase droplet. The buffer droplet was now passed out of the PTFE outlet tubing and tested by steps 2 and 3 above.

Droplets 7 to 9. For the third group of tests the droplet of catalase was halted in the main conduit for 1 minute, then returned to its resting place. The buffer droplet was dispensed and passed out of the device without stopping, and tested using steps 2 and 3 above.

Droplets 10 to 12. For the fourth group of tests the droplet of peroxidase was halted in the main conduit for one minute, and the buffer droplet was halted at the same site for one minute before being passed out of the device and tested using steps 2 and 3 above. The results of these experiments are given in table 8.

	Absorbance at 405 nm.			Mean
Droplets 1-3	0.000	0.004	0.001	0.002
Droplets 4-6	0.011	0.015	0.013	0.013
Droplets 7-9	0.056	0.043	0.072	0.057
Droplets 10-12	0.159	0.093	0.147	0.133

Table 8. An experiment designed to test the degree of transfer of enzyme between successive droplets passing through the block assembly conduits. Droplets of phosphate buffer, which had passed down the same conduit as droplets of peroxidase, were added to to assay tubes containing ABTS and hydrogen peroxide. See the text for the path of the various droplets within the block assembly.

4.3.c. Interpretation

It appeared that little or no enzyme was transferred if droplets are not allowed to remain stationary, as in droplets 1 to 3. If one droplet was allowed to remain stationary, the transfer of material was reduced as long as the other droplet was kept moving all the time. It was not clear at present why there was a difference between holding the buffer droplet stationary (droplets 4-6) and holding the enzyme droplet stationary (droplets 7-9), although this suggested that the rate of transfer was determined by the step of transferring the enzyme from the peroxidase droplet to the walls. When two droplets were held stationary at the same place, however, there was significant contamination as shown by droplets 10 to 12.

The amount of material transferred in droplets 10 to 12 was calculated, using the formula quoted by Putter (1983) as follows;

$$\text{Catalytic activity concentration of droplet} = b = \frac{A \times V \times 1000}{\varepsilon \times d \times t \times v}$$

ε = extinction coefficient = 1.86 l/mmol/mm.

V = assay volume = 2 ml.

v = droplet volume = 0.002 ml.

d = light path length = 10 mm.

t = incubation time = 360 secs.

A = mean absorbtion = 0.133

$$b = \frac{0.133 \times 2 \times 1000}{1.86 \times 10 \times 360 \times 0.002}$$

$$= 19.86 \text{ U/l}$$

Each buffer droplet contains $19.86 \times 2 \times 10 \text{ E } -6$ U

The peroxidase droplet contains $2\,500 \times 2 \times 10 \text{ E } -3$ U

Therefore the proportion of the peroxidase that is transferred

$$= \frac{19.86 \times 2 \times 10 \text{ E } -6}{2\,500 \times 2 \times 10 \text{ E } -3}$$

$$= 8 \times 10 \text{ E } -6$$

ie. about one part in 125 000 of the peroxidase in the droplet is transferred.

This experiment does not give any information about the time dependency of the transfer of material between the droplets. For example, other experiments would be necessary to determine whether significant transfer was still going on after the droplet had been held stationary for ten minutes, or whether most of the transfer had taken place during a much shorter time period. Another experiment which was not done was to determine the cumulative effect of a number of droplets, followed by one buffer droplets.

4.4. RADIOLABEL TRACING.

A similar set of experiments was performed using tritiated phenyl alanine as a label. A droplet of diluted tritiated phenyl alanine was passed through the main conduit, followed by a droplet of buffer, in a similar sequence to the above experiment using catalase. The radiation in the buffer droplet was now tested using a scintillation counter.

The results of this experiment were, however, inconclusive, and some difficulty was experienced in loading and manipulating the droplets without contaminating parts of the device that were not intended. I decided that before performing this experiment I would improve the loading and cleaning facilities of the device.

4.5. THE SENSITIVITY OF ENZYMES AND REAGENTS TO VARIOUS CARRIER LIQUIDS, COLORIMETRIC METHOD.

Another area of uncertainty was the effect of the carrier liquid on the reagents, especially enzymes, used in biochemical assays. I therefore tested two different assay systems by shaking the reagents with three potential carrier liquids. These were silicone oil, fluorocarbon fluid and washed white spirit.

4.5.a. Colorimetric Method.

The sensitivity of glucose oxidase to these carriers was first tested using the clinical assay for glucose of Trinder (1969). The glucose oxidase catalyses the reaction of glucose with molecular oxygen to give hydrogen peroxide and -gluconolactone, which reacts further to give gluconic acid. The hydrogen peroxide reacts with phenol and 4-aminophenazone to produce a coloured compound. Chemical details and the concentrations of reagents are given in Chapter 2. Two reagents are required; Buffer-Enzyme-Dye (BED) reagent which contains glucose oxidase, peroxidase and 4-aminophenazone, and saline phenol reagent which contains sodium chloride, phenol and polyoxyethylene sorbitan monolaurate.

These steps were followed:

1. 20 mls of buffer-enzyme-dye (BED) reagent were placed in each of four large glass test-tubes.

2. 20 mls of silicone carrier were added to the first of these tubes, 20 mls of fluorocarbon liquid to the second, and 20 mls of turpentine substitute to the third.

3. All tubes were thoroughly shaken for 30 seconds and allowed to stand for 30 minutes to separate the phases.

4. 30 mls of saline phenol were placed in a small beaker and one ml of 20 mM glucose solution was added.

5. 2 mls of the aqueous layer from each of the four test-tubes containing BED solution were pipetted into a separate test-tube, and 1 ml of the glucose - saline phenol solution of step 4 was added and mixed by inversion.

6. The contents of each test-tube was transferred to a 3ml. cuvette after 9 minutes, using a pipette to avoid transferring any remaining carrier, and the absorbance at 512 nm. was measured after exactly ten minutes.

Steps 4, 5 and 6 were repeated three times, ie a total of 12 tests were performed. Table 9 gives the results obtained.

	1	2	3	Mean	Std.dev	Coefficient of variation
No carrier	0.903	0.879	0.953	0.912	0.031	3.4%
Silicone Liquid	0.891	0.942	0.872	0.902	0.030	3.3%
Turpentine substitute	1.003	0.930	0.872	0.902	0.030	3.1%
Fluorocarbon	.922	0.862	0.960	0.915	0.040	4.4%

Table 9. A test of the stability of an enzymatic system for glucose measurement in the presence of the three potential carrier liquids, silicone liquid, turpentine substitute and fluorocarbon liquid. 20 mls. of "buffer-enzyme-dye" solution (which contains glucose oxidase, peroxidase and the dye 4-aminophenazone) were shaken with each of the carrier liquids, allowed to separate, and then added to a solution containing glucose and the other reagents of the assay (saline phenol solution.) The first 3 columns of the table show the results of three repetitions of the experiment.

4.5.b. Interpretation.

These results show no significant reduction in the activity of the glucose oxidase when it is exposed to any of these carriers. This suggests that the droplet reactor is suitable for carrying out this and perhaps other enzyme assays. However, before using any other assays in the device, compatibility of the reagents with the carrier to be used should be tested. It would also be advantageous to test for enzyme inactivation in this and other assays using conditions in which the enzyme is not in excess, as in the above experiment; if enzyme concentration was the limiting

factor any inactivation which did take place would be significant.

4.6. SENSITIVITY OF SEPARATE ENZYMES AND REAGENTS TO SILICONE LIQUID, AND THEIR SOLUBILITY IN SILICONE LIQUID, BIOLUMINESCENT METHOD.

4.6.a Introduction.

The bacterial bioluminescence system was chosen to test for sensitivity to carriers because it is a reasonably complex system (it involves two enzymes and two coenzymes) and so is a good test for any interference in enzymatic activity and also because it would be a suitable assay system for a variety of analytes. The sensitivity of all of the components of the system was tested by mixing each component in turn with silicone oil, then adding all the other components. After this the sensitivity of the two enzymes was determined by mixing with three different carriers. Chemical details of the bacterial bioluminescence system are given in chapter 2.

In the assay a long-chain aldehyde is oxidised by luciferase in the presence of $FMNH$ and O to give the corresponding carboxylic acid. The FMN produced is recycled to $FMNH$ by a reductase which reduces NADH. The system can therefore be used to measure the concentration of NADH.

4.6.b. Method, and Results.

The following solutions were prepared or obtained;

a) 50 mM. phosphate buffer pH 7.0

b) 0.5 mM. FMN in phosphate buffer pH 7.0

c) 0.1 mg/ml decanal in isopropanol

d) 0.5 mg/ml luciferase and 0.22 mg/ml reductase mixture

e) 10 uM NADH in phosphate buffer pH 7.0

These steps were then followed;

1. 50 microlitres of the four active components (b-e) were placed in four plastic test-tubes (one component per tube) and 1 ml. of silicone liquid was added to each. These were thoroughly shaken for twenty seconds, then centrifuged to separate the layers.

2. 10 microlitres of the aqueous layer of each of the tubes was placed in a separate plastic cuvette, and 960 microlitres of buffer and 10 microlitres each of the other three complimentary components in each case were added.

3. When the last component had been added the cuvette was inverted once and placed in the luminometer.

Steps 2 and 3 were repeated three times for each ingredient. The results of these tests are summarised in table 10. All of the components showed some loss of activity or concentration, but only the decanal was greatly reduced, presumably because it was dissolved in the silicone carrier. This is investigated further in the next experiment.

Component exposed to silicone.	Mean Luminescence Counts per second	Standard Deviation.
FMN	831	47.5
Decanal	431	28.6
Luciferase/ reductase mix	760	58.6
NADH	793	40.1
None (control)	842	44.5

Table 10. Testing the stability of each of the ingredients of the bacterial luciferase bioluminescence system by exposure to the silicone liquid. 50 ul of FMN, decanal, luciferase/reductase mixture, and NADH were separately shaken with 1 ml. of silicone, and then added to the other components of the system.

This experiment was now repeated except that a sample of the silicone liquid that had been shaken with the missing component was added to and shaken with a mixture of the three complimentary components. (Ie. instead of adding the missing component, a sample of silicone which might contain the missing component in a dissolved form was added.) This procedure was designed to test whether any of the components were soluble and stable in silicone liquid.

These solutions were prepared or obtained;

0.5 mM FMN

0.1 mg/ml decanal in isopropanol

5.1 mg/ml luciferase and 2.17 mg/ml reductase mixture

100 micromolar NADH

The last two ingredients were 10 times as concentrated as in the previous experiment. These steps were then followed;

1. To four tubes was added 500 microlitres of silicone fluid. Into each of these tubes 50 microlitres of a different component was added. These tubes were now vigorously shaken for thirty seconds, and centrifuged for three minutes to separate the silicone fluid.

2. Four small plastic test-tubes were set up, labelled 1 to 4. Into tubes 1 to 3 was placed 10 microlitres of NADH, into tubes 1, 2 and 4 was placed 10 microlitres of enzyme mixture, into tubes 1, 3 and 4 was placed 10 microlitres of decanal, and into tubes 2 to 4, 10 microlitres of FMN. Thus each tube was lacking a different component.

3. The luminescence of these tubes was measured in the luminometer.

4. 400 microlitres of silicone fluid from each of the tubes in step 1 was added to the tube in step 2 that was lacking the component that had been mixed with the silicone. The tubes in step 2 were then shaken for fifteen seconds, and measured immediately in the luminometer.

All experiments were repeated once. The results of the this experiment are summarised in table 11.

Component lacking from the system	Luminescence before shaking with silicone UNITS		Luminescence after shaking with the the appropriate silicone	
FMN	182	205	1 555	2 157
Decanal	545	480	4 800	Over load
Enzyme mix	0	0	0	0
NADH	2 - 0	5 - 0	23 - 0	7 - 0

Table 11. An experiment to measure the solubility of the components of the bioluminescence system in silicone liquid. Each of the components was shaken with the liquid, centrifuged, and the liquid was then mixed with the other components of the system. If any of the components had been soluble, they would have passed into the silicone, and thence into the complete system. Therefore, any increase in the level of light output in this experiment represents a transfer of reactant.

The tubes lacking NADH started at e.g. 2, and fell to 0 after about 30 seconds.

4.7.c. Interpretation

The tubes lacking FMN, decanal and NADH showed some background luminescence. This is typical for bioluminescence systems (Hastings, 1978), and may be due to non-specific side-reactions or contamination of the reactants used.

Obviously no luminescence was possible without the presence of luciferase enzyme, and since the previous experiment showed that it is not denatured by silicone liquid, it was apparently completely insoluble in it.

FMN and decanal were both significantly soluble, because enough of each of them could be transferred via the silicone to cause an increase of luminescence of at least an order of magnitude. In the case of decanal it can be shown that the solution of decanal in isopropanol is completely soluble in silicone by mixing the pure liquid with silicone carrier.

NADH is either insoluble or only slightly soluble, as the luminescence in this case was not clearly higher than the control, and rapidly fell to zero. Other experiments could be designed to determine the partition coefficients of these compounds between water and silicone. For example, the volume of the aqueous phase could made up to the volume of the silicone, and after shaking, the concentration of the component being tested in

4.7. SENSITIVITY OF LUCIFERASE TO VARIOUS CARRIER LIQUIDS.

4.7.a. Method and Results.

A similar experiment was now performed mixing three different carriers with the enzyme mix only, to determine the stability of the two enzymes in turpentine substitute, silicone liquid and fluorocarbon liquid. These steps were followed;

1. A cocktail of all the components except the enzymes was made. This comprised;

    20 microlitres FMN

    20 microlitres decanal

20 microlitres NADH

4 ml. 50 mM. phosphate buffer

2. 200 microlitres of the enzyme mixture was placed in three plastic test-tubes, and to each was added 1 ml. of one of the three carriers. 200 microlitres was also placed in a tube without carrier to act as a control.

3. 400 microlitres of the cocktail was placed in a cuvette, and 10 microlitres of the aqueous layer (containing enzyme) from one of the four tubes containing carrier was added.

4. The cuvette was inverted and the light emitted was measured in the luminometer.

The steps 2 to 4 were repeated four times. Table 12 summarises the results of this experiment.

Carrier shaken with enzymes	Mean light emission count / 3s	Standard deviation
Silicone liquid	578	43
Turpentine substitute	601	41
Fluorocarbon liquid	361	61
No carrier (control)	603	35

Table 12. The stability of the luciferase and reductase enzymes on exposure to a variety of carriers. The enzyme mix was shaken with each of the carriers, centrifuged, then added to the remaining components of the luminescence system, FMN, decanal, and NADH.

4.7.b. Interpretation.

Neither the silicone liquid nor the turpentine substitute significantly reduced the activity of the enzyme, within the limits of experimental error. These results were typical of those obtained when the experiment was repeated a number of times; the luminescence was reduced, but by less than the standard deviations of the readings.

Fluorocarbon liquid on the other hand clearly reduced the luminescence, in this case by nearly one half. Fluorocarbon liquid can dissolve protein in some circumstances, (Allington R.W, 1976) so this may be the explanation, rather than, or as well as, denaturation.

It is perhaps regrettable that plastic test-tubes were used in this series of experiments: there is the possibility that reactive substances may have been leached out of the plastic by the various carrier liquids.

CHAPTER 5. CLINICAL ANALYSIS.

CHAPTER 5. CLINICAL ANALYSIS.

5.1. INTRODUCTION.

Clinical chemistry is a major area of automatic chemistry, and some sectors, such as paediatric testing, are particularly suitable for this instrument because they require the handling of very small volumes.

In paediatrics the requirement for handling small volumes, often less than 10 microlitres, arises from the difficulty of removing blood from a neonatal baby. Blood is usually taken from a heel stab, which only yields about 50 microlitres. Squeezing the stab site to get more blood results in mixing blood with interstitial fluid. This means that at present only about four tests can be performed on each sample, whereas the average number of tests performed on an adult patient is around seven.

Although it would probably take at least five years to develop and test a device for clinical analysis, the potential market is very large. To illustrate, over 600 000 babies are born every year in England and Wales. Most automatic clinical testing is done with segmented flow or discrete analysers, with smaller contributions from flow injection and centrifugal analysers. However, these large analysers are very complex and expensive, and are not specifically designed for paediatric analysis.

5.2. REQUIREMENTS FOR DETECTION AND PRECISION.

By far the most commonly used detection system in clinical chemistry is photometry, where the absorbance of a solution in the infrared, visible, or ultraviolet regions of the spectrum is measured. If the droplet reactor could be adapted to photometry, the greatest range of existing chemical assays would therefore be available.

A high level of precision and repeatability are required for modern clinical analysis. Current analysers claim between-batch and within-batch coefficients of variation of 3 - 5% and 2 - 4% respectively (Fyffe 1985.) A more precise flow cell than that so far developed (the flow cell described in chapter 2) would be required.

Nevertheless it was important to demonstrate that the droplet reactor could actually be used to measure a clinically significant analyte. Two assays for glucose were selected, one based on colorimetry and one based on bioluminescence.

5.3. COLORIMETRIC ASSAY.

The colorimetric system used in chapter 3 to test the sensitivity of reagents to carrier liquids was adapted so that the reagents were dispensed and combined in the droplet reactor (but the absorbance was still measured in the spectrophotometer in the usual way.)

This system, the glucose oxidase / peroxidase method of Trinder (1969) and Pennock (1973), measures the amount of hydrogen peroxide produced when glucose is oxidised using glucose oxidase in the presence of molecular oxygen. Chemical details are given on page 49.

5.3.a. Methods and Results.

100 mM, 50 mM and 25 mM glucose solutions in water were prepared. The preparation of the buffer-enzyme-dye (BED) reagent and saline phenol solution and 0.1 M phosphate buffer are described in chapter 2.

These steps were followed:

1. The saline phenol solution was diluted (1:2) in 0.1M phosphate buffer pH 7.0.

2. 200 ul of BED reagent and 200 microlitres of saline phenol solution were each loaded into the two reservoirs of the device. The device was flushed out with carrier.

3. The end of the PTFE outlet tubing was placed in a tube of 25 mM. glucose solution and 2 ul were sucked into the tubing.

4. The tubing was removed from the solution, and air was sucked in until the droplet of glucose droplet reached the coalescing station.

5. 2 ul of the BED solution were dispensed and coalesced with the glucose droplet.

6. 2 ul of the saline phenol solution were dispensed and coalesced with the resulting droplet.

7. The resulting droplet was moved out of the outlet tubing with about 50 uls of carrier, into a 1 ml cuvette containing 1 ml. of phosphate buffer.

8. The contents of the cuvette were carefully stirred using a small spatula, and the cuvette was incubated at 37'c for 20 minutes.

9. The absorbance was measured at 515 nm.

Steps 2 to 9 were repeated four times each for 25 mM., 50 mM. and 100 mM. glucose solution. The results of this experiment are given in table 13, and plotted in figure 17.

Glucose solution	25 mM	50 mM	100 mM
Mean Absorbance	0.0187	0.0237	0.0267
Standard deviation	0.0017	0.0022	0.0022
Coefficient of variation	9%	9%	8%

Table 13. The measurement of glucose concentration by photometry, mixing the analyte and reagents in the block assembly. 2 ul of the solution to be measured were sucked into the outlet tubing, moved into the reaction conduit, and coalesced with microlitres each of "buffer-enzyme-dye" reagent and "saline phenol" reagent. The resultant droplet was passed out of the device, along with 50 ul of carrier, into a 1 ml. cuvette containing 1 ml. of phosphate buffer solution. The droplet was carefully stirred into the solution, leaving the carrier liquid floating on the surface, and the absorbance at 515 nm was measured.

Figure 17: Photometric measurement of glucose mixing the reactants in the Droplet Reactor.

5.3.b. Interpretation.

Accurate measurement was made difficult by the very low absorbances of the solutions, and uneven movement of the syringes, as discussed in chapter 3, is also contributing to the error. However this experiment demonstrates that it is in principle possible to measure clinical analytes in the appropriate concentration ranges, in the droplet reactor, since it was possible to distinguish the three glucose solutions in spite of these limitations. A more accurate design of syringe module has been built and is used in Embodiment III of the device, and it is hoped that when this is used in conjunction with the flow cell under development (which would obviate the need for diluting the reagents into a 1 ml. cuvette) it should be possible to achieve results with a coefficient of variation in the 2 to 4 % range.

5.4. BIOLUMINESCENT ASSAY, COMBINING REAGENTS WITHIN THE DEVICE.

4.3.a. Introduction.

As discussed in chapter 4 bioluminescence has great potential as an analysis system for it avoids the geometric problems inherent in the direct measurement of the absorbtion of droplets. The method also has the potential for sensitivity approaching that of radioisotope detection, and moreover it does not normally require a separation stage to remove unbound label.

5.3.b. Method and Results.

First it was shown that the components of the bacterial bioluminescence system could be dispensed and combined using the droplet reactor. The chemistry involved is described in detail in chapter 3. These steps were followed:

1. Two solutions were made up containing the following components;

Solution 1: 5 uM FMN
1 mg/ml. decanal
in 50 mM phosphate buffer pH 7

Solution 2: 36 mg/ml. reductase
44 mg/ml. luciferase
1 mM NADH
in 50 mM phosphate buffer

Note that the concentrations of NADH and the enzymes were about 10 x the concentrations normally used for these type of assays.

2. These two solutions was immediately loaded into each of the two reservoir syringes of the droplet reactor.

3. A 1.3 microlitre droplet of each solution was displaced and the two droplets were coalesced, then passed out of the outlet tubing into a cuvette containing 500 ul of silicone fluid.

4. The luminescence was measured in the luminometer, counting photons for 1 second.

Steps 3 and 4 were repeated 20 times.

The mean reading obtained was 256, with a standard deviation of 27. This reading is comfortably in the mid-range of the luminometer, avoiding the danger of overloading the device, and also avoiding readings which are so low that random variations become significant. The standard deviation of 27 shows that the system is reasonably accurate, giving a coefficient of variation (standard deviation / mean * 100) of just over 10.

The inactivation or dissolving of decanal (and FMN and NADH) did not significantly interfere with these results: in the first place, all of the movements of the reactants and their timing were controlled by a computer, so that each of the droplets was exposed to silicone under the same conditions, and each of the readings was comparable. In the second place, the decanal was present in excess, and it was found that only vigorous shaking with a large amount of silicone liquid (as in chapter 4) was sufficient to deplete it to levels where the luminescence was reduced.

5.4.c. Interpretation:

This experiment showed that it is feasible to use the bacterial luminescent system as an assay system in the droplet reactor. It would of course be possible, and indeed desirable, to use a photomultiplier mounted directly over the block assembly, as

described in Chapter 8, and so to measure the light output of a droplet while it remains within the device's main conduit. In this configuration the luminescence of the droplet could be measured as soon as the reactants are combined.

5.5. BIOLUMINESCENT ASSAY OF GLUCOSE, PERFORMED OUTSIDE THE DEVICE.

5.5.a. Method and Results.

The bioluminescence system was now combined with a protocol to measure a clinically important analyte. Glucose was again chosen as an analyte to demonstrate the feasibility of this detection system. The method was adapted from that of Lowry (1972) which also involves measuring reduced pyrimidines photometrically. These tests did not involve using the Droplet Reactor, but were performed to show that a suitable method of measuring glucose by bioluminescence could be devised, and that the enzymes and reagents involved were stable in the presence of silicone liquid.

Two reagents were used:

Reagent I; 1mM magnesium chloride
10 mM NAD+
10 mM ATP
0.4 U/ml glucose-6-phosphatase
1.44 U/ml hexokinase
in 50 mM phosphate buffer pH7.6

Reagent II; 1mM magnesium chloride

5 mM FMN

1 mg/ml decanal

8.8 ug/ml luciferase

7.2 ug/ml reductase

in 50 mM phosphate buffer pH 7.6.

These steps were followed:

1. These two reagents were prepared:

Reagent I:

Amount in 1 ml	Concentration	
0.095 mg	1 mM	MgCl2
6.4 mg	10 mM	NAD+
5 mg	10 mM	ATP
1.5 ug	0.4 U/ml	glucose-6-phosphate
4.5 ug	0.44 U/ml	hexokinase

in 50 mM phosphate buffer pH 7.6.

Reagent II

amount in 1 ml	concentration	
0.095 mg	1 mM	MgCl2
	5 uM	FMN
1 ug		decanal
8.8 ug/ml		luciferase
7.2 ug/ml		reductase

in 50 mM phosphate buffer pH 7.6

2. 50 microlitres each of reagent I and reagent II were placed in a cylindrical cuvette and the luminescence peak (after about 3 minutes) was measured in the luminometer.

3. To a cuvette were added 50 microlitres of reagent I and 10 microlitres of 5 mM glucose. This mixture was incubated for two minutes at room temperature.

4. 50 microlitres of reagent II were added and the luminescence was measured in the luminometer.

Steps 3 and 4 above were repeated three times each, using 5 mM, 10 mM and 20 mM glucose solution.

The stability of reagents I and II in the presence of silicone liquid were then tested as follows. 200ul of reagents I and II were each placed in two separate tubes, each containing 500ul of silicone. These were vigorously shaken for 30 seconds and centrifuged for 2 minutes. These two solutions were then tested, one at a time, by using them to replace the stock reagents I and II in steps 3 and 4 above. (10 mM glucose solution was used throughout.)

Table 14 summarises the results of these experiments, which are plotted in figure 18.

	Luminescence readings			mean	standard deviation	coefficient of variation
No glucose	15	12	23	16.7	4.6	28
5 mM glucose	295	203	229	242	39	16
10 mM glucose	325	350	316	330	14	4
20 mM glucose	413	398	416	409	7.87	2
10 mM glucose with reagent I shaken with Si liquid.	322	293	309	308	11.9	4
10 mM glucose with reagent II shaken with Si liquid.	283	321	303	302	15.5	5

Table 14. The measurement of glucose concentration using bioluminescence, mixing the analyte and reagents by conventional pipetting in test-tubes. Glucose is converted to glucose-6-phosphate using hexokinase, and thence to 6-p-gluconolactone and NADH using glucose-6-phosphate dehydrogenase. The NADH is measured by bioluminescence. In the second part of the experiment the stability of reagent I (MgCl, NAD+, ATP, glucose-6-phosphate and hexokinase) and reagent II (MgCl, FMN, decanal, luciferase and reductase) was tested by shaking with silicone liquid.

5.5.a. Interpretation.

The reading obtained when no glucose was present represents a background level of luminescence which can be attributed to impurities in the reactants and to side-reactions.

This method appears to be suitable for forming the basis for an assay for glucose. It is able to distinguish three glucose solutions in the micro-molar range, and reagent I appears to be

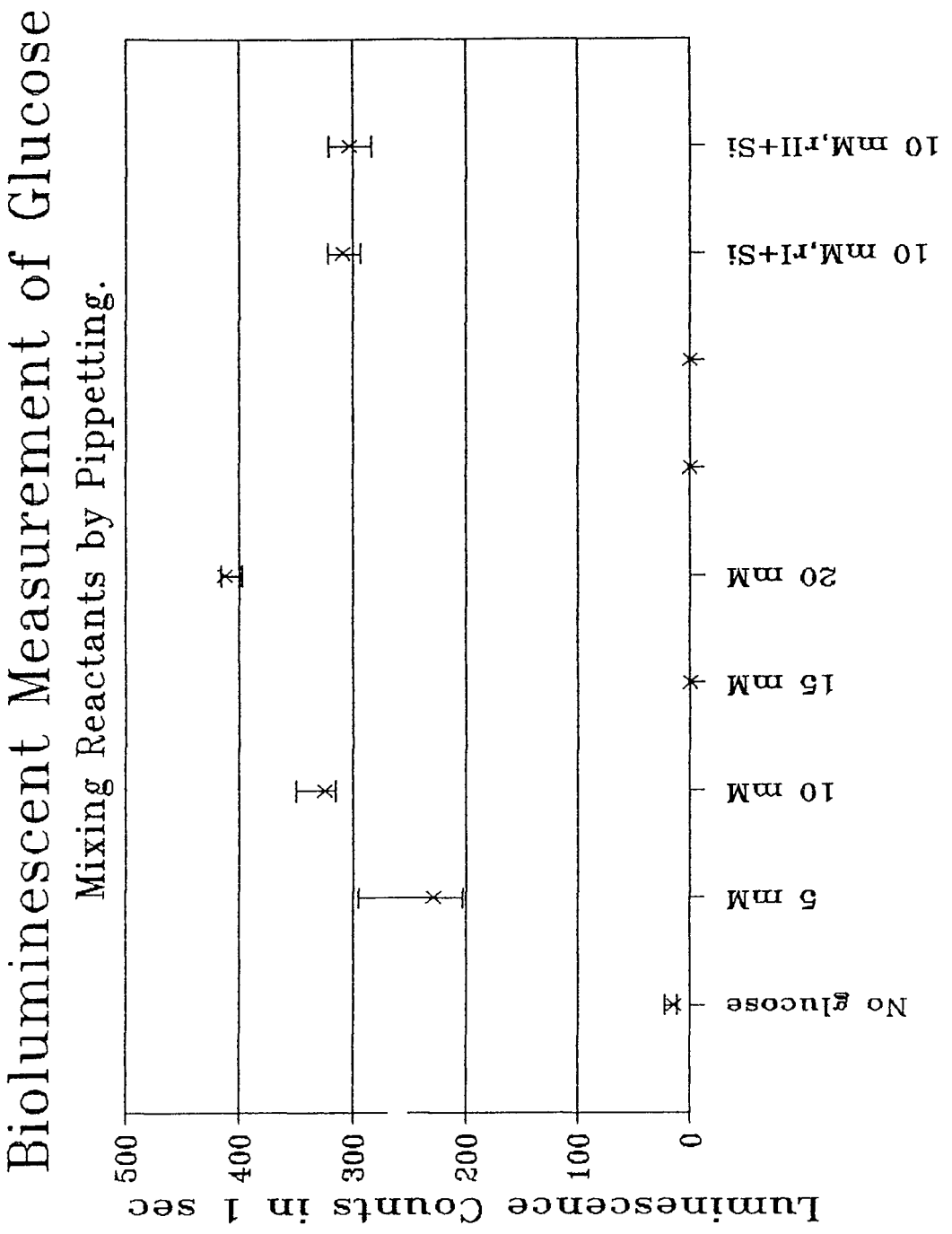


Figure 18: Bioluminescent Measurement of Glucose Mixing Reactants by pipetting.

stable to silicone fluid, while reagent II is only slightly effected. The light output is not greatly reduced when reagent II is shaken with silicone liquid in spite of the fact that it contains decanal, which was shown in chapter 3 to be soluble in silicone carrier. This is because the decanal is present in excess, and 200 ul of reagent II were shaken with only 500 ul of silicone carrier liquid. This compares with 50 ul of decanal solution which were shaken with 1000 ul of silicone liquid in the corresponding experiment in chapter 3. Note that the test of shaking and finely dispersing the reagents in silicone carrier is more rigorous than the conditions that would actually be encountered in the Droplet Reactor: the droplets will be at least 1 ul in the Droplet Reactor, which is very much larger than the size of the droplets formed during shaking. The coefficient of variation of the measurements would be unacceptable for a clinical assay. These variations are probably due to pipetting errors in the small volumes dispensed. Although this procedure was performed by dispensing the reagents with conventional liquid handling equipment, it shows that the method is chemically feasible, and that the reagents are substantially stable to silicone carrier liquid. These results, taken with the results of the previous section, (where all the components of the bioluminescent system were dispensed and combined using the Droplet Reactor) suggest that the device could be made into a viable clinical assay.

CHAPTER 6. CELL MANIPULATION.

CHAPTER 6. CELL MANIPULATION.

6.1. INTRODUCTION.

There are many situations where it would be advantageous to include in the droplets, not only chemicals, but also living eukaryotic or prokaryotic cells. This would be particularly useful for automating very repetitive procedures. For example, after combining the appropriate droplets containing DNA and enzymes so that DNA fragments and plasmids are digested and recombined, one might want to mix the recombined DNA molecules with "competent" bacterial cells which could absorb the DNA and become "transformed". In such a case these cells could be contained in a droplet, and coalesced with the droplet containing the recombined DNA. Or again, if it could be arranged that each droplet contained only one (or a few) bacteria, and nutrient broth was included in the droplet solution, a clone of bacteria could be grown within a droplet. Such a procedure could be used to screen clones to find a mutant which synthesised or broke down a particular metabolite.

The device could alternatively be used to manipulate living eukaryotic cells such as hybridomas or plant protoplasts. Hybridomas could be screened to find a clone which produces a particular antibody, and green protoplasts could be individually manipulated and fused without the need to produce cultured (white) cells.

6.2. STERILISATION AND CONTAMINATION OF CONDUITS BY BACTERIA IN DROPLETS.

6.2.a. Introduction.

For any application involving living cells it will be essential that the device can be thoroughly sterilised. Failure to achieve sterile conditions would result in contamination of bacterial cultures with bacteria from other sources, and the infection of cultured animal or plant cells by bacteria or fungi.

In these experiments the droplet reactor was sterilised with ethanol. This agent was chosen because it is partially miscible with silicone liquid and miscible with water, and so can be washed away. Also it is not highly toxic in small amounts, so that any traces which were left behind would be unlikely to kill cell cultures.

The effectiveness of sterilisation of the conduits and also the degree of contamination and transfer of bacteria was tested in a series of experiments.

6.2.b. Sterilisation method.

Detection of contamination before sterilisation;

1. The end of the outlet tubing was dipped in absolute ethanol, then in distilled water to remove the ethanol.

2. The end of the outlet tubing was placed in a plastic test-tube containing a solution of sterile Oxoid nutrient broth (13 g/l) and blue dextran (5 mg/ml). and 2 ul. was sucked into the tubing.

3. The end of the outlet tubing was now placed in (unsterilised) silicone liquid and the nutrient broth was sucked into the block, as a droplet, to a position upstream of one of the dispensing stations.

4. The broth droplet was moved out again to a position just before the end of the outlet tubing.

5. The broth droplet, together with about 10 ul of silicone liquid was ejected onto a nutrient agar plate, and spread across the surface with a bent glass rod. The plates were incubated at 37 c for 24 hours.

This procedure was repeated five times on nutrient agar plates without antibiotics (droplets 1 - 5 in table 15 below) and five times on nutrient agar plates containing the antibiotics tetracycline and kanamycin (droplets 6 - 10.)

Sterilisation procedure:

a. The four motorised syringes were disconnected from their luer connections.

b. Four 10 ml. syringes were loaded with distilled water and the

air bubbles were removed. These were connected to the four luer sockets, and each in turn was slowly emptied through the device, catching waste from the outlet tubing in a beaker.

c. Step 2 was repeated using absolute alcohol in the syringes in place of the water. The device was now left for 30 minutes.

d. Step b was repeated.

e. 20 mls. of silicone liquid were sterilised by the following procedure; 20 mls of silicone liquid and a beaker containing 200 mls of water were placed in a microwave cooker, which was switched on until the water boiled for one minute. The motorised syringes were loaded with sterile silicone liquid, all air bubbles were removed, and the syringes reconnected to the block assembly.

f. 2 mls of silicone from each of the two "carrier" (5ml) syringes and 200 ul of silicone from each of the two "reactant" (500 ul) syringes was passed through the device to remove any water from the device.

g. After this five droplets of buffer (droplets 11 - 15 on table 15) were passed through the device as in steps 1 to 4 in the previous section, to see if sterility had been achieved. These droplets were spread onto plates without antibiotics.

6.2.c. Contamination Detection Method.

A sequence of steps similar to that used in section 4.3.2, was

used to detect the transfer of peroxidase from one droplet to another, except that the droplet of strong peroxidase solution was replaced with an "overnight" culture of E. coli strain J53RP4 (a culture started from a single colony, shaken in 2 mls of oxoid nutrient broth at 37 c for 16 hrs.) The experiment works on the principle that a droplet containing bacteria is passed through the device, followed by a droplet of (initially sterile) nutrient broth. The bacteria picked up by the broth droplet are detected as before.

These steps were followed;

1. A 2 ul droplet of fresh "overnight" bacterial culture was sucked up into the reaction conduit of the device by following steps 1 to 4 above.

2. This droplet was allowed to stay in a position near the middle of the length of the reaction conduit for one minute, then passed out of the device and discarded.

3. A 2 ul droplet of sterile broth was sucked into the device by following steps 1 to 4 above, to a position above that reached by the droplet of bacterial culture, then immediately passed out of the device onto a plate containing tetracycline and kanamycin as in step 5 above.

This sequence was repeated 5 times, giving droplets 16 -20 in table 15.

	No. of colonies observed				
Droplets 1-5; before sterilisation, no antibiotics in plates.	1	3	0	1	2
Droplets 6-10; before sterilisation, Tet and Kan in plates.	0	1	0	0	0
Droplets 11-15; after sterilisation, no antibiotics in plates.	0	0	0	1	0
Droplets 16-20; after passing droplet of bacterial culture through device.	5	76	14	24	7

Table 15. Sterilisation of the device and contamination by micro-organisms passing between droplets. The numbers show the number of colonies formed when a (potentially contaminated) droplet is spread onto an agar plate. Droplets 1 to 5 show the results of spreading droplets that were passed through the device before it was sterilised, onto plates without antibiotics. Droplets 6 - 10 were passed through the device before sterilisation, and spread onto plates containing the antibiotics tetracycline and kanamycin. After sterilisation, droplets 11 - 15 were passed through the device and spread on plates with no antibiotics. After this, a droplet of an "overnight" bacterial culture, of a strain that is resistant to both tetracycline and kanamycin, was moved into the device allowed to stay in one place in the "reaction conduit" for one minute. The droplets 16 -20 were then dispensed and passed through the device and spread on plates with the antibiotics.

6.2.d. Interpretation.

The device was clearly not sterile at the start of the experiment because the buffer droplets that passed through the device carried bacteria which grew on the nutrient agar plates. These bacteria were not resistant to both kanamycin and tetracycline, as no growth occurred on the plates in the second row of the table. After sterilisation no bacteria (or very few) were present, as shown in the third row of the table.

When a droplet of bacterial culture was passed through the reaction conduit, and was allowed to remain stationary for one minute, significant contamination does occur. Other experiments have shown that when two droplets were allowed to remain stationary at the same site, contamination is around ten times greater. Also, when neither droplet is allowed to stop, contamination is reduced about an order of magnitude. As such experiments give rather variable results, however, they have not been described in detail.

The levels of transfer shown represent the transfer of perhaps one bacterium in 1 million. This level of transfer may be acceptable in some cases, for example where all droplets already contain large numbers of bacteria, or when the bacteria are not given the opportunity to multiply. If the device was to be used for the screening or selection of mutants a regime would be necessary where a droplet containing cells would be followed by one or several buffer droplets, or even a flush with a sterilising agent followed by a wash with buffer droplets.

6.3. CELL FUSION.

6.3.a. Introduction.

Cell electrofusion is an application which was considered in some detail. There is an important need for a device which could individually fuse green plant protoplasts, so avoiding the need

to produce white cell cultures from each of the plants to be hybridised. Protoplast fusion is one of most significant opportunities for producing new varieties of commercial crops until recombinant DNA technology becomes available (Van Brunt 1985).

To fuse two types of green plant cells it would be necessary to bring two individually chosen cells into intimate contact, then use electrodes to apply the appropriate potential gradient, using the electrical stimulation method of Senda et al. (1979). Senda's method involved the micromanipulation of individual cells. This has also been done, within a 50 nl. droplet of oil, by Koop et al. (1983). However, as this method is time-consuming and highly skilled it was decided to investigate whether the Droplet Reactor could be used to automate the process.

It was envisaged that droplets would be dispensed containing cells at a concentration where on average one cell is present in each droplet. Two droplets would be dispensed, each droplet containing one of the cells to be fused. The droplets would need to be checked visually or automatically scanned after dispensation to ensure that one cell was in fact present in each. If more or less than one cell was present the droplet would be discarded and replaced. The droplets would then be coalesced, and the resultant droplet moved into contact with two platinum electrodes. First an alternating current would be applied (1 MHz, V/cm), to bring the cells into contact with one another, followed

a DC pulse 1.5kV/cm for 50 us, as in the method of Zimmerman et al. (1981). After this the droplet containing the fused cell would be passed out of the device, and cultured to produce a new plant. It is essential that this process should be carried out either automatically or semi-automatically so that large numbers of plants can be produced.

6.3.b.Experimental.

The problems involved in handling protoplasts in the device were investigated in collaboration with Dr. Mike Jones of Rothampstead experimental station. Embodiment I the device was used, in which the reservoirs are machined in the block. Green protoplasts, which were prepared essentially according to the method of Senda et al. (1979), were loaded into the reservoirs, droplets were dispensed and coalesced. Although it was difficult to see the boundaries of the droplets, the protoplasts were clearly visible within the droplets (and within the reservoirs), using an ordinary dissecting microscope, without any special lighting.

Next, the difficulties involved in bringing the resultant droplet into contact with a pair of electrodes were investigated. It was felt that it was important that the droplets should not be much larger than those used by Koop, which were 50 nl. This is to minimise the distances that the protoplasts would have to travel in order to come into contact with each other. A block was therefore constructed which allowed 100 nl droplets to be coalesced. When droplets had been successfully coalesced using

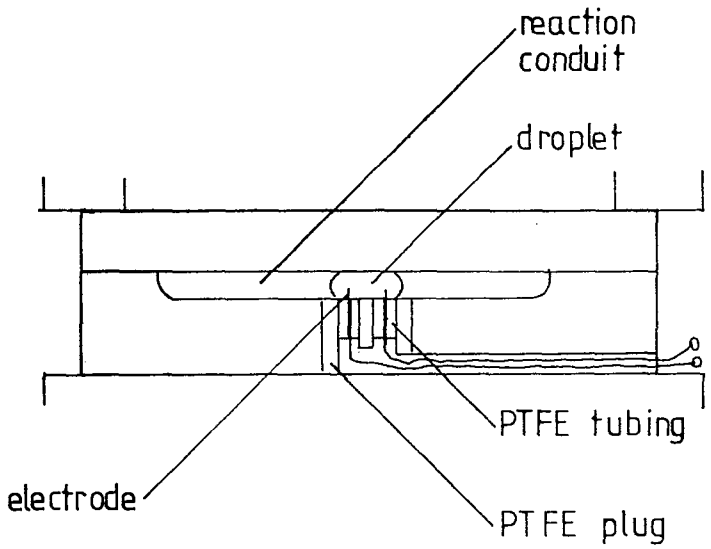


Figure 19. Electrode assembly for cell fusion.

this apparatus, an electrode assembly was constructed as shown in figure 19. This was situated downstream of the coalescing station and comprised two platinum electrodes arranged along the axis of the conduit. Each electrode was held in a short length of PTFE tubing which acted as a sleeve for the electrode and allowed the position of the electrodes to be adjusted in or out of the conduit. The two lengths of PTFE tubing were held in a cylindrical peg of PTFE which also formed the walls of the conduit in this region as shown. The tips of the platinum wire were cut to a sharp point using a sharp scalpel under a microscope. When the electrodes were in position they were sealed into the PTFE peg using plastic adhesive from an R.S. (tm) hot melt glue "gun". These electrodes could be connected to the standard apparatus for generating the electric currents required to fuse cells. The voltages applied would be lower than normal because the distance between the electrodes is lower than that normally used.

6.3.c. Results and Conclusions.

It proved to be extremely difficult to position the electrodes so that they made contact with the droplets as they passed through the electrode station, without causing the droplets to break up. It was concluded that it would be necessary to make the electrodes adjustable while the block assembly was in use, or to make electrical contact by some other method.

The Droplet Reactor clearly has potential for cell fusion. Plant protoplasts can be manipulated in 100nl droplets and visually inspected in the device during and after dispensation and coalescence. However, applying a current to the cells within the droplet using electrodes is not a trivial problem. The problem of automatically scanning the droplets before fusion to ensure that each contained one cell, could be overcome using lasers and light detectors arranged to detect light scattering or fluorescence, given sufficient financial resources. The technology is largely available, and was developed for cell sorters. As an alternative approach it is envisaged the aqueous and carrier phases could be replaced by two aqueous phases, each containing high concentrations of polymers. In such a case the carrier as well as the sample phase would conduct electricity, so that it would be possible to place the electrodes at remote sites on each side of the droplet. Thus the electrodes need not come into direct contact with the droplets.

CHAPTER 7. CRYSTALLOGRAPHY AND MOLECULAR BIOLOGY.

CHAPTER 7. CRYSTALLOGRAPHY AND MOLECULAR BIOLOGY.

7.1. INTRODUCTION.

Two other miscellaneous applications that were investigated are included in this chapter although they are not directly related to each other. These are crystallography and molecular biology. What they have in common is that neither requires any modification to the device or the development of any special detector.

7.2. CRYSTALLOGRAPHY.

7.2.a. Introduction.

Crystallography is a relatively specialised area of application of the droplet reactor, but it can be used in this area with a minimum of modification.

One of the most time-consuming procedures in forming crystals of a newly purified protein (or other material) for x-ray crystallography is identifying conditions where crystals will be formed. A number of workers (e.g. Ward 1986) have developed automatic methods using robotic sample preparation systems, but these are very complex and expensive. It is usually necessary to survey a range of conditions, varying the concentration of a precipitating agent or various other additives in each of perhaps 50 aliquots of the protein. Using the droplet reactor, this procedure can be performed automatically by coalescing a droplet

of protein with droplets of coalescing agents and additives, then storing the resulting droplets in transparent tubing or under oil in petri dishes or microtitre plates. The droplets can be periodically inspected with a microscope for crystal growth.

To test this system the droplet reactor was used to mix proteins with precipitating agents in conditions which are known to produce crystals. The volumes of the droplets were adjusted so that a range of concentrations could be tested.

7.2.b. Method and Results.

The method was applied to two proteins, ferritin and lysozyme, as follows;

1. The reservoirs and conduits of the droplet reactor were flushed out with deionised water.

2. 80 mg/ml ferritin solution was loaded into one of the reservoir syringes of the droplet reactor, and 5% cadmium sulphate into the other.

3. 9 "steps" of ferritin (ie. 1.5 ul.) were dispensed as a droplet, then 15 "steps" (ie 2.5 ul.) of cadmium sulphate were dispensed and the two droplets were coalesced.

4. This procedure was repeated about 20 times and the resulting droplets were passed out onto a petri dish containing 5 ml. of viscous liquid paraffin. (About 20 microlitres of the silicone

carrier liquid was passed out with each droplet. This mixed with the paraffin.) After a few seconds the droplets adhered to the bottom of the petri dish.

The computer program was modified and steps 2 and 3 above were repeated with different volumes so that a different proportion of ferritin and cadmium sulphate were mixed. In each series of 20 droplets the volume of the ferritin droplet was increased by one step, or 1/6 ul., and the cadmium sulphate droplet was decreased by 1/6 ul., until 2.5 ul. of ferritin were coalesced with 1.5 ul. of cadmium sulphate.

This entire sequence was now repeated using the protein lysozyme (60 mg/ml) with 10% NaCl as the precipitant. At the end of the experiment two sets of six petri dishes containing liquid paraffin, were produced each with 20 droplets adhering to the bottom. These dishes were stored for 24 hours, and the droplets were then inspected for crystals with a binocular microscope. Table 16 summarises the results of these experiments.

Volume of protein solution, ul.	Volume of precipitating agent, ul.	Presence of crystals	
		Ferritin	Lysozyme
2.50	1.50	No	Some
2.33	1.67	No	Yes
2.17	1.83	Some	Yes
2.00	2.00	Yes	Yes
1.83	2.17	Yes	Yes
1.67	2.33	Yes	Some
1.50	2.50	Some	Some

Table 16. The presence of crystals of the proteins ferritin and lysosyme when they were mixed, within the device, with the precipitating agents CdSO4 and NaCl respectively in the proportions shown. The total volume (4 ul) was kept constant in each case. 20 droplets of each mixture are passed out of the device into a petri dish containing liquid paraffin. These are stored for two days at room temperature then inspected for crystals with a dissecting microscope.

In another experiment, a siliconised glass capillary (I.D. 1 mm, length 70 mm) was connected to the outlet tubing with a length of silicone rubber. Silicone liquid with several coalesced droplets (each containing two microlitres of ferritin and cadmium sulphate) was passed into the capillary and the end was sealed with an RS (tm) "glue gun". The capillary was then disconnected and the other end was sealed. These capillaries were mounted on plasticine, stored overnight, and inspected for crystals. Most of the droplets contained crystals.

7.2.c.Conclusions.

These experiments demonstrate that protein crystals can be formed in aqueous droplets in silicone, and that the necessary components can be dispensed and coalesced in the droplet reactor. Most searching for conditions for the crystallisation of new proteins is done in "hanging drop" systems where a sample containing protein slowly equilibrates with a solution in a reservoir, usually with lower water content, from which it is separated by air in a closed system. This means that the sample passes through a series of concentrations, until a concentration is reached where crystal nucleation occurs. After this the concentration of protein may continue to rise, in spite of depositing some of the protein as crystal. This usually results in further nucleation of crystals so that one is usually left at the end with a few big crystals and many small ones. The alternative system is so-called "batch production", in which the protein, precipitating agent, water and additives are placed in a single sealed container. The problem with this approach is that it is very labour intensive, as a separate mixture has to be produced for every concentration that is to be tested. However, it has the advantage that it tends to produce a few, big crystals, provided appropriate conditions can be found. This is because once crystal growth starts, the concentration of protein goes down so that further nucleation is automatically inhibited.

The Droplet Reactor provides an automatic method of performing large numbers of batch crystallisations, and has the additional advantage of using very small amounts of protein. It is envisaged that the process could be taken a stage further by performing crystallisation in two steps: in the first step droplets containing a mixture with enough protein to initiate nucleation are dispensed. This mixture is incubated long enough to allow nucleation. In the second stage, these droplets are coalesced with a diluent to prevent further nucleation, and to provide ideal conditions for crystal growth. Thus the droplet reactor has the advantage of reducing the labour requirement and using less protein, and the potential for a two step process which would provide different conditions for crystal nucleation and growth. One area where these advantages may be particularly relevant is space research. In addition to a role as a general space biochemical analyser, the Droplet Reactor may be useful for performing crystallisation in space. The advantages of growing crystals in a microgravity environment, such as lack of distortion of crystal shapes by contact with the boundaries of the sample volume, and lack of convection currents, are already being investigated, but with far cruder apparatus.

7.3. MOLECULAR BIOLOGY.

7.3.a. Introduction.

Molecular biology is an area which could potentially make great use of automation and the versatile control of the droplet reactor. For example, one or a few DNA samples could be treated with a series of restriction endonucleases (singly or in "double digests") to carry out large scale restriction site mapping procedures. Several reservoirs could be loaded with restriction enzymes, while the remainder could contain the ingredients for buffers, so that the composition of the buffer for double digests etc. could be adjusted automatically. Since large numbers of digests with different combinations of restriction enzymes could be set up with a minimum of labour, the choice of the optimimum strategy in chromosome and gene mapping tasks would be less important. Instead, the researcher could perform a large number of digests and focus his attention in retrospect on the digests that produce relevant results. As discussed in chapter 6, the device could also be used to perform cloning experiments by carrying out transformations of bacteria in the presence of DNA fragments or plasmids. For cloning experiments, very small amounts of DNA could be used, with total reaction volumes of under one ul, since only one transformed cell is required. For gene mapping, larger amounts of material would be needed, but high quality electrophoresis gels can be run using only 5 ul sample volumes. Clearly there is great potential for the device in molecular biology, particularly the more labour intensive applications.

7.3.b. Experimental details.

The activity of restriction enzymes in the presence of silicone liquid was tested in collaboration with Carlos Flores in this laboratory. The object was to find out whether silicone liquid would inactivate the enzyme. A restriction digest of a plasmid preparation was set up, and the degree of DNA digestion in an aliquot to which silicone liquid had been added was compared with that in an aliquot with no silicone. The plasmid pFK::Tn7 5S (ccc) was used with the enzyme Hind III.

This procedure was followed;

1. To a test-tube was added

    30 ul plasmid DNA 0.8 mg/ml

    12 ul of Amersham's buffer E8, 1:10 dilution

    0.5 ul Hind III, 12 U/ml.

    77.5 ul of distilled water.

These components were mixed by inversion.

2. The solution was divided into 3 40 ul aliquots.

    Tube 1 was frozen immediately.

    Tube 2 was incubated normally at 37 c for two hours.

    To tube 3 20 ul of silicone fluid was added, the tube was shaken for 15 seconds and incubated at 37 c for two hours.

3. Tube 1 was thawed and 15 ul from each tube was loaded into a separate well of an agarose gel (0.8% agarose, length 14cms, volume 100 mls, containing 700 ngs of ethidium bromide per ml. The gel was run at 80 v for 4 hours and photographed under ultraviolet illumination.

7.3.c. Interpretation of Results.

Three tracks of a gel were used. Track I was the frozen aliquot, track II the aliquot that was incubated normally, and track III the aliquot that was incubated with silicone liquid. This plasmid gives four fragments with Hind III; 9.4, 3.5, 2.6 and 2.3 kilobases. Track I showed no digestion, and consisted of a heavy band of high molecular weight corresponding to covalently closed (ccc) DNA. Track II showed bands at 9.4, 3.5, 2.6 and 2.3 Kilobases, as well as lighter bands, which were interpreted as partial digests, which were present at 6.1 (=2.6 + 3.5) and 4.9 (=2.3 + 2.6). Track III, the aliquot containing silicone, follows the same pattern as track II, and no difference in the intensity of the bands, and in particular the partial digests, could be detected by eye. large lower band 1 of track I was covalently closed circular (ccc) DNA. The lighter upper band 2 was open circular DNA. On track II the first large band 3, somewhat smeared, was probably full length linear DNA, followed by the 9.4 kilobase fragment 4. Then followed two partial digests 5 (2.6 + 3.5 = 6.1 kilobases) and 6 (2.3 + 2.6 = 4.9 kilobases), followed

by the other three fragments 3.5 kilobases 7, 2.6 kilobases 8, and 2.3 kilobases 9. The partial digests bands 5 and 6 may be distinguished because they are lighter than the full digest band 7 although they occupy a higher molecular weight position.

7.3.d. Conclusions

The experiment shows that the silicone liquid has no significant effect on the rate at which the enzyme digests the DNA. This may be inferred because the relative strengths of the two partial digests with respect to the completely digested bands is apparently the same in the two tracks II and III. This means that the silicone liquid is not having any significant effect on the activity of the enzyme. This preliminary experiment could not detect marginal effects, but it does show that it would be practical to carry out restriction digest reactions in aqueous droplets in silicone liquid.

CHAPTER 8. SUMMARY AND FUTURE DEVELOPMENT.

CHAPTER 8. SUMMARY AND FUTURE DEVELOPMENT.

8.1. INTRODUCTION.

While each of the applications of the device that have been investigated in this project has shown significant potential, these investigations have also high-lighted certain technical weaknesses which need to be eliminated before more work is done on specific applications.

8.2. ACCURACY OF DISPENSATION.

A method of supporting the syringes more rigidly has been developed, and a coupling has been constructed which ensures that the leadscrew rotates accurately on its axis. Preliminary experiments have shown that these changes have significantly increased the accuracy of the motorised syringes. After this the design of the dispensing stations will receive more attention, for example use of smaller bore needles will be investigated.

8.3. FACILITIES FOR EASY LOADING OF THE REACTANTS.

Embodiment III showed a significant improvement over previous embodiments in the ease of flushing out reactant reservoirs and loading new reactants, but this design could be improved further. For example, it is not easy to remove air bubbles if these get into the reservoir line. They are most easily removed in Embodiment II by pulling out the plunger of the appropriate

syringe to a position where the syringe barrel becomes slightly wider, then pushing the carrier and bubble out of the opening of the motorised syringe with a disposable syringe attached to the inlet. Air bubbles would be more easily removed by a configuration which comprised two three-way valves in series between each inlet and motorised syringe, with a sample loop between them. Air bubbles would be removed by passing them into the sample loop, changing the position of the valves to connect the sample loop to the two side arms, which would each be equipped with a disposable syringe, and passing carrier liquid from one disposable syringe to the other. This configuration also allows the recharging of the motorised syringe without disturbing the reactant in the sample loop and block assembly, and flushing the block without disturbing the sample loop.

8.4.PHOTOMETRIC FLOW CELL.

The development of an effective photometric flow cell is regarded as an important priority. Once this is achieved a variety of chemical methods will become available immediately. This would also allow the accuracy of dispensation and the prevalence of contamination of the conduits etc. to be determined quickly. Figure 20 shows a new design for a photometer which it is hoped will overcome these problems. A length of PTFE tubing is passed through a bulkhead in a light-tight box, and one side of the bulkhead is illuminated with a laser. The sample droplet is passed into the tubing where it passes through the bulkhead. The diameter of the tubing should be narrow enough to elongate the

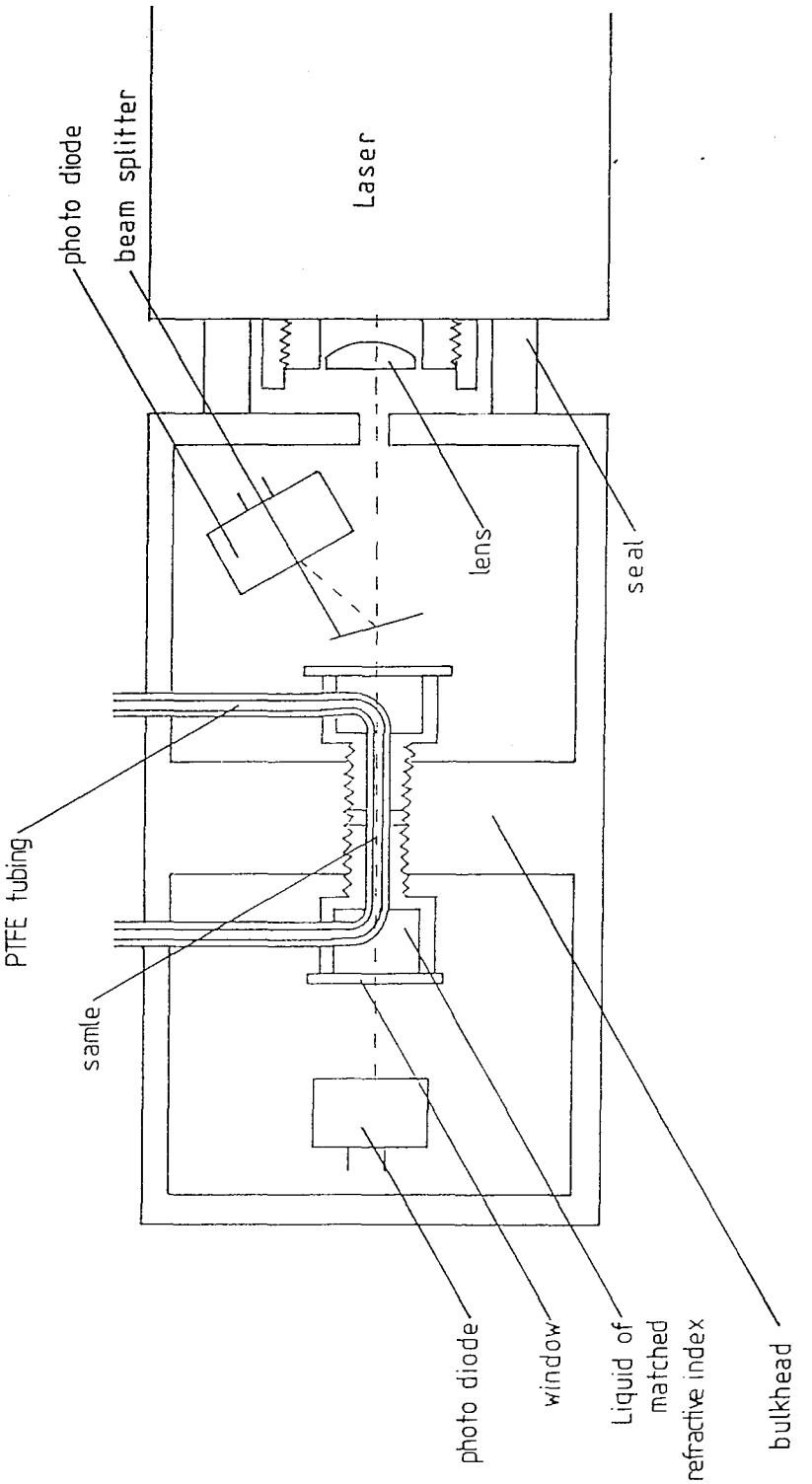


Figure 20. An Improved Design for a Photometer that would be Suitable for use with the Device.

droplet, so that a light-path of about 10 mm is obtained. The absorbance is related to the amount of light that is transmitted through the bulkhead. This light passes along the length of the sample, and is measured by a photodiode. Reflection and refraction at the surfaces of the tubing are minimised by bathing the tubing on both sides of the bulkhead in a liquid with a similar refractive index to the tubing and sample.

8.5.SPACE RESEARCH.

Obviously an important requirement for a device which is designed to be operated in outer space is that it should be small and light. It must also able to operate in very low gravity conditions, and it would be advantageous to have a sophisticated and flexible control system so that it could operate automatically, possibly including a data link to earth so that potential problems could be corrected remotely.

The present design of motorised syringes is not particularly suitable for this application, being unnecessarily large and heavy. Plastic syringes (but not with neoprene plunger tips as these absorb most carriers and swell and jam in the syringes) would be appropriate, and might have larger diameters and smaller lengths than those commonly used. The length of each syringe assembly could be further reduced by using linear actuators of the type sold by McLennans Servo Supplies Ltd. (Doman Road, Camberly, Surrey), or by The British Sonceboz Co. Ltd., (Victoria Road, Ruislip, Middlesex HA4 OLL.) These are stepper motors with

hollow shafts which are threaded internally, so that a threaded rod can travel right through the motor, eliminating the need for a slide member. The block assembly could be reduced in size by several modifications; a thinner PTFE block could be used, and a thinner sheet of glass. Also, the clamp and base components could be made of thinner material if the surface of the PTFE were more accurately machined, so that less pressure would be needed to seal the PTFE against the glass.

Finally a versatile analyser would be needed. Again a photometric analyser would be suitable as it would avoid the need for special chemical systems.

8.6. EVALUATION OF PARTICULAR APPLICATIONS AND REQUIRED DEVELOPMENTS.

8.6.a. Clinical Analysis.

As discussed in chapter 4 the device is a good candidate for a clinical analyser being easily automated, and having the potential for very flexible control. It is especially suited to paediatric testing because it has the capability of handling very small quantities. For this type of application it will be necessary to develop a detector suitable for either bioluminescent or photometric assays. Bioluminescent assays would require less innovation in designing a detector but would require more innovation in developing chemical methods, which are not yet available for a wide variety of analytes.

8.6.b. Cell Manipulation.

The Droplet Reactor has the potential to be used as a flexible, low-cost cell sorter. Unlike conventional cell-sorters, it can be used to test cells for characteristics that take minutes or hours to manifest themselves. This might include testing individual cells or clones to identify those that can synthesise or break down particular metabolites. For example, a series of droplets could be produced which contain bacterial cells at a low concentration, with only one or a few cells in each droplet, together with the appropriate nutrient mixture. Each droplet would be incubated for a predetermined period, then divided in two. One half would be coalesced with a droplet containing the reagents for a photometric or other chemical assay, and after further incubation, the absorbance etc. of the droplet would be determined. The other half of the droplet would be stored and used as a source of bacteria with the characteristics identified in the assay. Alternatively, the reagents for the assay could be included in the nutrient medium of the original droplet, providing that they are not toxic to the cells, so that no coalescence of droplets is required.

The use of the Droplet Reactor for plant protoplast fusion is another potential application. By insuring that two droplets, each containing different types of protoplasts, are brought together, the desired hybrids could be produced. The main problem

in this application is to make electrical contact between the coalesced droplet and a pair of electrodes. However, this problem could be overcome by adjustable electrodes, or possibly by using a conducting carrier liquid. This might be achieved without damaging the protoplasts by producing two separate aqueous phases containing polymers. One phase could be used as the carrier liquid, the other as the droplet medium, containing the protoplasts to be fused. The electrodes could be included in the carrier phase at some distance from the droplet, which could be exposed to electric fields by passing current through the carrier and the droplet together. In any event, this electrolysis station requires a significant amount of development work.

8.6.c. Crystallography.

Crystallography is the most promising application, and the one which is likely to be exploited commercially first. The Droplet Reactor can be used commercially in this role almost without any special modifications, requiring only the configuration described earlier in this chapter in which each channel is equipped with two three-way valves to allow easy loading, flushing and recharging with carrier liquid. Software for this application is being developed by Dr. Maeder in the Biophysics Department, Imperial College.

8.6.d. Molecular Biology.

The Droplet Reactor has another potential application in the field of molecular biology. By including DNA samples and restriction endonucleases in the droplets, it would be possible to carry out restriction digests. It has been shown in chapter 7, that restriction enzymes can operate in the presence of silicone liquid. It would also be possible to include ligase enzyme in the droplets, so that DNA fractions could be recombined. In this case the resulting molecules could be used to transform "competent" cells, by coalescing droplets containing these with droplets containing the appropriate DNA molecules. This application would make full use of the device's capacity to accurately combine very small volumes, because frequently large numbers of transformed cells are recovered, indicating that volumes below 1 ul would be adequate for such procedures.

For other applications in this field, such as mapping restriction sites, it is necessary to load the sample into the wells of an electrophoresis agarose gel. The samples and carrier could be loaded together into the wells of the gel, so that the sample, which should be denser than buffer, sinks into the well, while the carrier liquid would float upwards out of the well.

DNA sequencing is another potential application. The method of Sanger (1977), which uses chain-terminating inhibitors, is most suitable because it involves fewer liquid additions than that of

Maxam and Gilbert (1980), which uses base-specific chemical cleavages. The Sanger method would require that an enzyme cocktail is added to each sample, after which the sample would be passed into the device. Here, it would be divided into four segments to each one of which a different sequencing reagent would be added. These mixtures would be incubated for 20 minutes at 37 c as a series of droplets in PTFE tubing. These would then be returned to the block assembly where a reagent would be added to "chase" all the chains that were still active. Finally 100% formamide and dye solution would be added after further incubation, the droplets would be passed through tubing immersed in boiling water for two minutes, and loaded into the sequencing gel as described above.

Methods of adapting the Droplet Reactor to molecular biology can clearly be envisaged, but further resources would be required to develop a practical system.

PHOTOGRAPHIC PLATES.

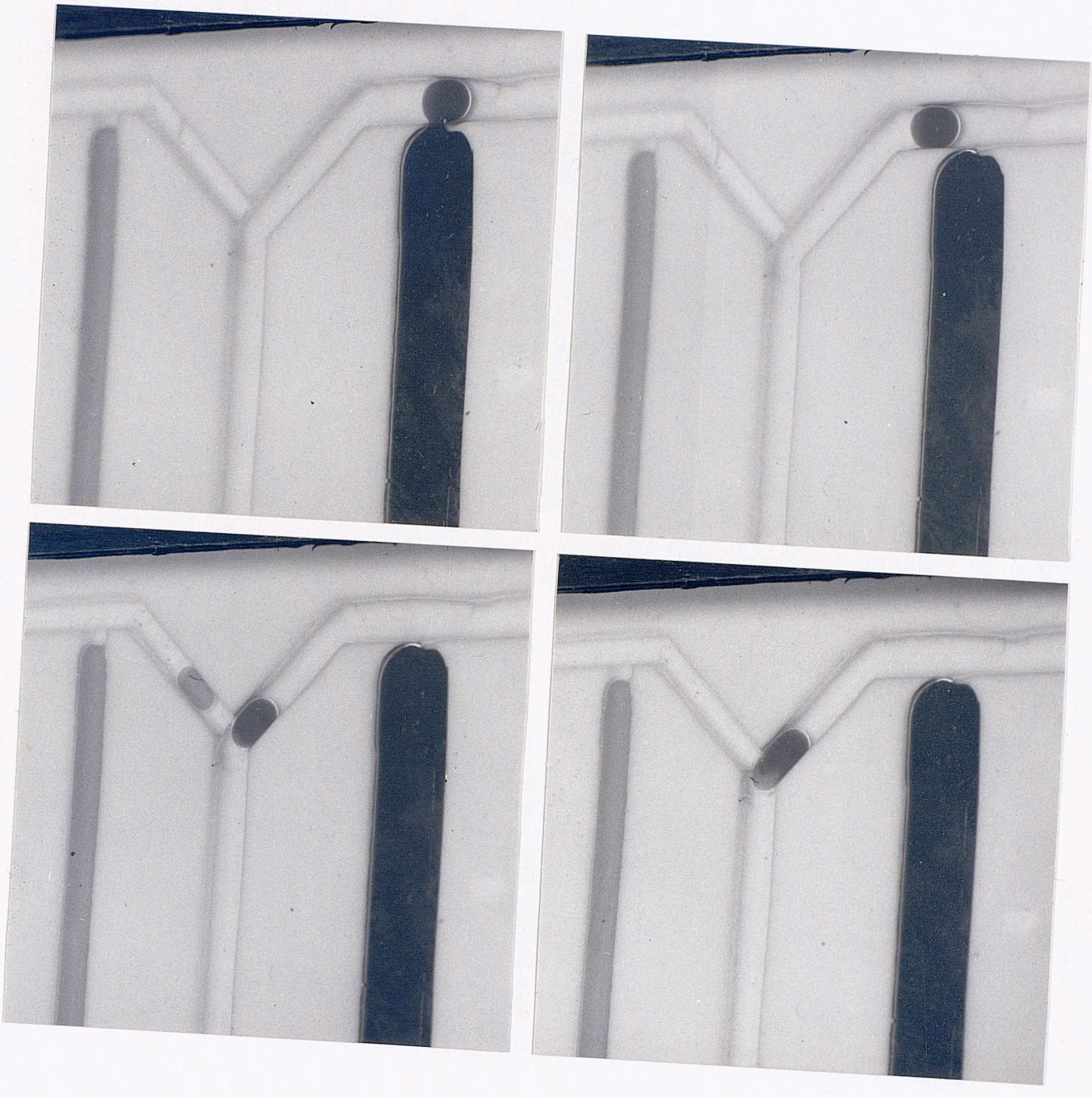


Plate 1a-d. The method of the Invention.

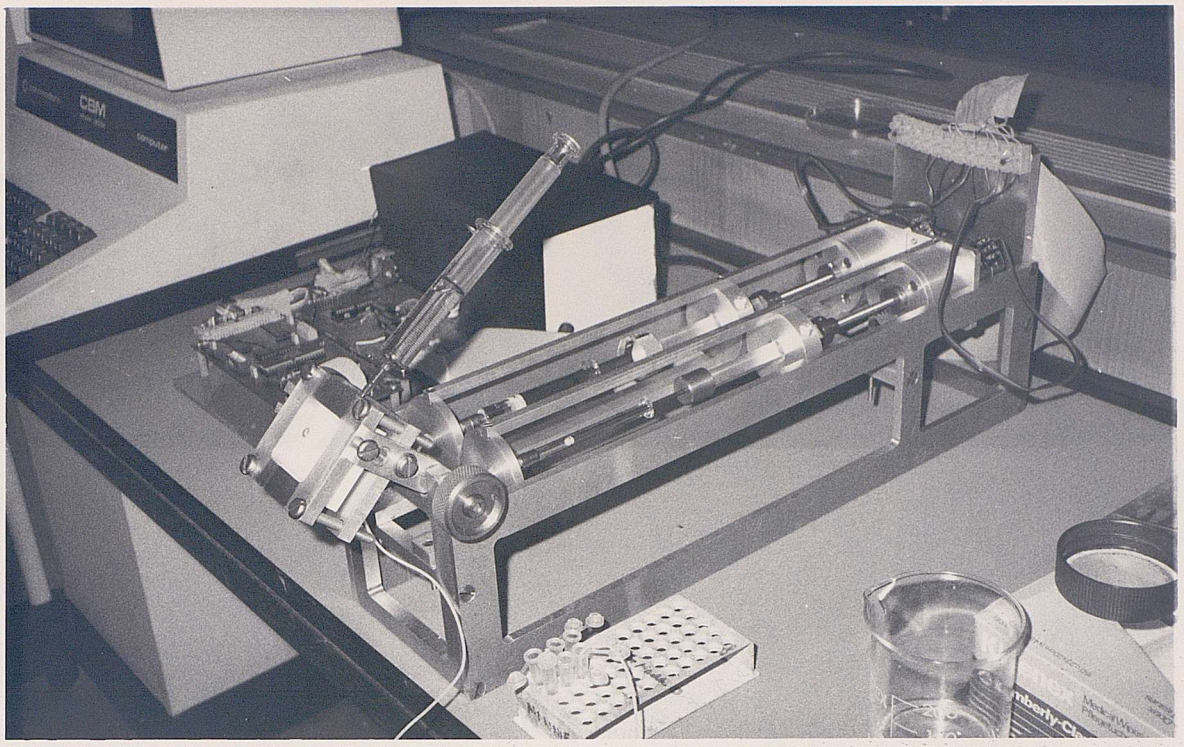


Plate 2. Embodiment I of the Device.

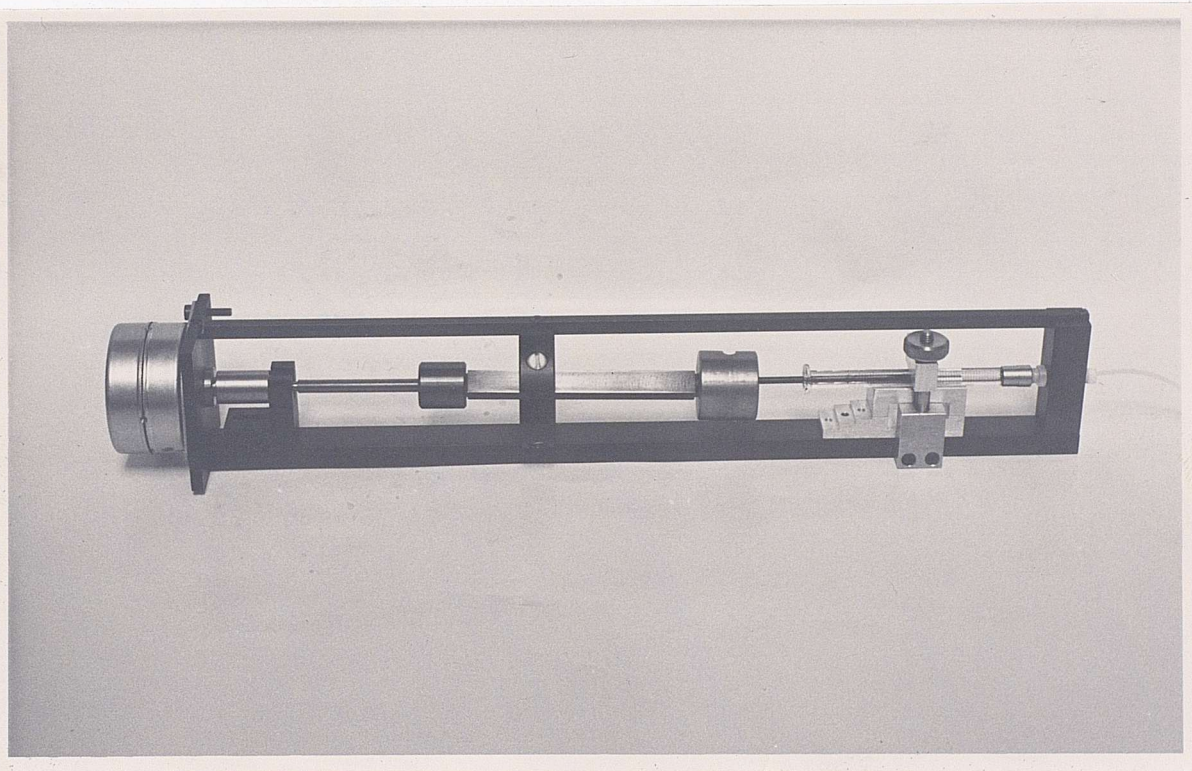


Plate 3. Motorised Syringe with Syringe Support.

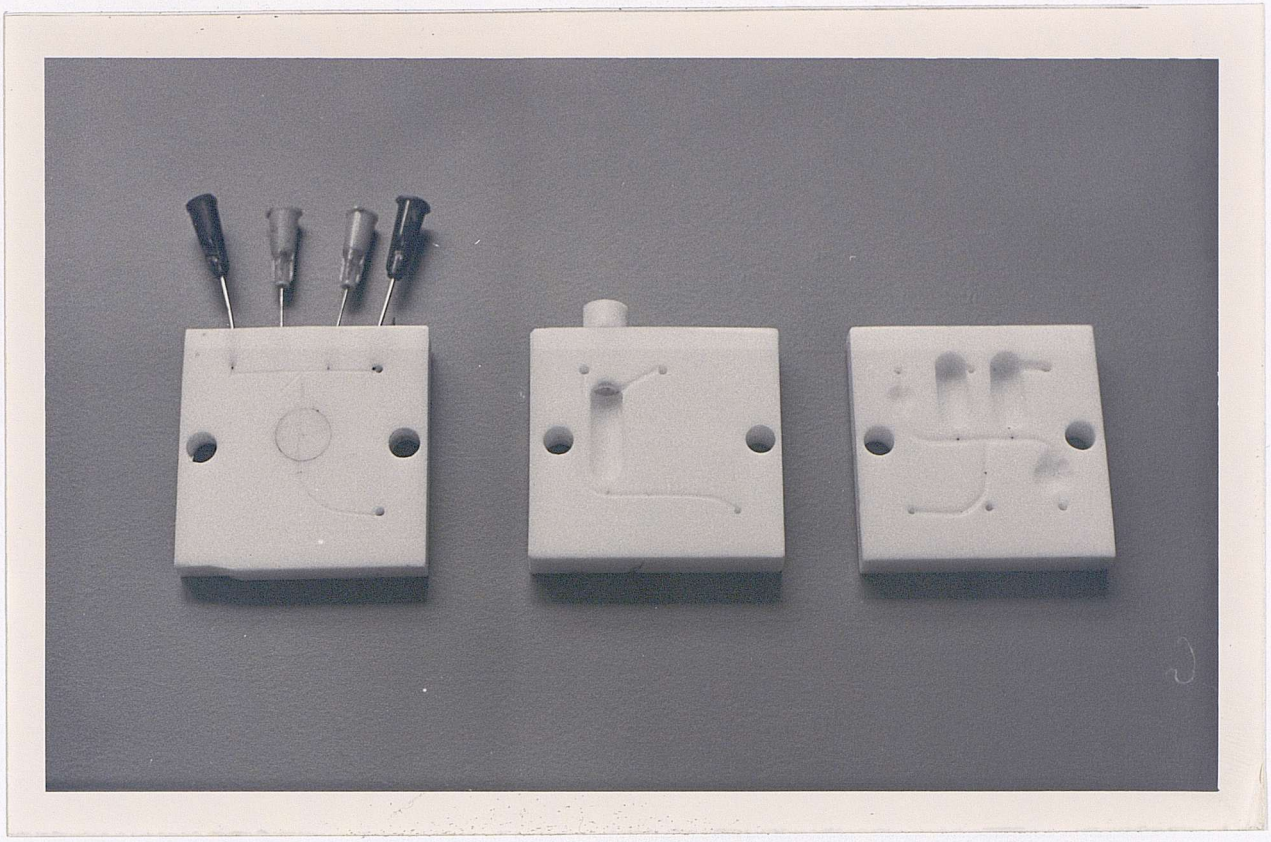


Plate 4 Alternative Blocks with Conduits for the Device.

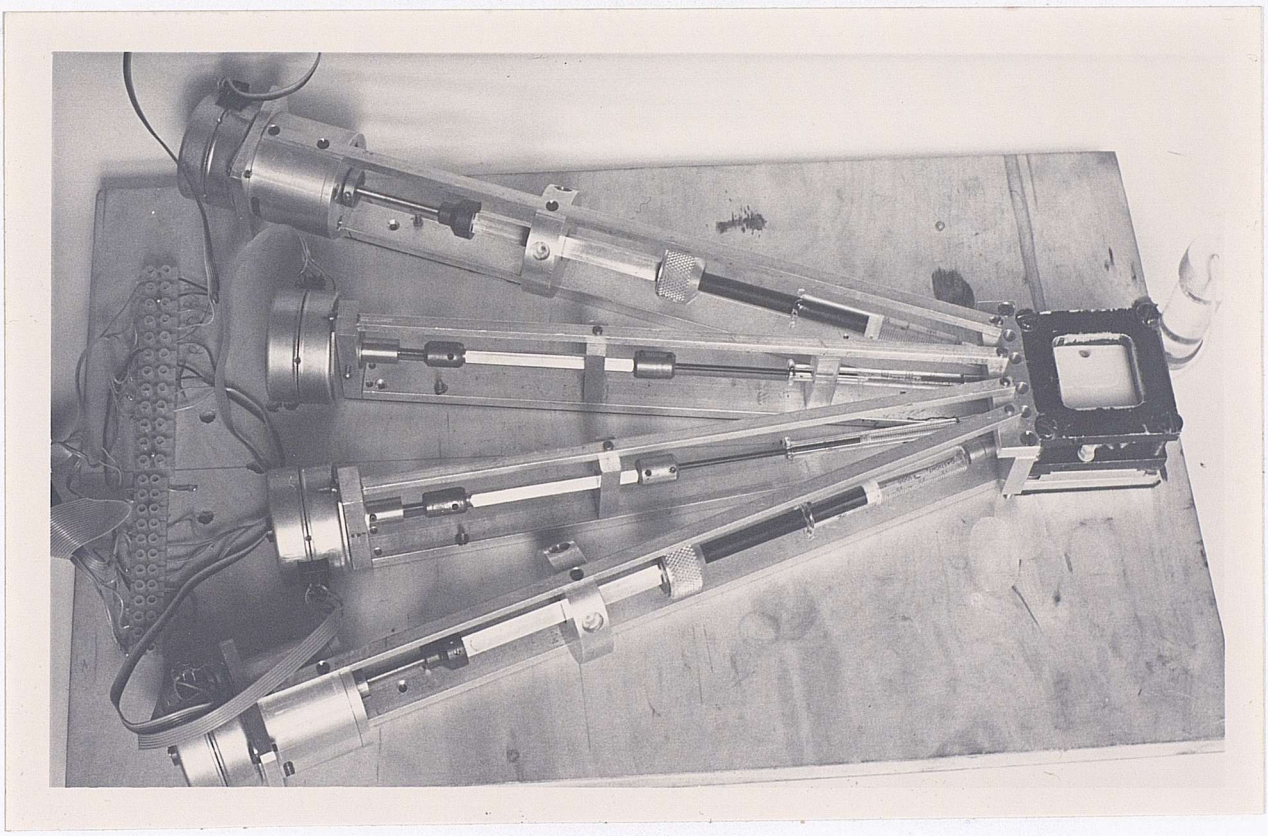


Plate 5. Embodiment II of the Device.

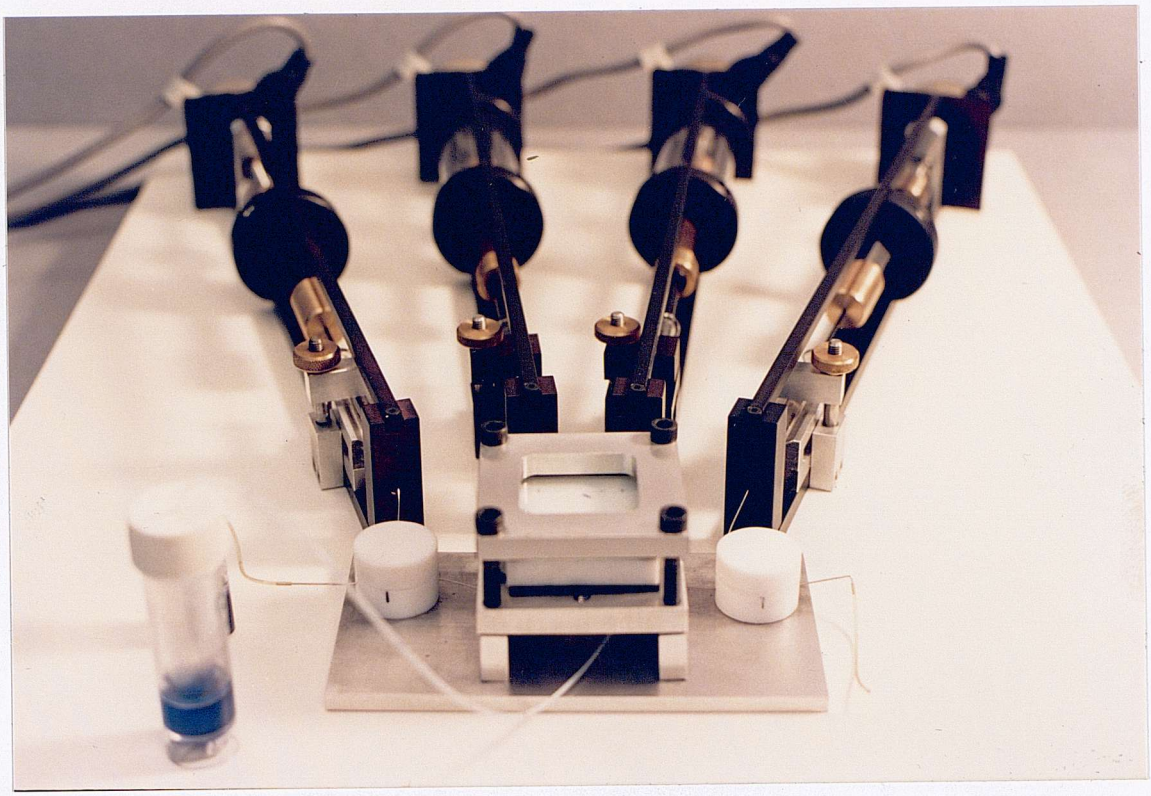


Plate 6. Embodiment III of the Device.

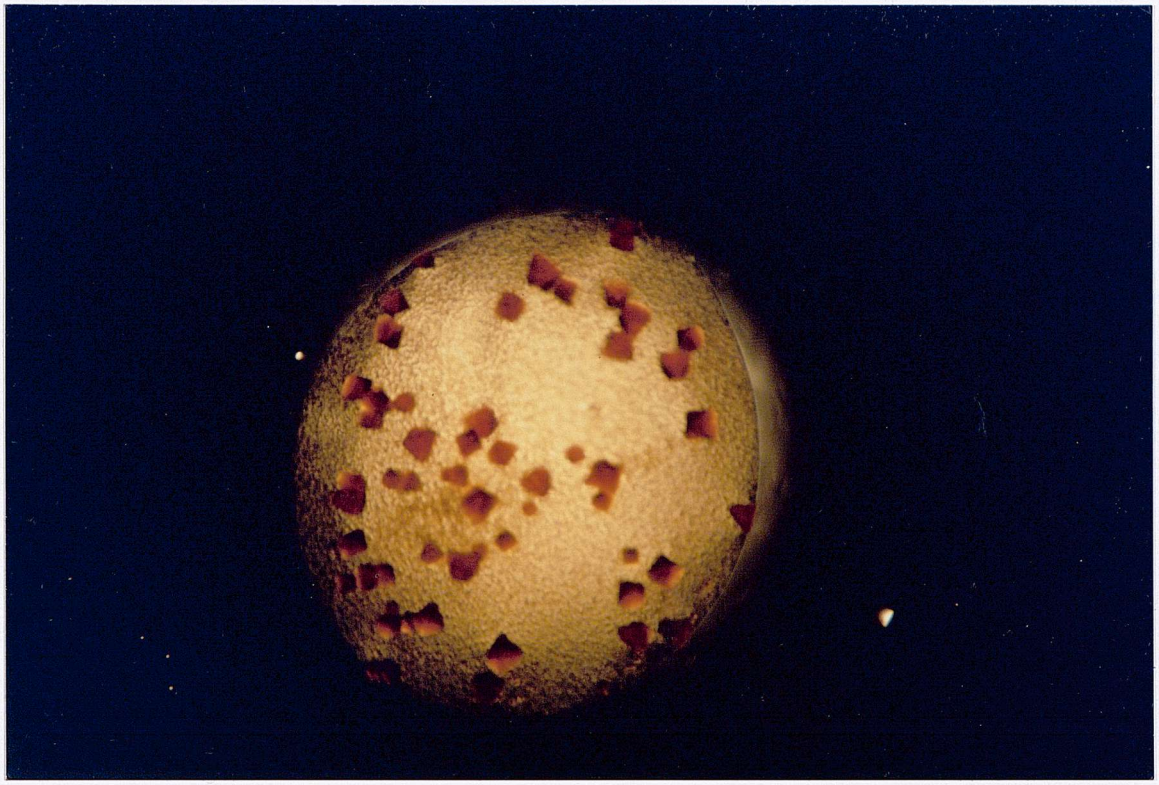


Plate 7 Crystals of Ferritin Produced Using the Device.

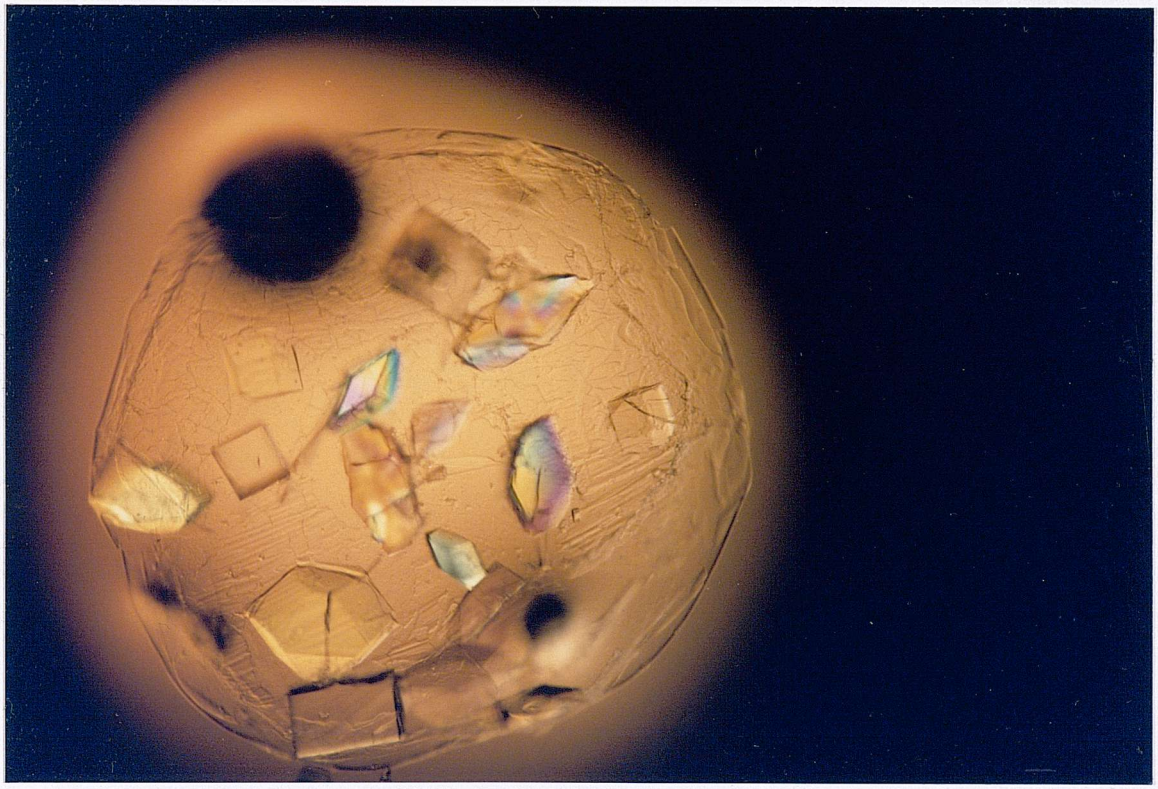


Plate 8. Crystals of Lysozyme produced using the Device.

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