Technical Report No. IRL 1082

CYTOCHEMICAL STUDIES OF PLANETARY MICROORGANISMS EXPLORATIONS IN EXOBIOLOGY

Status Report Covering Period April 1, 1968 to September 30, 1968
For
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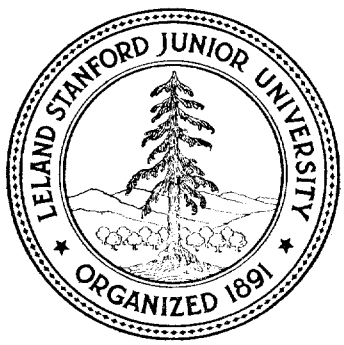

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Report to the National Aeronautics and Space Administration

"Cytochemical Studies of Planetary Microorganisms - Explorations in Exobiology"

NsG 81-60*

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A. INTRODUCTION

This Status Report covers the activities of the Instrumentation Research Laboratory from April 1, 1968 to September 30, 1968. Major technical efforts are described in separate technical reports and papers. The status report refers to these and summarizes continuing projects. Collaboration between the research carried out under this grant and other activities pointed out in the previous progress report has continued.

The general project areas of the program resume, part B of this status report, are:

I. Gas Chromatography and Optical Resolution
II. Mass Spectrometry
III. Computer Managed Instrumentation
IV. Cell Separator

During the six month period described above, ten papers were submitted or published in technical journals, one technical report was prepared and one paper was presented. A listing of these is included in this status report.

B. PROGRAM RESUME

I. Gas Chromatography and Optical Resolution

A. Gas Chromatography of Amino Acids

All the published literature and our previous work in this field (Tetrahedron Letters 319 (1968)) has always involved time consuming chemical manipulation (1-3 hrs) to convert the amino acids into volatile derivatives for gas liquid chromatographic analysis. We are now trying to obtain volatile amino acid derivatives by using the injector port of the gas chromatograph as a chemical reactor. Several chemical approaches are being investigated. The most promising system so far, uses amino acid derivatives which can be smoothly converted to the volatile alkyl esters in the injector port of the g.l.c. at 200°C. Preliminary work suggests that all neutral, acidic and basic amino acids can be volatilized this way, but some problems have been encountered with serine, threonine, cystine and proline.

During our work on amino acid analysis by g.l.c., we observed that amino acid benzyl esters can be transesterified with methanol or ethanol in the presence of a strong anion exchange resin at room temperature. Since in the "Merrifield Solid phase" peptide system the peptides are covalently bound to the polymer by a benzyl ester linkage, we have applied the transesterification procedure to the cleavage of peptides from the resin support. This procedure has now been used to remove several large peptides (up to octapeptides have been tried) from the inert support. The yields (approx. 60%) and the optical purity of the peptides (< 1% D) have been excellent, and in many cases the peptides have been obtained chromatographically pure. (For further details of the experimental procedure used see Tetrahedron Letters 5163 (1968)).

B. Sequence Analysis of Peptides by Mass Spectrometry

In continuation of our work aimed at the incorporation of chlorine into the N-terminal amino acid of the peptide chain by the NOCl reaction we have observed that the presence of a phenylalanine in the peptide results in the incorporation of a chlorine atom in the aromatic ring of the phenylalanine. Since the reaction conditions are quite mild (overnight at 5°C) we have examined the effect of this reagent on a series of cyclic peptides containing phenylalanine. In all the cases examined (c-gly-phe; c-leu-phe, c-phe-phe) analytically pure chlorinated products could be isolated. Finally the cyclic antibiotic Gramicidin "S" which contains 2 phenylalanine residues was treated in this fashion. An amino acid analysis of the crude product showed the absence of phenylalanine and the presence of a new amino acid which behaved as authentic p-chloro-phenylalanine on ion exchange chromatography.

C. Separation of Diastereoisomeric Compounds

G.L.C., N.M.R. and T.L.C. have been used to determine the steric purity and the absolute configuration of several diketopiperazines obtained from some microbiological sources. In all cases the compounds were shown to have the L-L configuration. In the case of echinulin, this conclusion is contrary to the published O.R.D. assignment, in which the tryptophan moiety was given the D-configuration. (This assignment has now been corrected to the L-configuration by the author.)

II. Mass Spectrometry

A. Analysis of Natural Products

Professor Djerassi's laboratory in the Department of Chemistry has yielded the results reported in the following papers:

Diekman, J.; Thomson, J. B.; and Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems. CLV. The Electron Impact Induced Fragmentations and Rearrangements of Some Trimethylsilyl Ethers of Aliphatic Glycols and Related Compounds. J. Org. Chem., 33, 2271 (1968).

Tokes, L.; Jones, G.; and Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems. CLXI. Elucidation of the Course of the Characteristic Ring D Fragmentation of Steroids. J. Am. Chem. Soc., 90, 5465 (1968).

Duffield, A. M.; Shapiro, R. H.: Mass Spectra of Quinoline and Isoquinoline N-Oxides. Tetrahedron, 24, 3139 (1968).

Buchardt, O.; Duffield, A. M.; and Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CLVII. A Study of the Fragmentation Processes of Some N-Acyl-2-indolinols Upon Electron Impact. Acta Chem. Scand., in press.

Duffield, A. M.; Djerassi, C.: Mass Spectrometry in Structural and Stereochemical Problems CLXVI. The Electron Impact Remoted Fragmentation of Some Aliphatic 1,2-Glycols. Org. Mass Spectry., in press.

B. Mass Spectral Microanalysis of Organic Solids.

The current study involves the use of a Bendix Time-of-Flight mass spectrometer to analyze the plume evolved from small portions of selected organic samples by incident focused ruby laser radiation.

Recent emphasis has been on the refinement of system operation technique with the goal of optimizing the reproducibility of results obtained with samples characterized by increasing variety and structural complexity.

The samples are generally deposited upon an oxidized stainless steel probe tip. The methods that have been used in placing the sample have involved passive deposition, dissolving in a solvent and then coating, and mixing in with sodium silicate and then coating. The latter has proved useful for securing in place classes of materials that would be expelled from the target area by the explosive expansion of gases prior to the attainment of an effective level of vaporization.

The reduction of raw data to a mass spectrum remains a manual operation. The mass spectrometer output signal is written on a CRO and photographed with Polaroid film. The combined CRO/film resolution capability and the writing format employed are such that it has not been satisfactory to write a mass range of more than 100 amu on a single firing. The attainment of a spectrum out to mass 400 has thus involved the taking of roughly 4 photographs, each entailing a different shot at a different spot on the target. The reading procedure involves certain subjective determinations that enable satisfactory though not complete recovery of the information content of the raw data. The mass spectrometer provides us with a complete mass spectrum every 100 microseconds. The vapor pressure of evolved material remains high enough to be detected for several hundred microseconds. The several spectral traces accompanying each shot are visually scanned and interpreted. The rigor of the reading procedure could be improved by devoting considerably greater time to the procedure, or to automating it. At present, approximately a half day is

required to make a single run. This sample rate does not conveniently lend itself to the class of experiments that are favored by the accumulation of extensive completely reduced spectra.

The reduced data are manually transcribed into a LINC computer for tape storage and bar graph plotting via a Cal-Comp Plotter. The spectra are also transmitted from the LINC to the ACME computer facility for disc storage and subsequent access for data manipulation and for computer driven "television" display and comparison of spectra.

Figure 1 is a full-sized reproduction of a Polaroid photograph of the "television" display of two spectra of the same sample of human red blood cells. The numbers 93 and 94, high and low, respectively, on the left hand side of the figure, are the spectrum identification numbers. The horizontal line across the center is the common base line for spectrum 93 displayed upward and 94 displayed downward. The mass range shown is 0-125 amu, as indicated by the numbers at both ends of the base line. Roughly 100 nanograms of material was vaporized for each of the shots associated with the spectra presented in this and the following figures.

In Figure 2 there is presented a comparison of two runs on the same sample of mouse lymphocytes. Figure 3 compares two runs on the same sample of human fibroblast cells. The spectra in Figures 1 and 3 are each characterized by an approximately 14 amu (CH_2) modulation on the signal strength. The fibroblast spectrum 100 and the red blood cell spectrum 94 are compared with one another in Figure 4.

An unsophisticated correlation program has been written and applied to the filed spectra as an initial effort to apply algorithmic procedures to the "finger print" comparison of spectra. The spectra are first normalized so that the sum of the peak heights is unity for all spectra:

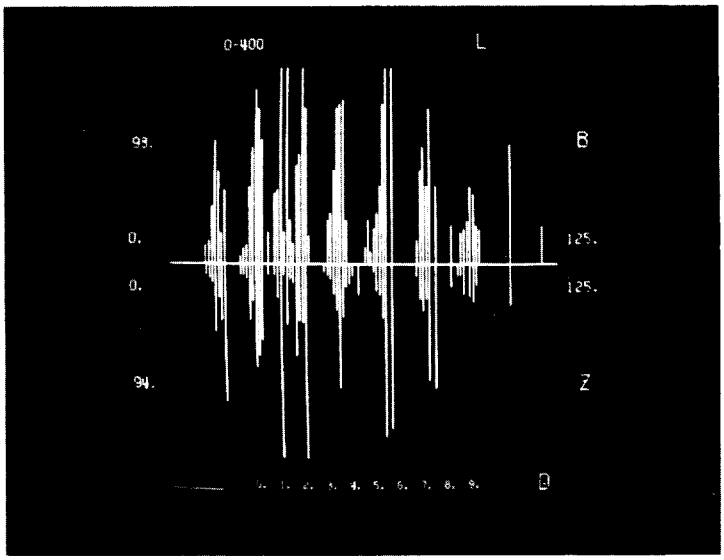


Figure 1. The spectra for two runs on the same sample of human red blood cells.

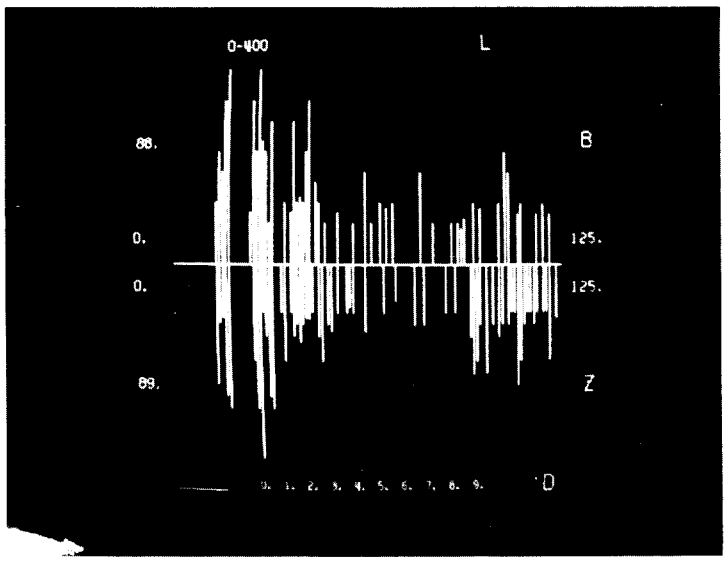


Figure 2. The spectra for two runs on the same sample of mouse lymphocytes.

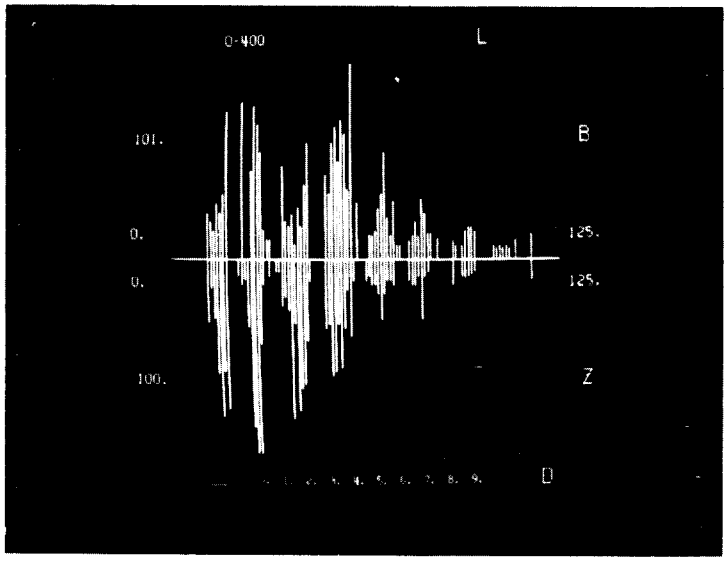


Figure 3. The spectra for two runs on the same sample of human fibroblast cells.

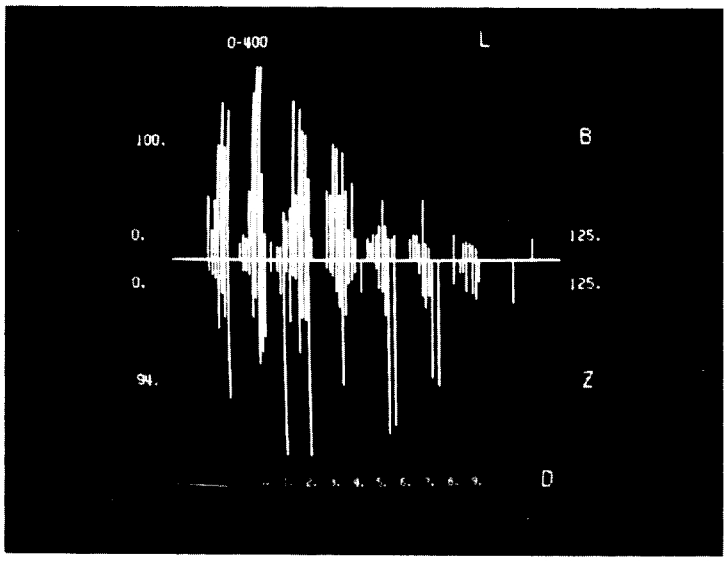


Figure 4. A comparison of human fibroblast cell spectrum 100 against human red blood cell spectrum 94.

$$\sum_{m=1}^{\infty} I_m(s) = 1,$$

wherein $I_m(s)$ denotes the normalized peak height corresponding to mass m in spectrum s. The degree of correlation of two spectra is then taken to be

$$C_1(p,q) = 100 \sum_{m=1}^{\infty} \min (I_m(p), I_m(q))$$

wherein the "min" function extracts the minimum value of the two signal intensities upon which it operates.

There are several factors that contribute "noise" corresponding to occasional ± 1 amu ambiguity in the mass determinations - above approximately 50 amu. These include flight time focusing uncertainties, multiply ionized heavier fragments (particularly doubly ionized odd masses), instrument instability, and reader error.

In an attempt to accommodate this noise, a second correlation procedure has been applied according to the following scheme. Above a selected mass number each normalized peak $I_m(s)$ is divided into three parts, two of which are displaced ± 1 amu. Thus, the modified portion of the new spectrum $I_m'(s)$ will have the general form

$$I_m'(s) = (1/3)(I_{m-1}(s) + I_m(s) + I_{m+1}(s)).$$

The correlation between two such modified spectra is referred to as $C_2(p,q)$.

The results of applying these correlation algorithms to a sequence of

filed spectra are presented in Tables I, II, and III. Table IV lists the sample descriptions associated with the spectrum numbers. The C_2 values have been derived by applying peak spreading to the full mass range.

It will be noted with reference to Table I that the red blood cell spectrum 94 correlates better with the other red blood cell spectra when peak spreading is involved. It is seen in Table II that spectra 87, 88, and 89 all of which are mouse lymphocytes correlate better in the peak spreading mode. Again, in Table III the fibroblast cell spectra 98, 99, 100, 101 correlate better in the peak spreading mode.

Table I also shows that the correlation algorithms, devoid of any sophistication, readily distinguish the fibroblast spectrum 100 from the red blood cell spectrum 94, which are compared with one another in Figure 4.

These data support the postulate that laser vaporization of organic material can sufficiently volatilize samples to give mass spectra while preserving a measure of the original structural information.

In connection with these efforts some software has been developed of general utility and made available to all ACME (Advanced Computer for Medical Research) users.

Two programs relating to the solution of ordinary differential equations by the Runge-Kutta procedure have been updated for inclusion into the ACME public file.

An author, title, key word store-search program has been written for information filing and reference retrieval purposes.

Two programs have been written and made available on the ACME public

file for application to user programs to achieve cleaning up, nested loop indention, and label and procedure identification in accordance with a standard format.

Table I

Spectrum 94 compared with spectra 87 through 101

Spectrum	C_1	Spectrum	C_2
94	100	94	100
93	75	93	83
91	67	91	79
101	58	95	70
100	56	101	67
98	54	100	63
95	53	98	61
99	45	88	55
88	43	99	53
87	42	96	52
89	37	87	51
96	33	92	47
92	26	89	46
97	20	97	28

Table II

Spectrum 88 compared with spectra 87 through 101

Spectrum	C_1	Spectrum	C_2
88	100	88	100
89	50	87	65
100	49	89	65
87	47	95	59
98	46	100	58
91	46	91	56
94	43	94	55
101	43	93	54
95	43	98	54
93	42	101	53
99	40	99	51
92	29	92	44
97	27	96	40
96	26	97	32

Table III

Spectrum 101 comared with spectra 87 through 101

Spectrum	C_1	Spectrum	C_2
101	100	101	100
100	70	98	76
98	70	100	74
93	63	99	72
99	60	93	69
91	60	94	67
94	58	91	66
95	53	95	64
88	43	90	55
90	43	87	54
87	42	88	53
89	40	96	53
92	36	92	52
96	31	89	47
97	30	97	26

Table IV

Spectrum	Description
87	mouse lymphocytes
88	mouse lymphocytes
89	mouse lymphocytes
91	human red blood cells
92	mouse lymphocytes
93	human red blood cells
94	human red blood cells
95	human red blood cells
96	human hair
97	human hair
98	human fibroblast cells
99	human fibroblast cells
100	human fibroblast cells
101	human fibroblast cells

III. Computer Managed Instrumentation

A. ACME, IBM 1800 development

ACME's main processor is an IBM 360/50, used in a time shared mode. One of the two main input/output channels for instrumentation data is a slave IBM 1800.

The IRL staff has spent a great deal of time assisting ACME personnel with debugging and evaluating the 1800 time shared data gathering system. Our involvement is the result of several factors: interest in using the 1800 for controlling the Finnigan Mass Spectrometer (see Section III, D), in particular and other instruments in general, and the availability of our LINC computer for generating test data and exercising control capabilities. Certain input-output functions appeared to work under limited data rates. However, interference between users was experienced as well as difficulties handling several buffer loads of data in rapid succession.

More development is required to make this system useful for most of our purposes. Discussions are regularly being held with the ACME staff and progress is being made to arrive at the results we desire.

B. 270X-270Y ACME Computer Connections

The 270X-Y is the other general access mode to the ACME 360/50 for digital instrumentation data. It was developed for higher speed, remote data transfers to and from ACME. The last technical report gave details on this system.

During the summer of 1968, IBM personnel did achieve "fixes" that have resulted in relatively trouble-free operation of the 270X-Y system. Troubles, when they do now rarely occur, seem to be of local user's

origin and do not cause interference with the other users of the time share ACME system.

The channel restraints remain essentially as reported last time. These concern large, over 64,000 byte, data transfers and relatively slow, 50 millisecond, channel turn around time (from write to read or vice versa). The 270X-Y system is now routinely used for three purposes:

1. Interconnection of the IRL's LINC computer with ACME. (This 270Y is manually multiplexed for other laboratory uses, one being plotting.)
2. Connection of the Genetics Department's Saunders character Display system with ACME.
3. Development of the computer connection of the high resolution mass spectrometer, the AEI MS-9, with the time shared ACME computer.

A fourth 270Y connection and unit is as yet uncommitted.

C. Mass Spectrometer-Computer Instrumentation

A survey of the various mass spectrometers involved and the instrumentation approaches involved may be found on page 26 of the prior status report, IRL 1076. Details on the subsequent development are given in the following paragraphs.

D. GLC/MS Computer Systems

The development of an ACME 360 computer control system for the Finnigan mass spectrometer has become somewhat more involved than the straightforward concept of the last status report. Now most of our computational equipment has become involved in this project in some way.

The existing computer control system has been well documented in IRL 1062. In that system instrument control was exercised by the LINC

computer: valving in a reference gas, performing a calibration, controlling the mass spectrometer while a spectrum of the unknown sample is taken, and finally processing the data for online presentation to the researcher. However due to the initial programming and hardware considerations, the mass range was limited to m/e 256. The connection is diagrammed in Figure 5a.

It was decided that a mass range of 500 was required and to explore the application of time shared computation to this problem. Initially we desired to also check the capability of the 270X-Y to the application. This would be the connection of Figure 5b. However the inability of the 360/50, via the 270X-Y, to communicate efficiently with the mass spectrometer in the alternating, single word, read-write mode required became apparent. See Section III, B, pages 23 and 24 in the previous status report IRL 1076 and Section III, B of this report. Two 270Y's could be employed, but changes would be necessary in the ACME operating system to concurrently use two 270Y's. The 270Y adaptor needed would require extensive development. Instead a different approach was conceived.

The IBM 1800 channel could be employed. The 1800 has analog inputs, and a wider word, 16 bits. Both of these characteristics would allow simplification of the adaptor. In this concept the 1800 would transmit control data from the 360/50 to the mass spectrometer interface and performs the A to D conversion of the output of the mass spectrometer. This is diagrammed in Figure 5c. In order to evaluate the IBM 1800 capabilities for this purpose we designed a use of the LINC to simulate either of the foregoing more sophisticated systems (1800 or double 270Y).

This present system is diagrammed in Figure 5d. It has the advantage that the hardware is built, and is working. Also, it follows from the general purpose syntax of the ACME PL/1 input output command (for the 1800 and 270Y), that an ACME program written for the LINC simulator

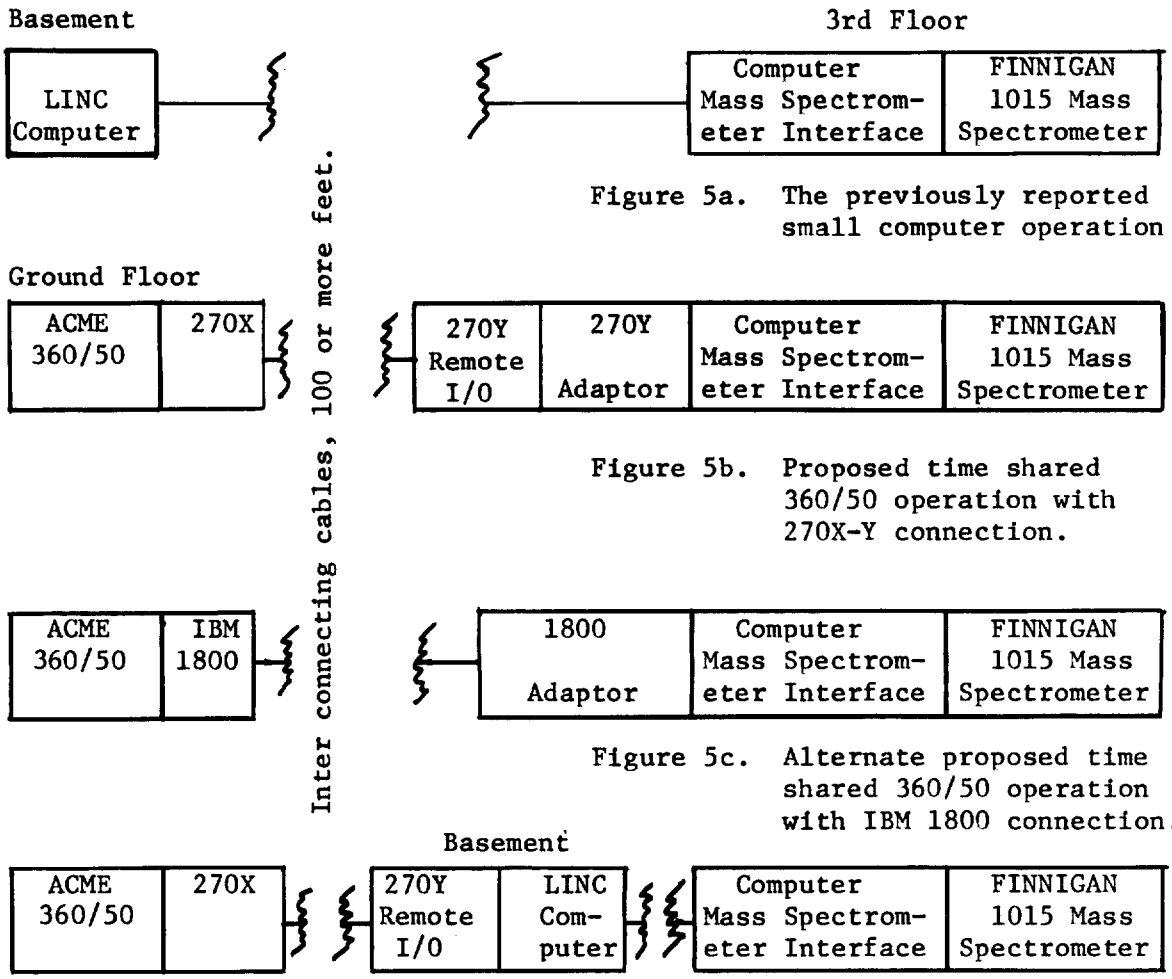


Figure 5a. The previously reported small computer operation

Figure 5b. Proposed time shared 360/50 operation with 270X-Y connection.

Figure 5c. Alternate proposed time shared 360/50 operation with IBM 1800 connection.

Figure 5d. Connection of existing hardware to simulate b or c above. Or, by more extensive LINC programming it may act as a satellite computer with a central large computer system.

Figure 5. Existing and proposed methods of computer control and data acquisition from the Finnigan 1015 quadrupole mass spectrometer.

system may be employed in either the system of Figure 5b or c. This is true since the LINC is simply programmed to simulate an adaptor to the computer interface.

Currently about 70% of the PL/1 coding has been written for this ACME implemented control system, utilizing the LINC as an adaptor simulator. However, needed changes in the LINC-mass spectrometer interface have delayed until recently the online debugging of much of this coding. This need for debugging, as well as completion of coding, leads to an estimate of 50% completion of the software system.

If the time-shared system is successful, and the IBM 1800 channel becomes operational or the dual 270Y channel is implemented, the appropriate adaptor may be built and the LINC removed from the operation. The LINC then could be used for other applications.

Conversely, if direct time-shared operation is proven unworkable or impractical, the system may be reprogrammed to allow joint computer action; the LINC computer may be programmed as a satellite computer.

There are indications that the slow response time of ACME 360/50 system may be somewhat of an inconvenience to the MS operator. The data collections and presentation functions appear to take two to four times as long (depending on the ACME system load) as they did on the LINC based system. However this estimate is based on rather limited experience and with programs which could be made more efficient with more attention to detail.

The evaluation of the ACME 360/50 system using PL/1 programming in a time-shared environment will certainly be very important to the further design concepts of computer systems for laboratory support. When this evaluation with the present hardware is possible, i.e. the system works, a three-way comparison may be made. These will be:

a. A stand alone small computer.
b. A time-shared large central computer.
c. A system of one large time-shared computer with satellite small laboratory computers.

The application, the data rates and conditions, are quite typical of the average laboratory instrumentation need.

E. Bendix TOF

There has been no change in the basic data acquisition system used. The previously described system (IRL 1063) with the LINC computer remains the basic data reduction vehicle.

F. Atlas CH-4

No direct development has been done on the Atlas CH-4 during this period. The system demonstrated and reported in the last status report consisting of a voltage to current transmission system and reception at a remote site. Then data reduction was by the "BENDIX" system at the LINC computer. This has not been practical as an operational system. The access to the LINC was limited and the physical locations further complicated matters. However, the method worked and a similar approach is anticipated with the ACME time shared system and the CalComp plotter which are accessible to the CH-4 Chemistry Department users. These CH-4 plans are dependent upon MS-9 developments (see the next paragraph).

The Atlas CH-4 and another Finnigan quadrupole are in the same room with the MS-9. This is in the Chemistry Building and about 1500 feet cable distance from the ACME computer. The MS-9 instrumentation is more than a special purpose interface: By its very nature, portions of it constitute a rather general purpose data input station. When the particular problems of the MS-9 are solved it is expected to make use of its data station capability and automate the data acquisition for all

three instruments with suitable PL/1 ACME programs.

G. MS-9

With the advent of reliable 270Y data transmission in September the MS-9 interface could be exercised as a complete system. Complete low resolution data runs were accomplished on September 13, 1968.

Initial data acquisition runs furnished sufficient operating data to progress on to the next necessary steps. These consist primarily in development of peak position identification procedure routines and a study of the function (mass position - function of time) for this particular instrument. Signal level amplitudes have been observed and recorded whose values are necessary to the design of thresholding and/or data compression which may be necessary.

Some interesting results have already been apparent. A theoretical model of peak shape is emerging that seems to be unique and not reported in the literature. Work is progressing on this, and if it indeed is successful, it may allow better estimates of peak position from digital data.

Good analog signal-to-noise results have been obtained with the new electrometer installed by this laboratory. The signals from single ions have a signal height of 2 to 6 times the peak to peak heights of coherent noises (i.e., 60 hz and induced noises of the digital interface). The borad band Johnson noise is less, perhaps half the coherent noise. Since a minimum of 10 ions is usually considered necessary to obtain useful peak information, this does suggest the system does have ample operational analog signal to noise margin.

However the installation of the new electrometer has interfered with some of the existing modes of operation. The existing electronics is

so marginal and so complexly designed that it is difficult to attach anything, or take a signal out, without some degration of existing functions or rebuilding much of the existing electronics console.

Programming of the system has progressed through the data acquisition and data filing system (a number of initial runs are now filed, accessible by ACME PL/1 programs). Peak finding and portions of the analysis system have been written.

Scan synchronization has been accomplished, but at the end of this reporting period a digital interference has developed that has not yet been solved.

The interface uses resistor-transistor-logic (RTL) micrologic extensively. RTL has been found to have only marginal digital noise immunity. This project, and others lately completed, have caused this laboratory to reconsider the choice of RTL vs transistor-transistor-logic (TTL) micrologic. TTL, while 50 to 100% more costly in components, promises to be more reliable.

The MS-9 developments, up to the original design goals, have been within the proposed budget. It is now apparent that additional work would be desirable.

1. a. Added digital capability to threshold signal and condense data points that are below threshold. This would greatly conserve the ACME 360/50 memory requirements.
   b. Digital capability to recognize missed data points (caused by the time shared computer environment) and preserve data integrity in such a case.
   (This latter would be a new and unique development in the field of time shared computer data acquisitions.)
2. Extend the computer adaptability that exists to other mass spectrometers and analysis instruments in the immediate area.

H. Computer Manipulation of Chemical Hypotheses

This work is covered in two papers: "Applications of Artificial Intelligence for Chemical Inference I. The Number of Possible Organic Compounds: Acyclic Structures Containing C, H, O and N." by J. Lederberg, G. L. Sutherland, B. G. Buchanan, E. A. Feigenbaum, A. V. Robertson, A. M. Duffield and Carl Djerassi; and "Applications of Artificial Intelligence for Chemical Inference II. Interpretation of Low Resolution Mass Spectra of Ketones" by A. M. Duffield, A. V. Robertson, Carl Djerassi, B. G. Buchanan, G. L. Sutherland, E. A. Feigenbaum and J. Lederberg, submitted to the Journal of the American Chemical Society for publication.

The first paper describes the use of the computer program DENDRAL in constructing the total number of possible acyclic structures of C, H, N and O. Those structures containing either chemical absurdities or undesired functional groups are not constructed if these substructures are explicitly listed. Conversely, if it is desired to restrict the output to any functional group(s) then this can be accomplished. Examples of the linear notation used are given. Semilog plots of total numbers of isomers vs carbon content for selected compositions summarize the results. Some broader implications of the program are discussed which forms the basis for the computer aided interpretation of mass spectra to be reported in subsequent articles from our laboratories.

The second paper presents a general approach to computer interpretation of the mass spectra of aliphatic ketones. Given the low resolution mass spectrum plus the composition of the molecular ion, the computer program decides upon the most probable structure(s) consistent with the unknown's mass spectrum. The program makes extensive use of the DENDRAL ALGORITHM.

This research was financed by the Advanced Research Projects Agency of the Office of the Secretary of Defense (Grant SD-183), the National

Institutes of Health (Grants GM-11309 and AM-04257), in addition to receiving support from this National Aeronautics and Space Administration grant.

IV. Cell Separation

A. IBM Cell Separator

We have continued work on application of the IBM rapid cell spectrophotometer to fluorochromasia, as discussed in the previous progress report.

Cell suspensions used for this work included rat thoracic duct lymphocytes, mixed mouse spleen cells (including lymphocytes and red blood cells), ascites tumor cells, and cultured chinese hamster ovarian cells.

Fluorochromasia was developed by adding a small amount (usually 2 microliter per ml) of 0.5% FDA in acetone to the cell suspension, allowing to incubate, diluting to an appropriate concentration with normal saline, Eagles, Hanks, or other appropriate media, and passing the suspension through the instrument. The current pulses caused by passage of cells through the field of view were amplified and displayed on the X-Y oscilloscope built into the instrument. They were also displayed separately on a dual trace oscilloscope, and counted with a Beckman EPUT meter.

Experimental results

1. Flow rate

The average flow rate was about 0.005 ml/sec but it varied from a low of about 0.0038 ml/sec to a high of about 0.0062. Momentary obstruction in the channel caused even larger short term changes. If accurate count rates were needed a volumetric measurement of flow rate would be required. In addition, in some older samples of large

cells count rate decreased with time by as much as 20%/minute, probably because of settling. Thus continuous stirring or agitation would also be desirable.

2. Signal to noise ratios and counting accuracy

The accuracy of the method depends on the ability to discriminate between voltage pulses caused by passage of cells through the field, and those from random noise or from passage of other particles. If cell signals are all about the same amplitude and are much greater than those from the latter causes the cell count should stay relatively constant over a relatively wide range in the level of the voltage above which pulses are counted. ("Threshold level") This was not the case for the scatter channel in these experiments, as shown by the curves of Figure 6, showing relative scatter count as a function of threshold level. It can be seen that the scatter signals varied greatly in amplitude for a given cell population. This is probably partially due to variations in cell size. It may also reflect different scatter from similar cells, particularly if they pass through different parts of the channel.

The variation was greatest for the small cells (curves 1, 2 and 3) which gave no indication of the existence of a plateau in cell count as threshold level was changed. However the counts at minimum threshold - a level such that the scatter count without flow through the channel was close to but not quite zero - was within about ± 30% of the expected number as calculated from the flow rate and the concentration measured by hemocytometer or Coulter counter. It appears that small lymphocytes and red blood cells are close to the lower size limit for reliable detection by scatter in this instrument using the 100 μ channels available. Smaller channels should permit detection of smaller cells.

Fluorescent channel noise varied with the experimental conditions. This is a result of the fact that the noise is proportional to the square root of the light level, and the light level depends on the total amount of free fluorescein in the solution. Here again use of a smaller channel

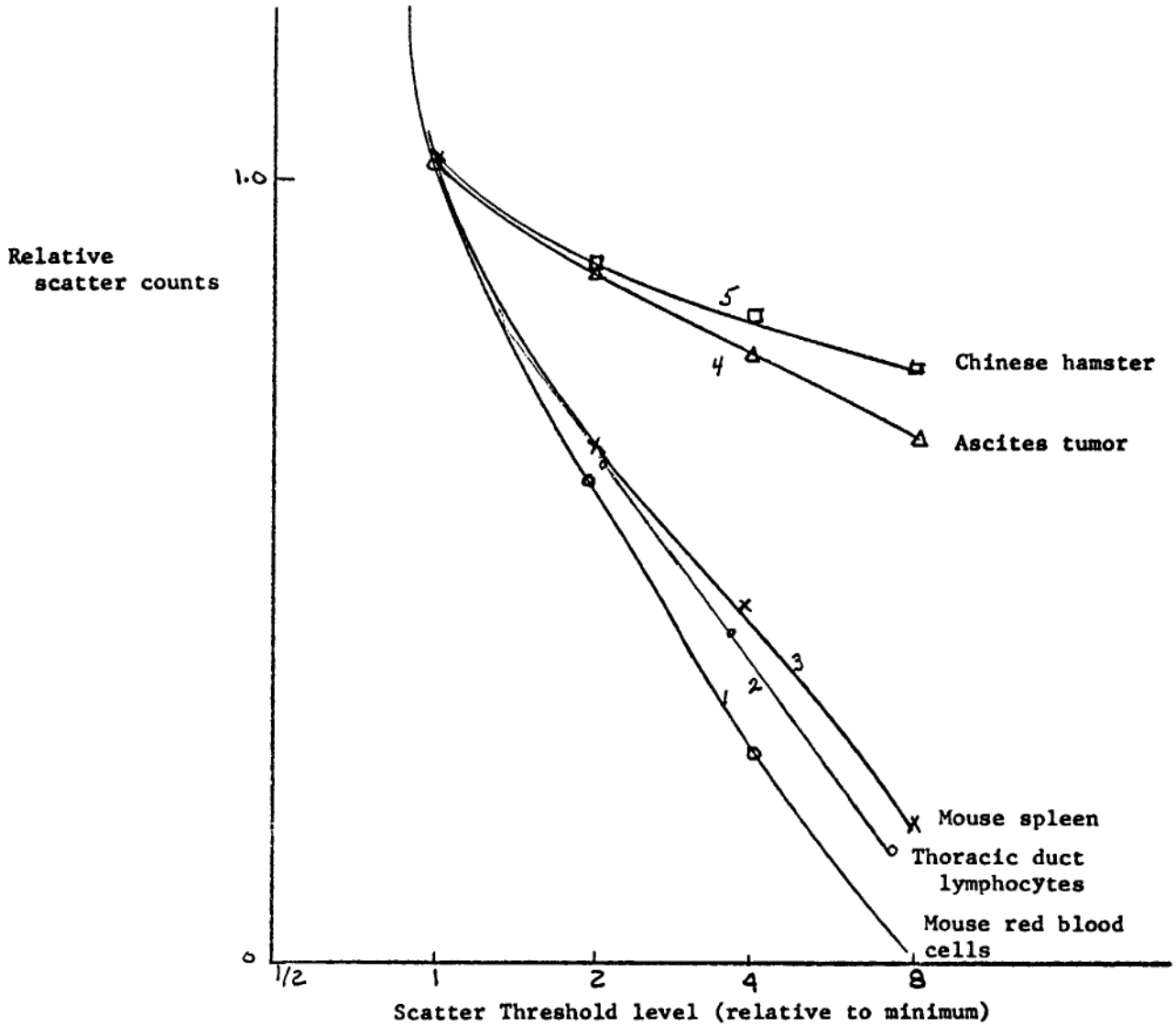


Figure 6. **Scatter count as a function of scatter threshold level.**

would be desirable. Reducing channel size from 100 to 50 μ should improve S/N ratio by about $\sqrt{8}$. In addition it should permit use of higher cell concentrations, more nearly reproducing physiological conditions. We have done some work on production of smaller channels but no usable ones have been produced as yet.

In no case did all the cells counted in the scatter channel give fluorescent signals above the noise. However in many cases, as shown in Figure 7, curves 1 and 2, nearly all the fluorescent cells gave quite high signals indicating that those cells not fluorescing were qualitatively different from those that did. In most small cell suspension (curve 3, Figure 7) the variation in fluorescent signal was similar to the variation in scatter signal.

2. Effects of dilution

The scatter count was always relatively higher at low concentrations. Some of this increase was caused by scatter from particles in the suspending medium, even after filtering through 0.2 μ filters. As a result all counts had to be corrected for this background. In addition relative count at higher levels was lowered because coincident passage of two or more cells through the field of view results in a single count. However in some cases, the count at concentrations below about 50,000 cells per ml was much higher than could be accounted for by these effects. This problem could be remedied by adding low concentrations of fetal calf serum (about 0.05 to 0.5%). Probably these cells were breaking up at extreme dilution, giving large numbers of scattering fragments, and the protein in the fetal calf serum prevented this. Another effect should be noted - large scatter counts were observed where the sample run through the machine was not diluted after the original treatment with FDA. This was apparently caused by the presence of small crystals of FDA, which is very sparingly soluble in water. To prevent this problem the FDA should be added to concentrated samples and then diluted to a concentration of about 1 part in ten million or less before running through the unit.

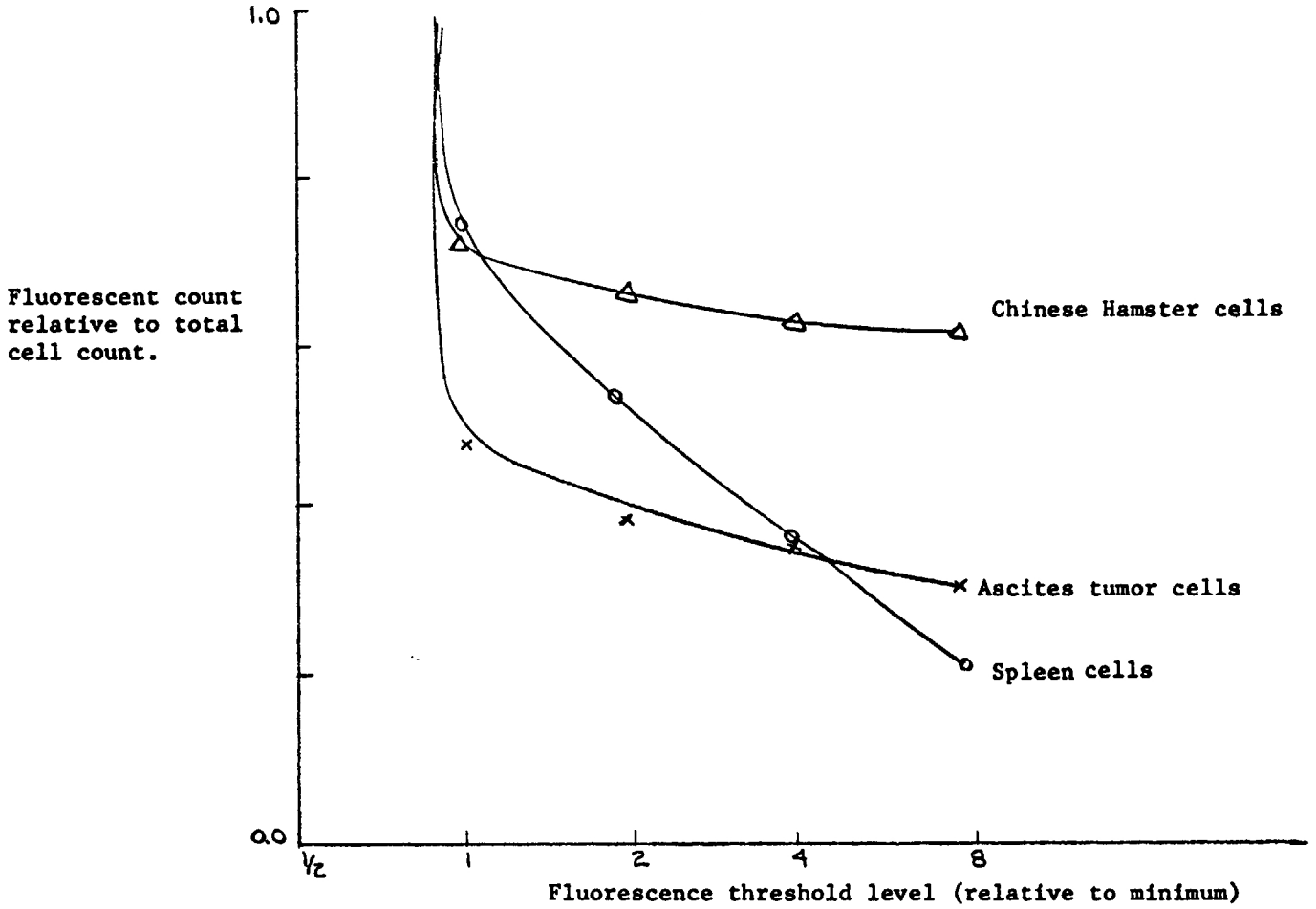


Figure 7. Fluorescent counts as a function of fluorescence threshold level.

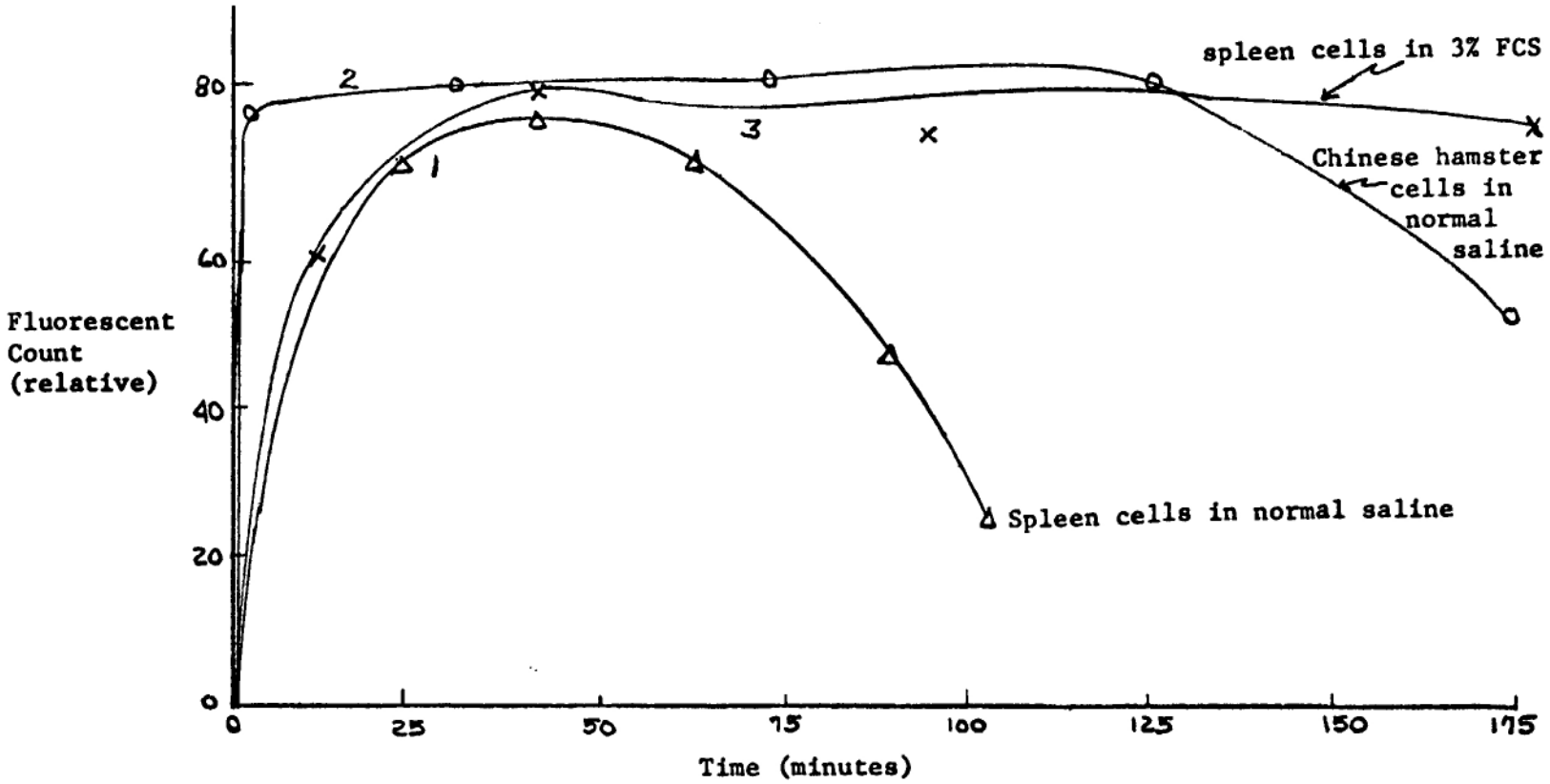


Figure 8. Fluorescent count as a function of time

3. Time course of fluorochromatic reaction

As shown in Figure 8, the incubation period necessary for maximum development of fluorochromasia was only a few minutes for large cells, up to 30 minutes for small ones. Decay was more rapid in the small cells with a rather sharp maximum (Curve 1, Figure 8) compared to a long flat maximum in samples of large cells (Curve 2, Figure 8). This decay time could be varied by changing the suspending medium. Decay was much slower in solutions containing a few percent of fetal calf serum (Curve 3, Figure 8).

Decay was more rapid in suspensions which had been centrifuged after development of fluorochromasia. There is probably a balance between FDA uptake, hydrolysis, and passage of fluorescein back across the cell wall which is destroyed when FDA is removed from the suspending medium.

4. Relation to viability

In order to exhibit fluorochromasia cells must possess a mechanism for transport of the fluorescein into the cell, esterases capable of splitting the FDA, and an intact cell membrane to prevent rapid leakage of internal fluorescein out of the cell.

Viable cells containing nuclei seem to fulfill these requirements, as evidenced by the fact that of all the cells tested, only red blood cells did not become fluorochromatic under appropriate conditions. However, this does not mean that all fluorochromatic cells are viable. Viability is usually considered synonymous with ability to replicate. It is apparent that cells considered nonviable under this definition could become fluorochromatic if the basic requirements listed above are fulfilled. Thus chinese hamster cells given doses of radiation sufficient to prevent reproduction in 99% of the cells were perfectly competent with respect to fluorochromasia. The same condition held with respect to incubation in alcohol, in acetic acid, and at high temperature. Figure 9 shows the effect of the latter on fluorochromasia.

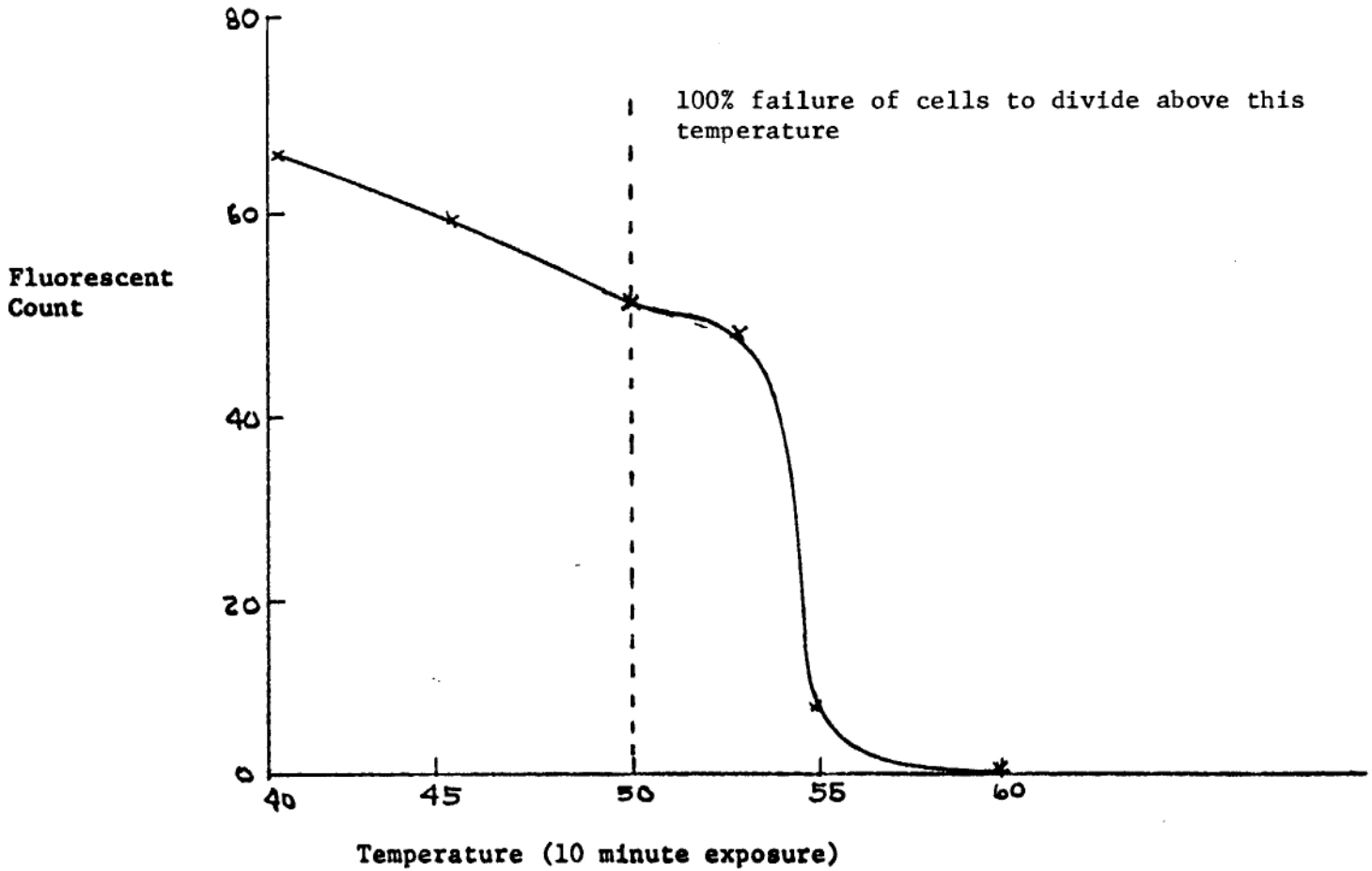


Figure 9. **Fluorescent count as a function of incubation temperature.**

Heat treatment for ten minutes at 50°C was sufficient to prevent reproduction but even at 53° most of the cells still exhibited fluorochromasia. It is interesting to note that after treatment at 55° the hydrolysis of the FDA is as rapid as before, indicating that the enzymes participating in the reaction are fully active after the treatment. Probably the cell wall has been damaged.

Effect on viability

Tests on plaque forming ability of chinese hamster cells which had been treated with FDA and run through the unit showed that the treatment had little effect on viability. Radioactive uridine uptake measurements on thoracic duct lymphocytes after FDA treatment were inconclusive, but there may have been some loss of viability as a result of the exposure to FDA.

B. Applications of fluorochromatic assay

A fluorochromatic cytotoxicity assay, developed by Bodmer, Tripp and Bodmer (3) appears to be adaptable to automation using this instrument. The assay depends on the fact that intact cell membranes are needed to develop fluorochromasia. It determines the fraction of human leucocytes whose ability to develop fluorochromasia remains after treatment with one of a number of different sera in the presence of complement. It thus gives evidence on the immunological compatibility (or lack thereof) of the specimen and the serum with which it is treated. It is desirable to check many samples, which currently requires counting of fluorescent cells on a number of microscope slides for each specimen, a procedure which would be very time consuming on a large scale.

Since sera taken at different times may need to be checked against the sample, current practice is to add FDA to the sample, (in 3% bovine serum albumin) allow fluorochromasia to develop, centrifuge to remove excess FDA, and keep at liquid nitrogen temperatures until used. The

cells are incubated with serum and complement at room temperature for two or three hours. Thus it appeared desirable to compare the performance of the instrument on fresh specimens with that on cells which had been frozen

Results are shown in Table V.

Table V

Effect of freezing, incubation, and restaining on fluorochromasia of human leucocytes

Sample		Fresh	Frozen and Thawed				
			Immediate	Incubated		Stained after 5 hours	
				2 hrs	4 hrs	Previously FDA treated	Not previously treated
H	1	74	56	23	9	52	66
G	2	71	66	23	15	52	54
P	3	80	66	39	14	65	77

As indicated by columns 2 and 3 the fluorochromatic count immediately after thawing was of the same order as that when freshly stained. However when incubated at room temperature for several hours the count was much lower (columns 4 and 5). It was possible to bring the count back close to its original value by another FDA treatment (columns 6 and 7).

Since the effect of incompatible serum in the manual test is to reduce the count to a small fraction of its initial value it appears that this instrument should be sensitive and accurate enough for the assay, particularly since comparison can be made between treated samples and controls containing the same cells but no serum or complement. Further testing of treated cells exposed to serum and complement will be done in the near future. If these tests are successful, design changes will be initiated in the sample collection portion of the instrument to permit better adaptation to the small volumes and large numbers of samples required in the tests.

Other applications

The instrument is also being used to investigate heparin in mast cells in conjunction with Dr. A. M. Saunders (4). Heparin is a high molecular weight polysaccaride containing carboxyl and sulfate groups and thus binds to basic dyes such as acridine orange. The binding depends on ionic strength, and the fluorescent spectrum of the resulting complex is affected by the extent of binding. Quantitative measurements of the relationship between fluorescence, cell size, and ionic strength should provide information on the characteristics of the heparin at various stages in cell life.

Initial tests showed that where proper filters and a red sensitive photomultiplier tube were used the system could detect the acridine orange stained mast cells. However there was some doubt about the quantitative relationships between cell size and fluorescence and the photomultiplier output signals in the scatter and fluorescence channels. Polystyrene resin beads used in ion exchange columns are spherical, the smallest beads are of the same order of size as the cells, and such beads are fluorescent and can be stained by fluorescent dyes. Thus they should be usable to provide reference signals. Small bead fractions were obtained by allowing -400 mesh samples to settle for periods of several minutes and retaining the supernatant. The beads were stained and passed through the channel. Good signal to noise ratios were obtained. We are presently attempting to correlate the signal output with the size. Results appear to be reproducible, with the fluorescence of stained beads appearing to depend on the square of the diameter. This is similar to that found in tests using a microscope equipped with a photomultiplier, where the fluorescent signals from individual particles mounted on microscope slides were compared with the measured sizes. It thus appears that the quantitative relationships of signal to size are maintained in the system.

Further experiments with the stained mast cells will be conducted soon.

References

1. L. A. Kamentsky, M. R. Melamed and H. Dermean, Science 150, 630, 1965.
2. B. Rotman and B. W. Papermaster, Proc. Natl. Acad. Sci. 55, 134, 1966.
3. W. Bodmer, M. Tripp and J. Bodmer, Histocompatibility Testing 341, 1967.
4. S. L. Meyer and A. M. Saunders, Analytical Biochemistry 22, 493, 1968.

C. Volumetric Cell Separator

1. Introduction

We are continuing the development of a high speed volumetric cell or particle separator as has been reported in technical reports IRL 1056, 1061 and 1076.

Certain modifications have been incorporated in the apparatus in order to facilitate easier handling of the solutions and operation of the apparatus.

We are presently conducting preliminary biological experiments to validate the performance of the apparatus. Preliminary indications show that separation of cells according to size can be achieved; however the separated cells tend to clump thus limiting, at the present time, culturing these separated cells for biological experimentation. It is believed that due to the low concentration of cells in the experimental sample, cells die, releasing protein molecules and consequently the intact cells tend to attach to these molecules forming clumps. These cell clumps are more easily observable in the separated sample due to the high concentration of cells.

2. Present Configuration

As has been reported previously the high speed volumetric separator can be divided into the following functional blocks.

1. Reservoir and filtering
2. Detection Cavity
3. Drop Generation System
4. Signal Amplification and Discrimination
5. Digital Signal Derivation and Delay
6. Charging and Deflection
7. Collection

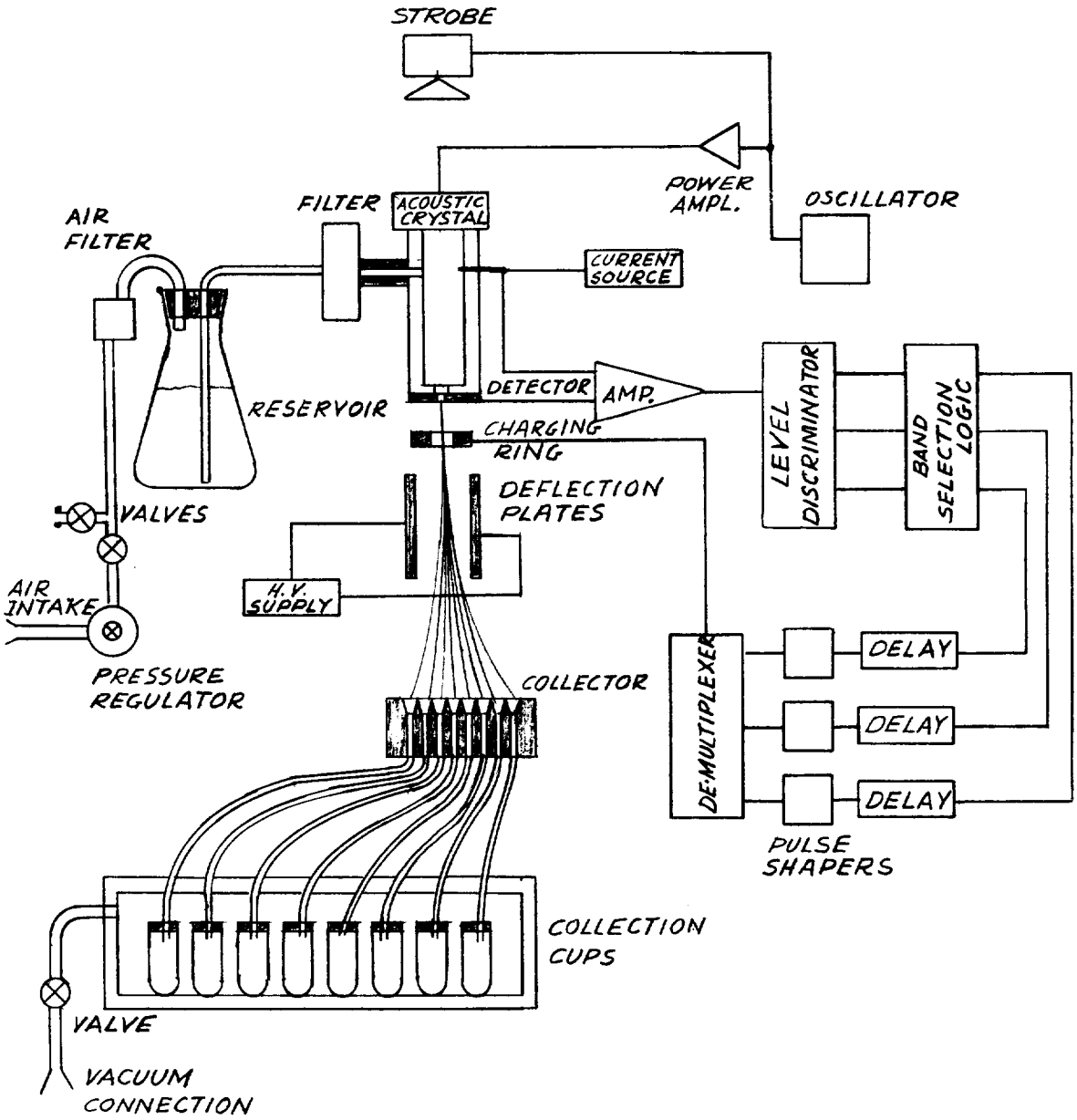
A block diagram of the separator is shown in Figure 12 where all the above functional blocks are identified. The construction of parts 1, 2 and 7 have certain critical points that will be briefly stated. The reservoir is composed of a pressurized chamber. This chamber (laboratory flask) is pressurized at a specific controlled pressure. The air is filtered to prevent any large particles from entering the test solution. The output of the reservoir is connected to the filter housing. This housing encloses a 20 micron nickel filter which in turn is connected to the detection cavity. The detection cavity encloses the insulating orifice (80 micron in diameter by 100 microns in length) and a metal orifice (70 micron in diameter by 100 micron in length). Alignment of the two orifices is of major importance. Since removal of the both orifices is necessary for cleaning purposes, mechanical means have been incorporated enabling the operator to remove both orifices and replace them if necessary without too much difficulty.

The drop generating system shown in Figure 12 consists of a variable frequency oscillator, a variable gain power amplifier, and, an ultrasonic crystal generator. This crystal vibrates the liquid column, at the oscillator frequency, forcing the emerging stream to break into uniform drops at a rate defined by the oscillator frequency.

The collection device is a stainless steel plate with machined cavities. There are eight cavities in total with a distance between center of 2 millimeters. These collection cavities are coupled with teflon tubes to a partially evacuated container enclosing eight test tubes.

The electronics incorporated in the cell separator are shown in Figure 13. The constant current source drives the variable detection resistance.

The signal generated, due to changes in the detection resistance, is amplified and coupled to the level discriminators. The three discriminators facilitate the selection of three levels of electrical signal



VOLUMETRIC CELL SEPARATOR
BLOCK DIAGRAM

FIGURE 12

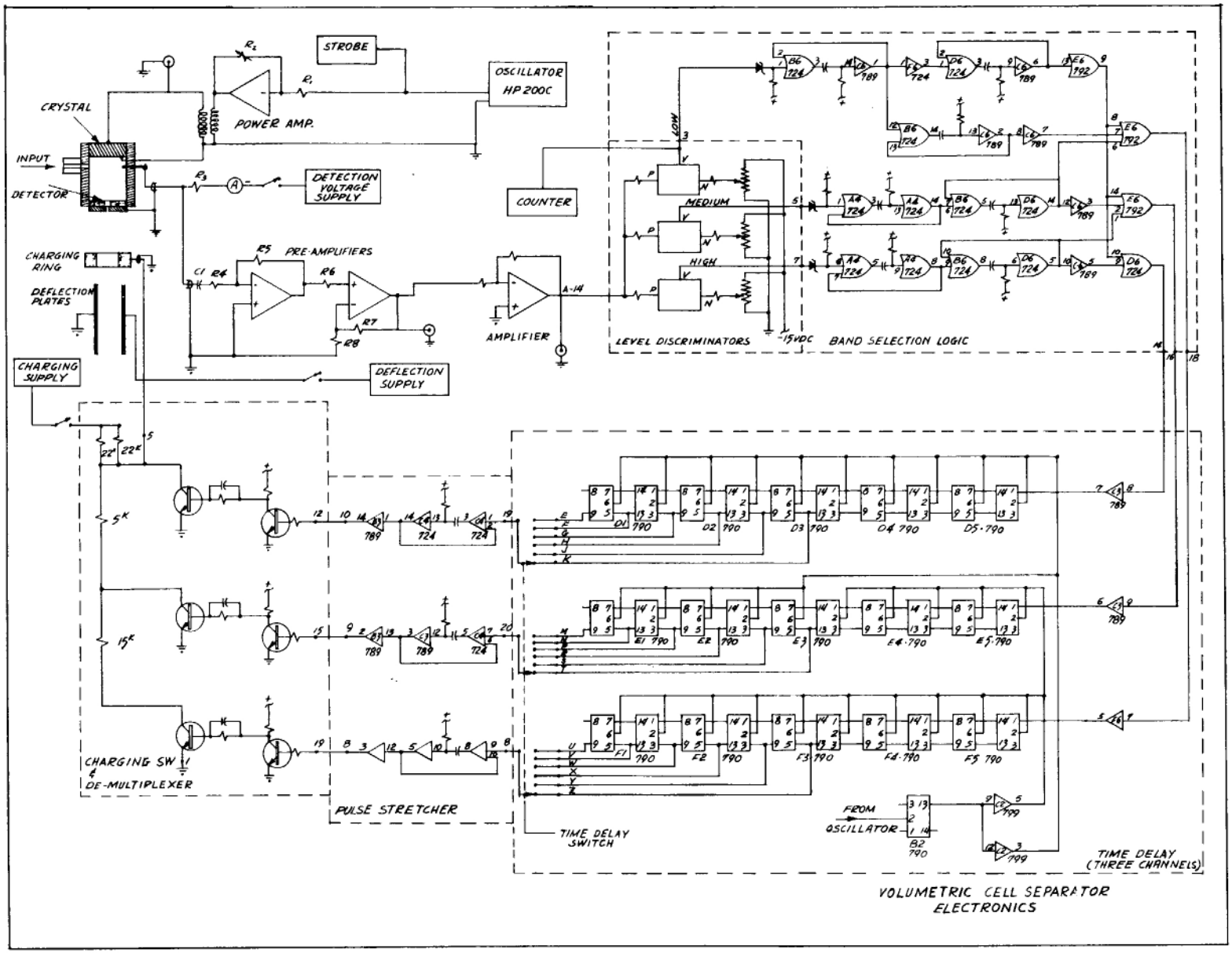

STROBE
OSCILLATOR HP 200C
POWER AMP.
CRYSTAL
INPUT
DETECTOR
DETECTION VOLTAGE SUPPLY
COUNTER
CHARGING RING
DEFLECTION PLATES
PRE-AMPLIFIERS
AMPLIFIER
CHARGING SUPPLY
DEFLECTION SUPPLY
LOW
MEDIUM
HIGH
LEVEL DISCRIMINATORS
-15VDC
BAND SELECTION LOGIC
CHARGING SW & DE-MULTIPLEXER
PULSE STRETCHER
TIME DELAY SWITCH
FROM OSCILLATOR
TIME DELAY (THREE CHANNELS)
VOLUMETRIC CELL SEPARATOR ELECTRONICS

FIGURE 13

which corresponds to three distinct volume thresholds of particles or cells. From here on, the original analog signal is converted into three digital signal lines that contain, at any given moment, the size information of any given particle. In the following digital block these signals are multiplexed in order to arrive at distinct signals corresponding to specific size-bands of cells. The resulting digital lines carry these signals to the three digital delay lines (shift registers). These delays provide the time interval between detection and application of the charging pulse. Since the time difference between detection and charge depends on the velocity of the jet stream, the ultrasonic generator power and, the viscosity of the diluting media, this delay has been made variable. The exact delay can be predetermined, and for any given set of condition the appropriate switch position can be selected.

The clock for this digital delay is derived from the oscillator driving the ultrasonic generator. Thus the digital time delay has been quantized into segments proportional to the drop generating frequency. This characteristic enables the experimenter to adjust the delay by intervals equal to the drop generation frequency. The output of the delay units are fed to three monostable flip-flops that provide adjustable length pulses determining the number of drops to be deflected. These stretched signals are de-multiplexed and connected to the charging voltage switches. In this unit the maximum charging voltage in 200 volts. The deflection voltage is 2000 volts.

3. Calculation of operating parameters.

In previous reports the various parameters of the volumetric cell separator have been presented. These parameters included the differential resistance detection as a function of orifice configuration and detection current; charging voltage as a function of drop size; and deflection voltage as a function of the deflection plate configuration, velocity of stream and size of drops. To summarize the above the

following formulas are repeated below:

Resistance: (Technical report No. IRL 1074, p. 40)

(a) $$\Delta R = \rho \frac{4\ell_1}{\pi d^2} \cdot \left(\frac{d_1}{d}\right)^2$$

where ΔR = change in resistance
ℓ_1 = length of particle
d_1 = diameter of particle
d = diameter of detecting orifice
ρ = resistivity of the medium ($\sim$ 50 ohm-cm for normal saline)

It was pointed out in the above mentioned report that with a detection current of 0.4 ma the noise level of the apparatus was equivalent to a 26 μ^3 (sphere) (Red blood cells have a volume of 54 μ^3).

Charging: Technical Report No. IRL 1056, p. 37

The charge of a column of liquid when passed through an electric field formed in the center of a metal ring is

(b) $$Q_m = \frac{V_{12}\, 2\pi\epsilon_o}{\ell n\left(\frac{r_2}{r_1}\right)}$$

where
Q_m = charge per Unit Mass
V_{12} = potential difference
r_2 = radius of charging ring
ϵ_o = Permittivity (8.88 x 10^{-12} farads/meter)
for a column of liquid of length nr_1

(c) $$Q_T = nr_1 \cdot \frac{2\pi\epsilon_o V_{12}}{\ell n\left(\frac{r_2}{r_1}\right)}$$

where

Q_T = total charge.

Deflection: Technical Report No. IRL 1056, p. 37

The horizontal velocity of a drop having a mass corresponding to a length of nr_1 and radius r_1 when charged with Q_t is

(d) $$v_h = \frac{Q_T V_d \ell}{n S v_v \gamma \pi r_1^3}$$

where

v_h = horizontal velocity

v_v = vertical velocity

γ = density of liquid

ℓ = length of deflection plates

S = separation of deflection plates

V_d = deflection voltage.

When all the above parameters (charge and deflection) are lumpted into one expression, giving the ratio of the horizontal to vertical velocities of a particle of mass m, we have

(e) $$k \equiv \frac{v_h}{v_v} = \frac{Q_T V_d \ell}{S v_v^2 m} = \frac{4 \epsilon_o \ell V_{12} V_d}{7 \cdot 10^3 P S r_1^2 \ln\left(\frac{r_2}{r_1}\right)}$$

where P = Pounds (weight) per square inch.

Another critical parameter of the cell separator is the time separation between detection and the application of the charge. This parameter is a function of ultrasonic frequency, stream size, stream velocity and liquid viscosity. With the present orifice dimensions at an ultrasonic frequency of 30,000 Hz the minimum time difference between detection and charging is attained. For this reason the various parameters mentioned above are plotted in Figure 14 with an excitation frequency of 30,000 Hz.

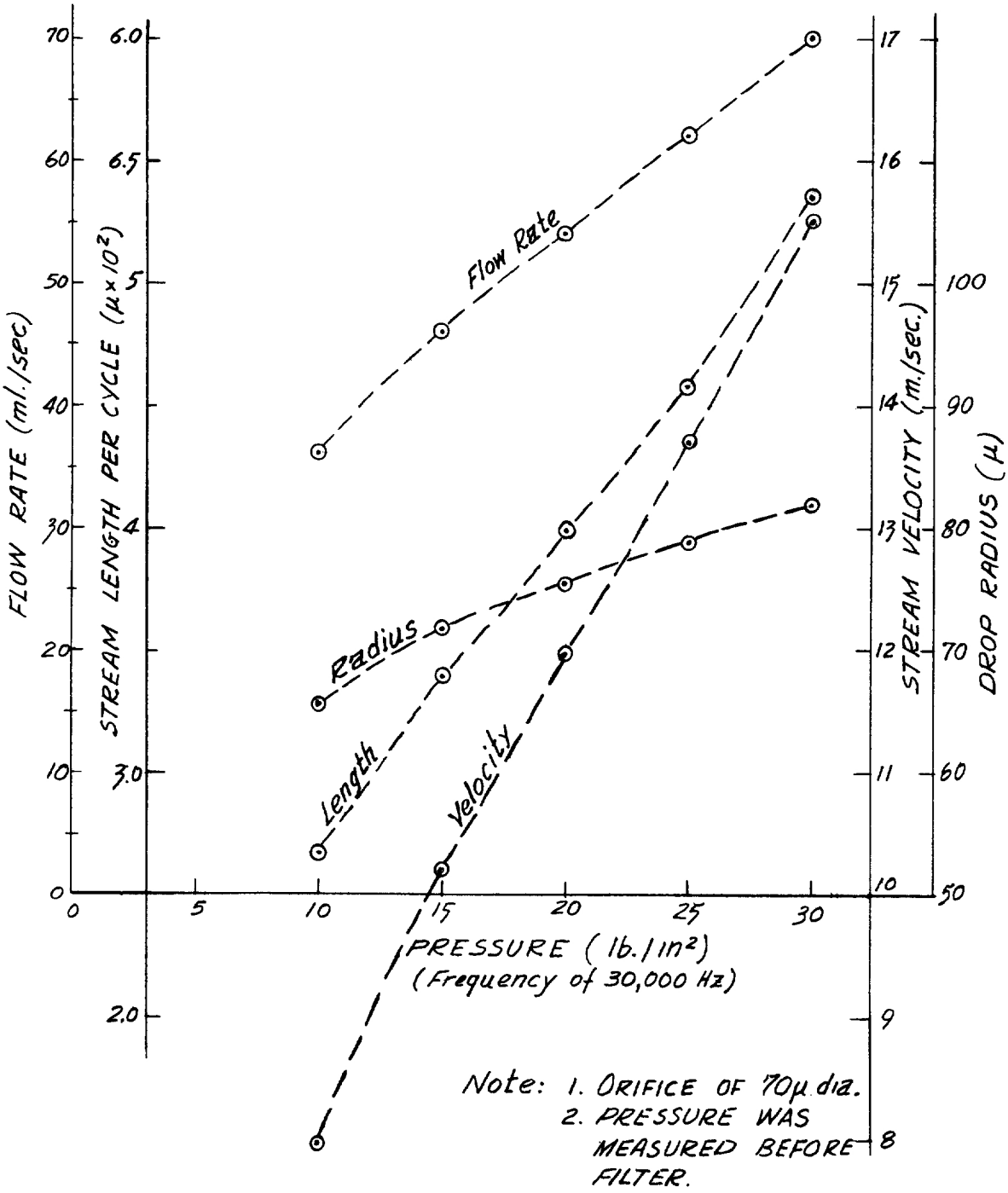

FLOW RATE (ml./sec)
STREAM LENGTH PER CYCLE (μ × 10²)
STREAM VELOCITY (m./sec.)
DROP RADIUS (μ)
Flow Rate
Radius
Length
Velocity
PRESSURE (lb./in²)
(Frequency of 30,000 Hz)
Note: 1. ORIFICE OF 70μ dia.
2. PRESSURE WAS MEASURED BEFORE FILTER.

FIGURE 14

The breaking point of the stream into drops is experimentally measured and the number of drop-delay from the detection cavity is deduced. This in turn defines the total delay required between detection and application of charging voltage for subsequent separation.

4. Noise Level

The noise level of the cell separator has been discussed in previous reports (IRL No. 1076, pp. 41-42). The observed noise level is

$$R_{\text{noise/unit volume}} = 9.3 \times 10^{-4} \text{ ohms}/\mu^3$$

or a noise level equivalent of a 26 μ^3 particle.

This noise is mainly composed of RF signal due to the ultrasonic generator. This noise could be, with some difficulty, suppressed but this is not warranted since it represents a small fraction of the signal to be detected.

An additional high level noise is also present. This noise is generated due to electrolysis in the detection cavity or the intermediate reservoir. The formation of bubbles in the system has been observed to occur at a rate of 100-500 per second depending on the physical conditions. These bubbles cause a signal to appear at the output equivalent to a 15 to 20 micron diameter particle. It is believed that this fictitious signal does not limit the usefulness of the instrument since a given bubble will be thought of as an appropriate size particle and thus deflected accordingly. The only error will occur only when particle and bubble coincide. However, to minimize this effect, the concentration of the solution can be reduced in such a manner so that, the sum of the generated signals due to particles and bubbles per unit time coincide with the maximum permissible count ($\sim$ 2000 counts per second).

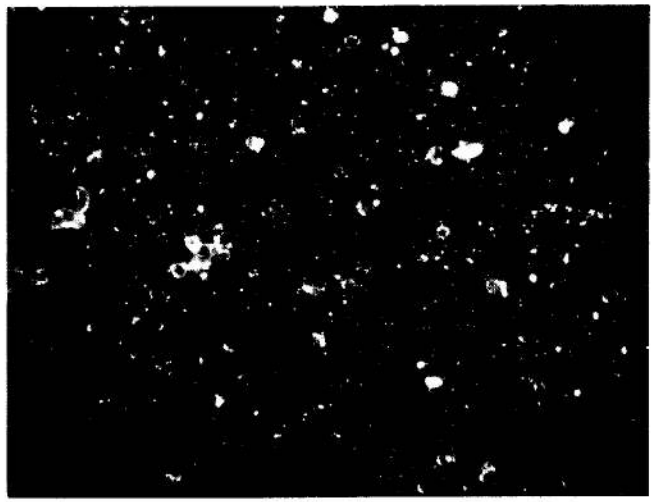


Figure 15

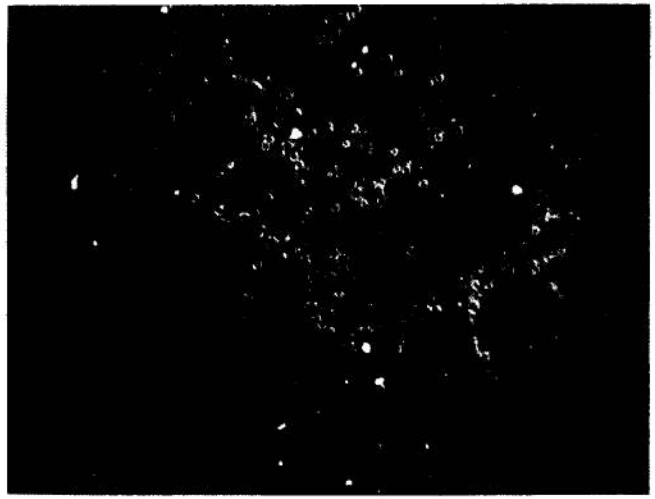


Figure 16

5. Preliminary Results

Several experiments were performed in order to prove separation and viability of cells. Different types of cells have been chosen for these experiments including mixtures of red blood cells and bone marrow cells, tumor cells, and spleen cells. These various types of cells were diluted in different media; normal saline, Puck's saline or medium 199 to bring the concentration down to about 10^5 cells per milliliter. This concentration corresponds to a cell for every ten drops formed at an oscillator frequency of 30,000 Hz and an upstream pressure of 13 pounds per square inch, thus minimizing coincidence. These various diluting media are frequently biologically used for preparation of standard solutions. However due to the low concentration required by the cell separator it has been found that clumping of cells occurs in a manner objectionable for further biological processing of the separated solution. We are looking into the possibility of developing a diluting media that will overcome the clumping problem and result in a biologically stable cell suspension. Figures 15 and 16 show cell smears that have been passed through the separator. Figure 15 is the unseparated sample while figure 16 is the separated one. It is obvious that the concentration in figure 16 is much larger than the one in figure 15 . An increase of about 40 to 50 times in concentration have been observed. Also obvious in figure 16 is the clumping problem mentioned above.

Preliminary experimental results indicate that at low pressures (up to 20 pounds per square inch) the bone marrow cells are not damaged by the separator even if multiple passes of the suspension are performed.

A normal saline solution containing 10^5 bone marrow cells per milliliter was prepared and was passed through the separator several times at fixed time intervals. Figure 17 shows a plot of counts versus time. Although the count rate, after the sixth pass, has dropped to one half of that of the original count when this count is compared with the count of the

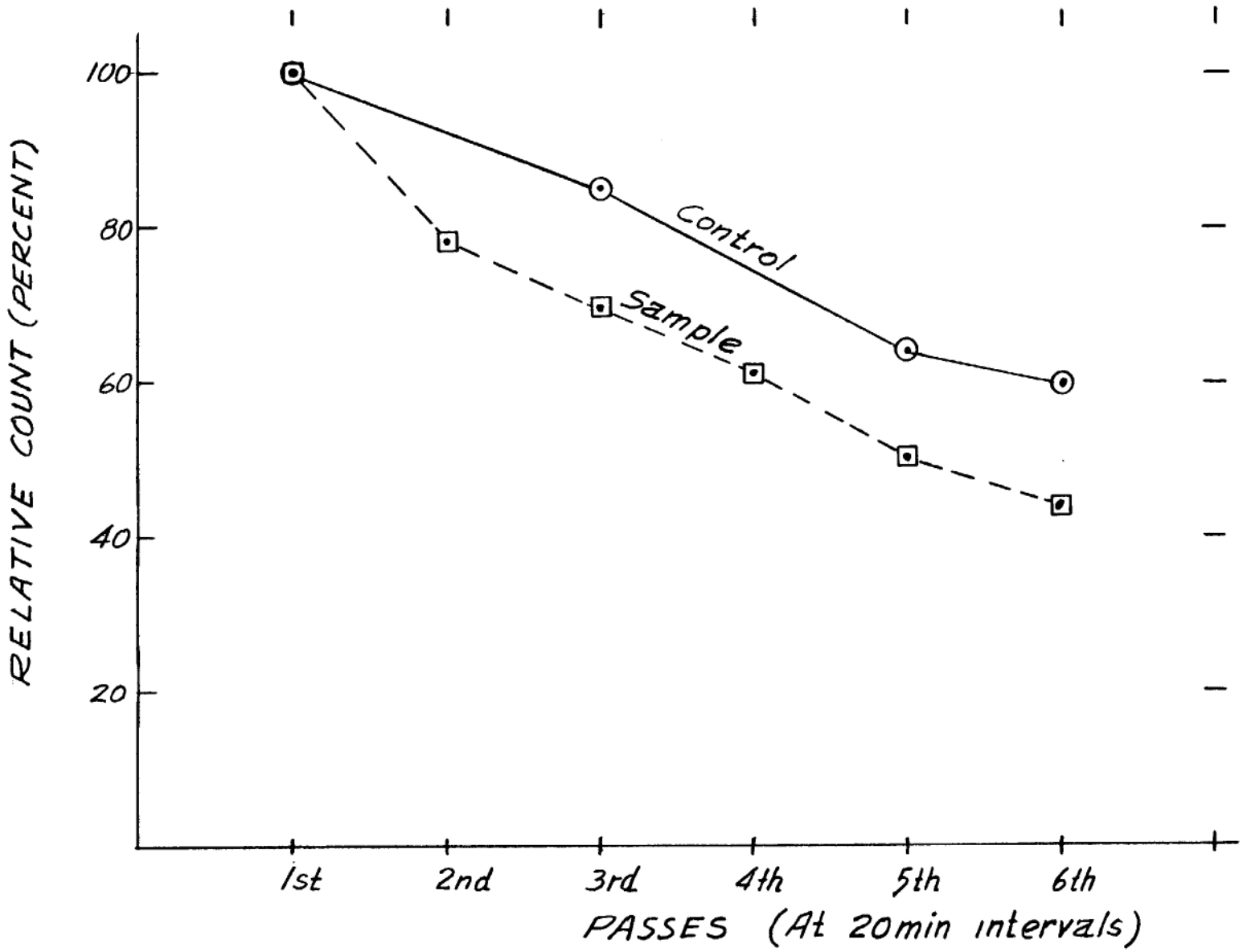

RELATIVE COUNT (PERCENT)
100
80
60
40
20
Control
Sample
1st
2nd
3rd
4th
5th
6th
PASSES (At 20min intervals)

Figure 17

control it is obvious that, within the accuracy of experimental error, the drop in the experimental sample count is not due to the subjection of the cells to the radical changes of the physical environment experienced when passed through the separator. Similar findings regarding bone marrow cell viability were reported by A. L. Carsten and V. P. Bond in Nature (Sept. 7, 1968, p. 1082).

6. Conclusion

The volumetric separator in its present configuration promises to be a useful apparatus in discriminating and separating cells or particles according to their size. Our main interests lay in the separation of cells according to their size. At this stage of development the appropriate suspension media for conserving cells at dilutions required by the separator must be developed. The engineering development of the apparatus seems to be at the point where such basic biological experiments can be performed. Further technological improvements could only contribute to the ease of operation of the machine in a clinical laboratory.

D. High Speed Fluorescent Cell Separator

Summary: Developmental work has continued on the cell separator system described in previous reports. Attempts have been made to effect separation of fluorescent from non-fluorescent cells, and similar tests have been carried out with microscopic resin beads. Results have been encouraging in that the intrinsic capability of the system has been validated, but poor reproducibility has indicated the need for improvement in some areas. These areas are well defined, and remedial action is in process.

Using the cell separator, substantially as described in IRL 1076, several attempts were made to separate cells based on their fluorescence characteristics. In one experiment about 1.5×10^7 chinese hamster cells

in 26 mls of saline were passed through the system. It had been estimated that one-third of these cells would demonstrate detectable fluorescence as a result of incubation for 20 minutes in F.D.A. (fluorescein di-acetate). A Hewlett-Packard counter registered every pulse of greater than 0.5V amplitude obtained as a result of fluorescent cells passing the system's detector. After the entire 26 ml had flowed through the system a total fluorescent cell count of 1.6×10^6 was noted. As each count occurred a + 60V, 60 microsecond charging pulse was applied to the emerging liquid stream. Droplets formed bearing this charge were deflected to a separate part of the collector unit by passage between 10KV charged deflection plates. Approximately 2.5×10^6 droplets were deflected, and formed about 1 ml of fluid separated from the main stream. This 1 ml of fluid, and 16 ml of fluid from the undeflected stream were passed through the I.B.M. cell counter by Dr. R. Hulett. The 1 ml separated fraction contained sixty times the number of fluorescent cells that 1 ml of the unseparated fluid contained. One further test yielded similar results but with a diminished ratio of separation. Two other tests indicated that a random separation of droplets had apparently occurred, accompanied by a large loss of cells both fluorescent and nonfluorescent.

There has been a manifest lack of reproducibility of results, not all of which can be attributed to system errors. In each of the experiments it was noted that the average amplitude of signals fell off sharply as a function of time. It is not known whether this is entirely due to loss of fluorescence by the cells, or because smaller and presumably less fluorescent cells remain in suspension longer, and so pass through the system later. The terminal phases of each experiment were marked by a progressively depressed counting rate, followed by a dramatic increase as the last 1-2 ml cleared the supply tubing and filter. This last observation indicates a differential retention of cells within the system, and a possibility of some degree of permanent retention of cells on the walls of the delivery tubing and filter.

One other observed phenomenon merited closer study. In each experiment a loss of fluid was noted, the summed collected fractions being 3 to 15 percent less than the original volume. In further experiments using only saline, some attempts were made to determine the loss mechanism. Computer calculations predicted a loss by the in flight droplet of only 0.7 of one percent; observation and measurement of liquid samples maintained under reduced atmospheric pressure showed a similar small loss. It was noted that small and fairly predictable losses not exceeding 3 percent occurred only when the droplet stream was uncharged and undeflected.

When the stream was charged and deflected at various synchronous on-off ratios fluid loss varied and approached the larger values mentioned. In particular, if the charge one-off ratio was low, such that the collected fraction was small, then that fraction was always well below the predicted value. It seems most probable based on these observations that erratic aiming, inconsistent charging and imprecise droplet formation could account for most of the fluid loss involved.

The fluid losses could not account for all the apparent cell losses, because these losses appeared to be occurring in both undeflected and deflected fractions.

In an effort to calibrate the performance of the instrument some microscopic fluorescent resin beads were used as test objects. Such beads as did pass under the detector yielded large and reproducible signals. The number of counts however was drastically below that predicted. It became evident from subsequent microscopic examination of the filter and input delivery tubing that beads were being retained in very large numbers.

In one test run to demonstrate this source of error a few million beads were added to 30 mls of saline and passed through the system. A total of only 3.23×10^5 counts was obtained. Four successive additions of

saline without beads were made to the reservoir of 15 mls each and the additional counts of: 5.17×10^4, 4.63×10^4, 1.91×10^4 and 2.1×10^4, were obtained. At this point the filter and supply tubing were microscopically examined and found to contain very large numbers of beads. It is likely that an identical condition existed in the collector module and tubing; of the counted total of 4.6×10^5 beads none could be located in the collector storage tube.

The problems of adhesion to the walls of the system by the beads, and of retention by a filter of adequate pore size will have to be solved before the beads can be regarded as a useful calibration standard.

It is not known how much data obtained from the test objects can be validly applied to cells. Certainly some of the observations are common to both sets of experiments, and may be attributable to the same causes. Some changes are in progress on the instrument to improve the stability and accuracy of separation. An improved charging ring has been devised which does not occlude the high aperture optical beams required. A special long working distance objective has been acquired which will relieve optical, mechanical and electronic constraints in the formerly severely limited area of the forming jet stream.

A small spread in position of deflected droplets was noted, due to overshoot in the transferred charge, and has been corrected by a compensating network. Upon completion of the necessary mechanical and optical improvements, further separation experiments will be conducted.

PUBLICATIONS

PAPERS AND REPORTS

April 1, 1968 to September 30, 1968

REPORTS

1. J. Lederberg, "Online Computation of Molecular Formulas from Mass Number, C.R. 95977 (1968). IRL 1081.

PUBLICATIONS

1. Carl Sagan, E. C. Levinthal, J. Lederberg, "Contamination of Mars" Science, 159, 1191-1196 (1968). IRL 1072

2. J. Lederberg and E. A. Feigenbaum, "Mechanization of Inductive Inference in Organic Chemistry", in Formal Representation of Human Judgment (B. Kleinmuntz, ed.) John Wiley & Sons, New York, p. 187-218 (1968). IRL 1084.

3. W. E. Reynolds, J. C. Bridges, R. B. Tucker and T. B. Coburn, "Computer Control of Mass Analyzers", Paper 33, page 77-84. Sixteenth Annual Conference on Mass Spectrometry and Allied Topics, Pittsburgh, Pa., May 12-17. ASTM Committee E-14, 1968. IRL 1080.

4. B. Halpern, L. Chew, V. Close, W. Patton, "Removal of Peptides from "Merrifield Solid Phase" by Transesterification with an Anion Exchange Resin", Tetrahedron Letters, 5163 (1968). IRL 1083.

5. J. W. Westley, D. Nitecki, V. A. Close and B. Halpern, "Determination of Steric Purity and Configuration of Diketopiperazines by GLC Thin Layer Chromatography and Nuclear Magnetic Resonance Spectroscopy," J. Anal. Chem., 40, 188 (1968). IRL 1077.

6. J. W. Westley, B. Halpern,"The Use of (-)-Menthyl Chloroformate in the Optical Analysis of Asymmetric Amino and Hydroxyl Compounds by Gas Chromatography," J. Org. Chem., 33, 3978 (1968). IRL 1078

7. J. W. Westley and B. Halpern, "Determination of the Configuration of Asymmetric Compounds by Gas Chromatography of Diastereoisomers" 7th International Symposium on Gas Chromatography and Its Exploitation. Edited by C.L.A.Harbourn and R. Stock. Paper No. 7, 1968. IRL 1074.

8. E. C. Levinthal, J. Lederberg, Carl Sagan, "Relationship of Planetary Quarantine to Biological Search Strategy" COSPAR, Plenary Meeting, 10th, London, England, Paper 15, pp. 136-145 (1968). IRL 1071

9. B. Halpern, V. A. Close, A. Wegmann and J. W. Westley. "Gas Chroma-

tography of Amino Acids as N-Thiocarbonyl Ester DErivatives" Tetrahedron Letters, No. 27, 3119 (1968). IRL 1075.

10. D. E. Nitecki and B. Halpern, "The Synthesis of the Pentapeptide Related to the GM(a) Antigen of Human Gamma G-Globulin." Submitted to Aust. J. Chem., 1968.

11. J. Lederberg, G. L. Sutherland, B. G. Buchanan, E. A. Feigenbaum, A. V. Robertson, A. M. Duffield and Carl Djerassi, "Applications of Artificial Intelligence for Chemical Inference I. The Number of Possible Organic Compounds: Acyclic Structures Containing C, H, O and N," (submitted to Jour. Am. Chem. Soc. 1968).

12. A. M. Duffield, A. V. Robertson, Carl Djerassi, B. G. Buchanan, G. L. Sutherland, E. A. Feigenbaum and J. Lederberg, "Applications of Artificial Intelligence for Chemical Inference II. Interpretation of Low Resolution Mass Spectra of Ketones," (submitted to J. Am. Chem. Soc., 1968).

PAPERS

1. E. C. Levinthal, "The Role of Molecular Asymmetry in Planetary Biological Exploration" Gordon Research Conferences, Nuclear Chemistry Section, 1968.