TRN AU8004750

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INTERACTION OF ERYTHROCYTES AND HEXAVALENT URANIUM COMPOUNDS - AN AUTOANALYTICAL STUDY

by

W.I. STUART
M.E. SHYING

May 1980

ISBN 0 642 59688 3

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ABSTRACT

An automated analytical system was devised to measure the kinetics of hemolysis by uranyl compounds. Accurate plots of percentage hemolysis *v.* time were obtained; these, together with the corresponding differential curves, show that hemolysis of plasma-free erythrocytes is a two-stage process. The first stage of hemolysis is particularly affected by pH and anion content of uranyl solutions, and also by incubation of cell suspensions at 37°C before mixing with lysing solution.

Complementary studies involving Coulter counting and microscopic observation established the general pattern of hemolysis and showed that cell agglutination is a prominent feature of the interaction of cells with uranyl solutions.

National Library of Australia card number and ISBN 0 642 59688 3

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ANIMAL CELLS; IN VITRO; CHEMICAL ANALYSIS; TOXICITY; URANYL COMPOUNDS; ERYTHROCYTES; HEMOLYSIS; BIOLOGICAL EFFECTS

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1. INTRODUCTION

This report is about automated chemical analysis (ACA) and how this technique can be adapted to study the _in vitro_ interaction of cells and potentially toxic materials. Work described in the report arose as part of a wider program aimed at defining the physicochemical factors that contribute to the biological effects of uranium particulates and other industrial dusts such as asbestos and silica.

Development of the ACA technique was planned around a sequence of three objectives, namely:

(i) To adapt automated analysis and data processing techniques for measuring kinetics of hemolysis by soluble uranium compounds.

(ii) To extend these techniques to the study of processes either preceding or accompanying hemolysis, e.g. potassium transport, uranium absorption, cell agglutination.

(iii) To study kinetic aspects of cellular damage by insoluble particulate material (for example, insoluble uranium oxides, silica, asbestos) where surface properties of the material determine the nature and degree of cell damage.

As a pilot study, ACA was adapted and combined with data-processing techniques to measure kinetics of _in vitro_ hemolysis by soluble uranyl compounds; this study complements earlier work by Stuart et al. [1979a,b] which showed that hemolysis of washed erythrocytes by certain uranium particulates arises from the reaction of dissolved uranium with constituents of the cell membrane.

The report deals with the technical innovation and preliminary experiments carried out from June 1976 until cancellation of the program in May 1977. Although the results are incomplete, they show, nevertheless, some important features related to hemolysis and cell agglutination induced by soluble uranium; they also demonstrate the usefulness of automated analytical techniques in the study of biological effects by potentially toxic materials.

2. EXPERIMENTAL

2.1 Preparation of Erythrocyte Suspensions

Samples of human blood were obtained by venipuncture during routine medical examinations. Standard hematological measurements, using EDTA as a anticoagulant, included hemoglobin content, red cell count and hematocrit.

For hemolysis experiments, cells from whole blood were washed three times with isotonic saline (0.15 mol NaCl L^{-1}) and then mixed in saline to give a 1 vol.% cell suspension.

2.2 Measurement of Hemolysis

The automated analytical scheme shown in Figure 1 was developed, in the first instance, for continuous measurement of hemoglobin released from cells when incubated at 37°C in the presence of uranyl compounds. The entire flow system is maintained by means of a multichannel proportioning pump, which ensures that:

(i) lysing solution and cell suspension are mixed homogeneously, and the volume of each is accurately metered;

(ii) mechanical stress imposed on cells is reproducible for each experiment;

(iii) analytical reagents are introduced continuously and in precise amounts at appropriate parts of the flow system; and

(iv) throughout the system, cells are subjected to mild agitation in order to maintain homogeneity and to prevent sedimentation of cells before centrifugation.

The system operates as follows. A freshly prepared cell suspension is stored at ambient temperature in vessel A and stirred by an accurately controlled stream of air bubbles, introduced into the vessel by the proportioning pump. The suspension is raised to reaction temperature (37°C) by its passage through the heating coil HC which is immersed in a thermostatted water bath; lysing solution (that is, saline containing

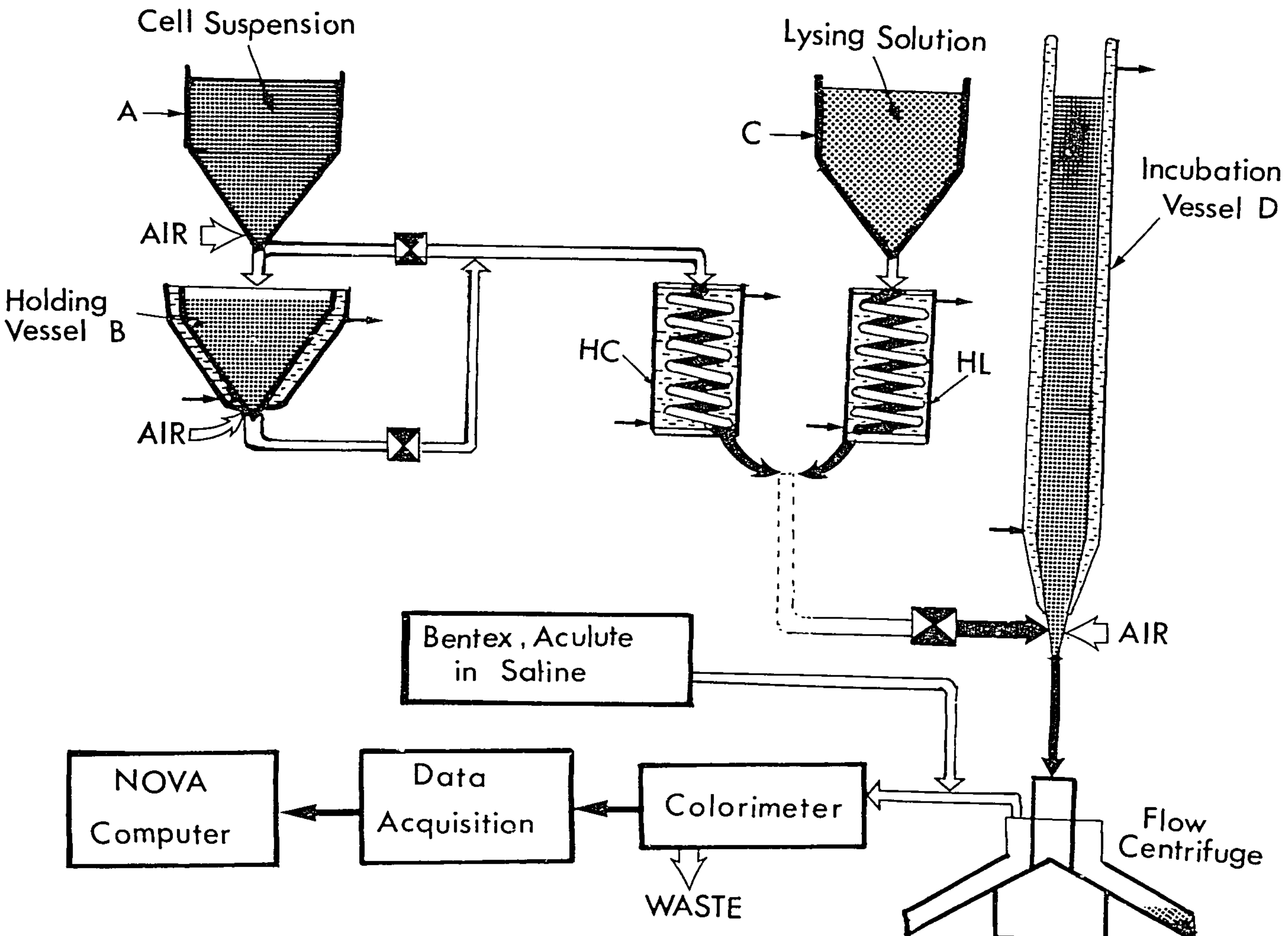


FIGURE 1. AUTOANALYTICAL SYSTEM FOR CONTINUOUS MEASUREMENT OF KINETICS OF HEMOLYSIS

dissolved uranium) is likewise heated in coil HL. The two streams are then mixed homogeneously before passing into the water-jacketed incubation vessel D. Homogeneity of the reaction mixture is maintained by pumping air bubbles into the base of vessel D. When the reaction vessel is loaded (loading time was 8 minutes in our experiments), the reaction mixture is agitated for a further 2 minutes before sampling begins. A sampling stream then passes from the base of vessel D to a continuous-flow centrifuge E, which removes cells from lysate. Saline, containing Drabkin's reagent (Aculute) and detergent (Bentex), is then added as a reagent stream to the lysate which then passes to a colorimeter equipped with a continuous-flow optical absorbance cell.

The data acquisition system enables data output from the colorimeter to be monitored at regular intervals and recorded on paper tape.

2.3 Conditioning of Cell Suspension at 37°C

The bypass system through holding vessel B enables the cell suspension to be maintained at 37°C for various periods before mixing with lysing solution.

2.4 Reagent Stream

All forms of hemoglobin in the lysate are converted to cyanmethemoglobin by Drabkin's reagent. The presence of 1 per cent Bentex detergent prevents a build-up of organic matter (lipids, protein) as a film deposited in the flow cell.

2.5 Continuous Flow Centrifuge

The centrifuge shown in Figure 2 enables cells to be removed continuously from the sampling stream. Concentric glass tubes A and B are joined to glass side arms as shown. The glass assembly is mounted on an aluminium base C and fitted to the centrifuge head. Cell suspension from incubation vessel D drops into the inner tube A at a constant rate determined by the proportioning pump, and passes sequentially to the base of each side arm. About 95 per cent of the cells are collected in the first side arm and the remainder are deposited in the second side arm. Cell-free lysate is then withdrawn from outer tube B through a stainless steel needle. The exit flow rate is necessarily greater than the inlet flow rate, and air is therefore drawn into the exit stream. Air bubbles are removed from the lysate stream by a bubble extractor of

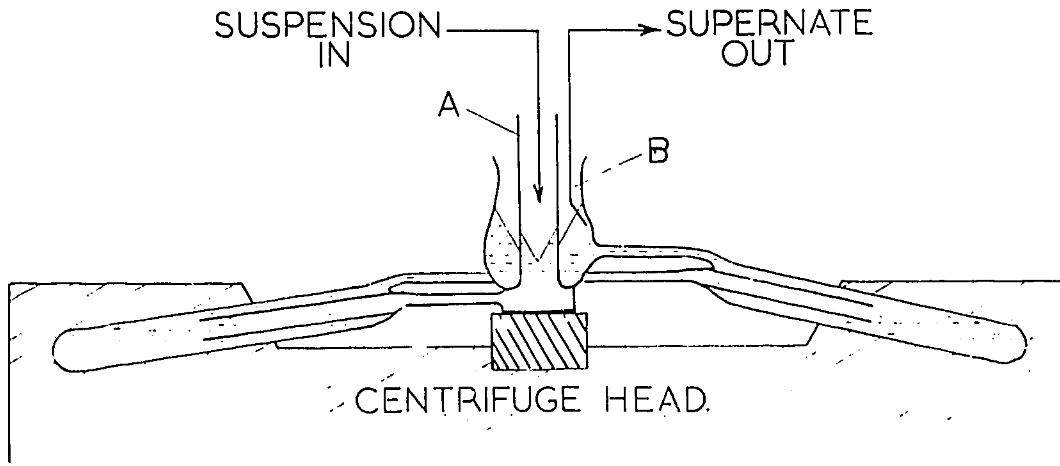


FIGURE 2. CONTINUOUS FLOW CENTRIFUGE

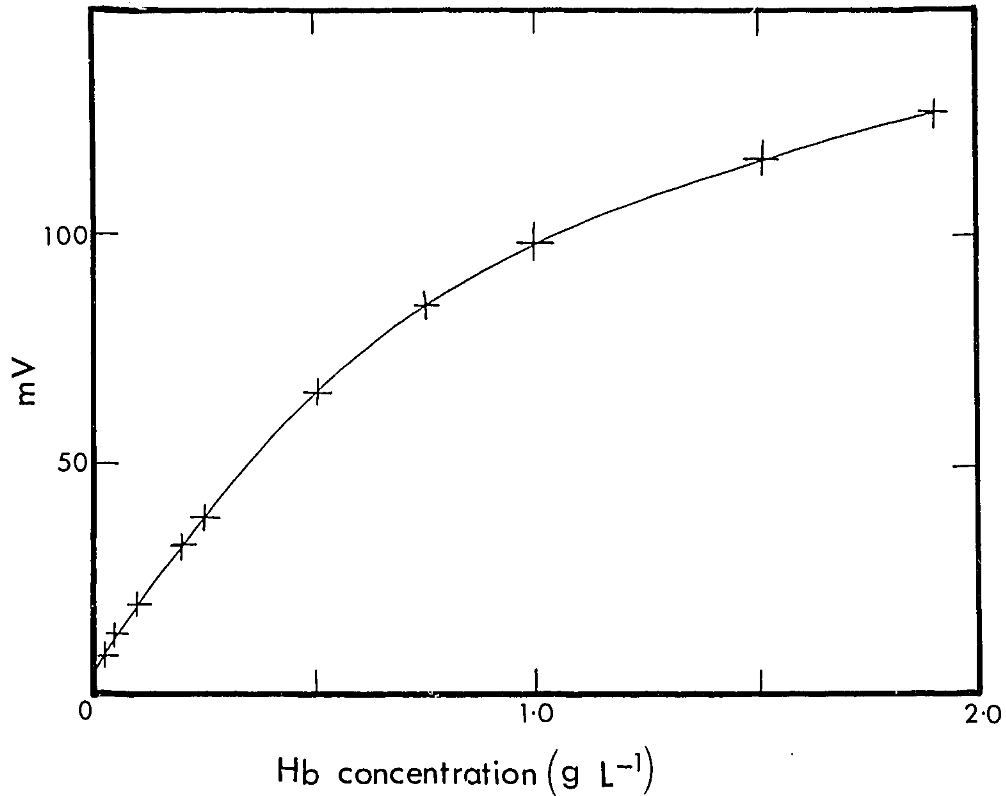


FIGURE 3. ANALYSIS OF HEMOGLOBIN; CALIBRATION CURVE. CROSSES ARE EXPERIMENTAL POINTS; UNBROKEN CURVE IS LINE OF BEST FIT TO FOURTH POWER POLYNOMIAL

conventional design.

2.6 Description and Calibration of the Colorimeter

A Technicon double-beam colorimeter, employing a filter (for the transmission range 300-650 nm) and equipped with a continuous-flow optical cell, was used to measure hemoglobin concentration.

Standard solutions of cyanmethemoglobin were prepared by dissolving weighed amounts of methemoglobin in saline which contained 2 per cent Drabkin's reagent and 1 per cent Bentex. The colorimeter output voltage V (denoting optical absorbance) was measured for various concentrations [Hb] of hemoglobin.

When processing the colorimetric data by digital computer, it is convenient to express [Hb] as a continuous function of V, i.e. [Hb] = f(V). Optical absorbance was measured for a range of wavelengths rather than at a single wavelength (e.g. 540 nm), hence Beer's Law does not apply; however, a polynomial in V of degree four was fitted satisfactorily to the data using non-linear regression analysis. Parameters were refined using full-matrix least squares techniques.

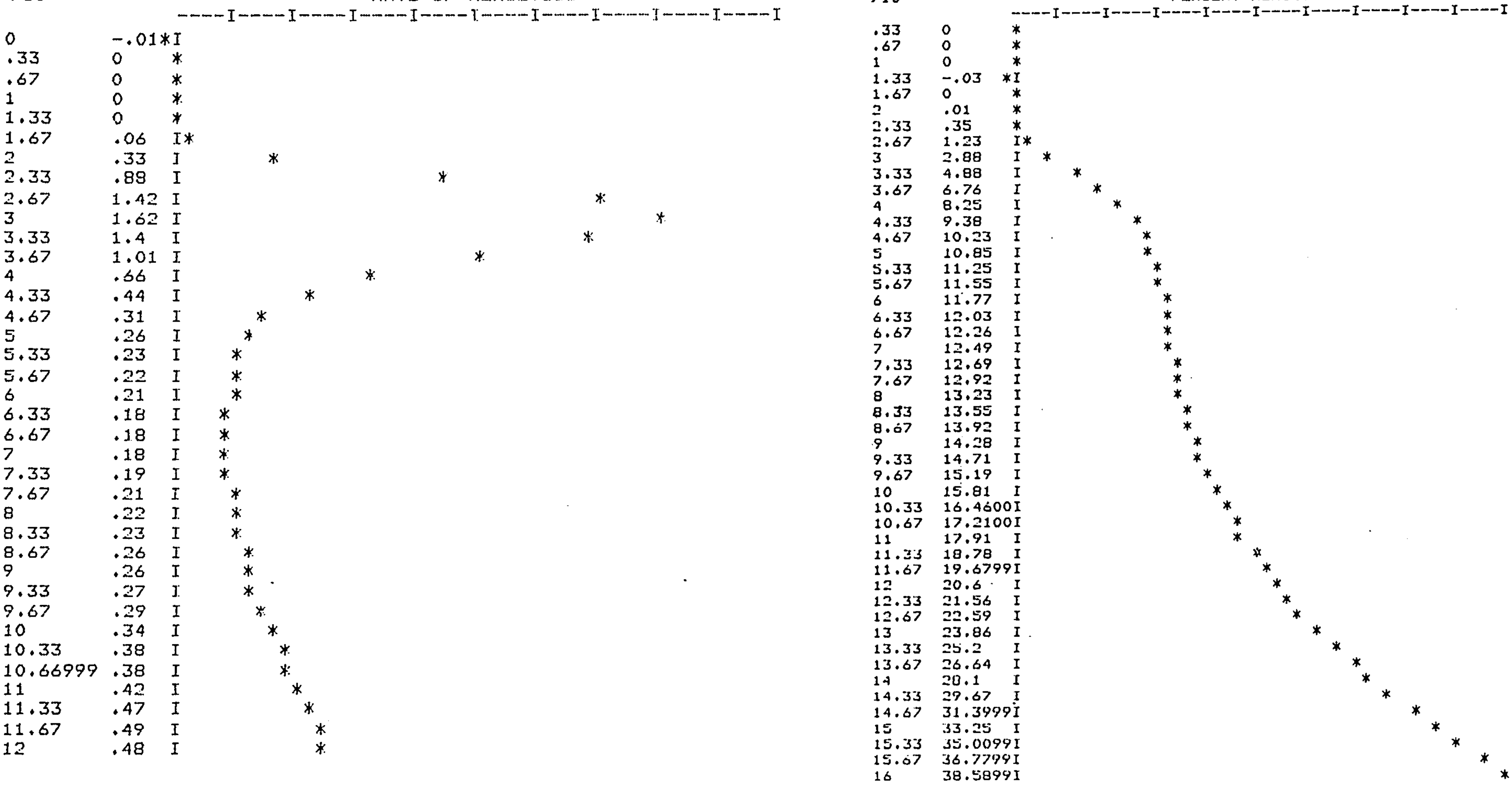
Figure 3 compares experimental data points with the plot of [Hb] v. V calculated from [Hb] = f(V) and demonstrates the excellence of fit.

The degree of hemolysis was expressed as a percentage of a fully lysed suspension. Complete hemolysis was achieved by incubation of a cell suspension with Drabkin's reagent.

2.7 Data Acquisition and Processing

Data acquisition equipment, which can be operated as a two-channel system, consists of:

- Two Ortec model 702 parallel-to-serial converters, with each converter providing an interface between a digital panel meter and a serial BCD acquisition system.


NO CELLS * E8/ML=.6378653
DOSE FMOL/CELL=.4703189

EXPERIMENT:R305 — RATE OF HEMOLYSIS

TIME MIN /10	RATE OF HEM
0	-.01
.33	0
.67	0
1	0
1.33	0
1.67	.06
2	.33
2.33	.88
2.67	1.42
3	1.62
3.33	1.4
3.67	1.01
4	.66
4.33	.44
4.67	.31
5	.26
5.33	.23
5.67	.22
6	.21
6.33	.18
6.67	.18
7	.18
7.33	.19
7.67	.21
8	.22
8.33	.23
8.67	.26
9	.26
9.33	.27
9.67	.29
10	.34
10.33	.38
10.66999	.38
11	.42
11.33	.47
11.67	.49
12	.48

EXPERIMENT:R307 — PERCENT HEMOLYSIS

TIME MIN /10	% HEM
.33	0
.67	0
1	0
1.33	-.03
1.67	0
2	.01
2.33	.35
2.67	1.23
3	2.88
3.33	4.88
3.67	6.76
4	8.25
4.33	9.38
4.67	10.23
5	10.85
5.33	11.25
5.67	11.55
6	11.77
6.33	12.03
6.67	12.26
7	12.49
7.33	12.69
7.67	12.92
8	13.23
8.33	13.55
8.67	13.92
9	14.28
9.33	14.71
9.67	15.19
10	15.81
10.33	16.4600
10.67	17.2100
11	17.91
11.33	18.78
11.67	19.6799
12	20.6
12.33	21.56
12.67	22.59
13	23.86
13.33	25.2
13.67	26.64
14	28.1
14.33	29.67
14.67	31.3999
15	33.25
15.33	35.0099
15.67	36.7799
16	38.5899

FIGURE 4. KINETICS OF HEMOLYSIS : EXAMPLES OF COMPUTER PRINTOUT

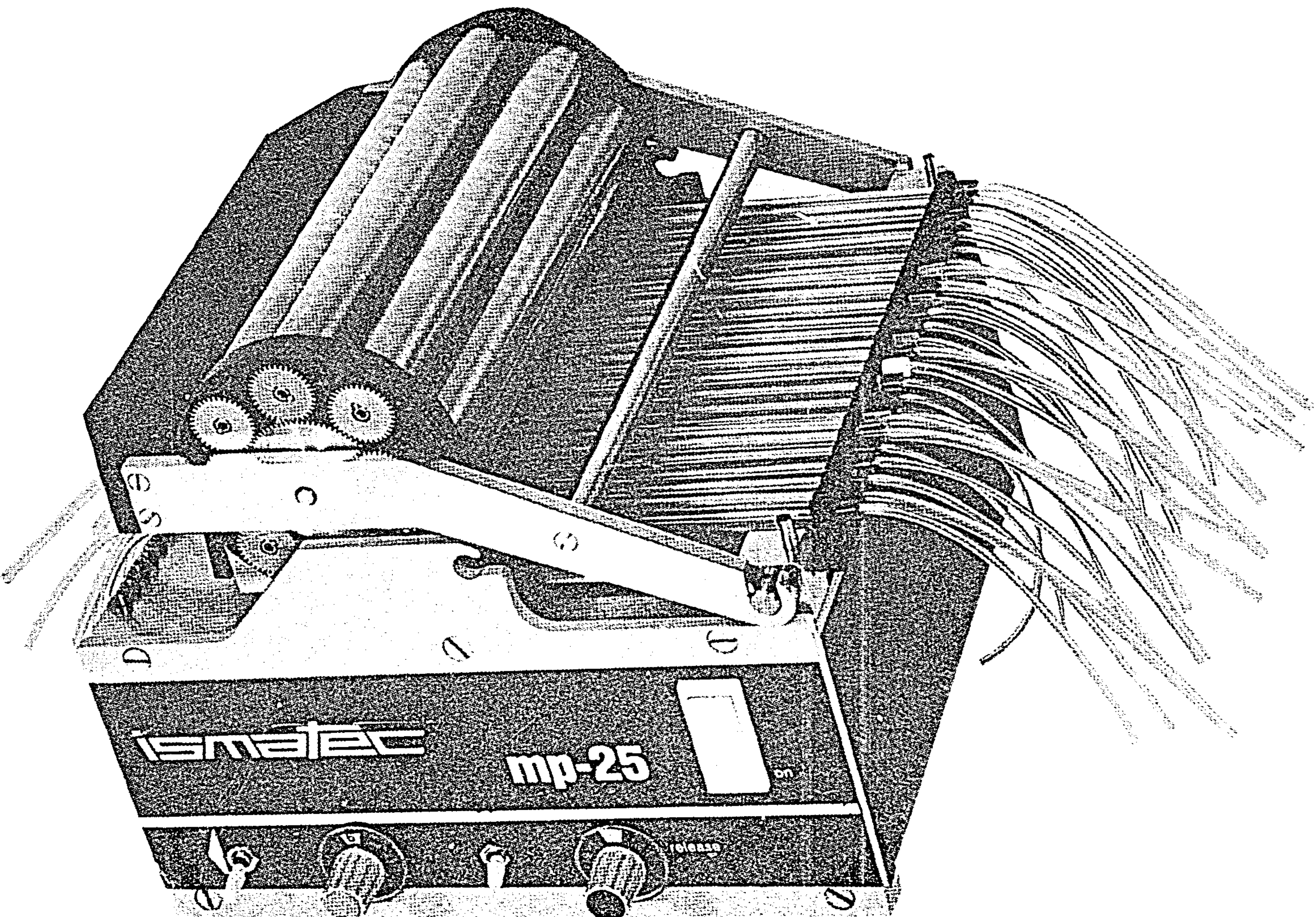


FIGURE 5. MULTICHANNEL PROPORTIONING PUMP

- One Ortec model 773 timer unit with a time base of either 0.1 sec or 1.0 min to give time intervals in the range 0.1 s to 9×10^5 min.

- One Ortec model 224 ASR33 teletype page printer with paper tape punch.

Data were analysed with a NOVA digital computer having a teleprinter output (Figure 4) and input in the form of punched paper tape.

2.8 Proportioning Pump

The pump used was an ISMATEC model MP-25 25-channel peristaltic pump fitted with variable speed control. As shown in Figure 5, this pump operates by means of rollers on a fixed platen.

2.9 Non-automatic Sampling

In some experiments, samples of cell-uranium mixture were withdrawn by syringe from the incubation vessel D and the samples were used for measuring absorption of uranium by cells, for studies of cell agglutination and for microscopic observation.

2.10 Cell Counting

Cell counts and particle size distributions were obtained by means of a Coulter Counter model ZF.

3. RESULTS AND DISCUSSION

3.1 Kinetics of Hemolysis

Curves of percentage hemolysis, H_u, *v.* time of incubation, t, are given in Figure 6; these, together with differential curves of dH_u/dt (Figure 7), illustrate some general kinetic features of hemolysis by uranyl compounds. There are two stages of hemolysis: Stage 1 is characterised by a rapid increase in dH_u/dt from 20 min up to a maximum at about 30 min, and a subsequent decrease to a low minimum at 50 min; a slower process (Stage 2) then ensues for $t > 60$ min.

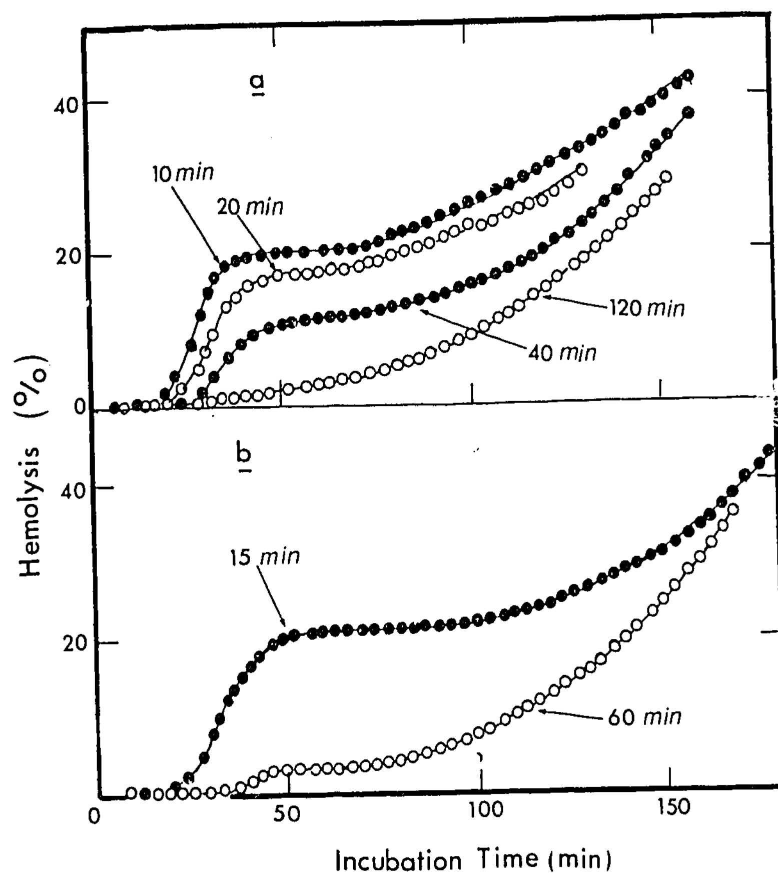


FIGURE 6. KINETICS OF HEMOLYSIS BY URANYL ACETATE; EFFECT OF CONDITIONING TIME t_c ON KINETICS

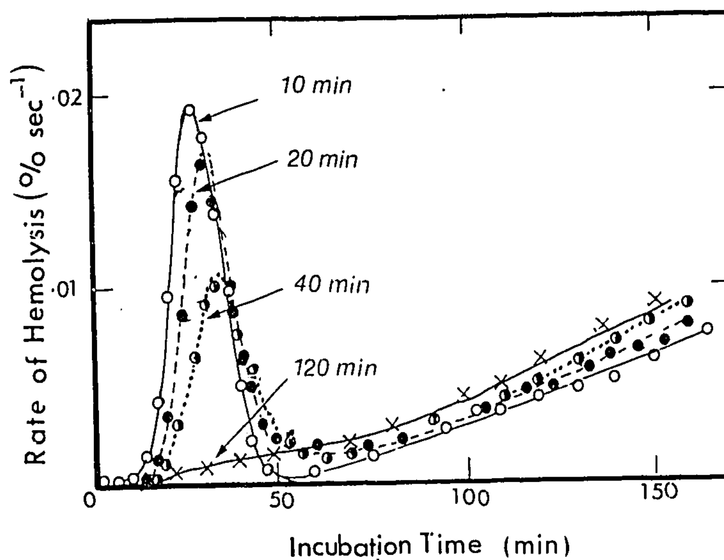


FIGURE 7. EFFECT OF CONDITIONING TIME t_c ON RATES OF HEMOLYSIS BY URANYL ACETATE

Some factors affecting hemolysis are as follows:

(i) Conditioning of cell suspension As depicted in Figures 6 and 7, conditioning (that is, heating the cell suspension at 37°C for a certain period before addition of lysing solution) can affect the course of subsequent kinetics quite substantially; with increasing time of conditioning, t_c, the extent of hemolysis encountered during Stage 1 steadily diminishes.

(ii) pH of uranyl solutions The differential curves given in Figure 8 refer to hemolysis by uranyl acetate and uranyl nitrate dissolved in saline; they show that, for a given uranium dose and conditioning time, the initial pH of the uranyl solution is an important factor in modifying Stage 1 kinetics, so that rates of hemolysis are enhanced by decreasing the initial pH of the uranium solution.

(iii) Anion of the uranyl compound Differential curves in Figure 9 compare hemolysis by saline solutions containing respectively uranyl chloride, uranyl acetate and uranyl nitrate, but with the same conditions in terms of total uranium dose, initial pH and conditioning time. The influence of the anion on Stage 1 kinetics is particularly striking, as is indicated by substantial differences in the maximum values of dH_u/dt, which are in the order NO_3^- > acetate > Cl^-.

3.2 Agglutination of Erythrocytes

The development of cell agglutination is a prominent feature of the reaction between erythrocytes and uranyl compounds [Wyatt 1977]. Evidence of cell agglutination is given by the results of visual observation, particle counting and measurement of particle size distribution.

Figure 10 shows, for four different systems, the progressive decrease in particle count (that is, Coulter count expressed as a percentage of total cell count) following the addition of uranyl compounds to erythrocyte suspensions. These results indicate a wide variation from one system to another, both in the extent of cell agglutination and the rapidity with which agglutination occurs. There is however no obvious correlation of agglutination and hemolysis; for example, the data of Figure 10(b,c) show that cell agglutination for certain systems occurs within the first 20 min of incubation and substantially before the onset of hemolysis, whereas Figure 10(a)

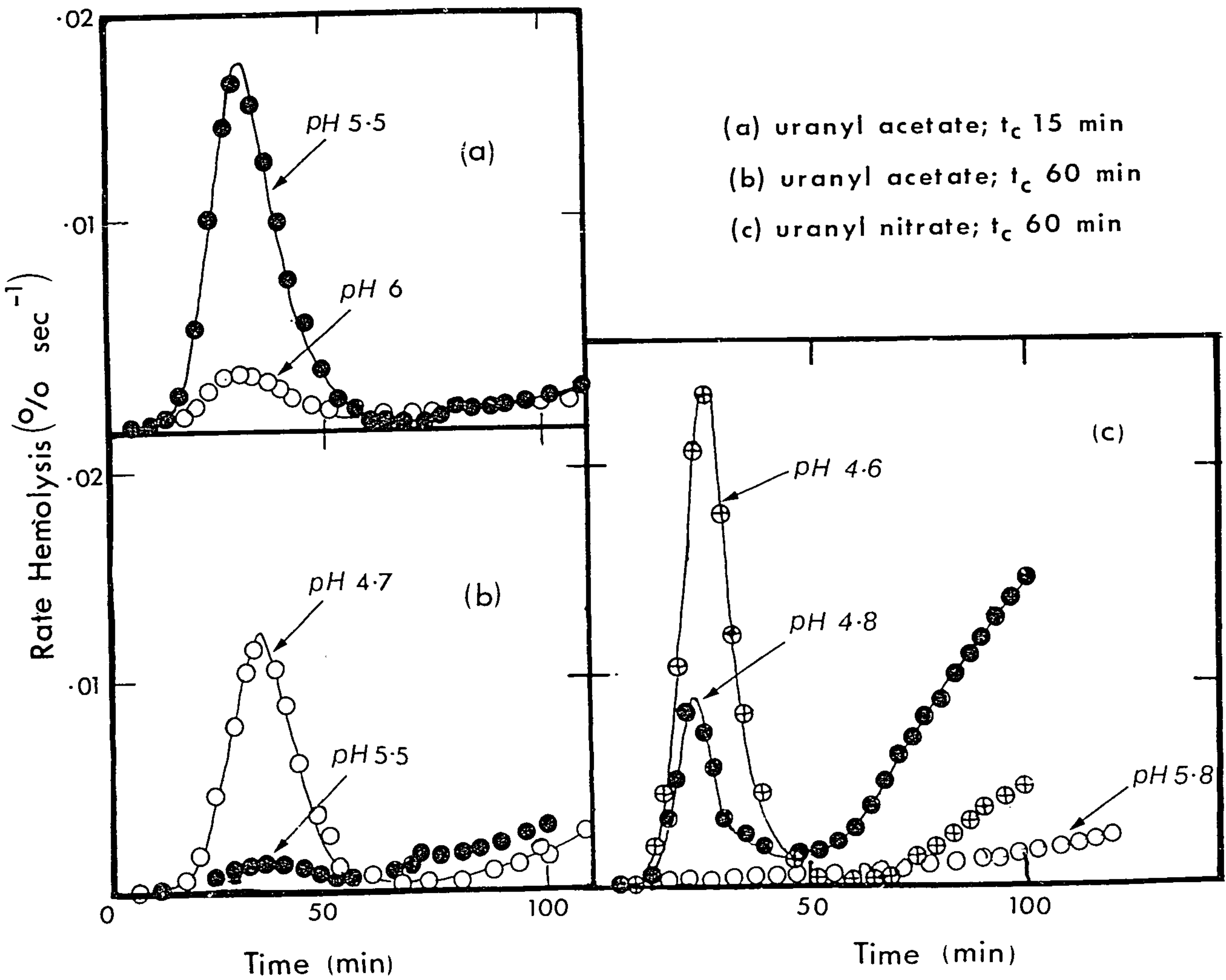


FIGURE 8. KINETICS OF HEMOLYSIS BY URANYL COMPOUNDS — EFFECT OF INITIAL pH OF URANYL SOLUTION DOSE: 0.4 FMOL/CELL

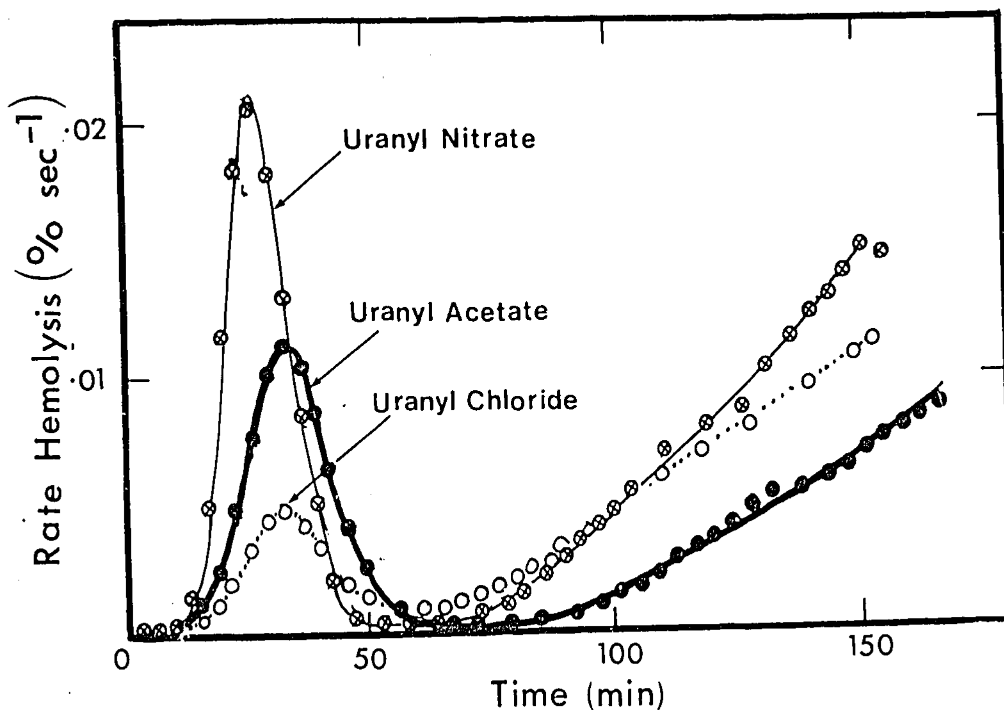


FIGURE 9. EFFECT OF ANION ON KINETICS OF HEMOLYSIS BY URANYL COMPOUNDS

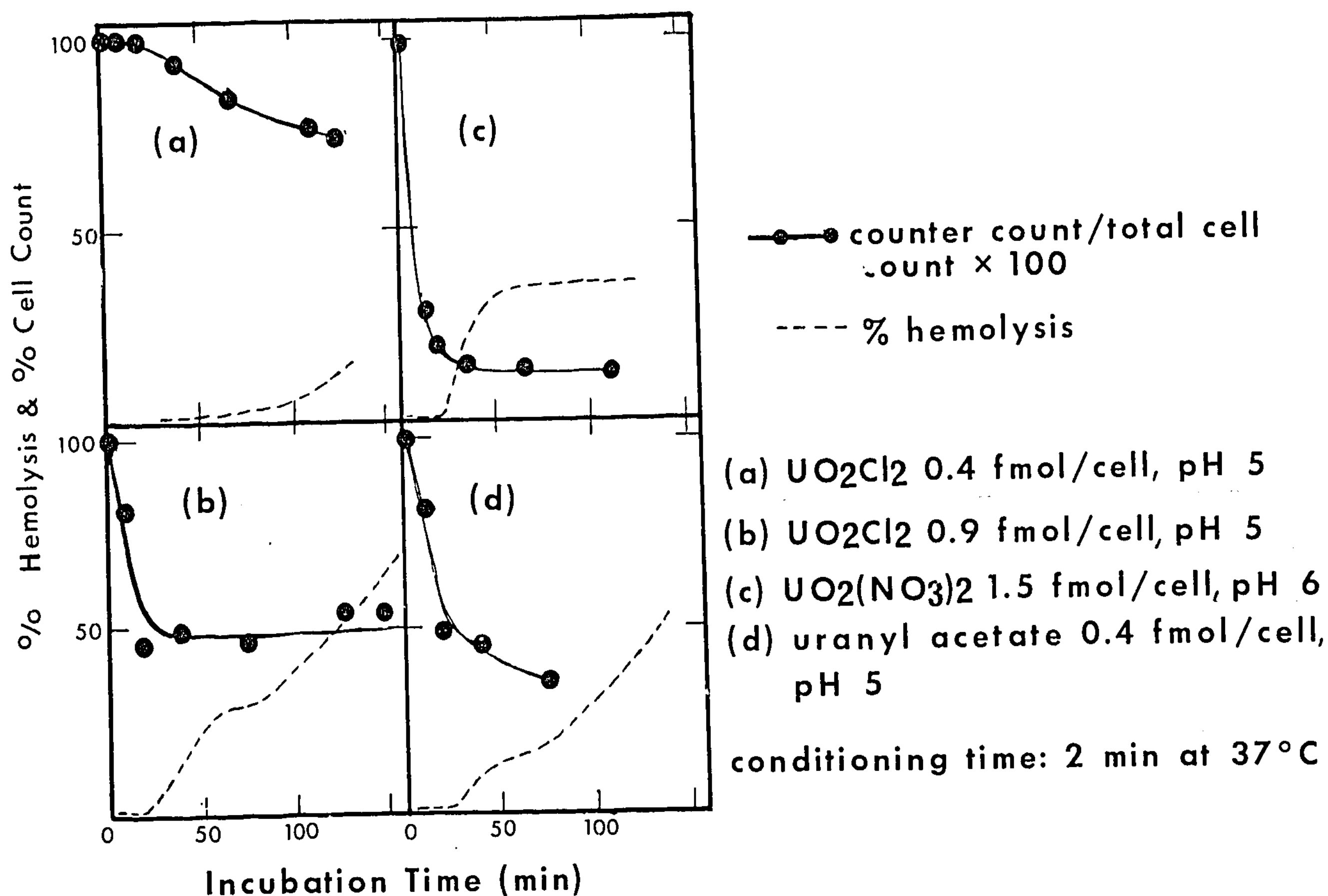


FIGURE 10. HEMOLYSIS AND AGGLUTINATION OF ERYTHROCYTES BY URANYL COMPOUNDS

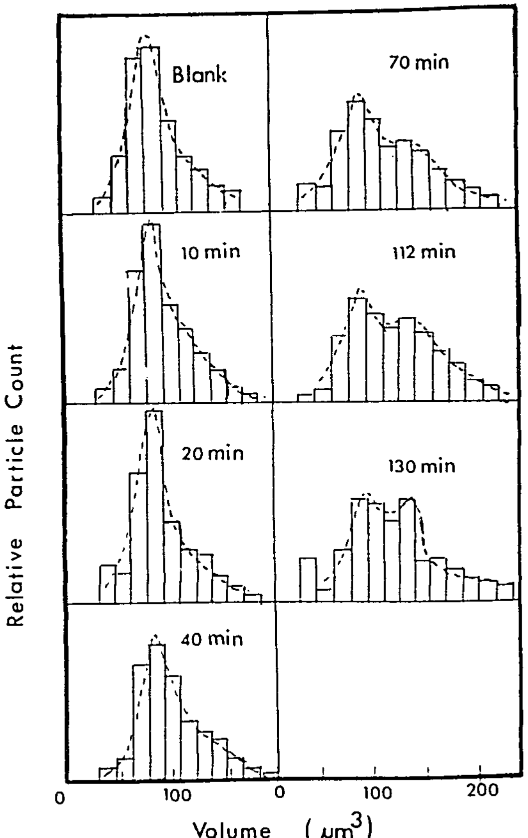


FIGURE 11. AGGLUTINATION OF ERYTHROCYTES BY URANYL CHLORIDE ; SIZE DISTRIBUTION HISTOGRAMS FOR VARIOUS TIMES OF INCUBATION, 0.4 fmol/cell, pH 5

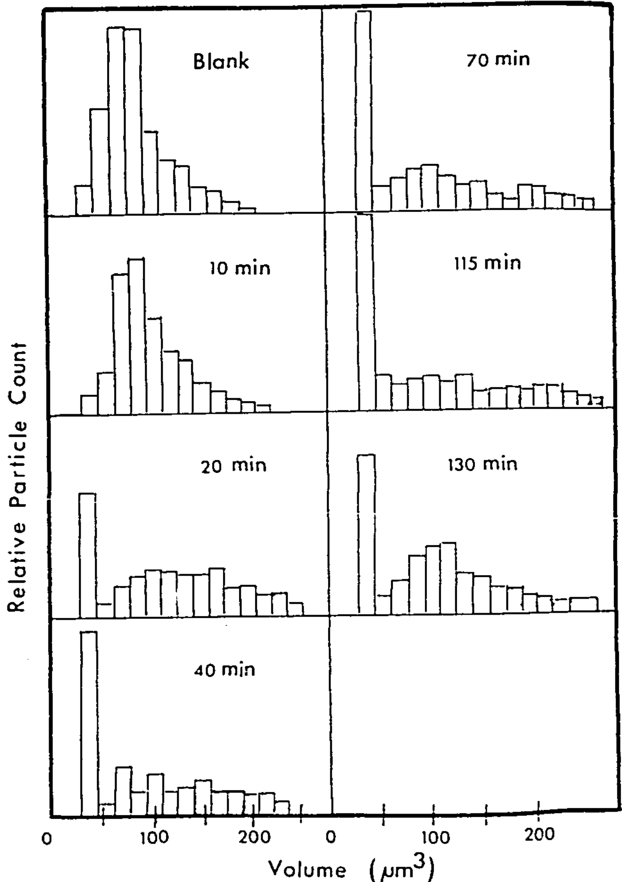
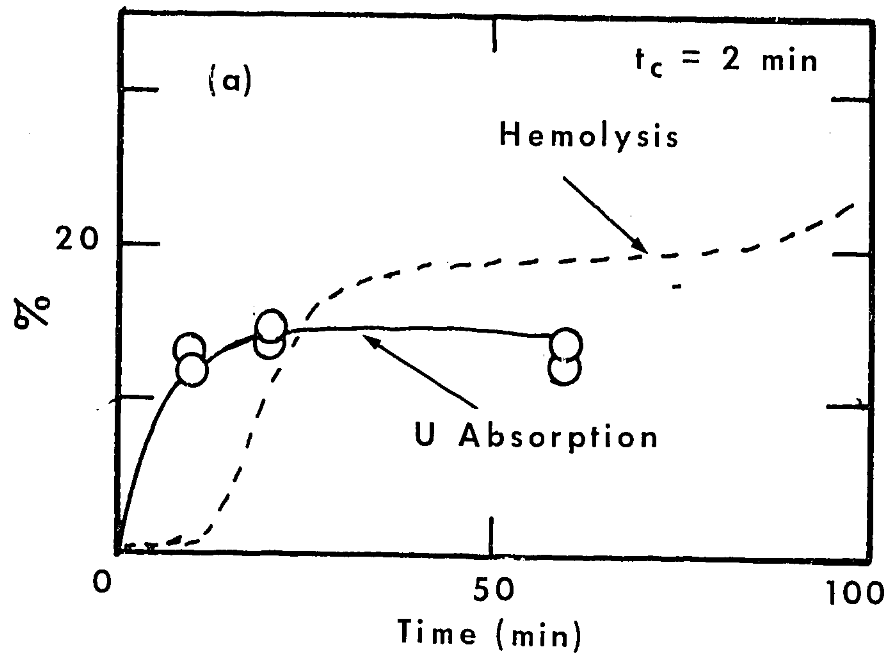


FIGURE 12. AGGLUTINATION BY URANYL CHLORIDE; SIZE DISTRIBUTION HISTOGRAMS FOR VARIOUS TIMES OF INCUBATION, 0.9 fmol/cell, pH 5



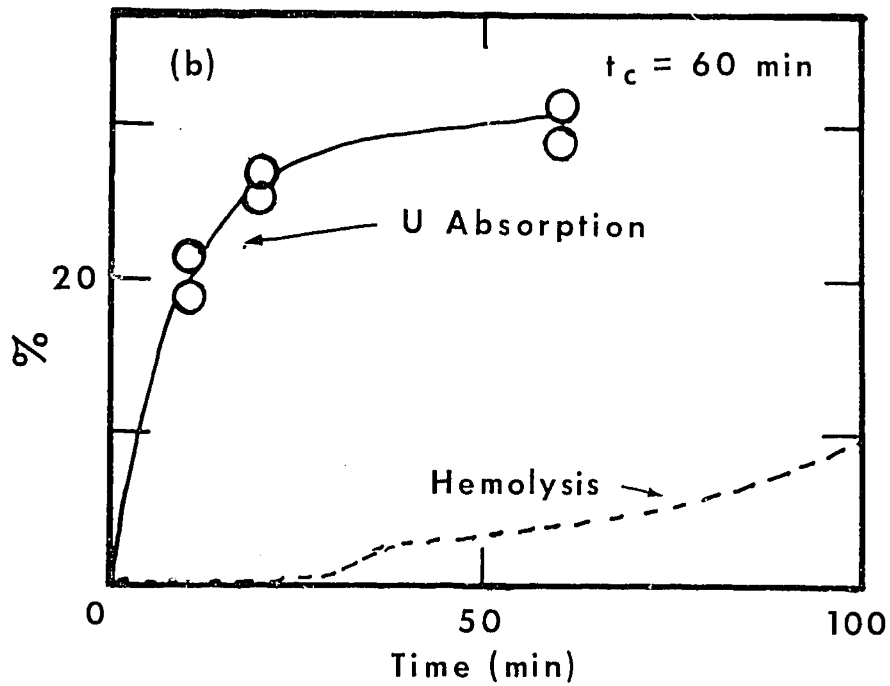


FIGURE 13. INTERACTION OF ERYTHROCYTES AND URANYL ACETATE; ABSORPTION OF URANIUM BY CELLS (AS % OF TOTAL DOSE). TOTAL DOSE: 0.4 fmol/cell

(a) Stage 1 hemolysis present
(b) Stage 1 hemolysis absent

describes a slower process that extends to > 120 min and takes place simultaneously with hemolysis.

Features of cell agglutination are confirmed and extended by size distribution analysis (as shown in Figures 11 and 12) and also by microscopic evidence. Figure 11 refers to the slow agglutination initiated by UO_2Cl_2 (0.4 fmol/cell) as shown in Figure 10(a); the series of distribution histograms specifies a change after 40 min incubation from the skewed distribution about a mode of 80 μm^3, typifying suspended single cells, to a bimodal distribution with maxima at about 80 μm^3 and 130 μm^3 respectively; these results are consistent with the microphotographic evidence of G.H. Price [private communication], which shows, after exposure to UO_2Cl_2, a decrease in the number of single cells and an increase in the proportion of agglutination pairs; multicell aggregates are not formed.

In contrast, the rapid agglutination (Figure 10(b)) initiated by a higher dose of UO_2Cl_2 (0.9 fmol/cell) leads to aggregates of many cells, as shown by videoscan photomicrography [G.H. Price, AAEC report in preparation]. As one might expect, the distribution histograms in Figure 12 indicate a rapid reduction in the proportion of single cells and a corresponding shift in distribution to particles of large size, i.e. > 200 μm^3.

3.3 Absorption of Uranium by Erythrocytes

Figure 13(a) shows the development of two-stage hemolysis and, conjointly, the uptake of uranium by cells as a function of incubation time. Similar data in Figure 13(b) describe the absorption of uranium or a system in which Stage 1 hemolysis is absent. In each case, uptake of uranium occurs almost totally within the first 20 min after mixing, and precedes hemolysis; thus the rate of uranium absorption does not directly control the rate of hemolysis.

It can be seen from Figure 13 that, although the effect of conditioning is to reduce Stage 1 hemolysis, this is not due to a decrease in the amount of uranium absorbed by conditioned cells; indeed, an extension of conditioning time appears to promote absorption of uranium. Now, investigations using electron microscopy have shown that hexavalent uranium reacts principally with constituents of the red cell membrane [Goodford and Wolowyk 1972; Wyatt 1975]; in our view, therefore, the effect of conditioning in altering uranium

absorption and subsequent hemolysis arises through a change in the nature of reaction sites at the cell membrane.

3.4 Pattern of Hemolysis

Two patterns of hemolysis can now be discussed:

(i) The case in which hemolysis of a single cell leads to complete liberation of hemoglobin [Chan et al. 1958], so that two types of cell can be observed; these are unaltered cells which are clearly distinguishable by optical microscopy, and faintly visible ghost cells. The fraction of hemoglobin from a cell population is therefore an indicator of the fraction of cells transformed into ghosts.

(ii) The case of partial hemolysis in which liberation of hemoglobin from individual cells is incomplete. This has been suggested as the pattern of post-hypertonic hemolysis in the presence of certain inhibiting agents such as dextran [Zade-Open 1968; Davis et al. 1968].

The evidence of optical microscopy obtained by Price [AAEC report in preparation] shows hemolysis by uranyl compounds to be of category (i) as indicated by the presence both of ghost cells and optically opaque unhemolysed cells. Furthermore, the same pattern of hemolysis applies within cell aggregates as for non-agglutinated cells.

Consider now the sequence leading to hemolysis of an individual cell in a population of cells: absorption of uranium occurs rapidly within time t_a, and changes certain properties of the cell membrane, thereby inducing prolysis and, finally, hemolysis which begins at time t_h. We define t_h as the hemolysis time, with $t_h \gg t_a$. Now, observation of hemolysing cells by videoscan shows that hemolysis is accompanied by a rapid decrease in optical density, although the shape and size of cells do not change during the liberation of hemoglobin. Cell disintegration or membrane rupture do not occur; hemoglobin is released over a period of about 60 s by a rapid diffusion across a cell membrane, the properties of which have been modified by reaction with uranium and subsequent changes during prolysis. From the evidence of optical microscopy, it can be assumed that if Δt is the time interval in which

hemolysis of any one cell occurs, then $\Delta t \ll t_h$. Thus, within our experimental limits, the prolytic stage is rate-controlling for hemolysis of the cell population. Stuart et al. [1975] interpreted hemolysis by uranium in terms of a deterministic process, assuming a two-compartment system with diffusional exchange of hemoglobin between the two as the rate-controlling step. But clearly, for the systems that we have examined, hemolysis of the individual cell is, comparatively, a sudden catastrophic element of a stochastic process. This process is in effect continuous for our cell population, and its rate is given by dN/dt, where N is the number of hemolysed cells at time t.

For a population of cells there is a distribution of hemolysis times which we take as evidence of a difference in properties of individual cells. In principle then, we may postulate a joint probability density function (PDF) given by $\phi(x_1, x_2, \ldots x_n, t_h)$, where the hemolysis time t_h gives the duration of prolysis for an individual cell; $x_1, x_2 \ldots x_n$ are properties which determine the magnitude of t_h and may or may not be time-dependent.

We have insufficient data to define $x_1, x_2 \ldots x_n$. Certainly the extent of uranium absorption and the nature of reactive sites at the cell membrane are properties that must affect the magnitude of t_h; but in effect, nothing is known of the events that attend the prolytic stage for the systems studied here. However, Davson and Danielli [1938], who examined the action of various lysins on erythrocytes, observed that some cell potassium escapes before the loss of hemoglobin, and that the amount lost depends upon the nature of the lysin. The results described by Ponder [1971] for a number of hemolytic systems, indicate that release of K^+, changes in cell volume and diffusion of water are important prolytic phenomena.

Although we cannot define $x_1, x_2 \ldots x_n$, the data yield an experimental PDF, $\phi(t_h)$, for t_h alone as the following argument shows. From the pattern of hemolysis established by visual observation, it is evident that the unhemolysed lifetime of an individual cell in vessel D is the incubation time, t, for which that cell survives until hemolysis, and is also the hemolysis time, t_h, for that cell. Thus the probability that a cell will have an unhemolysed lifetime of duration between t and t+dt is also the probability $\phi(t_h)dt_h$ that it will be hemolysed in the interval between t_h and $t_h + dt_h$, where $t = t_h$ and $dt = dt_h$. For the cell population, the former probability can be equated with the statistical estimator $dH_u/100$ to yield

$$dH_u/100 = \phi(t_h)dt_h = \phi(t_h)dt$$

whence

$$dH_u/dt = 100\phi(t_h) \tag{1}$$

so that, apart from the factor 100, any curve of dH_u/dt in Figures 7 to 9, is also a curve of $\phi(t_h)$.

Now, the kinetic data do not follow hemolysis to completion, but a curve of dH_u/dt _v_. t shows a low minimum between t~5 min and t~75 min, followed by a rise that must lead to at least one other maximum between t~100 min and completion of hemolysis. Therefore, according to Equation 1, it is at least clear that the complete curve of $\phi(t_h)$ is divided by a low minimum into two definite regions corresponding to the two stages of hemolysis. Assuming that the two regions are indicators of two mutually exclusive processes, we may write

$$\phi(t_h) = \phi_1(t_h) + \phi_2(t_h) \tag{2}$$

where $\phi_1(t_h)$ and $\phi_2(t_h)$ refer to Stages 1 and 2 respectively, and $\phi(t_h) \sim\phi_1(t_h)$ for $t \leq 50$ min. As shown in Figures 7 to 9, that part of the differential curve encompassing Stage 1 hemolysis exhibits a degree of symmetry that suggests a Gaussian function as an appropriate description of dH_u/dt and hence of $\phi_1(t_h)$.

A Gaussian function was fitted to experimental data for times t up to ~45 min (that is, data covering Stage 1 but excluding transitional data points). This was done with the aid of a Data General Nova computer, using a program that allows the parameters in a general non-linear regression problem to be refined using full-matrix least squares techniques.

Experimental data-points of dH_u/dt are plotted in Figure 14, together with the Gaussian function curve of best fit for each of four cases. The plots show that Stage 1 hemolysis conforms well to a Gaussian function, i.e.

$$dH_u/dt = A \exp\left[-(t-T)^2/2S^2\right] \tag{3}$$

Combining Equations 1, 2 and 3 then yields

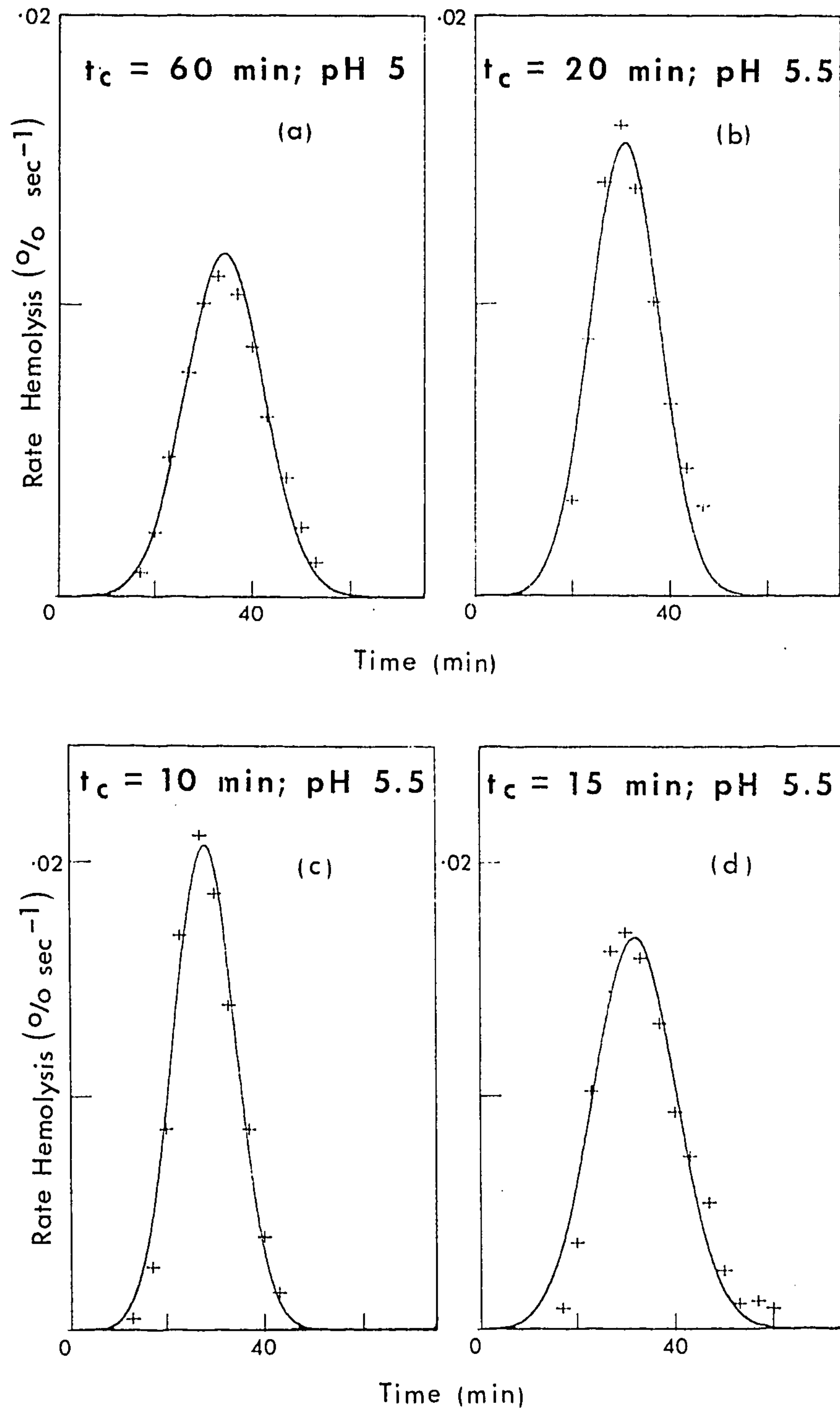


FIGURE 14. RATE OF HEMOLYSIS BY URANYL ACETATE; CROSSES ARE EXPERIMENTAL POINTS; UNBROKEN CURVE IS GAUSSIAN CURVE OF BEST FIT TO EXPERIMENTAL POINTS.
DOSE: 0.4 fmol/cell

$$\phi_1(t_h) = 10^{-2} \cdot A \exp\left[-(t-T)^2/2S^2\right] , \qquad (4)$$

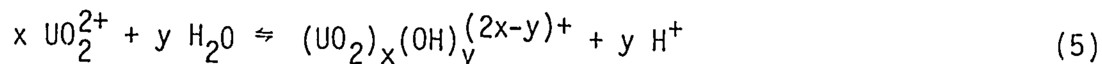
where A is maximum rate of hemolysis for Stage 1 and T is the incubation time at which $dH_u/dt = A$.

The influence of conditioning on Stage 1 hemolysis can be demonstrated graphically in terms of the Gaussian parameters; as shown in Figure 15, the magnitude of A diminishes rapidly with increasing conditioning time, t_c, whereas T increases. There does not appear to be any systematic change in S for $t_c \leq 60$ min.

The results described here point to the usefulness of automated analysis in providing accurate kinetic data for <u>in vitro</u> hemolysis so that a quantitative interpretation can be attempted. The data draw attention to certain factors affecting the interaction of hexavalent uranium and erythrocytes which could be profitably studied in more detail. For example, it has been shown that pH of uranyl solutions and the presence of anions (NO_3^-, acetate even in an excess of Cl^-) markedly influence the course of hemolysis. This may arise in two ways:

(i) Through changes in cell properties, and especially by changing the nature of reactive sites at the cell membrane.

(ii) By altering the nature of U(VI) species in solution, either by complex formation involving anions or through hydrolysis reactions denoted by



$$x\,UO_2^{2+} + y\,H_2O \rightleftharpoons (UO_2)_x(OH)_y^{(2x-y)+} + y\,H^+ \qquad (5)$$

The relative importance of these various effects could be established readily by further autoanalytical studies, particularly if the pH and anion content of cell suspensions were to be varied systematically before mixing with uranium solution.

Our kinetic data and microscopic evidence demonstrate the importance of prolysis as the rate-controlling process in hemolysis by uranyl compounds. A logical extension of the present work would therefore require the simultaneous measurement of K release, Na exchange, uranium absorption and hemoglobin release to provide differential rate curves for all four phenomena. This can

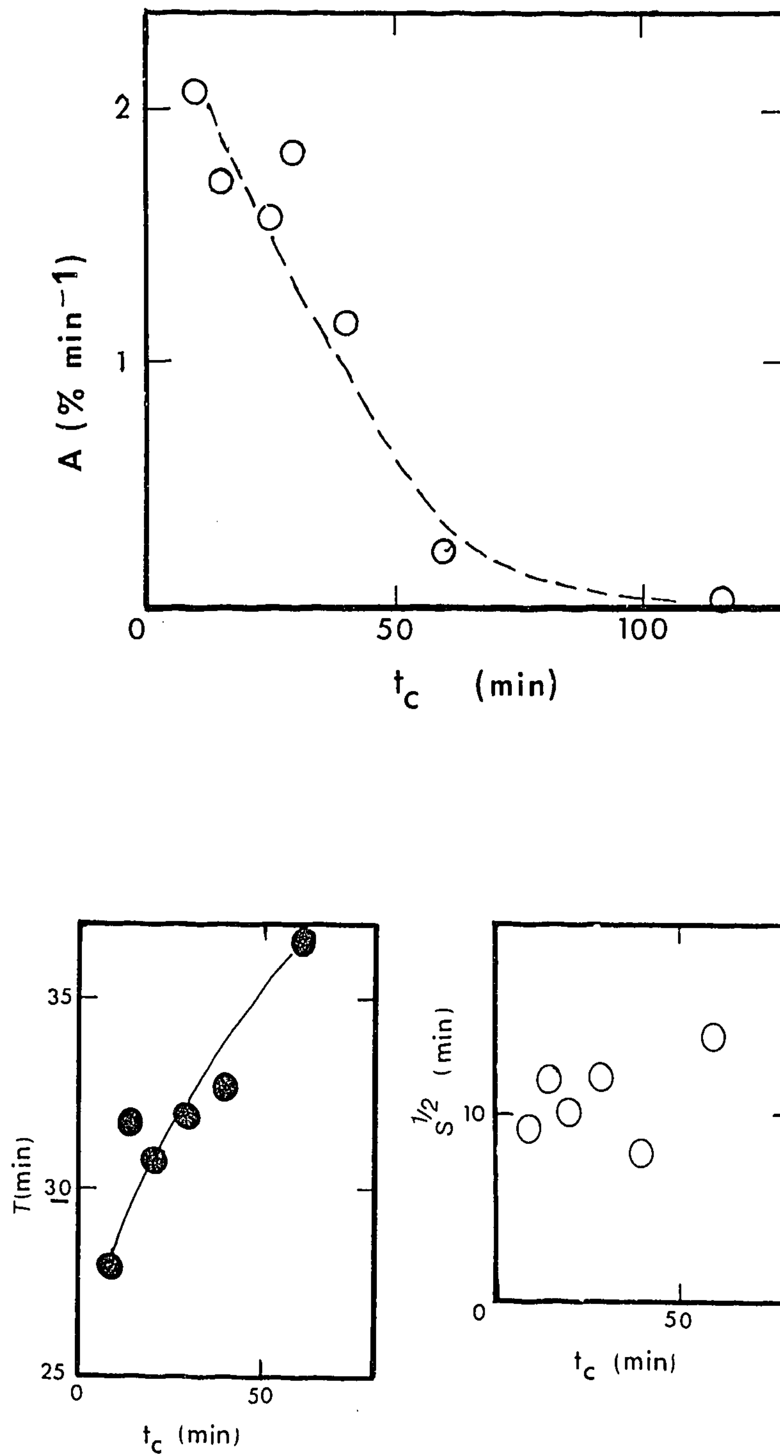


FIGURE 15. KINETICS OF HEMOLYSIS BY URANYL ACETATE; EFFECT OF CONDITIONING TIME t_c ON GAUSSIAN PARAMETERS. DOSE: 0.4 fmol/cell

readily be done by splitting the output from the flow centrifuge E into four controlled streams and adding appropriate detectors for continuously monitoring concentrations of Na, K and U.

A potentially important application of ACA techniques is in the study of cellular damage induced by mineral dusts and fibres. *In vitro* hemolysis by mineral substances has often been investigated, with particular attention being directed to asbestiform materials and the various polymorphs of silica. There has been much discussion on the use of hemolytic techniques for screening potentially harmful dusts, and two differing viewpoints emerge.

Firstly, it can be argued that hemolysis of washed erythrocytes by dusts is too elementary a system to be correlated even qualitatively with pathogenic effects in humans or intact animals, so that *in vitro* studies must be directed towards macrophages or related tumour cells, the properties of which are more likely to correlate with *in vivo* effects.

On the other hand, it has been suggested by Hefner and Gehring [1978] and Morgan et al. [1977] that a correlation may exist between hemolytic activity of mineral dusts or fibres and their fibrogenic potential. There is however some difficulty in defining hemolytic activity. Hemolysis by mineral substances is commonly determined by mixing a standardised amount of material with washed erythrocytes in saline; the amount of hemoglobin released after a fixed period of incubation is then determined photometrically. But, as was shown by a number of investigators [Harington, et al. 1971; Morgan et al. 1977; Ottery and Gormley, 1978; Stuart and Price, 1980], the measured degree of hemolysis depends upon various factors such as particle size and concentration, surface area of the mineral, time of incubation, extent of fibre dispersion, and degree of agitation of the lysing mixture. As pointed out by Ottery and Gormley [1978], these factors may also be important for other *in vitro* methods.

Application of automated analysis would give precise control over the experimental parameters that we have discussed as well as providing in each single experiment accurate kinetic data not only for the release of hemoglobin but also for prolytic events such as release of potassium.

4. ACKNOWLEDGEMENT

We acknowledge the valuable help provided by Mr R.A. Phillips who designed and assembled the data acquisition system.

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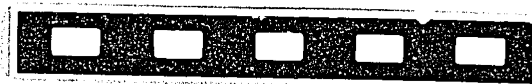

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