ANALYSIS OF PROTEIN DISINTEGRATION UNDER PROTON BOMBARDMENT

by

Frank Stewart Eadie

A thesis submitted to the Faculty of Graduate Studies and Research of McGill University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

August, 1952.

TABLE OF CONTENTS

Page No.

SUMMARY i

ACKNOWLEDGEMENTS. ii

CHAPTER I - INTRODUCTION. 1

CHAPTER II - THEORY

I PROTON DOSIMETRY 6

II ULTRACENTRIFUGE THEORY 10

CHAPTER III - APPARATUS

I THE ULTRACENTRIFUGE . . . . . .

1. General 15

2. The Svedberg Cell. 17

3. The Mechanical System. 19

4 The Vacuum System 24

5. The Electrical System. 25

6. The Optical System 25

II PHOTOGRAPHIC PLATES 28

III THE MICROPHOTOMETER 29

CHAPTER IV - EXPERIMENTAL WORK

I CYCLOTRON IRRADIATIONS

1. The Target 31

2. Dosage Measurements. 31

II ULTRACENTRIFUGE MEASUREMENTS

1. Calibration of Plates and the Concentration Scale 34

2. Absorption Measurements. 36

3. Sedimentation Measurements 37

TABLE OF CONTENTS (Cont'd.)

Page No.

III ELECTRON MICROSCOPE EXAMINATION OF PROTEINS . . . 40

CHAPTER V - RESULTS

I DOSAGE MEASUREMENTS

1. General 42

2. Experiments with Urease 42

3. Experiments on Serum Albumin 45

II ABSORPTION MEASUREMENTS

1. Absorption Curves and Concentration Scales. . 47

2. Serum Albumin 52

3. Haemoglobin 54

III UNIRRADIATED PROTEINS

1. Serum Albumin 57

2. Haemoglobin 58

3. Urease 58

IV SEDIMENTATION EXPERIMENTS ON IRRADIATED PROTEINS

1. General 58

2. Serum Albumin 67

3. Haemoglobin 82

CHAPTER VI - CONCLUSIONS

I IRRADIATED SERUM ALBUMIN 85

II IRRADIATED HAEMOGLOBIN 86

III EXPERIMENTAL TECHNIQUES 87

SUMMARY

Dry bovine serum albumin and bovine haemoglobin have been irradiated with 50 Mev cyclotron protons. The measured dosage rate was $6.1 \pm 2.7 \times 10^5$ rep/second. Irradiation times varied from one to eight minutes.

Fragments of gram molecular weight estimated at 1000 were found in sedimentation experiments on serum albumin irradiated with 7.3×10^7 rep. These fragments did not absorb at 2920 Å. It was concluded that molecules of serum albumin in which a single primary ionization occurs are broken preferentially at one point to give fragments of this size.

Sedimentation experiments on haemoglobin irradiated with dosages of protons up to 3×10^8 rep showed that little fragmentation had occurred.

For serum albumin, the ultra-violet absorption at 2576 Å and 2920 Å increased by a factor of three after a primary ionization had occurred in 93% of the irradiated molecules. The absorption of haemoglobin at the same wavelengths increased by 50%. These changes were attributed to a shift in the maximum absorption band towards the visible end of the spectrum. At 3906 A the absorption of haemoglobin decreased by 50%.

ACKNOWLEDGEMENTS

The author expresses his appreciation to Dr. J.S. Foster, director of the research, for his interest and encouragement in this work.

Discussions with Dr. J.H. Quastel on the biological aspect of the work, and with Dr. D. Jackson on the energy losses of fast charged particles, were most helpful.

Thanks are expressed to Mr. L. Fridhandler for his measurements of the enzyme activity of irradiated urease.

In early stages of the work Dr. J.A. Carruthers gave valuable advice on the operation of the ultracentrifuge.

The help of Dr. S. Wagner in using the electron microscope was greatly appreciated.

Special thanks are extended to Mr. Stanley Doig for his advice and assistance on mechanical problems associated with the ultracentrifuge.

The able operation of the cyclotron by Mr. R.H. Mills was greatly appreciated.

CHAPTER I - INTRODUCTION

One way in which the internal structure of large molecules can be studied is by the examination of the effects on the molecules of various ionizing radiations. In the present work the breakdown products of bovine haemoglobin and serum albumin irradiated in the dry state with 50 Mev protons have been studied using an ultracentrifuge. Dosages varied from 4×10^7 rep[#] to 3×10^8 rep in irradiations of from one to eight minutes duration. The rate of energy loss of protons at this energy is low enough so that not more than one primary ionization is likely to be produced by a proton crossing a molecule of either of these proteins. The secondary electron ejected in a primary ionization initiates, on the average, two other secondary ionizations within a distance equal to the radius of a serum albumin molecule.

By using an ultra-violet spectrograph as a camera for the ultracentrifuge, the sedimentation of the fragments was studied in several regions of the absorption spectrum at one time.

The irradiated proteins were also examined with an electron microscope for aggregation products.

These experiments show that a single primary ionization occurring in molecules of serum albumin produce small fragments whose gram molecular weight is estimated at 1000. Aggregation products with gram molecular weights estimated at 5×10^5 and 4×10^6 were also found. For two and three primary ionizations per molecule a large

---

[#] The rep has been defined by Parker[(1)] as that "dose" of any ionizing radiation which produces energy absorption of 83 ergs/cc. of tissue. Rep is an abbreviation for roentgen equivalent physical.

number of very small fragments are produced and the production of large aggregates ceases.

No effects were found in haemoglobin which could be ascribed to single primary ionizations occurring in individual molecules. Instead, the material became increasingly polydisperse with increasing dosages of radiation.

It is concluded that in serum albumin molecules there are regions which would be described by Lea[2] as radiosensitive. From these, fragments of fairly uniform size are broken off. In haemoglobin, on the other hand, there is little fragmentation. Ionizations within the molecules merely cause rearrangement of the internal chemical bonds.

An extensive review of the effects of ionizing radiations on viruses and other large molecules has been given by Lea[2]. He has shown that in small and medium sized viruses (diameters from 16 mμ to 70 mμ) an ionization anywhere in the molecule is sufficient to inactivate the virus. In the case of large viruses (diameters of the order of 200 mμ) large discrepancies were found between the size estimated from irradiation experiments and the actual sizes. He postulates that in the latter case the whole volume of the molecule is not radiosensitive. He denotes as radiosensitive those parts of the molecule essential to the infectivity of the virus, so that chemical change in those parts following ionization leads to loss of infectivity.

A group at Yale University[3-9] has studied various proteins, enzymes and viruses bombarded by 4 Mev deuterons, X-rays and low voltage electrons (0-100 volts). Measurements have been made on molecular deactivation cross-sections and compared with those computed from molecular size.

Pollard and Dimond[4], by measuring the infectivity of tobacco mosaic virus after irradiation with deuterons, have shown that the whole molecule is radiosensitive. However, they also found that the infectivity may be reduced below one percent without affecting the ability of the molecules to combine with anti-serum[9]. They attribute this large difference in molecular cross-section to the difference in the number and energy of the chemical bonds which are specific for the two reactions.

Svedberg and Brohult[10] irradiated solutions of haemocyanin, haemoglobin and serum albumin with alpha particles from radon dissolved in the solutions at the temperature of liquid air. They then studied the sedimentation of the molecules in an ultracentrifuge and concluded that every haemocyanin molecule situated in the path of an alpha particle was split into half-molecules. No effect on the serum albumin and haemoglobin solutions was observed. In 11.8 days the number of alpha particles per cubic centimeter of solution was 5.8×10^{12}. The average energy of the alpha particles from Rn, RnA, and RaC is 6.4 Mev. The dosage calculated from these figures is approximately 7×10^{5} rep. Twenty-eight percent of the haemocyanin molecules were changed by the radiation. The molecular cross section of haemoglobin and serum albumin is approximately one tenth that of haemocyanin so that no effect on these proteins would be expected with this dosage of alpha particles.

Carruthers[11] studied the breakdown of dry bovine serum albumin under alpha particle bombardment. The irradiations were made by mixing the protein with powdered boron and irradiating the mixture with slow neutrons in the nuclear pile of the National Research Council at Chalk River, Ontario. The neutrons interacted with the boron to produce alpha

particles and recoil lithium nucleii. With this method dosages estimated at 5 x 10^7 rep were obtained. The 5 Mev alpha particles produced up to 16 ionizations in crossing a molecule of serum albumin.

Carruthers found that, after alpha particle irradiation, the absorption of ultra-violet light by bovine serum albumin was increased and extended into the visible region of the spectrum. He attributed the large increase in absorption to small fragments broken off the protein molecule. It was also evident that large aggregation products were present. He did not, however, attempt to find out whether the rate of sedimentation in the ultracentrifuge was the same in all regions of the absorption spectrum.

Many experiments have been performed on proteins irradiated in solutions. These are not comparable with the work described here because of the effects of the ionizing radiations on the solvent. The subsequent chemical reactions between the solute and solvent mask the direct effects of radiation on the protein. A large bibliography on this subject is given by Lea[2]. Recent experiments on the effect of ionizing radiation on heat denaturization of protein have been described by Friche[12]. The protein was irradiated in solution with 33,000 r. of X-rays. By raising the concentration he has been able to show that damage by the direct action of radiation on the molecule was more profound than that due to secondary interaction with the solvent.

Techniques used in ultracentrifuge experiments are largely those developed by Svedberg and his co-workers[13] between 1923 and 1933, using centrifuges with oil turbine drives. The ultracentrifuge at McGill is of the type developed by Beams[14] between 1933 and 1940. The use of

the spectrograph as a camera for the ultracentrifuge dispenses with the necessity of ultra-violet filters and was first described by Gunton[15] and Carruthers[11].

Before experiments were started it was decided to move the apparatus from the Physics Building to the Radiation Laboratory at McGill University. While it was disassembled for this move many improvements were made on the centrifuge. The most important of these was in the bearings, which were completely redesigned.

CHAPTER II - THEORY

I PROTON DOSIMETRY

The breakdown of large molecules subjected to ionizing radiation is due to chemical changes initiated by ionizations occurring within the molecule. From the comparison of biological effects of ultra-violet light and ionizing radiations Lea(2) concluded that the effects of excitation are small compared with those of ionizations. Breakdown due to nuclear collision may also be neglected.

High energy protons dissipate most of their energy by the ejection of secondary electrons from atoms in the matter through which they pass.

The average energy lost per secondary electron is of the order of twice the average ionization potential of the atomic electrons. Half of the energy lost by the primary particle appears as kinetic energy of the secondary electron. For proteins, composed of light elements hydrogen, carbon, oxygen and nitrogen, the average ionization potential is approximately 100 ev. Electrons of this energy, according to Lea(2), create 1.7 ion pairs per milli-micron of path and have a range of 3 mμ in tissue of unit density. Hence, a secondary electron ejected from an atom in a serum albumin molecule of radius 2.2 mμ is likely to cause other ionizations within the molecule.

Considering the spread in energy of the secondary electrons, from one to five ionizations might be found in single molecules due to the effect of a single secondary electron. The average number used by Lea(2) is three.

For 50 Mev protons the distance between successive primary ionizations is much larger than the diameter of molecules used in this work. Aron, Hoffman and Williams[16] have calculated the rate of energy loss of high energy protons in air. For protein composed of elements of low atomic number the rate of energy loss may be taken as approximately proportional to density.

In Table I the rates of energy loss of 14 Mev, 50 Mev and 90 Mev protons are shown for both air and protein. The number of primary ionizations per micron of path in protein is also shown, calculated for an energy loss of 160 ev. per secondary electron ejected. For the value of the average ionization potential of the protein, that of air--80 ev.-- has been used.

TABLE I

Proton energy	Rate of energy loss in Air 15°C 760 mm. Hg	Rate of energy loss in Protein Density 1.0 gm/cc.	No. of primary ions produced per micron of path in protein
Mev	Mev/cm.	Mev/cm.	ions/μ
14	3.7×10^{-2}	30.2	19
50	1.3×10^{-2}	10.6	6.6
90	0.83×10^{-2}	6.8	4.3

In the present experiments with 50 Mev protons, a single molecule is broken by internal formation of a cluster of ion pairs. Since the separation of these clusters is large compared to molecular dimensions, the rate of breakdown can be calculated on the basis that clusters are formed uniformly throughout the material.

The rate at which molecules are broken down is then given by the expression

$$\frac{dn}{dt} = -\lambda n \tag{1}$$

where n is the number of unbroken protein molecules present,

t is the time,

and λ is the probability per unit time of a cluster forming inside a molecule.

The solution of this equation is

$$n = n_o e^{-\lambda t} \tag{2}$$

where n_o is the original number of molecules.

The number, n_1, of molecules with at least one primary ionization is given by

$$n_1 = n_o(1 - e^{-\lambda t}) \tag{3}$$

The rate of formation of molecules, n_2, in which two or more primary ionizations have occurred is given by the expression

$$\frac{dn_2}{dt} = \lambda (n_o - n_o e^{-\lambda t} - n) \tag{4}$$

The solution of this equation is

$$n_2 = n_o(1 - e^{-\lambda t} - \lambda t e^{-\lambda t}) \tag{5}$$

The number of molecules, n_1', in which one, and only one, primary ionization has occurred is then given by

$$\begin{aligned} n_1' &= n_1 - n_2 \\ &= n_o \lambda t e^{-\lambda t} \end{aligned} \tag{6}$$

The fraction of the molecules $\frac{n_1'}{n_o}$ has a maximum at irradiation time t_{max}, where

$$t_{max} = \frac{1}{\lambda} \tag{7}$$

The constant λ is related to the volume, V, of a single molecule by the relation

$$\lambda = V\mathcal{J} \tag{8}$$

where $\mathcal{J}$ is the number of clusters formed per cubic centimeter per second.

$\mathcal{J}$ is related to the current of protons and their rate of energy loss by the equation

$$\mathcal{J} = \frac{i \frac{dE}{dx} \Delta x}{v \times 160} \tag{9}$$

where i is the number of protons per second passing through the target,

$\frac{dE}{dx}$ is the rate of energy loss of protons in protein in electron volts,

Δx is the length of the proton path in the protein,

v is the volume of the protein target

and 160 is the average energy lost by the protons per primary ionization in electron volts.

The unit of dosage, the rep, has already been defined as an energy absorption in tissue of 83 ergs/cc. Dosage rate is related to proton current and rate of energy loss by the equation

$$r = \frac{i \frac{dE}{dx} \Delta x}{v \times 83} \times 1.6 \times 10^{-12} \tag{10}$$

$$= \frac{\mathcal{J} \times 160}{83} \times 1.6 \times 10^{-12}$$

$$= \frac{\lambda}{V} \times 3.09 \times 10^{-12} \tag{11}$$

where r is the proton dosage rate in rep/sec.

and 1.6×10^{-12} is the conversion factor from electron volts to ergs.

The volume of the serum albumin molecule is 4.5×10^{-20} cc., giving for the dosage rate the expression

$$r = 6.8 \lambda \times 10^{7} \text{ rep/sec.} \qquad (12)$$

Hence, by measuring the probability per unit time of a primary ionization occurring within a molecule of serum albumin, the dosage rate can be determined.

II ULTRACENTRIFUGE THEORY

A complete derivation of the theory of the ultracentrifuge has been given by Svedberg[15]. This synopsis has been abstracted from his book.

Svedberg considers the motion of particles of uniform size suspended in a rotating liquid. Under the action of the centrifugal field these move to the periphery of the liquid. A definite boundary is established between the inner portions of the liquid, with zero concentration of particles, and the outer portions where the concentration of particles is constant from boundary to periphery.

The molecular weight of the particles can be calculated from the velocity with which the boundary moves, using the expression

$$M = \frac{RTs}{D(1 - V\rho)} \quad \frac{dx/dt}{w^2 x} \qquad (13)$$

In this expression

M is the molecular weight of the particles,

x is the distance of the boundary from the center of rotation,

t is the time,

w is the angular speed of rotation,

R is the gas constant,

T is the absolute temperature,

D is the diffusion constant of the particles,

V is the partial specific volume of the particles

and ρ is the density of the solution.

The constant $\frac{dx/dt}{w^2x} = s$, the sedimentation rate in unit centrifugal field, is the sedimentation constant of the particles.

Usually diffusion blurs the sedimenting boundary. According to diffusion theory, point x is then that point where the concentration is 50% of the maximum. From the movement of this point the sedimentation constant may be calculated, using the approximation

$$s = \frac{\Delta x/\Delta t}{w^2 x_m} \tag{14}$$

or the exact expression

$$s = \frac{\log (x_2/x_1)}{w^2(t_2 - t_1)} \tag{15}$$

In these expressions

x_m is the mean position of the 50% point on the boundary between the times t_1 and t_2 at which measurements are made.

x_1 and x_2 are the positions of the 50% point at the times t_1 and t_2, and $\Delta t = t_2 - t_1$.

To get convection-free sedimentation the cell containing the solution must have the shape of a sector. In such a cell the maximum concentration at the periphery of the cell decreases with time. The concentration, C_{t_1}, in this part of the cell may be calculated at any time, t_1, after sedimentation has started, from the expression

$$C_{t_1} = C_o \, e^{-2t_1 w^2 s}$$

where C_o is the original concentration of the solution.

In deriving this formula, account has been taken of the variation of centrifugal force with distance from the center of rotation. Rearranged, the formula may be used to calculate the sedimentation constant.

$$s = \frac{\log C_o/C_{t_1}}{2 w^2 t_1} \qquad (16)$$

Agreement between the values of s, calculated from equations (14) and (16), is a sensitive test as to the homogeneity of the particle sizes. The value of the sedimentation constant derived from equation (16) is particularly sensitive to the presence of particles of larger than average size. The value of the diffusion constant, D, can also be derived from sedimentation experiments.

Knowing the values of the sedimentation and diffusion constants, the concentration of solution, C, at any distance, x, from the center of rotation, can be calculated from the expression

$$C = \frac{1}{2} C_o e^{-2w^2 st} \left\{ 1 - \frac{2}{\sqrt{\pi}} \int_o^y e^{-y^2} dy \right\} \qquad (17)$$

where $y = \frac{x_o e^{w^2 st} - x}{\sqrt{4 D t}}$

and x_o is the distance from the center of rotation at which sedimentation starts.

Other symbols are the same as those already used.

The expression

$$\Phi (y) = \frac{2}{\sqrt{\pi}} \int_o^y e^{-y^2} dy$$

is the well-known probability integral and is tabulated by Svedberg[13].

If the curves of concentration, C, against distance, x, plotted from equation (17), agree with the experimentally determined curves, the solute is certainly a monomolecular species.

The expressions given above are all derived assuming a monodisperse material as solute. In studying polydisperse systems the values of s found by the two different methods described are average values. An average value of D must be assumed or measured in a separate experiment to plot the curves of equation (17). In the experiments on polydisperse material described in this thesis, the concentration at any point in the Svedberg cell is measured by the ultra-violet absorption at that point. However, no exact method is known for calculating from sedimentation experiments the distribution of particle sizes present in the cell for cases in which the sedimentation constants, diffusion constants, ultra-violet absorption constants and concentrations of the individual particle sizes are all unknown.

The sedimentation constant varies with viscosity, hence with temperature. It also depends upon the solvent in which the material under study is dissolved. It is usual to correct experimentally determined values of s to the values which would be obtained with water as a solvent at 20°C, designated s_{20}.

The first correction applied is for temperature variations between exposures.

$$s'_{20} = \frac{\Delta x/\Delta t}{w^2 x_m} \quad \frac{n_T^o}{n_{20}^o} \tag{18}$$

$$s'_{20} = \frac{\log (C_o/C_{t_1})}{2\, w^2 t_1} \quad \frac{n_T^o}{n_{20}^o} \tag{19}$$

where n_T^o is the viscosity of water at the mean temperature, T, between exposures

and n_{20}^o is the viscosity of water at 20°C.

A correction is then made for the solvent, using the expression

$$s_{20} = s'_{20} \frac{n_T}{n_T^o} \frac{0.2523}{1 - V_T \rho_T} \qquad (20)$$

where n_T is the viscosity of the solvent at the mean temperature, T, of the sedimentation experiment,

n_T^o is the viscosity of water at the mean temperature, T,

V_T is the partial specific volume of the solute in the investigated medium, at the mean temperature, T

and ρ_T is the density of the medium at the mean temperature, T.

The constant 0.2523 has been calculated by Svedberg for aqueous protein solutions. The last correction factor for the acetate buffer solution used in the present experiments is equal to 1.07 ± 0.01 for all temperatures between 25°C and 30°C.

Svedberg[13] lists the molecular weights and corresponding sedimentation constants of a large number of proteins. In Fig. 27, page 80, molecular weight has been plotted as a function of sedimentation constant from this data. By interpolation and extrapolation on this curve molecular weights were estimated from the sedimentation constants measured in the present work.

CHAPTER III - APPARATUS

I THE ULTRACENTRIFUGE

1. General

The electrically-driven ultracentrifuge used in the present work is similar to that designed by Skarstrom and Beams[17]. As detailed descriptions of the apparatus have been given by Rotenberg[18], Gunton[15] and Carruthers[11], only a general description will be given here, except where improvements have been made.

A schematic diagram of the cross section of the ultracentrifuge is shown in Fig. 1. The Svedberg type rotor (A) rotates in the armour-plate vacuum chamber (B). The rotor is directly connected to the armature (C) of the motor by a 1/8" tubular steel shaft (D). The rotating system is supported by the solenoid (E). The motor bearings are indicated by (F) and (G); the bottom bearing for the Svedberg rotor, which also acts as a vibration damper, is marked (H).

The two-phase induction motor (K) is driven by a transition oscillator, the frequency of which is variable in steps of approximately 200 c/s from 430 to 1420 c/s. The output of the oscillator is fed into a power amplifier capable of supplying 1,000 watts to the motor.

The speed of the motor is measured by reflecting light from the armature, which is partially blackened, into a photo-electric cell. Output pulses from the photo-electric cell are amplified and fed into a counting rate meter which gives a direct reading of the speed in revolutions per second.

The Svedberg cell, (P), in the spinning rotor is illuminated once in each revolution by a quartz mercury arc. Light from the arc,

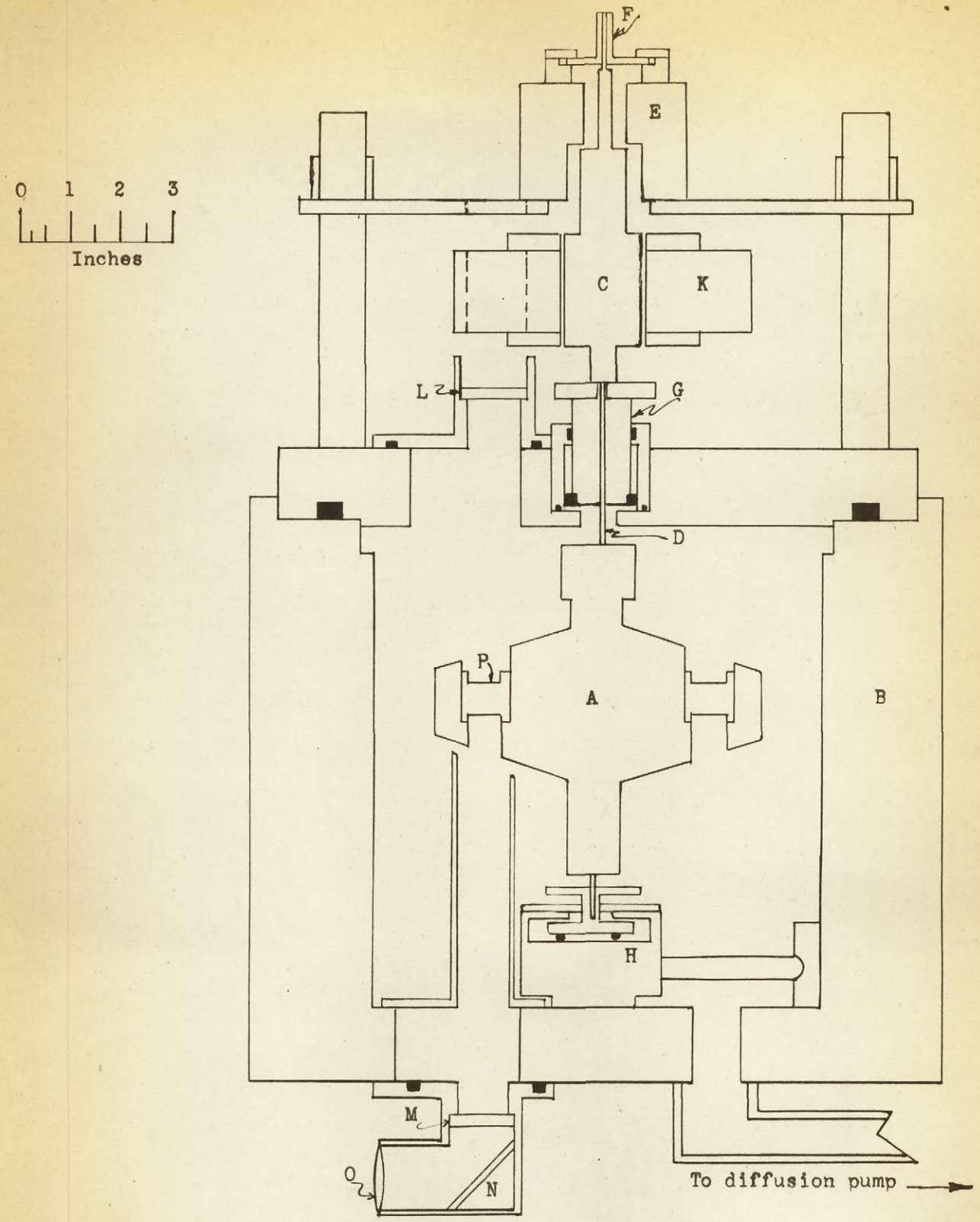


FIG. 1

after passing through the illuminating lens (O), is reflected through quartz window (M) by the mirror (N). The light from the cell then passes through quartz window (L) through a hole in the stator (K) to a mirror not shown in Fig. 1. This mirror reflects the light through another lens which focusses an image of the cell onto a slit of the spectrograph after reflection at another mirror.

In Fig. 1 the supports for the solenoid are shown at right angles to their true position with respect to the light beam.

Pictures of the ultracentrifuge laboratory in the Annex of the Radiation Laboratory are shown in Figs. 2, 3 and 4. Fig. 2 shows the centrifuge, Fig. 3 the centrifuge and part of the control table and Fig. 4 the control table and spectrograph.

2. The Svedberg Cell

The sample to be centrifuged is held in a sectorial cell which is placed opposite a counter-balance in the Svedberg Rotor. The cell is 1.5 cm. long and 0.3 cm. thick. The angle of the sector is 3°. The center of the cell is 5.8 cm. from the center of rotation. The sides of the cell are enclosed by crystalline quartz plates in order that the concentration of protein throughout the cell can be measured by ultra-violet absorption while the rotor is spinning.

The quartz discs are liable to breakage due to the tremendous pressures exerted by the solution inside the cell. This trouble is accentuated by the high pressure which the discs must exert on the pliofilm gaskets to seal the solution within the cell. Pressure to seal the cell is applied to the discs by a nut screwing into the cell holder. To keep this pressure to the minimum necessary for a good seal, a torque-

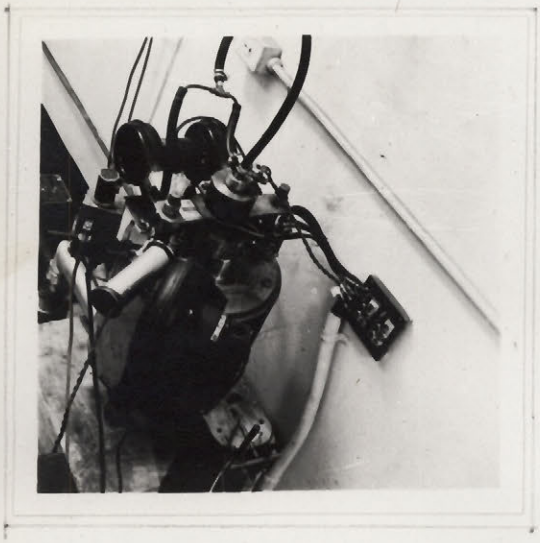


Fig. 2

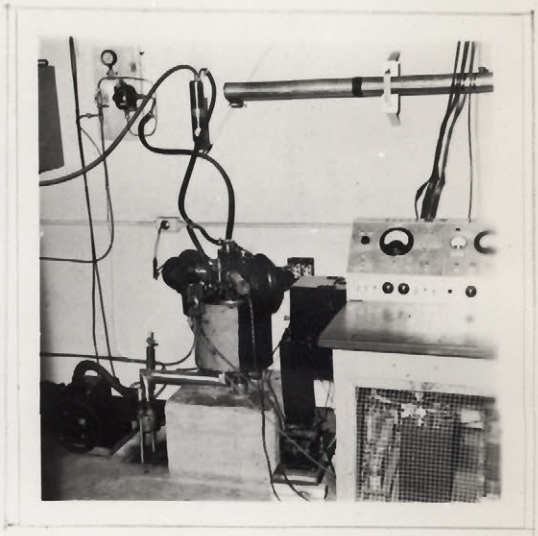


Fig. 3

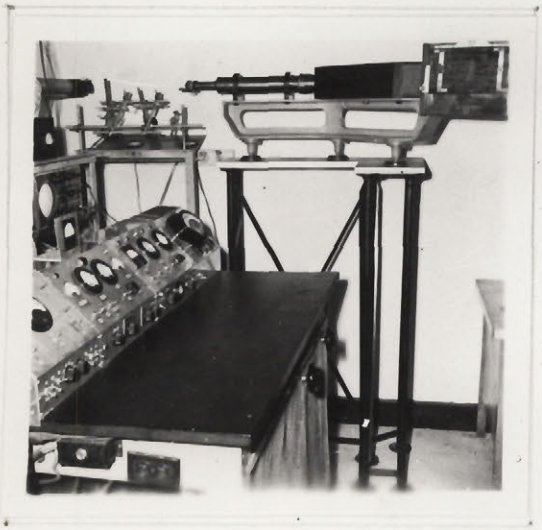


Fig. 4

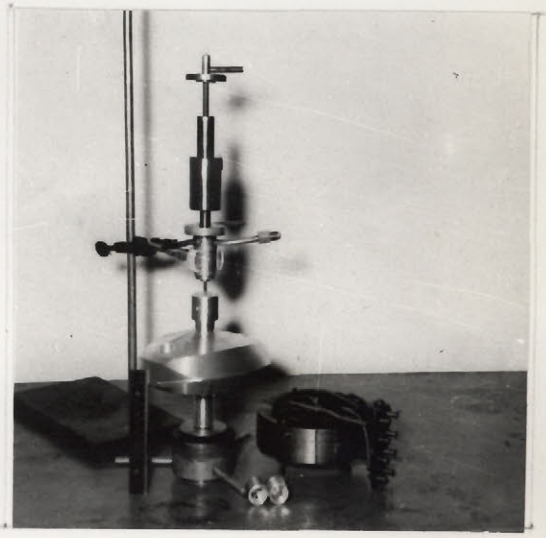


Fig. 5

measuring wrench was obtained to tighten the nut. For an operating speed of 730 r.p.s. a torque of 75 inch pounds was found to be sufficient to ensure a good seal.

To obtain undisturbed sedimentation in the cell, and uniform illumination along the length of the spectrograph slit, the sides of the sector should be radial. A jig was made for holding the rotor so that two slits 0.015 inches wide were radial from the center of rotation. The cell then could be rotated in the rotor until the two slits and either edge of the cell sector were in line.

3. The Mechanical System

A picture of the disassembled rotating system is shown in Fig. 5. At the bottom is the vibration damper and above it the Svedberg rotor. Over the rotor is the main bearing, then the motor armature and above this the top bearing.

(a) The Svedberg Rotor. The experience of Svedberg and Beams[13] has indicated that an oval shaped rotor will remain intact at higher speeds than one circular in cross section. This is due to reduction of stresses at the center by the removal of excess material.

To insure safe operation at speeds from 800 to 1000 r.p.s. the rotor already in use was cut to an oval shape. After approximately twenty hours' operation at 800 r.p.s. it was found that the diameter of the rotor through the cell and counterbalance had increased by 0.003 inches. After this the rotor was not run above 730 r.p.s. The rotor was made as accurately as possible but was not balanced statically or dynamically. A quarter of a gram of solution leaking out of the cell at high speed caused no noticeable precession.

Table II shows the radial accelerations at a radius of 5.8 cm. at various speeds of rotation.

TABLE II

Speed of Rotation rev./sec.	Radial Acceleration g
500	58,500
700	115,000
730	125,000
800	150,000
1000	234,000

(b) The Main Bearing. The main bearing on the top of the vacuum chamber has four important additional functions. It

i) provides an exit for cooling water from the armature,

ii) acts as a shock absorber to damp resonance vibrations of the rotor,

iii) provides a vacuum seal between the rotating shaft and the lid of the vacuum chamber, and

iv) acts as a thrust bearing to support part of the weight of the rotating system.

The bearing formerly used in this position was in two separate parts. These were clamped in place on rubber gaskets with cooling water flowing between them. Trouble from these bearings came from two sources:

1) The rubber gaskets were required to act as both shock absorbers and vacuum seals. Rubber under compression, as required in a

vacuum seal, loses its elastic properties so that it is not a good shock absorber.

2) There was no way to ensure that the two bearings were aligned.

A sketch of the new main bearing is shown in Fig. 6. The clearance between the hard babbit[11] bearing surfaces and the shaft is 0.0003". The bearings were made by pressing babbit slugs into brass bushings. To bore a babbit slug axially, a jig was turned in a lathe holding a single brass bushing with a tight fit. Using a small boring tool the hole was then bored to a diameter of $0.1250 \pm .0002$" measured by fit and no fit gauges. For final reaming by hand to 0.1260" the bearings were mounted in the brass cylinder as shown in Fig. 6. Once assembled the bearings are permanently aligned and were not removed from the cylinder until they had to be replaced.

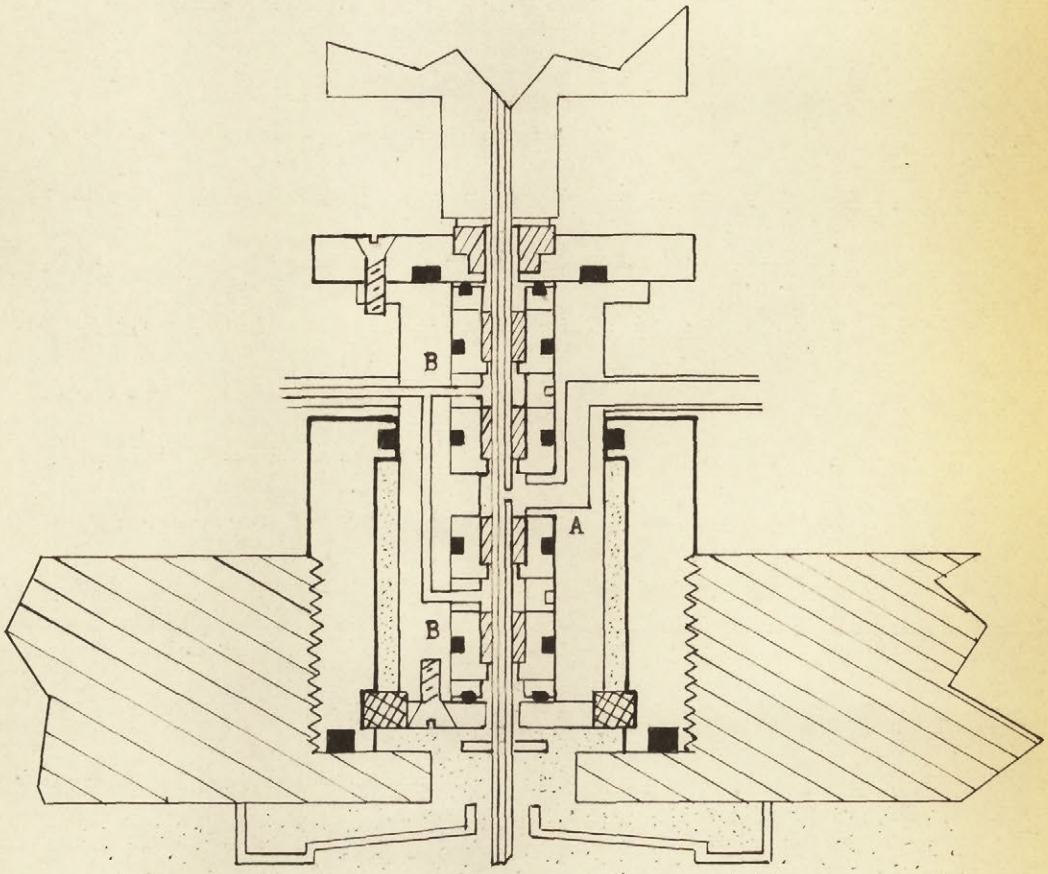
The cylinder in which the bearings were mounted was held near its upper end by an O-Ring which allowed it to tilt two or three degrees from the vertical. The bottom of the cylinder was supported by a rubber ring bonded to the brass tube which also held the upper O-Ring. This holder screwed into the cover of the vacuum tank. As the bearing was quite elastic in its mount it damped all vibrations excellently.

Referring to Fig. 6. Water flows from a hole in the tubular shaft into the bearing through the channel marked "A". It is prevented from flowing between the bearing surfaces and the shaft by lubricating oil under pressure, introduced between pairs of bearing surfaces through channels marked "B". For speeds up to 700 r.p.s. an oil pressure of 10 lb/in^2 has been found satisfactory. To prevent seizing at higher speeds it is necessary to increase this to 15 lb/in^2, depending on the

Legend

- O-Ring Seals
- Rubber
- Evacuated Space
- Babbit

Scale - Full size



Main Bearing

Fig. 6

tightness of the bearing. Pressures less than 10^{-3} mm. of mercury are easily maintained in the vacuum chamber at all rotor speeds.

(c) The Vibration Damper. The bearing on the lower end of the Svedberg rotor has also been redesigned. This bearing is designed to damp any precessional vibrations of the rotor. With the old design the bearing occasionally caused the lower pin of the rotor to bend or break.

The bearing surface is a leather washer 1/8" thick, allowing considerable tilt to the rotor. The leather washer is fixed in a brass disc mounted on ball bearings and placed in an oil cup. Over the oil cup is a neoprene sheet moving with the disc. Surface tension of the oil between the neoprene and the top of the cup keeps the flexible sheet adhering to the surface, preventing sudden large movements of the rotor. Together with the flexible mounting of the main bearings this device reduces resonant precessional vibrations of the rotor until they are barely perceptible. The oil in the cup must be renewed after two or three runs. Its level, however, is not critical so long as the surface between the neoprene and the top of the oil cup is well lubricated.

(d) The Top Bearing. Originally this bearing was mounted on top of the adjustable core of the solenoid supporting the rotating system. This had the disadvantage of making the total distance between the main bearing and the top bearing dependent upon the adjustment of the solenoid. The bearing has now been mounted on the top of the solenoid. Provision was made for a screw adjustment of the core without movement of the bearing.

A flexible mount was not found to be necessary in the top bearing so long as the armature of the motor was made sufficiently true.

The present armature is a right angled circular cylinder, true to less than 0.0001" along its outside length. The inner 1/4" hole for water cooling is probably truly axial to 0.001" along its whole length. The upper shaft is off-center 0.0001" and the lower shaft 0.0004".

(e) Alignment of Bearings. To reduce precessional vibrations it is necessary that the rotor hang vertically. The vacuum chamber is provided with levelling screws fitting into pipes in a concrete base. To ensure that the chamber cover was always in the same position with respect to the chamber, the loose rubber ring which was used as a vacuum seal between the cover of the vacuum chamber and the chamber itself was replaced. A rubber gasket allowing contact between cover and vacuum chamber when the cover is screwed down tightly was used instead.

Using the levelling screws in the vacuum tank, the main bearing was made vertical by levelling a plate mounted on the shaft supporting the rotor.

To align the top bearing the centrifuge was assembled except for this bearing. With no water or oil lines in place, or supporting magnetic field, the rotor was allowed to coast at about 200 r.p.m. The top bearing was then put into place and screwed down tightly.

4. The Vacuum System

The rotor turns in a vacuum at a pressure less than 10^{-3} mm. of mercury. This vacuum is achieved by using an oil diffusion pump with a speed of 10 litres per second at 10^{-3} mm., backed by a mechanical pump. The overall speed of the system was increased from approximately 1/2 litre per second to 5 litres per second by using large diameter pumping leads. The pressure is measured continuously with a Pirani gauge. The vacuum

gauge usually gives the first indication of trouble in the rotating system.

All seals on holes into the vacuum chamber were redesigned to allow the use of O-Ring gaskets where possible. Wax seals were made on the quartz discs to allow the light beam to traverse the vacuum chamber. These were found to be more convenient than gaskets, which tend to deposit grease on the quartz.

5. The Electrical System

No changes were made in the driving and control circuits[11] other than those necessary to keep them in good repair.

The automatic speed control was not used in the present work, since it was found that the 60-cycle hum from surrounding apparatus was of the same order of magnitude as the slip speed voltage induced in the pick-up coil. So long as the power input to the motor was kept constant variations in speed were negligible.

6. The Optical System

(a) General. The Svedberg cell was illuminated by a 250-watt quartz mercury arc. The light from the cell was then focussed on the slit of the spectrograph by a quartz fluorite achromatic lens of focal length 100 cm. and aperture f/36. The system was used at unit magnification.

To achieve uniform illumination of the cell an image of the mercury arc was made on the quartz fluorite achromat by the illuminating lens. This condition ensures that each point on the image of the cell receives light from all points of the mercury arc.

The system was found to be astigmatic even for points near the axis of the lens, so that the sides and ends of the cell did not come into focus at the same time. To get even illumination of the slit the image at the circle of least confusion was used. The slit was easily placed at this point by looking into the back of the spectrograph and noting whether all the collimating lens disappeared at once at the ends of the image. If the slit was not at the circle of least confusion the lens disappeared as though a diaphragm were drawn across it. The cause of the astigmatism has not yet been determined, as it was undesirable to disturb the lens and the two mirrors after they had been aligned.

Weak tails appear at the ends of the spectrographic lines. These are caused by scattering of the light by air bubbles in the fluorite of the achromatic lens. This scattered light has been reduced as much as possible by using diaphragms.

Several changes were made in the optical system.

i) A mirror was introduced to bend the light into the spectrograph. This was necessary to fit the apparatus into the room available in the Radiation Laboratory Annex.

ii) Larger windows were put into the vacuum tank. These were made of fused, instead of crystalline, quartz to allow the windows to be waxed into place.

iii) As much stray light as possible was eliminated by blackening all parts of the apparatus near the arc. This allowed the speed indicator to be used while the arc was operating.

iv) The magnification of the image in a prism spectrograph varies with wavelength. Thus it is necessary to be able to find the center

of the image on each spectral line and its magnification. The intensity across the image varies so that the cell length is not suitable for use as a standard of length. Instead, two holes were drilled in the counterbalance equidistant from its center. These gave images just beyond the ends of the image of the cell. By comparing the distance between the images with the distance between the holes, the magnification at any wavelength can be found. The center point between the images was coincident with the center of the cell image. By measuring the distance of any point in the image from this center point, and correcting for magnification, the corresponding position in the cell could be obtained.

v) A constant voltage transformer was used to keep the mercury arc voltage, and hence the light intensity, constant.

(b) The Spectrograph. Light from the cell, illuminated by the mercury arc, is focussed on the slit of a Hilger E2 quartz prism spectrograph. Sedimentation can then be followed in several regions of the absorption spectrum of the protein at the same time.

The slit width used on the spectrograph was 0.40 mm. This width of slit was necessary in order to keep the microphotometer beam on the curved spectrographic line while photometering along its length. For the same reason mercury lines chosen for sedimentation measurements could not be overlapped by other lines.

With a slit width of 0.40 mm., six lines in the mercury spectrum were chosen which did not overlap. Curves of photometer reading versus exposure time were plotted for these lines. Of the six, the three lines at 3906 Å, 2920 Å and 2576 Å were chosen for measurements. They were fairly well spaced in the absorption region of the proteins. Being of roughly equal intensity, only a single exposure time of one minute was required.

Maximum blacking on all lines, corresponding to zero concentration of protein, was determined solely by the exposure time. Minimum blackening was determined by the absorption of the protein. The minimum on lines on which measurements were to be made had to be clearly distinguishable from clear plate. The concentration of protein in the Svedberg cell was adjusted so that the spectral line on which absorption was greatest could be distinguished from clear plate. For haemoglobin this line was at 3906 Å; for serum albumin at 2576 Å.

II PHOTOGRAPHIC PLATES

The spectra from sedimentation experiments were recorded on Kodak Spectrographic Analysis #1 plates, 4" x 10". These are very fine grain, high contrast plates, sensitive in the ultra-violet down to wavelengths of 2300 A.

Plates were developed singly in Kodak Developer "D19" for five minutes, placed in a stop bath (Kodak SB.5) for 45 seconds and fixed for 20 minutes in Kodak "Liquifix". They were then washed in water for 1/2 hour. The temperature of the developer was kept constant at 20°C (± 0.2°C) during development. The temperatures of the stop bath, fixing bath and washing were all kept at 20°C to ensure that the gelatine would not be damaged due to sudden changes in temperature. The plates were not developed until at least twenty-four hours after exposure to avoid errors due to growth of the latent image. During development they were brushed continuously with a soft nylon brush to ensure uniform development.

With the 4" x 10" plates, three exposures of spectra with lines 1.5 cm. long and eleven calibration marks 3 mm. long could be placed on

each plate. Making five exposures per centrifuge experiment meant using two or three plates.

Plates from the same box showed wide variations in blackening. However, those which had been cut from the same sheet in manufacture could be distinguished easily because of their uniform blackening characteristics. These showed that the precautions taken during development produced very uniform development.

III THE MICROPHOTOMETER

A Kipp & Zonen Type A Moll recording microphotometer was used to measure the specular density of blacking on the photographic plates.

Operation of this instrument is as follows: the image of the filament of a lamp operated at constant voltage is focussed on a slit and a reduced image of this slit focussed on the photographic plate whose blacking is to be measured. A magnified image of the light transmitted by the second slit actuates a vacuum thermopile and the thermal current is measured using a Moll galvanometer whose deflection is registered by a drum camera.

The plate is propelled across the beam of the photometer lamp by a very accurately made screw which is also geared to the drum camera. The instrument also records on the sensitive paper of the camera, lines marking every tenth of a millimeter travelled by the plate, and a centimeter scale for measuring the galvanometer deflection.

The distance moved by the plate was magnified by four, and later by eight, in making the photometric curves. The distances measured between points on a line in a plate were consistent within $\pm$ 0.02 mm.

In using the photometer, the minimum reading for no light transmitted was set by adjustment of the zero setting of the galvanometer. The maximum reading for clear plate was determined by the intensity of the light in the photometer beam. The probable error in the galvanometer reading was 0.05 cm. This gave a probable error in concentration of about 2% for low contrast lines.

CHAPTER IV - EXPERIMENTAL WORK

I CYCLOTRON IRRADIATIONS

1. The Target

As the proton beam of the McGill synchro-cyclotron is not deflected out of the vacuum chamber it was necessary to mount the protein on the end of a probe which projected into the beam.

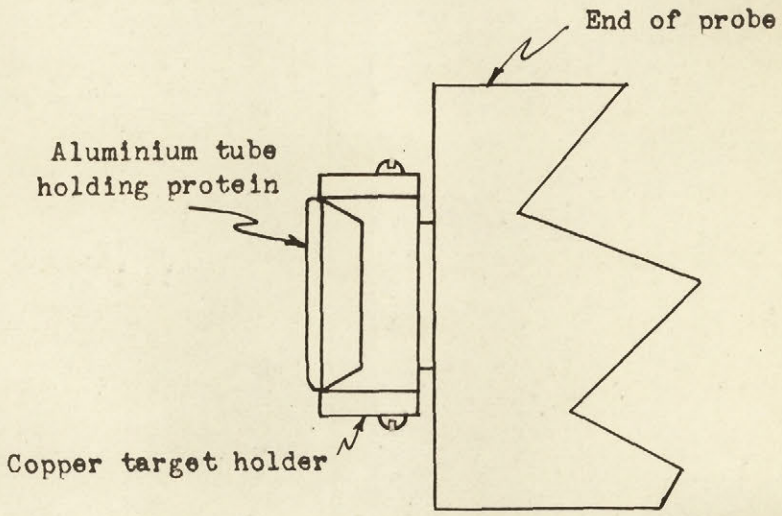
Aluminium tubes 1/16" in diameter, with walls 0.005" thick, were filled with the protein to be irradiated. The ends of the tubes were flattened, leaving a center portion 3.5 cm. long filled with protein. The ends were then clamped to a copper holder fastened to the end of the probe, which was water cooled. The target and holder are shown in Fig. 7. The protons in the cyclotron move in a spiral with an average difference in radius per revolution of about 0.002". However, according to Henry[19] radial oscillations make the difference in radius between successive revolutions as much as ± 0.01", so that irradiation is fairly uniform across the aluminium tube.

The beam current at 10 Mev could be measured by swinging a copper block into its path. This block absorbed all the protons and was connected to a galvanometer. The current at this energy for all irradiations was 0.09 ± 0.02 micro-amperes and was checked intermittantly during exposures.

2. Dosage Measurements

The actual dosage of protons delivered to the target could not be measured but was calculated from λ_s, the fraction of the serum albumin molecules in which primary ionizations occur per unit time.

The actual proton beam current hitting the protein cannot be



Target Assembly

FIG. 7

measured at 50 Mev as the protons all pass completely through it. Of the 0.09 μa., measured at 10 Mev, 0.03 $\pm$ 0.005 μamperes are lost due to protons hitting the dees and the target holder. Of the remainder it is estimated that one-sixth are lost due to scattering from the walls of the aluminium container. This leaves a proton current of 0.05 μ amperes hitting the protein.

A value of λ for serum albumin was obtained by measuring the rate of decrease in the enzyme activity of urease with time of irradiation. Under irradiation this enzyme loses its power to hydrolyse urea to carbon dioxide and ammonium hydroxide. Urease samples were irradiated for 5, 15, 30 and 60 seconds with 50 Mev protons. The measured beam current at 10 Mev was 0.09 μamp. The enzyme activity after irradiation was measured by Mr. L. Fridhandler and compared with that for unirradiated material. The method used for determining the activity was to measure the rate of evolution of carbon dioxide. To do this the enzyme was dissolved in a solution of 10% urea at pH 5. The change of pressure with time was then measured at constant temperature and converted to micro-litres of carbon dioxide evolved per minute. The activity of the enzyme can be expressed in terms of micro-litres of carbon dioxide evolved per minute per milligram of urease.

From these measurements a value of λ for urease was obtained. The value of λ for serum albumin was then found by correcting for the size of the molecules.

The value of λ for serum albumin was also measured directly by the following experiment.

It was observed that the portion of serum albumin, irradiated for five minutes, which was soluble in distilled water did not coagulate

on heating. Serum albumin unaffected by ionizing radiations is completely soluble in water and coagulates on heating. The coagulant can be removed easily by centrifuging. Assuming that serum albumin molecules affected by the irradiation did not coagulate, a measurement could be made of the number of unaffected molecules by weighing the coagulant.

Samples of serum albumin were irradiated for 10, 15, 30, 90, 120, 150 and 180 seconds. These were mixed with water (10 mg/cc.) and the insoluble material removed by centrifuging. The solution was then heated to 100°C. After two minutes the coagulated protein was removed by centrifuging, and weighed. The percentage of the original protein recovered was plotted against time of irradiation on a logarithmic scale. The slope of this curve gave a value of λ for serum albumin.

The temperature of the target during irradiation was 80 ± 20°C. This was measured by irradiating finely powdered napthalene (melting point 80°C) using the same method as for the protein. With a beam current of 0.006 microamperes at 10 Mev, no sign of melting was found. At 0.009 microamperes the crystals in the centre of the tube melted and adhered to those at the sides.

Carruthers[11] found that heating serum albumin for ten hours at 80°C caused no noticeable change in sedimentation.

II ULTRACENTRIFUGE MEASUREMENTS

1. Calibration of Plates and the Concentration Scale

The concentration of protein at any point in the rotating cell was found using the photometer, by comparing the galvanometer reading at that point with a curve of galvanometer reading versus concentration.

This curve is referred to as the concentration scale.

A concentration scale was prepared for all the spectrum lines on which measurements were made; also for each plate used in sedimentation experiments because of variation of the blackening characteristics among the plates.

In making concentration scales the rotating cell was filled with water. Another cell, filled with the solution of protein to be investigated, was placed immediately in front of the spectrograph slit. A sufficient number of exposures were made with varying concentrations of protein in this cell to plot a concentration scale. While making exposures the rotor was kept spinning at 500 r.p.s. to avoid any intermittancy effect[23]. Exposure times and light intensity were the same as those used in sedimentation experiments. In making exposures during sedimentation experiments the cell in front of the spectrograph was kept filled with water.

It was not practical to put a concentration scale directly on each plate when more than one plate was used per sedimentation experiment. Instead, the blackening characteristic of each plate was plotted as a curve of galvanometer reading versus logarithm of the time of exposure, from calibration marks made by varying the exposure time from six seconds to one minute. In making these exposures the rotor was spinning as before and both cells were filled with water.

According to the reciprocity law of Bunsen and Roscoe[20], the blackening of a plate in time, t, due to intensity of light, I, is specified by the product Ixt, independently of I and t. Hence, curves of photometer readings versus the logarithm of t with I constant are the same as those that would be obtained by plotting against logarithm of I

with t constant. These curves can then be called curves of photometer reading versus logarithm (I x t).

Each photometer reading for the blackening obtained from a known concentration of protein then corresponds to a value of $\log_{10}(I \times t)$ taken from the calibration curve of the same plate. Plotting values of log (I x t) against the corresponding concentrations gives a curve whose equation is

$$I = I_o \, e^{-kc} \tag{21}$$

$$\text{or} \qquad \log_{10} I = \log_{10} I_o - kc \log_{10} e \tag{22}$$

where I_o is the original intensity of the light,

I is the intensity after absorption,

C is the concentration of absorber

and k the absorption constant.

These absorption curves were independent of the plate characteristic. To obtain the concentration scale for any other plate the absorption curves were used with the calibration curve for that plate in the reverse manner. The straight lines obtained for the absorption curves of equation (22) show that the reciprocity law was obeyed for the exposure times and intensities used in these experiments.

2. Absorption Measurements

Absorption curves were drawn for each spectral line on which sedimentation measurements were made, in order to make concentration scales. The slope of these lines gives the absorption constant "k". Concentrations were computed from the original amount of protein available

disregarding the portion which was insoluble. The real absorption constant, k_1, is related to that measured by the expression

$$k_1 = \frac{k}{\alpha} \qquad (22a)$$

where α is the fraction of irradiated protein which was soluble.

The absorption of haemoglobin for mercury lines at 3906 A, 2920 A and 2576 A was measured for proton irradiation times of 1/4, 1, 2, 4 and 8 minutes.

Absorption measurements on serum albumin were made for irradiation times of 1, 1.5, 2, 3, 4 and 8 minutes.

3. Sedimentation Experiments

Bovine serum albumin and bovine haemoglobin were obtained from Armour Laboratories in Chicago; urease from Bios Laboratories, New York.

The sedimentation of the unirradiated material was measured at 2920 A for serum albumin and urease, and at 3906 A for haemoglobin. Irradiated samples for sedimentation and absorption experiments were kept in their aluminium tubes and preserved in solid carbon dioxide until used. The period of storage was never more than forty-eight hours.

For all sedimentation and their associated absorption experiments, the proteins were dissolved in acetate buffer solution of pH 4.75. This solution was made up of 0.1 M sodium acetate and 0.05 M acetic acid. The concentrations of protein used in the various sedimentation experiments are shown in Table III, page 39. Any insoluble material associated with the irradiated protein was removed by centrifuging before putting the solution into the Svedberg cell.

Sedimentation experiments were performed with serum albumin irradiated 1, 1.5, 2, 3, 4 and 8 minutes, on haemoglobin irradiated 2, 4 and 8 minutes, and urease irradiated 15 and 60 seconds.

The procedure used in operating the centrifuge was as follows in all cases.

As soon as the rotor was in place the vacuum pumps were started and the rest of the apparatus prepared. When the vacuum pressure was 10^{-3} mm. of Hg the centrifuge was started and the quartz mercury arc turned on. To start, the power was slowly increased until input to the motor was 1000 watts at a frequency of 600 cycles/second. After five minutes the speed had risen to 500 r.p.s. The driving frequency was then increased to 840 c/s with 1000 watts input. Ten minutes after starting the rotor speed was 730 r.p.s. Acceleration was stopped by lowering the power input to 600 watts. The speed of rotation was monitored constantly by comparing it with the 60-cycle mains frequency on an oscilloscope. So long as power input was constant the rotational speed would not vary by more than $\pm$ 2 r.p.s.

As soon as the rotor had reached top speed an exposure was made using the spectrograph. Subsequent exposures were made at half-hour intervals.

The temperature of the rotor was measured before and after each run. To determine the temperature between exposures it was assumed that the temperature rose linearly with time as the run progressed. Table III gives the main data for each run in this series.

Protein	Irradiation Time	Concentration	Duration of experiment	Time between Exposures	Mean Speed of Rotation	Mean Rotor Temp.	Temperature Rise
	minutes	mg/cc.	hours	minutes	r.p.s.	°C	°C
Serum Albumin	0	8.94	2.7	40	703	27.2	3.7
Haemoglobin	0	0.5	2.5	30	734	27.1	3.4
Urease (1)	0	5	1	15	505	25.3	1.5
Urease (2)	0	3	1	15	504	28.8	0.7
Serum Albumin	1	4.5	2	30	735	29.0	2.2
Serum Albumin	1.5	6	2	30	732	27.3	2.5
Serum Albumin	2	8	2	30	731	27.9	1.6
Serum Albumin	3	10	2	30	735	29.6	1.5
Serum Albumin	4	10	2	30	735	29.6	1.1
Serum Albumin	8	10	2	30	730	26.8	2.4
Haemoglobin	2	0.75	2	30	732	25.1	2.5
Haemoglobin	4	0.75	2	30	735	27.3	2.2
Haemoglobin	8	0.75	2	30	733	27.5	2.4
Urease (1)	0.25	3	2	30	734	27.2	2.3
Urease (1)	1	3	3	30	733	26.3	3.3

TABLE III

III ELECTRON MICROSCOPE EXAMINATION OF PROTEINS

Haemoglobin, serum albumin and urease were all examined in the McGill electron microscope both before and after irradiation.

The electron microscope is an R.C.A. type EMU, capable of magnifying to 25,000 diameters and with a resolving power of 3 mμ.

Formvar films were used to support the specimens. These were made and placed on wire mesh supports using the method described by Zworykin[21].

The proteins to be examined were made up into 0.1%, 0.01% and 0.001% solutions in distilled water. Any insoluble material was removed by centrifuging. Distilled water was used as a solvent to avoid any confusion which might occur due to crystals from a buffer solution. Single drops of each solution were placed on separate mounted formvar films and allowed to dry. The specimens were then ready for examination.

Unirradiated serum albumin and haemoglobin could not be seen using the microscope at any magnification between 6000 and 25,000 diameters. Unirradiated urease, however, consistently showed concentrations of spots. These spots were assumed to be individual molecules as their number varied with varying concentration of urease in the solution, although their size remained constant.

Samples of serum albumin irradiated for five minutes (2×10^8 rep) and examined in the electron microscope showed particles of two sizes, attributed to aggregation products resulting from irradiation. The number of particles in any field of view varied with the concentration of the sample solution.

In the electron microscope examination of haemoglobin irradiated for five minutes no particulate matter could be seen under any conditions.

Urease irradiated for two minutes showed particles with a wide distribution in size, mostly larger than the original molecules.

CHAPTER V - RESULTS

I DOSAGE MEASUREMENTS

1. General

All irradiation experiments were performed using 50 Mev cyclotron protons. The beam current at 10 Mev was $0.09 \pm 0.01\,\mu$ amp., giving an estimated $0.05\,\mu$ amp. on the target. The dosage rate was calculated from equation (12), using an average value of λ for serum albumin. Values of λ for serum albumin were obtained from measurements on the rate of decrease under irradiation of the enzyme activity of urease, the amount of serum albumin recovered by coagulation and by calculation from the estimated beam current.

2. Experiments with Urease

Fig. 8 shows the percentage of the original enzyme activity of urease remaining after irradiation, plotted against time of irradiation. The unirradiated urease evolved 21.0 μl/min./mg. of carbon dioxide when mixed with 10% urea at pH 5, at 38°C.

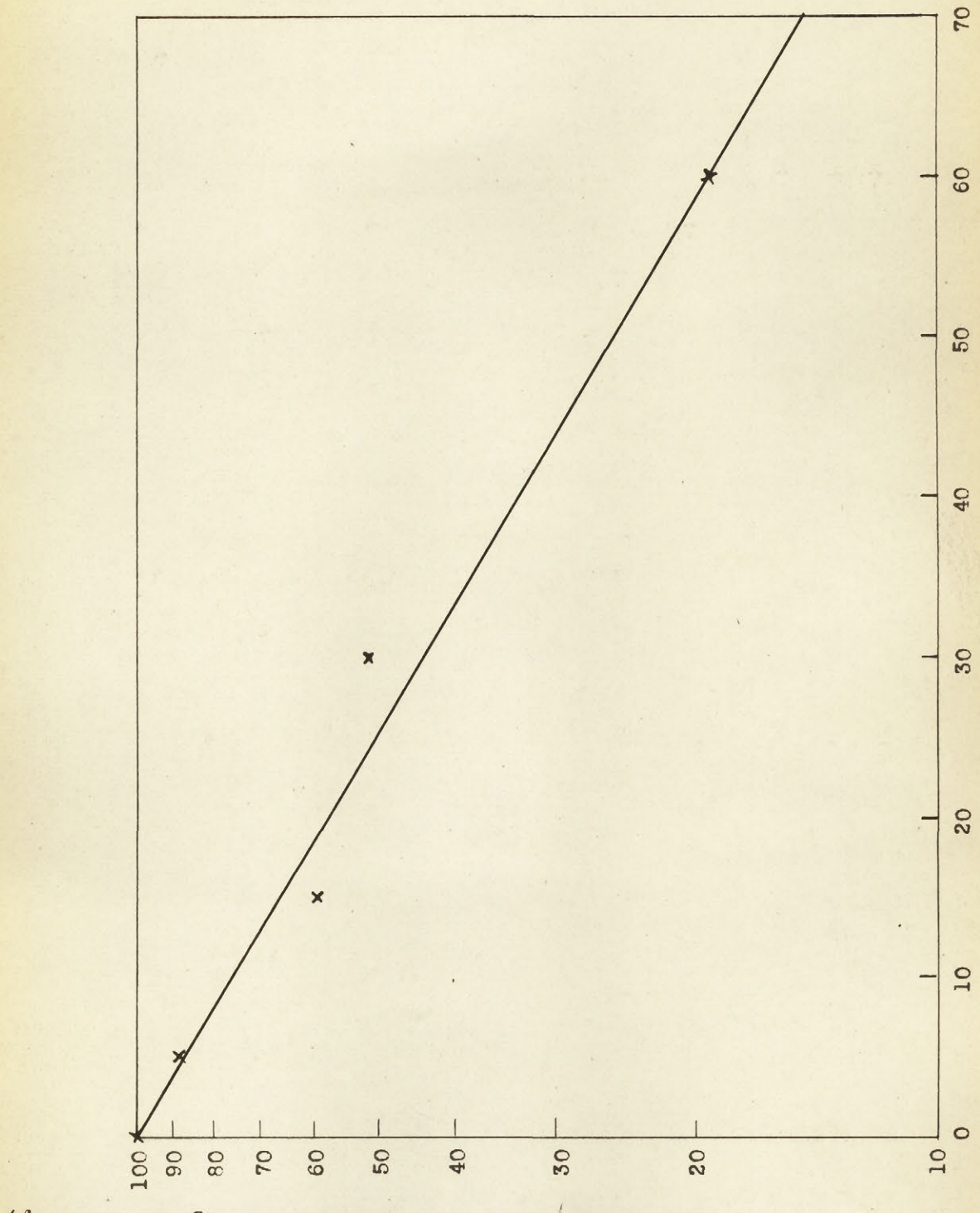
The value of λ_u for urease was found from the time, $T_{\frac{1}{2}}$, at which the activity of the urease had decreased to one-half its original value, using the equation

$$\lambda_u = \frac{0.693}{T_{\frac{1}{2}}} \qquad (23)$$

$$= 0.028 \text{ seconds}^{-1}$$

λ has been related to the volume, V, of the molecule by the relation (8)

$$\lambda = V\sqrt{\nu} \qquad (8)$$



Decrease of enzyme activity of urease under proton irradiation

FIG. 8

where $\mathcal{J}$ is the number of primary ionizations formed per cubic centimeter per second in the target.

Hence, λ_u for urease is related to λ_s for serum albumin by the equation

$$\frac{\lambda_u}{\lambda_s} = \frac{V_u}{V_s} \tag{24}$$

where V_u and V_s are the volumes of the urease and serum albumin molecules respectively.

Assuming the volumes of the molecules in equation (24) to be proportional to molecular weights,

$$\lambda_s = \lambda_u \frac{M_s}{M_u} = 0.028 \times \frac{68,000}{480,000}$$

$$= 0.004 \text{ Sec.}^{-1}$$

Setlow[9,25] has indicated that the whole molecule of urease may not be radiosensitive, as measured by enzyme deactivation, to formation of a single ion pair anywhere in the molecule.

In equation (8)

$$\lambda_u = V \mathcal{J} \tag{8}$$

λ_u is the probability per unit time that a primary ionization will occur in a urease molecule.

As measured experimentally, λ_u' is the probability per unit time that a urease molecule will be deactivated.

In the present case an average cluster of three ion pairs is formed per primary ionization. Since the fraction of the geometrical volume of the urease molecule which is radiosensitive is unknown, it has been assumed that it is not less than one-third, so that the two

values of λ can be considered equal.

If the sensitive volume is less than this the value of for serum albumin has been underestimated.

3. Experiments on Serum Albumin

Fig. 9 shows the percentage of serum albumin recovered by coagulation from solutions of irradiated material in distilled water plotted against time of irradiation. Equation (23) was used to determine λ_s. This gave

$$\lambda_s = 0.016 \text{ seconds}^{-1} .$$

Another value of λ_s can be calculated from equations (10) and (12) and the estimated beam current.

$$r = \frac{i \frac{dE}{dx} \Delta x}{v \times 83} \times 1.6 \times 10^{-12} \qquad (10)$$

$i = 5 \times 10^{-8}$ amperes

$= 3.1 \times 10^{11}$ amperes/sec.

$\frac{dE}{dx} = 10.6 \times 10^{6}$ ev./cm., from Table I,

$\Delta x = 0.12$ cm., the average path length across the 1/16" diameter target,

$v = 2 \times 10^{-2}$ cc., the volume of the protein target,

giving $r = 3.8 \times 10^{5}$ rep/sec.

From equation (12)

$$r = 6.8 \lambda_s \times 10^{7} \text{ rep/sec.} \qquad (12)$$

$$\therefore \lambda_s = \frac{3.8 \times 10^{5}}{6.8 \times 10^{7}}$$

$$= 0.006 \text{ seconds}^{-1} .$$

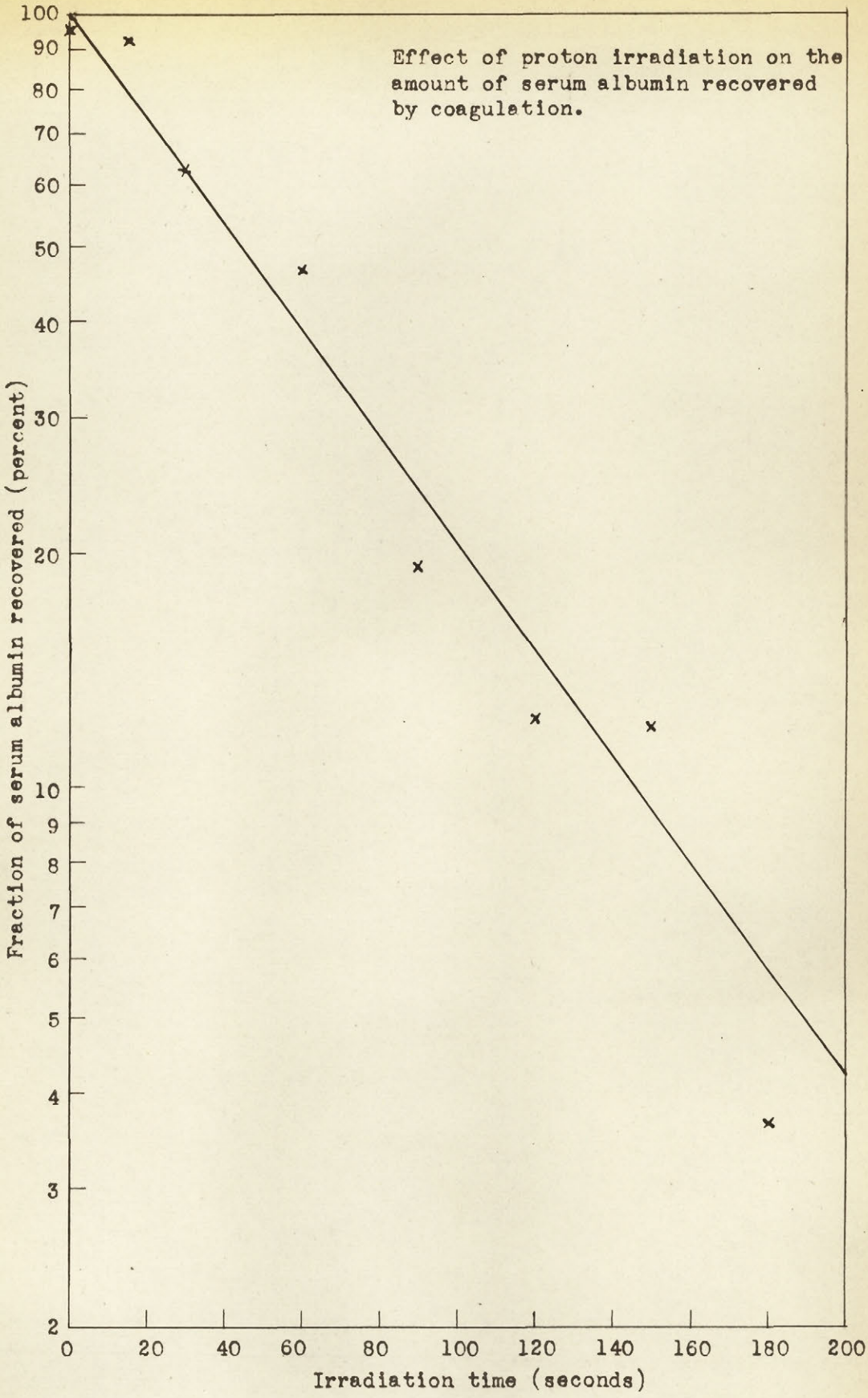

Effect of proton irradiation on the amount of serum albumin recovered by coagulation.
Fraction of serum albumin recovered (percent)
100
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
0
20
40
60
80
100
120
140
160
180
200
Irradiation time (seconds)

FIG. 9

The average of the three values of λ_s gives

$$\lambda_s = 0.009 \pm 0.004 \text{ seconds}^{-1}.$$

Using equation (12) this gives for the dosage rate, r,

$$r = 6.1 \pm 2.7 \times 10^5 \text{ rep/sec.}$$

The fractions of serum albumin molecules in which one and two primary ionizations have taken place are plotted against irradiation time in Fig. 10 from equations (3) and (5), using $\lambda = 0.009 \text{ Sec.}^{-1}$

A curve plotted from equation (6) for the fraction of molecules in which one, and only one, primary ionization has occurred is also shown. This curve has a maximum at irradiation time

$$t_{max} = \frac{1}{\lambda_s}$$
$$= 111 \text{ seconds}.$$

II ABSORPTION MEASUREMENTS

1. Absorption Curves and Concentration Scales

Absorption curves for haemoglobin irradiated two minutes are shown in Fig. 11, for wavelengths 2576 Å, 2920 Å and 3906 Å. Concentration scales drawn from these curves and the calibration curves of Fig. 12 are shown in Fig. 13.

The concentration scales show the absorption of a homogeneous mixture of protein fragments. The solutes whose concentrations were measured by ultra-violet absorption, however, consisted of particles of different sizes distributed in the rotating cell according to their molecular weights. The concentration scales therefore gave only approximate relative concentrations of solute.

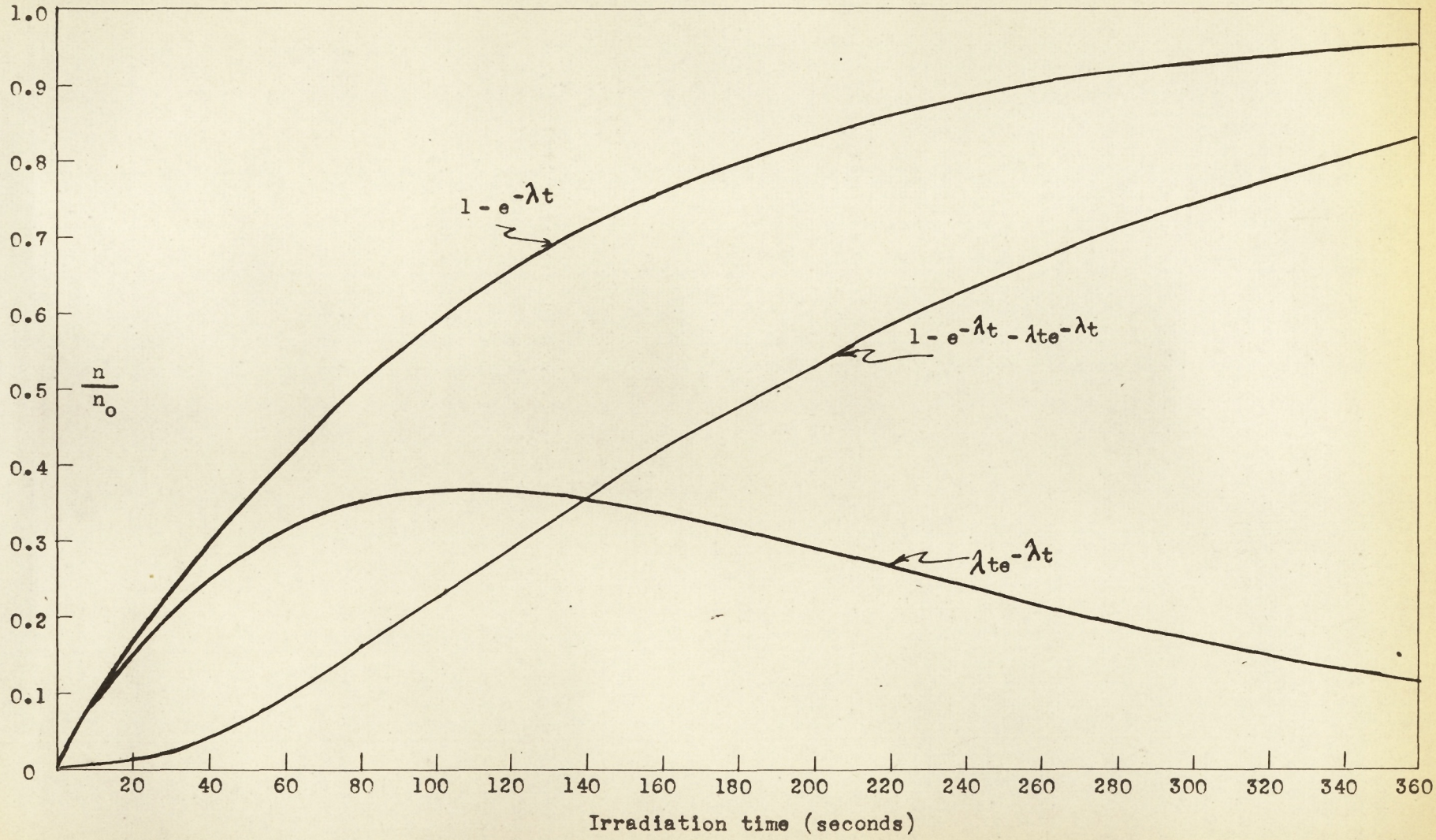
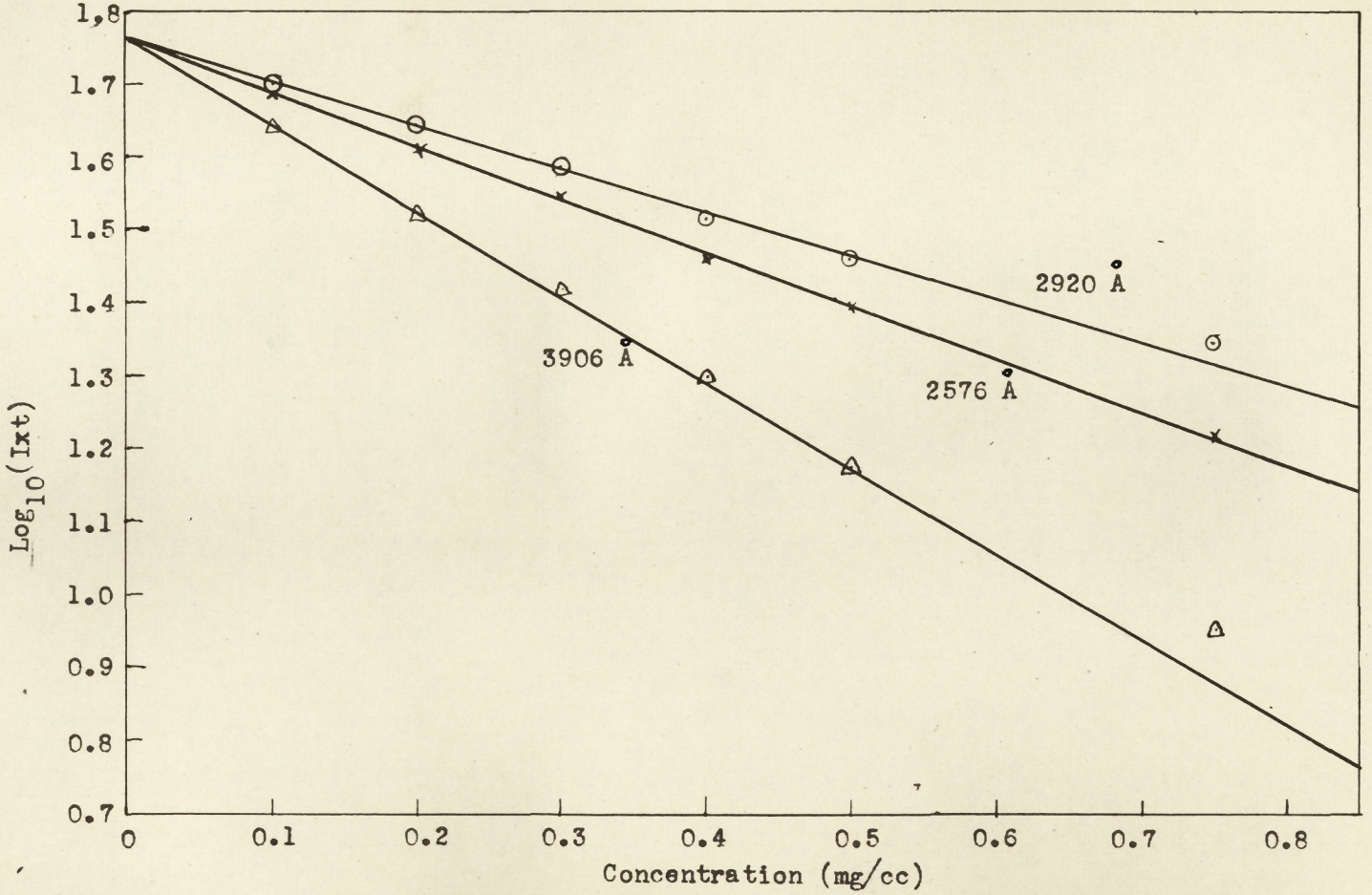


FIG. 10

Fraction of molecules in which one or more primary ionizations, a single primary ionization, and two or more primary ionizations have taken place. $\lambda = 0.009$ seconds^{-1}

FIG. 11



Absorption curves for haemoglobin irradiated two minutes.

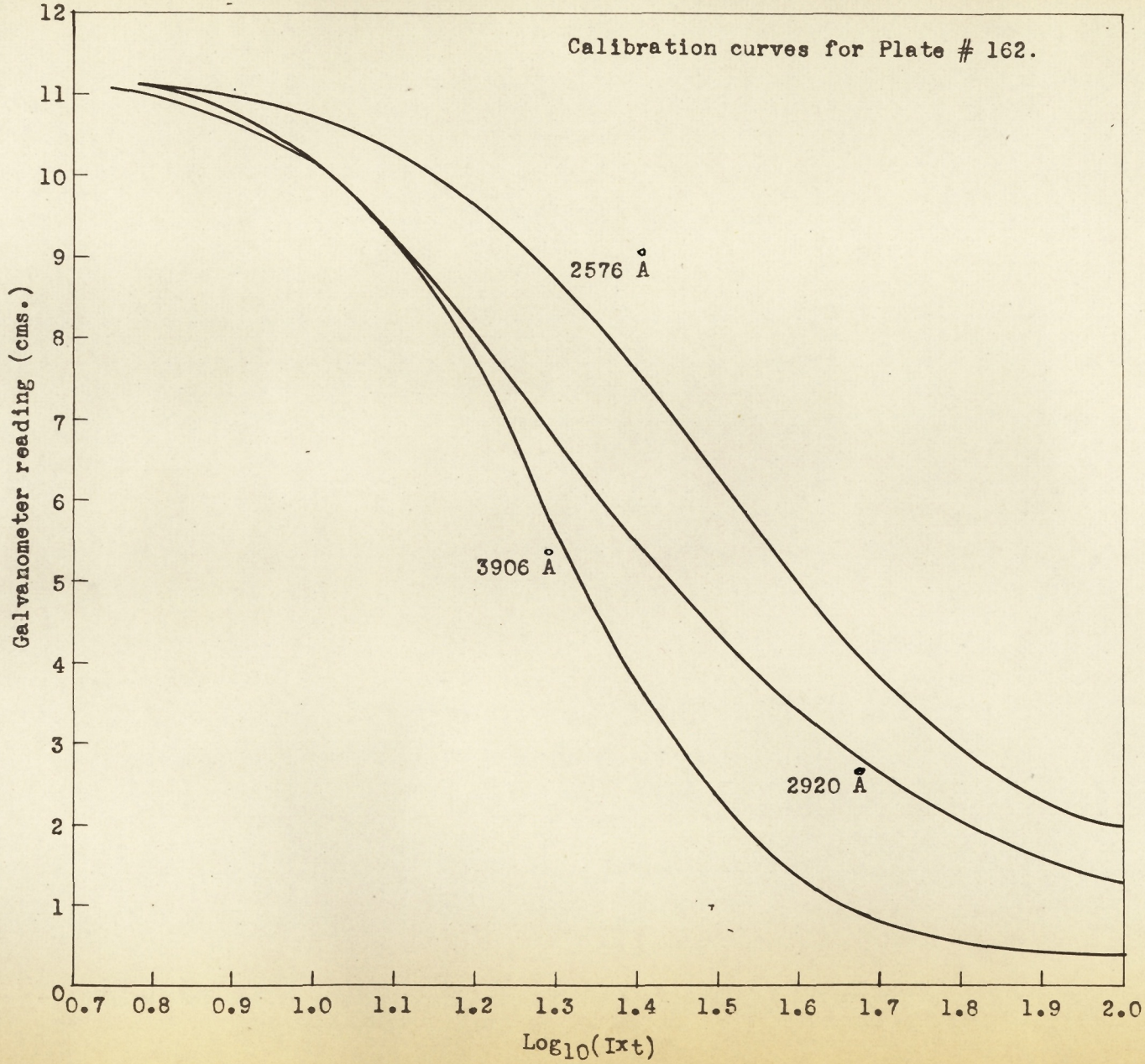
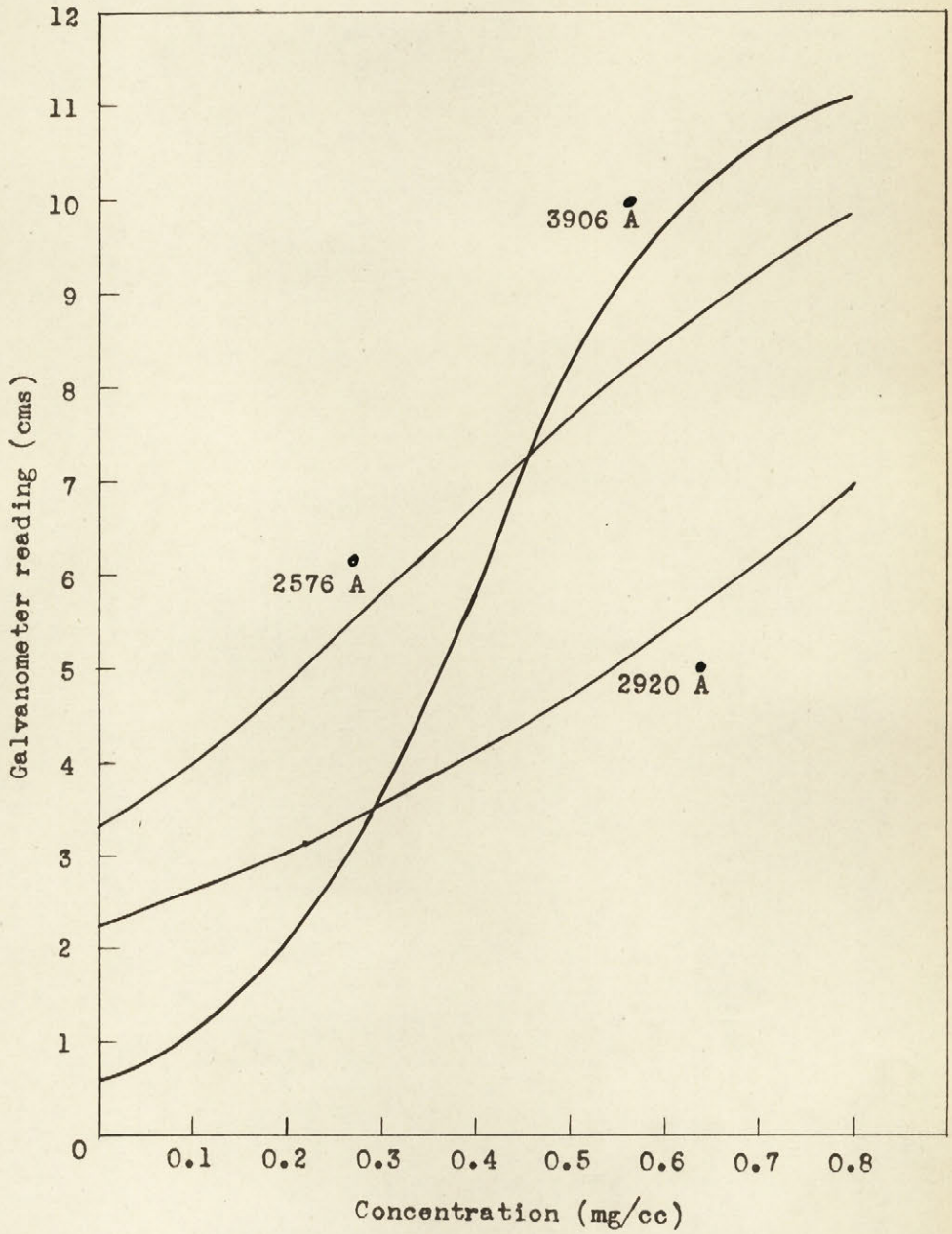

Calibration curves for Plate # 162.
Galvanometer reading (cms.)
$Log_{10}(Ixt)$
2576 Å
2920 Å
3906 Å
0
1
2
3
4
5
6
7
8
9
10
11
12
0.7
0.8
0.9
1.0
1.1
1.2
1.3
1.4
1.5
1.6
1.7
1.8
1.9
2.0

FIG. 12



Concentration scales for Plate # 162.

Haemoglobin irradiated two minutes.

FIG. 13

The approximation is accurate for high concentrations of solute where separation into particles of different sizes is small. For low concentrations of small particle sizes, found near the meniscus in the Svedberg cell, the approximation gives relative concentrations liable to error. The magnitude of these errors depends upon how the ultra-violet absorption of the small particles differs from that of the remainder of the solute.

2. Serum Albumin

Absorption constants, k, measured at wavelengths 2576 A and 2920 A, are shown in Table VII, page , for serum albumin irradiated 0, 1, 1.5, 2, 3, 4 and 8 minutes. The percentage changes in k are plotted against irradiation time in Fig. 14. Errors of ± 10%, indicated on the individual points, are estimated from the reproducibility of the measurements. These variations were attributed to the non-uniformity of the irradiations; measurements made on unirradiated material were consistent within two or three percent.

The maximum decrease in absorption with irradiation of 40% was reached after five minutes of irradiation. After five minutes' irradiation only 20% of the albumin was soluble. Thus, the absorption of the protein which was soluble must have increased by a factor of three at the two wavelengths where measurements were made.

The ultra-violet absorption spectrum of serum albumin has a maximum at 2800 A. Absorption decreases to zero at 3000 A, to a minimum at 2600 A and then rises again at lower wavelengths. Absorption below 2600 A is due mainly to peptide bonds. At 2800 A the absorption

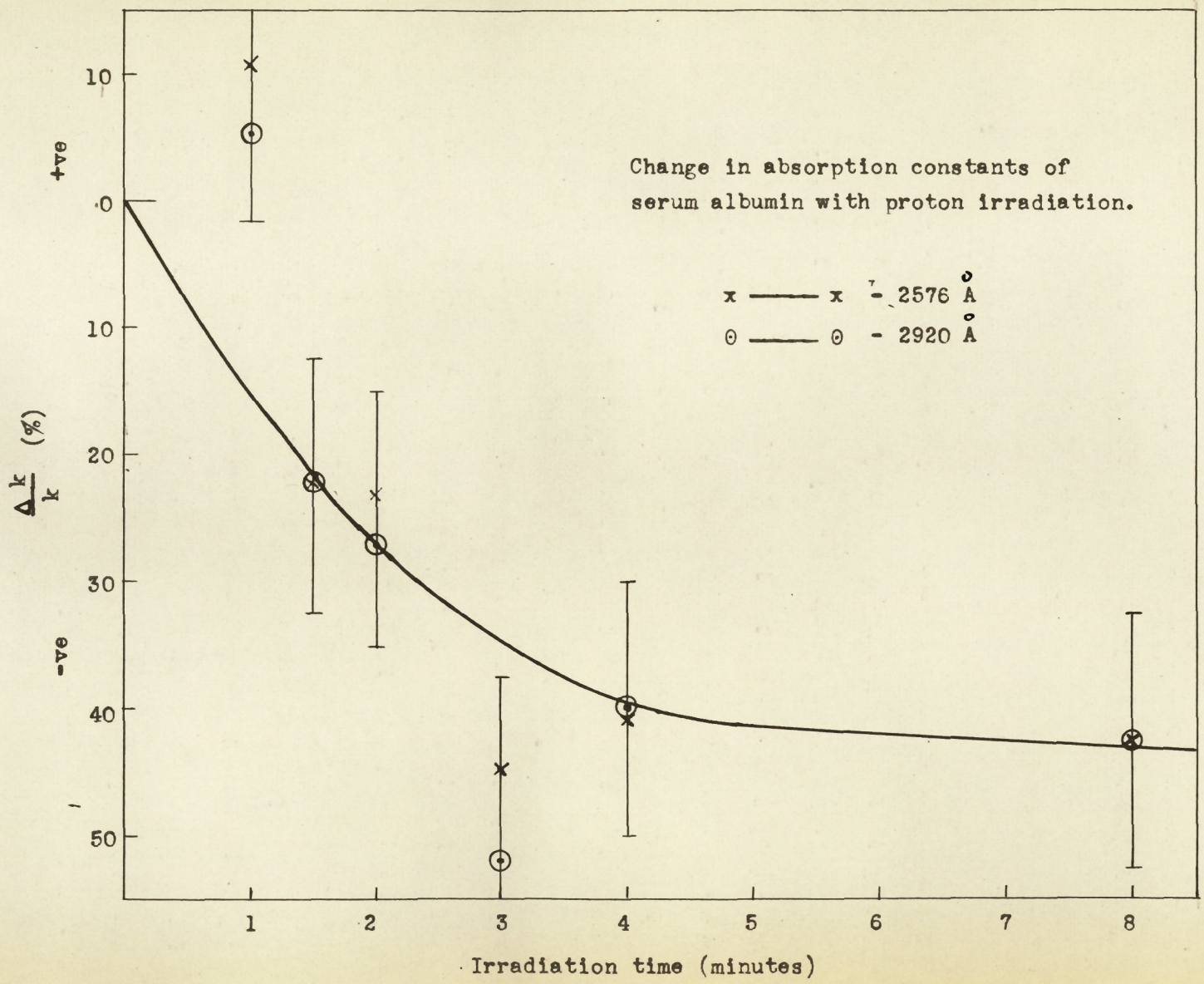

Change in absorption constants of
serum albumin with proton irradiation.
x —— x - 2576 Å
⊙ —— ⊙ - 2920 Å
+ve
-ve
10
0
10
20
30
40
50
$\frac{\Delta k}{k}$ (%)
1
2
3
4
5
6
7
8
Irradiation time (minutes)

FIG. 14

is due to the benzene ring structure of the amino acids tryptophane and tyrosine. The increased absorption at 2576 Å and 2920 Å of the irradiated material in solution is probably due to a shifting of the whole absorption spectrum toward the higher wavelengths. After irradiation with alpha particles Carruthers found that the absorption spectrum of serum albumin extended into the visible region.

The absorption of water vapour by albumin before irradiation had a profound effect on the absorption constant. On a particularly humid day this effect lowered the absorption constants at both wavelengths by a factor of two. Consistent results were again obtained when the protein was dried under vacuum for five hours before irradiation.

3. Haemoglobin

The absorption constants, k, at wavelengths 2576 Å, 2920 Å and 3906 Å are shown in Table IX, page 84, for haemoglobin irradiated 0, 1/4, 2, 4 and 8 minutes. The percentage changes in k are plotted against irradiation time for wavelengths 2576 Å and 2920 Å in Fig. 15, and for 3906 Å in Fig. 16. The probable errors indicated are the same as those for serum albumin.

The solubility of haemoglobin was unaffected by proton irradiation for times up to eight minutes. The absorption constants, k and k_1 of equation (22a) are therefore the same. It is seen that at 2576 Å and 2920 Å the absorption constant rises with increasing dosage as do the corrected absorption constants for serum albumin. At 3906 Å, however, the absorption constant is decreased by irradiation.

The ultra-violet absorption spectrum of haemoglobin below 3000 Å is similar to that of serum albumin. Above 3000 Å, however, it

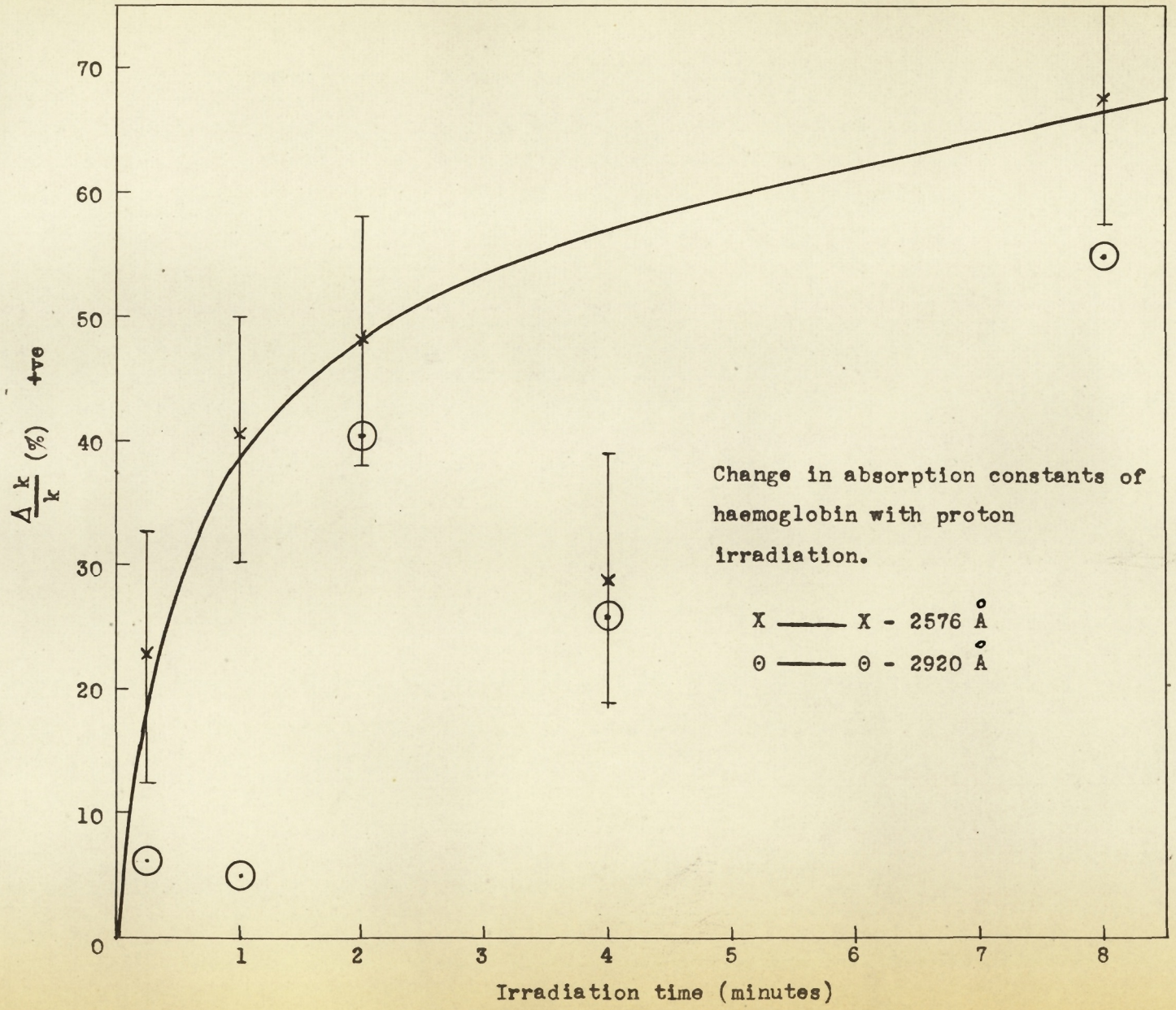

70
60
50
40
30
20
10
0
1
2
3
4
5
6
7
8
$\frac{\Delta k}{k}$ (%) +ve
Irradiation time (minutes)
Change in absorption constants of haemoglobin with proton irradiation.
X —— X - 2576 Å
⊙ —— ⊙ - 2920 Å

FIG. 15

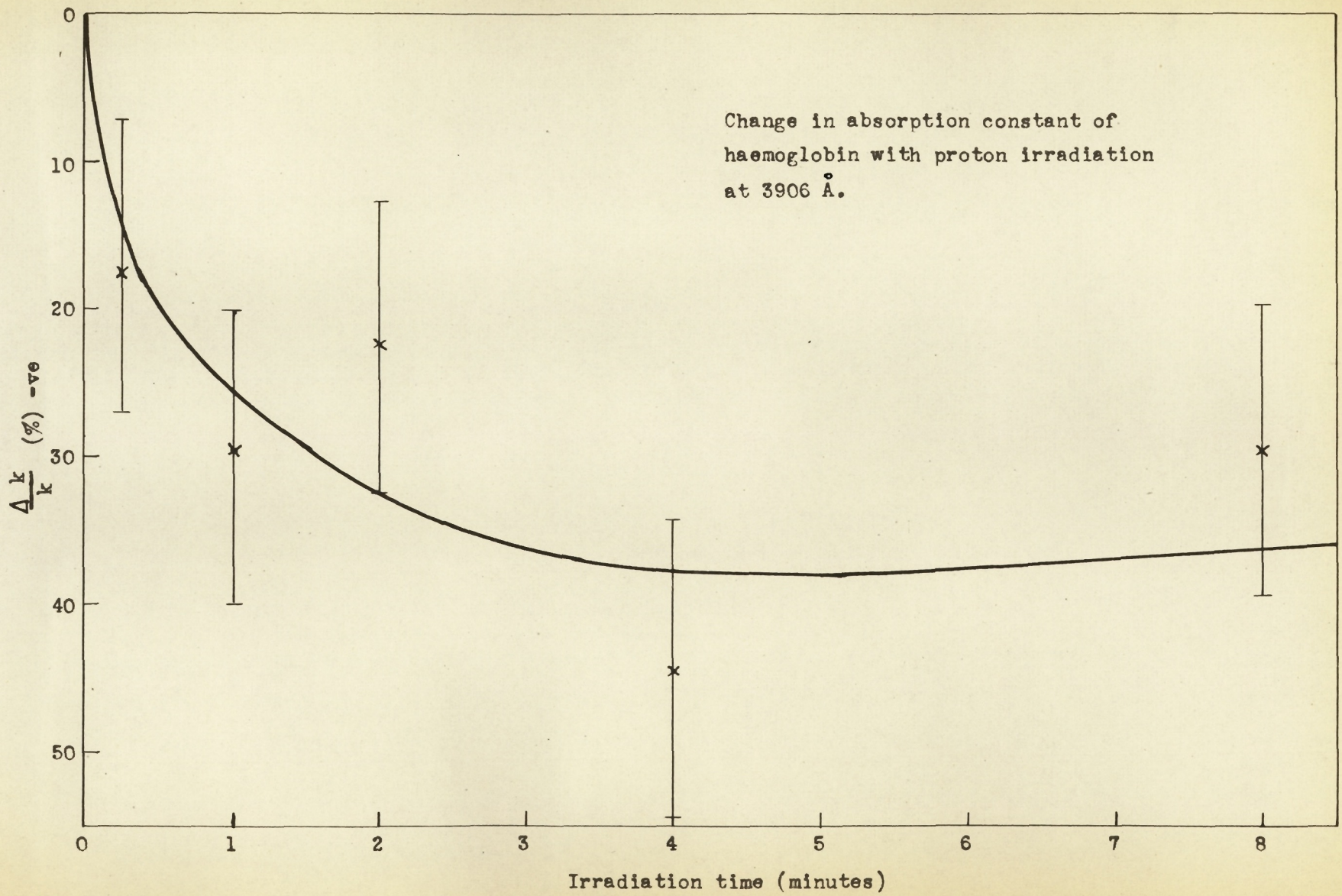

Change in absorption constant of haemoglobin with proton irradiation at 3906 Å.
$\frac{\Delta k}{k}$ (%) -ve
0
10
20
30
40
50
0
1
2
3
4
5
6
7
8
Irradiation time (minutes)

FIG. 16

absorbs far into the visible region, giving the protein its red color. The absorption above 3000 Å is due to the pyrrole group containing iron. Decrease in absorption at 3906 Å with irradiation may be due to a breakage in some bonds in this group or to a change in valence of the iron atom in the group.

The increase in absorption at 2576 Å is probably as in serum albumin due to a shifting of the whole absorption spectrum to higher wavelengths. At 2920 Å, however, it is observed that the points showing the change in absorption with irradiation time do not fall on a smooth curve as do those at 2576 Å and 3906 Å. This is due to the shift in the absorption maximum of globin to higher wavelengths, tending to increase the absorption at 2920 Å , and the decrease in absorption of the pyrrole group tending to decrease it.

III UNIRRADIATED PROTEINS

1. Serum Albumin

The bovine serum albumin was prepared by Armour Laboratories according to methods described by Cohn, Hughes and Wease[22].

The sedimentation constant measured from the movement of the diffusion boundary at 2920 Å was 4.4×10^{-13} seconds. This agrees with the sedimentation constant of 4.5×10^{-13} seconds found by Oncley[22] within the limits of experimental error. The value 5.3×10^{-13} seconds, found from the decrease in concentration at the periphery of the cell indicates the presence of some heavier impurities.

2. Haemoglobin

The bovine haemoglobin obtained from Armour Laboratories was a crude preparation. The sedimentation constants at 3906 Å, measured from movement of the diffusion boundary and decrease in concentration at the periphery of the cell, were 3.3×10^{-13} seconds and 6.5×10^{-13} seconds respectively. The value, given by Svedberg[13] for horse and human haemoglobin, is 4.4×10^{-13} seconds.

The solution was thus polydisperse with impurities present whose molecular weights are both smaller and larger than that of haemoglobin. No non-sedimenting material was detected.

3. Urease

The sedimentation constant of urease, measured by Sumner[23], is 18.6×10^{-13} seconds. Two different lots of urease, however, showed no sedimentation at all after two hours of centrifuging at 58,500 g (500 r.p.s.), due to the presence of impurities of low molecular weight. These impurities masked any effect of irradiation on the urease molecules when examined in the ultracentrifuge; therefore no results were obtained from sedimentation experiments with this protein.

IV SEDIMENTATION EXPERIMENTS ON IRRADIATED PROTEINS

1. General

All three proteins had a disagreeable odor after irradiation. Serum albumin had a yellowish tint and the color of haemoglobin was darkened. Color changes were not obvious in solution. Duggar[24]

mentions similar odors and changes in color associated with the irradiation of proteins with ultra-violet light. The odor was associated only with the irradiated material and hence was not due to bacterial action.

Serum albumin irradiated with 50 Mev protons for five minutes, at a dosage rate of 6.1×10^4 rep/sec., was only 20% soluble in acetate buffer solution pH 4.75. The solubilities of haemoglobin and urease irradiated for times up to eight minutes at the same dosage rate were unaffected.

Mercury lines at 2576 Å and 2920 Å were photometered for sedimentation measurements on serum albumin irradiated 1, 1.5, 2, 3, 4 and 8 minutes. For haemoglobin irradiated 2, 4 and 8 minutes these two lines, and also that at 3906 Å, were photometered.

The following measurements were made on the photometer curves:

(i) The sedimentation constant was calculated from the rate of movement of the diffusion boundary, using equation (18).

Sample calculations on the photometer curves of Fig. 20, for serum albumin irradiated two minutes, are shown in Table IV and Table V. The following symbols are used in these tables:

I_1 is the position on the photometer sheet of the first index in the counter-balance.

I_2 is the position of the second index on the photometer sheet.

y is the position on the photometer sheet of the center of the cell; this is equal to $\frac{I_1 + I_2}{2}$.

max G.R. is the maximum galvanometer reading associated with the maximum concentration of protein at the periphery of the cell.

max. conc. is the maximum concentration found from the maximum galvanometer reading, using the concentration scale.

50% conc. is the mean of the maximum concentration and the minimum concentration associated with non-sedimenting material.

$G.R._{50}$ is the galvanometer reading obtained from the concentration scale for the mean concentration above.

x_{50} is the position of the point on the photometer sheet where concentration on the diffusion boundary is the mean concentration. This is referred to as the 50% point.

r'_{50} is the distance from the 50% point to the center of the cell. This is calculated from the equation

$$r'_{50} = \frac{(y - x_{50})}{m}$$

where m is the magnification of the spectrograph at this wavelength. At 2576 Å, m = 0.934.

r_{50} is the distance from the center of rotation to the 50% point.

Δr is the difference between values of r for a difference in time between exposures, Δt.

r_m is the mean value of r in the time interval Δt.

Other symbols are as used on pages 10 to 14.

TABLE IV

Plates 176, 177, 178.

Bovine Serum Albumin irradiated 2 minutes, 50 Mev protons.

Dosage rate = 6.1×10^5 rep/sec.

Line 2576 Å

Minimum Concentration 1.4 mg./cc.

Plate No.	Exposure No.	I_1 mm.	I_2 mm.	y mm.	max.G.R. cm.	max. conc. mg/cc.	50% conc. mg/cc.	$G.R._{50}$ cm.	x_{50} mm.	r'_{50} mm.
176	1	0.18	14.32	7.25	11.30	8.00	4.70	9.55	1.02	6.66
177	2	0.56	14.71	7.63	10.90	6.90	4.15	8.80	2.75	5.33
177	3	0.38	14.58	7.48	10.55	6.20	3.80	8.45	3.70	4.05
177	4	0.32	14.45	7.39	9.95	5.36	3.38	7.98	4.65	2.94
178	5	0.19	14.35	7.27	9.70	5.00	3.20	7.60	5.04	2.39

TABLE V

Exposure No.	r_{50} cm.	Δr cm.	r_m cm.	w^2 $(rad/sec)^2 \times 10^{-6}$	T °C	Δt sec.	n^o_T/n^o_{20}	S'_{20} sec. $\times 10^{13}$
1	5.134							
		0.133	5.200	20.9	27.4	1740	0.842	5.92
2	5.267							
		0.128	5.331	21.0	27.8	1800	0.839	5.34
3	5.395							
		0.111	5.450	21.0	28.1	1800	0.835	4.50
4	5.506							
		0.055	5.533	21.1	28.5	1800	0.831	2.18
5	5.561							

Using figures for exposures #1 and #2,

$$S'_{20} = \frac{\Delta r/\Delta t}{w^2 r_m} \times \frac{n_T^o}{n_{20}^o} \qquad (18)$$

$$= \frac{0.133 \times 0.842}{20.9 \times 10^6 \times 5.200 \times 1740}$$

$$= 5.92 \times 10^{-13} \text{ seconds.}$$

$$\text{Mean } S'_{20} = 4.5 \pm 1.1 \times 10^{-13} \text{ seconds.}$$

$$S_{20} = 1.07 \times S'_{20} \qquad (20)$$

$$= 4.8 \pm 1.1 \times 10^{-13} \text{ seconds.}$$

The decrease in the sedimentation constant with time as the solute sediments towards the periphery of the cell shows that the solute is polydisperse. The sedimentation constants for irradiated proteins have been listed with the probable errors. The probable error gives a measure of polydispersity for the solute. The uncertainty in probable error calculated from four measurements is 25%.

(ii) The sedimentation constant calculated using equation (19) was attributed to aggregation products. The molecular weights corresponding to these sedimentation constants, taken from Fig. 27, page 80, are in good agreement with those estimated from electron micrographs of irradiated serum albumin.

The concentration of aggregation products was estimated by comparing the decrease in concentration at the periphery of the cell, after two hours' sedimentation, with that of normal protein.

Table VI shows calculations made at 2576 Å on the decrease of concentration in the cell.

TABLE VI

Exp. No.	Max. Conc. mg/cc.	T °C	n_T^o/n_{20}^o	w^2 $(rad/sec)^2 \times 10^{-6}$	$\frac{C_1'}{C_i}$	$\log_{10}\frac{C_1}{C_i}$	$\frac{n_T^o/n_{20}^o \log_{10} C_1/C_i}{w^2}$ $(rad/sec)^{-2} \times 10^9$	t sec.
1	8.00	27.2	0.844	20.9	1	0	0	720
2	6.90	27.6	0.840	20.9	1.160	0.0645	2.60	2460
3	6.20	28.0	0.837	21.1	1.290	0.1106	4.39	4260
4	5.36	28.3	0.833	21.1	1.495	0.1746	6.89	6060
5	5.00	28.7	0.829	21.2	1.600	0.2041	8.00	7860

The following symbols have been used in Table VI.

T the average temperature of the rotor from the first exposure to the time, t.

w average angular frequency from the first exposure to the time, t.

$\frac{C_1}{C_i}$ ratio of the initial maximum concentration to the maximum at exposure, i.

In Fig. 24, page 78, $\frac{\log_{10} C_1/C_i \ (n_T^o/n_{20}^o)}{w^2}$ has been plotted against t. The slope of this curve, tan ϕ, gives the sedimentation constant using the equation

$$S_{20}' = \frac{\tan \phi}{2 \log_e 10}$$

$$= \frac{10.50}{10{,}000} \times 10^{-9} \times \frac{1}{0.869}$$

$$= 12.1 \times 10^{-13} \text{ seconds}^{-1}.$$

$$S_{20} = 1.07\ S'_{20}$$

$$= 13 \times 10^{-13} \text{ seconds.}$$

The concentration at the periphery of the cell after two hours of centrifuging at 125,000 g was 63% of the original concentration. After sedimenting for the same time under the same conditions, the relative concentration of unirradiated serum albumin decreased to 81%. The difference of 18% is ascribed to aggregation products. The assumption that after two hours of sedimentation the bulk of the aggregation products have been removed from the solution is supported by the fact that the distribution in the cell of these particles was approaching equilibrium, as shown by the increase in back diffusion.

Tables VII and IX show the results obtained from absorption and sedimentation experiments on serum albumin and haemoglobin respectively. Table VII is divided into two parts and Table IX into three parts, showing the results obtained at the two different wavelengths for serum albumin and the three different wavelengths for haemoglobin.

Results have been listed under the following headings:

S_{20} - diffusion boundary - column 3, refers to the average sedimentation constant measured from the rate of movement of the diffusion boundary. The uncertainty listed is the probable error, showing the spread in the four values measured as these decreased as sedimentation progressed.

S_{20} - aggregates - column 4, refers to the sedimentation constant calculated from the decrease in concentration of the protein in the periphery of the cell.

Relative concentration of aggregates - column 5, is obtained by comparing the decrease in concentration after two hours of sedimentation with the decrease in concentration of normal serum albumin under the same conditions.

Relative concentration of non-sedimenting material - column 6 - shows the relative concentration of non-sedimenting material in the rotating cell. This material remained at the meniscus after two hours of centrifuging. Only small fragments were present here. The concentration scale gave a good approximation to the relative concentration only when the distribution in size of fragments in solution was not too different from that in the solution which had not been centrifuged. The absorption constant probably varies directly as some positive power of the molecular weight of the particles. This would raise the true relative concentration of these particles, measured as a percentage by weight. Their percentage by number would be considerably higher.

Results of sedimentation and absorption experiments on serum albumin

Irrad. Time	Results at 2576 Å					Results at 2920 Å				
	k	S_{20} Diffusion Boundary	S_{20} Aggreg.	Rel.Conc. Aggreg.	Rel.Conc. Non-sed. Material	k	S_{20} Diffusion Boundary	S_{20} Aggreg.	Rel.Conc. Aggreg.	Rel.Conc. Non-sed. Material
minutes	$(mg/cc)^{-1}$	sec x 10^{13}	sec x 10^{13}	%	%	$(mg/cc)^{-1}$	sec x 10^{13}	sec x 10^{13}	%	%
0	0.261	4.4 ± 0.3	5.2	0	0	0.177	not measured			
1	0.289	4.5 ± 0.2	12	21	0	0.186	4.6 ± 0.5	12	21	0
1.5	0.203	4.6 ± 0.7	15	29	2	0.137	4.5 ± 1.3	20	33	0
2	0.200	4.8 ± 1.1 0.25	13	27	10	0.129	4.9 ± 1.3	17	31	12
3	0.144	3.4 ± 0.5	14	30	12	0.085	3.7 ± 0.7	20	42	8
4	0.154	3.0 ± 1.0	14	19	30	0.107	3.2 ± 1.0	14	28	30
8	0.150		not measured		50	0.102	not measured	6.1	9	50

TABLE VII

2. Serum Albumin

a) Photometer Curves. Photometer curves of the line at 2920 Å, showing the sedimentation of unirradiated serum albumin, are shown in Fig. 17. Figs. 18 and 19 show photometer curves of the lines at 2576 Å and 2920 Å for serum albumin irradiated one minute, Figs. 20 and 21 for two minutes' irradiation, and Fig. 22 and 23 for four minutes' irradiation.

Considering the photometer curves of albumin irradiated one minute, shown in Figs. 18 and 19, there was clearly a difference in the sedimentation near the meniscus at the two different wavelengths.

Exposure # 1 in Figs. 18 and 19 corresponds to the distribution of protein in the cell when the rotor has been running at full speed for one minute. At 2920 Å the meniscus at the point marked "m" was clearly defined, while at 2576 Å it was barely evident as a slight bend in the diffusion boundary. In Exposure # 2 (one-half hour later) at 2920 Å sedimentation was well clear of the meniscus and the concentration of material absorbing at this wavelength next to the meniscus was zero. At 2576 Å the concentration of material next to the meniscus was 5%.

The difference in the sedimentation of small fragments measured at the two wavelengths is shown more distinctly in Figs. 20 and 21. These figures show photometer curves at 2576 Å and 2920 Å for serum albumin irradiated for two minutes.

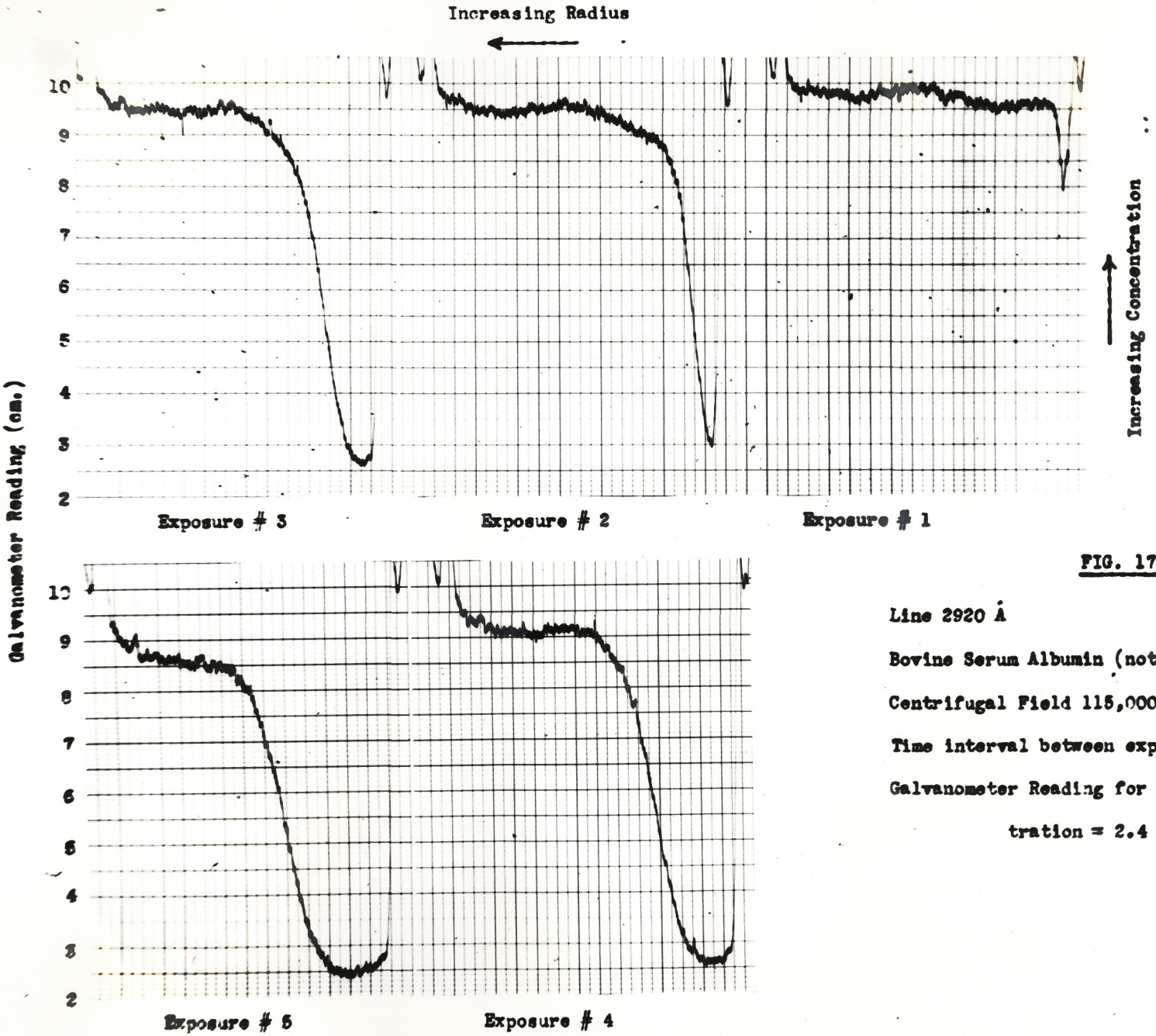


FIG. 17

Line 2920 Å

Bovine Serum Albumin (not irradiated)

Centrifugal Field 115,000 g.

Time interval between exposures 30 min.

Galvanometer Reading for zero concentration = 2.4 cm.

The space between vertical lines is equivalent to 0.5 mm. on the photographic plate.

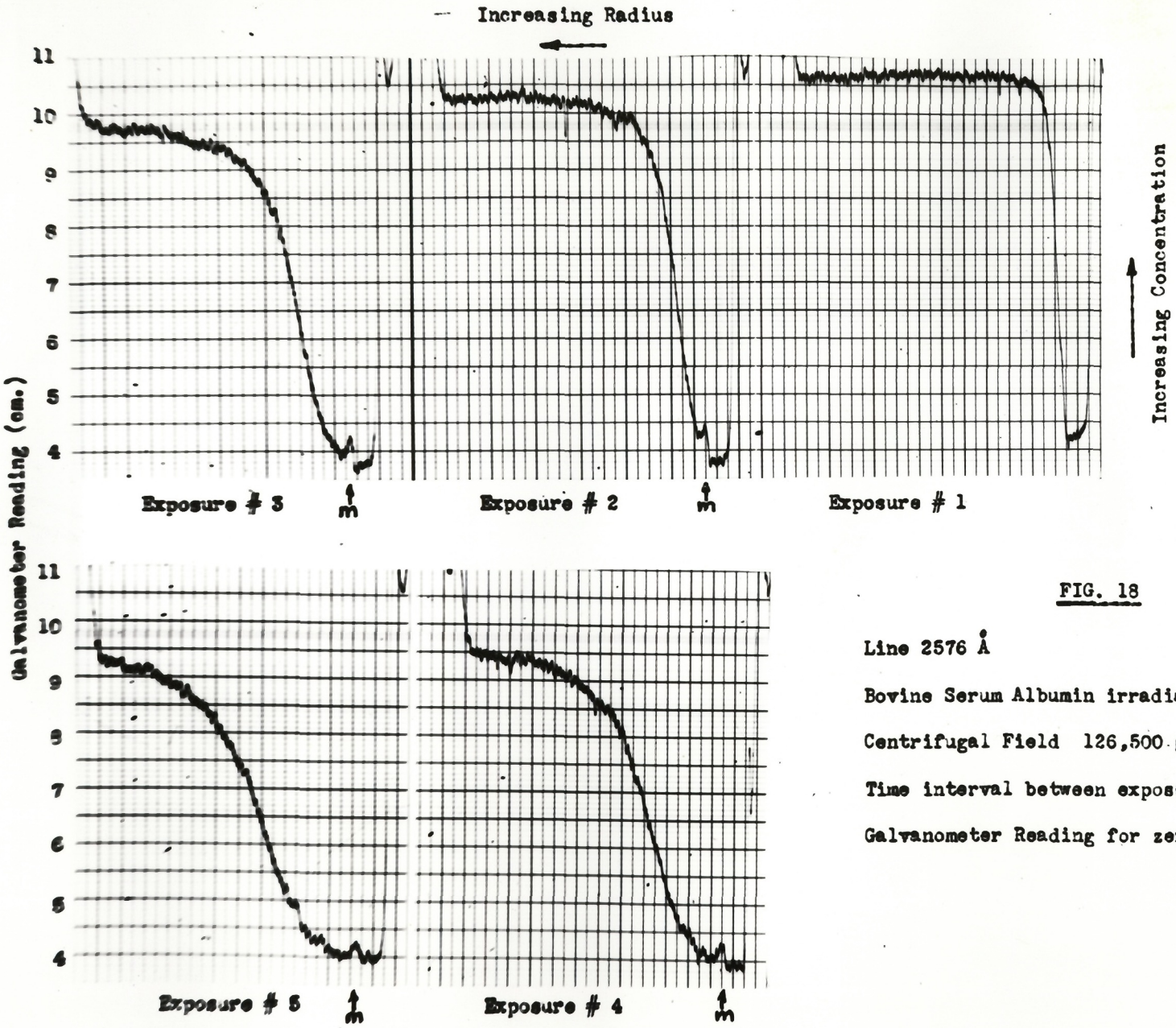


FIG. 18

Line 2576 Å

Bovine Serum Albumin irradiated 1 minute

Centrifugal Field 126,500 g.

Time interval between exposures 30 minutes

Galvanometer Reading for zero concentration = 4.0 cm.

The space between vertical lines is equivalent to 0.5 mm. on the photographic plate.

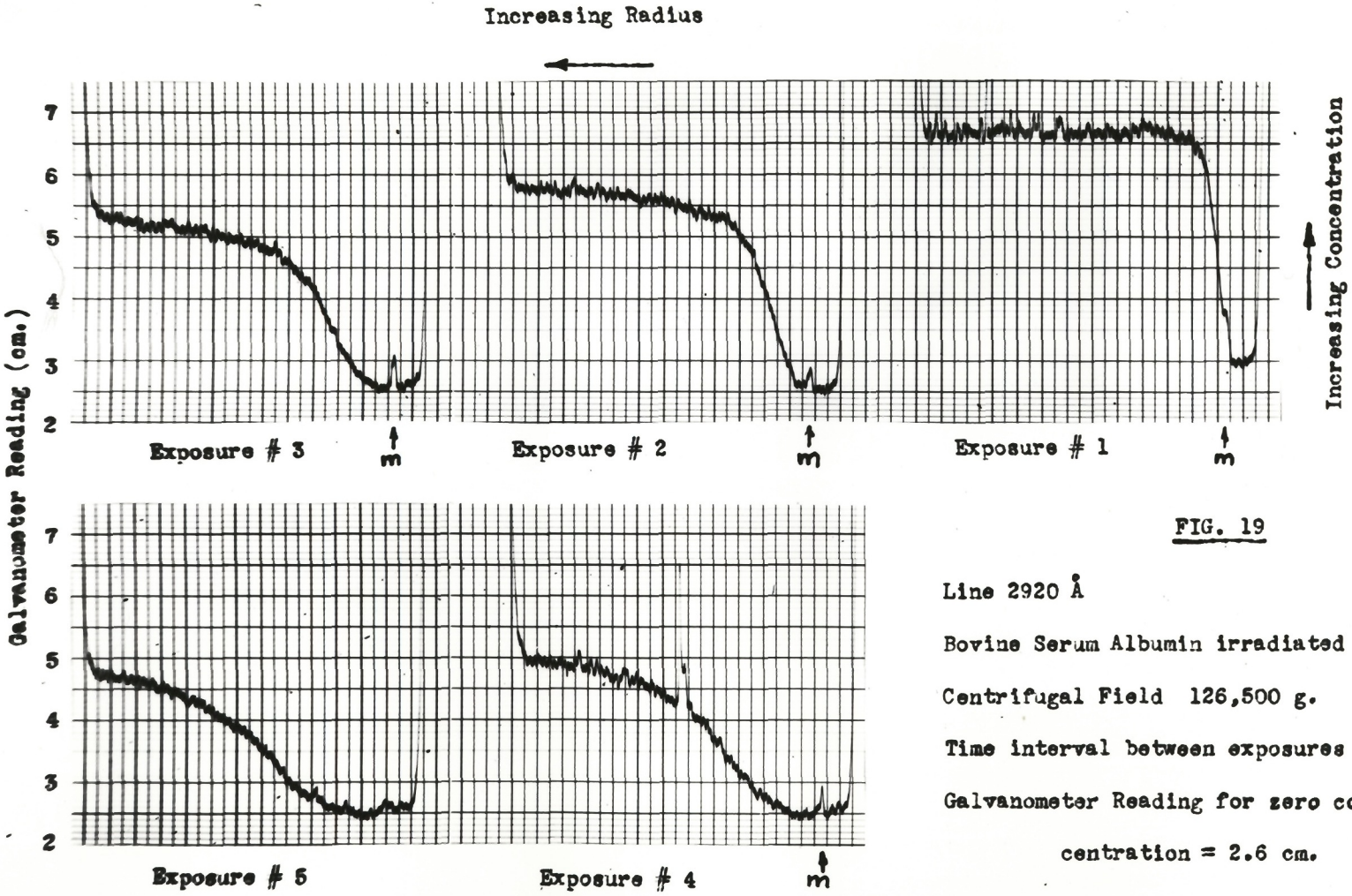


FIG. 19

Line 2920 Å

Bovine Serum Albumin irradiated 1 min.

Centrifugal Field 126,500 g.

Time interval between exposures 30 min.

Galvanometer Reading for zero concentration = 2.6 cm.

The space between vertical lines is equivalent to 0.5 mm. on the photographic plate.

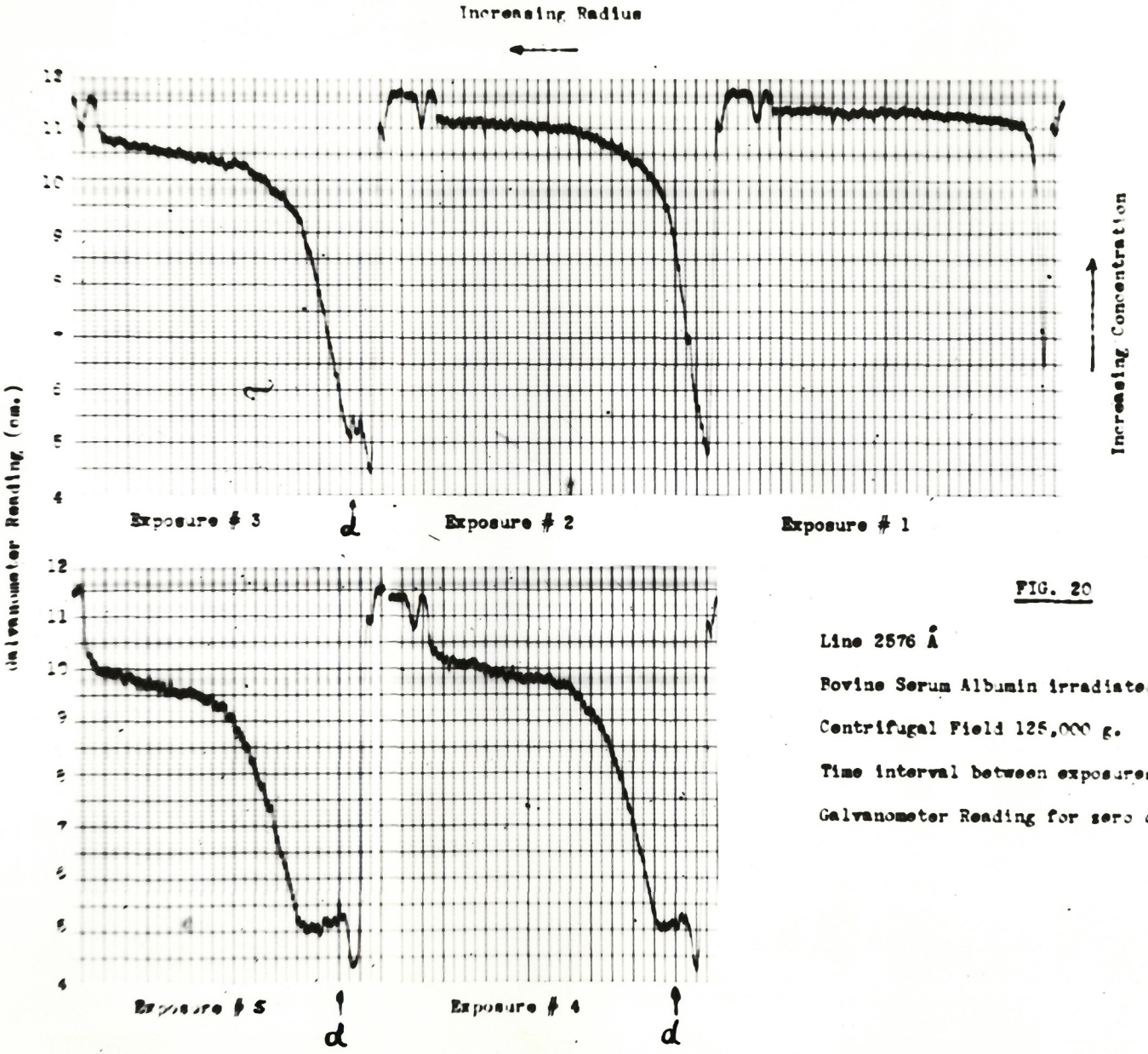


FIG. 20

Line 2576 Å

Bovine Serum Albumin irradiated 2 minutes

Centrifugal Field 125,000 g.

Time interval between exposures 30 minutes

Galvanometer Reading for zero concentration = 3.5 cm.

The space between vertical lines is equivalent to 0.5 mm. on the photographic plate.

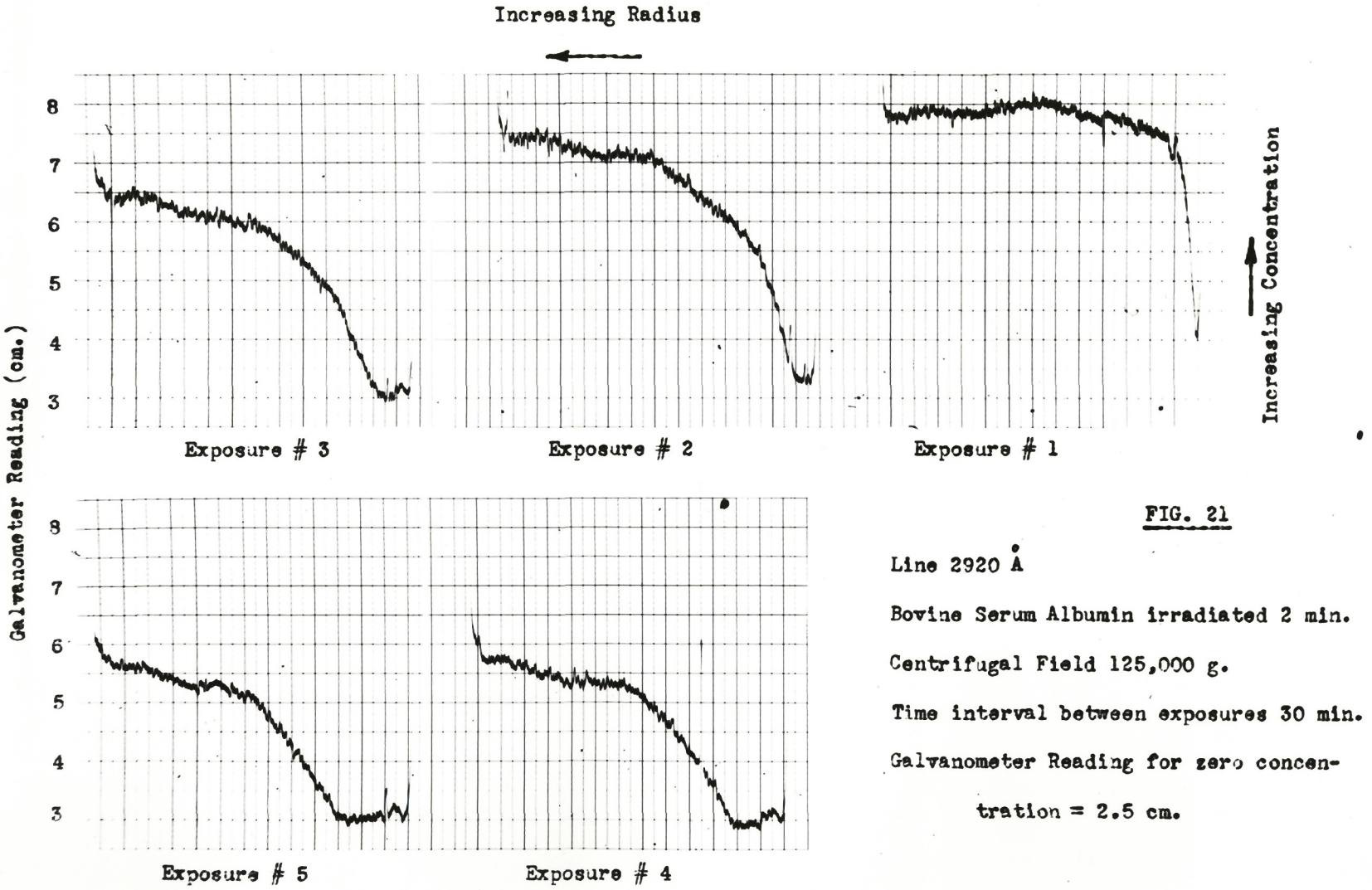


FIG. 21

Line 2920 Å

Bovine Serum Albumin irradiated 2 min.

Centrifugal Field 125,000 g.

Time interval between exposures 30 min.

Galvanometer Reading for zero concentration = 2.5 cm.

The space between vertical lines is equivalent to 0.5 mm. on the photographic plate.

At 2576 Å, particles of two distinctly different sizes sedimenting at different speeds caused a discontinuity in the diffusion boundary. The large particles in high concentration had a sedimentation constant of $4.8 \pm 1.1 \times 10^{-13}$ seconds, giving them a distribution in molecular weight about that of unirradiated serum albumin. The lowest sedimentation constant measured on this part of the boundary was $S'_{20} = 2.0 \times 10^{-13}$ seconds.

The second diffusion boundary which separated from the main diffusion boundary approximately one hour after sedimentation began is shown in exposures 3, 4 and 5 of Fig. 20, marked "d". The particles causing this second boundary had a sedimentation constant of 2.5×10^{-14} seconds. This was measured by the movement of the boundary between exposures 3 and 5.

The gram molecular weight corresponding to a diffusion constant of 2.5×10^{-14} seconds, from Fig. 27, is 1000. The relative concentration of these fragments was 6%.

There is no discontinuity in the diffusion boundary at the wavelength 2920 Å, shown in Fig. 21. If the fragments absorbed equally on this line a dip in the photometer curve of 0.5 cm. would be expected.

Photometer curves for the sedimentation experiments on serum albumin irradiated 1.5 minutes resembled those for serum albumin irradiated 1 minute. Photometer curves for experiments on albumin irradiated 3 minutes resembled those for the 2-minute irradiation, except that the average size of fragments present decreased. This made the separation of the two diffusion boundaries smaller.

Figs. 22 and 23 show photometer curves for serum albumin irradiated four minutes. In this case the average size of the fragments

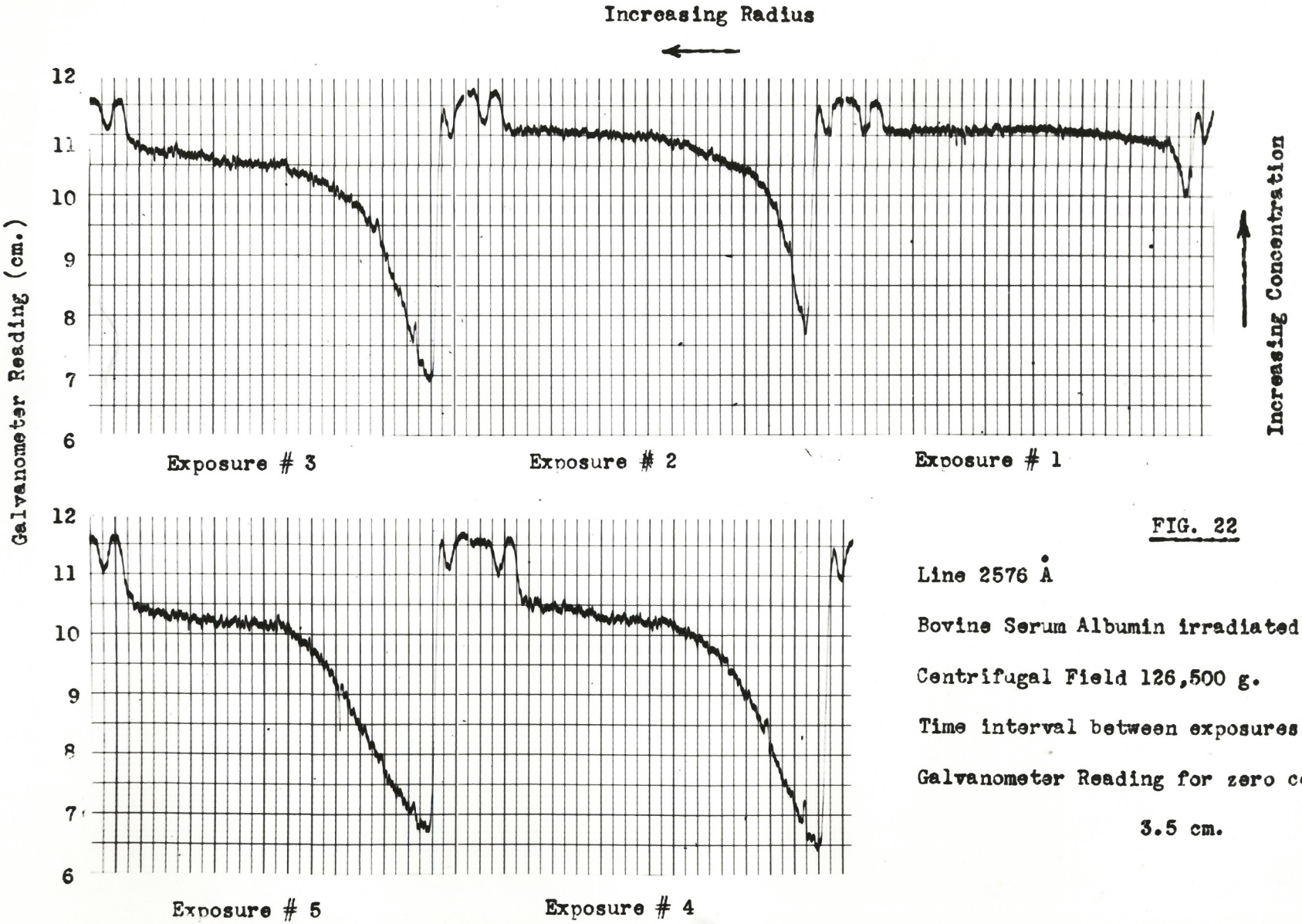


FIG. 22

Line 2576 Å

Bovine Serum Albumin irradiated 4 minutes

Centrifugal Field 126,500 g.

Time interval between exposures - 30 min.

Galvanometer Reading for zero concentration = 3.5 cm.

The space between vertical lines is equivalent to 0.5 mm. on the photographic plate.

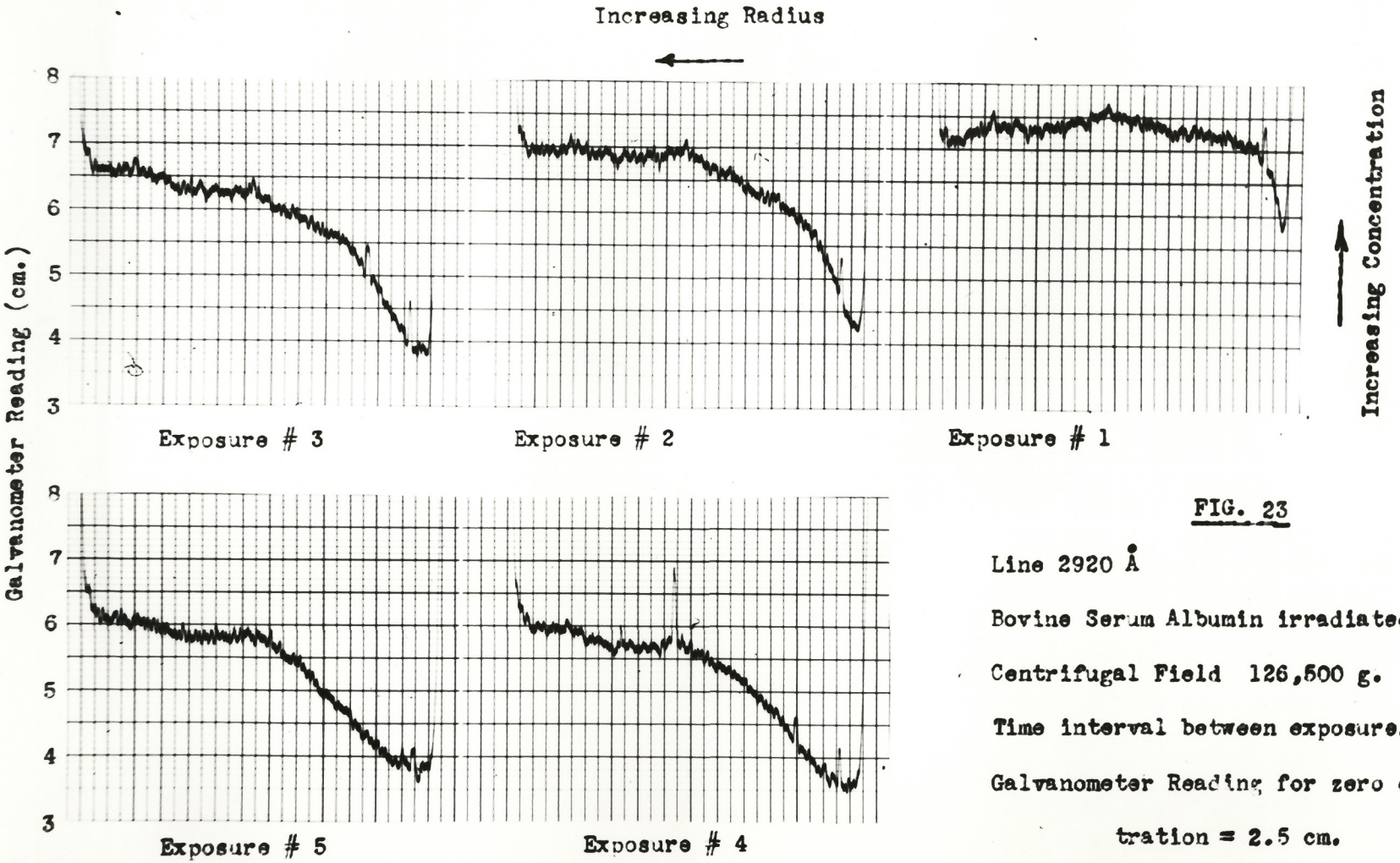


FIG. 23

Line 2920 Å

Bovine Serum Albumin irradiated 4 min.

Centrifugal Field 126,500 g.

Time interval between exposures 30 min.

Galvanometer Reading for zero concentration = 2.5 cm.

The space between vertical lines is equivalent to 0.5 mm. on the photographic plate.

in solution again decreased. The distribution in size of the fragments was continuous from particles which did not sediment at all to normal serum albumin molecules.

b) Diffusion Boundary Measurements. The average sedimentation constant measured from the rate of movement of the diffusion boundary remained equal to that for serum albumin for irradiation times up to three minutes although the polydispersity, as measured by the probable error, increased. This effect was attributed to the presence of albumin unaffected by the irradiation which for a two-minute irradiation was estimated at 73% of the solute. This percentage was estimated in the following way.

From Fig. 10, fraction of molecules unaffected by irradiation = 34%. This fraction is completely soluble.

Of the molecules affected by irradiation, only 1/5 are soluble from measurements on solubility after irradiation for five minutes.

This gives for the fraction of the protein which was soluble - 13%.

Thus, the fraction of the original protein in the solution was 47%, and of this the relative concentration of unaffected serum albumin molecules was 73%.

After four minutes' irradiation the relative concentration of unaffected molecules in solution was only 38%.

The decrease in concentration of unaffected albumin molecules was, however, not the only cause of the decrease in average sedimentation constant. The number of small fragments, both non-sedimenting and sedimenting, also increased. The concentration of non-sedimenting

fragments became so high after irradiation for eight minutes that no measurement could be made on the movement of the diffusion boundary.

The average sedimentation constants measured at 2920 Å were a little higher than those measured at 2576 Å. The probable errors are also higher. In general, smaller fragments did not absorb as much at 2920 Å as at 2576 Å. In addition the sedimentation constant, as measured from the decrease in concentration at the periphery of the cell, was higher at 2920 Å, indicating that the absorption of aggregation products was higher at this wavelength. These aggregates would also have an effect on the sedimentation constant measured from the diffusion boundary.

c) Aggregation Products. Aggregation products were found to be present in high concentration for all irradiation times of less than eight minutes. The average of the ten measurements gives a value for the sedimentation constant measured from the decrease in concentration at the periphery of the cell, of 15×10^{-13} seconds. This corresponds to a gram molecular weight of 420,000, from Fig. 27, page 80.

Electron micrographs of unirradiated urease and serum albumin irradiated for five minutes are shown in Figs. 25 and 26. The magnification is approximately 17,000 diameters.

In Fig. 26, the electron micrograph of irradiated serum albumin, three different sizes of particles can be distinguished.

1) Very small particles appear faintly in the background. The diameter of these particles is comparable with the limit of resolution of the electron microscope, 3 mμ. Their size is thus comparable to that of normal serum albumin molecules, diameter 4.4 mμ.

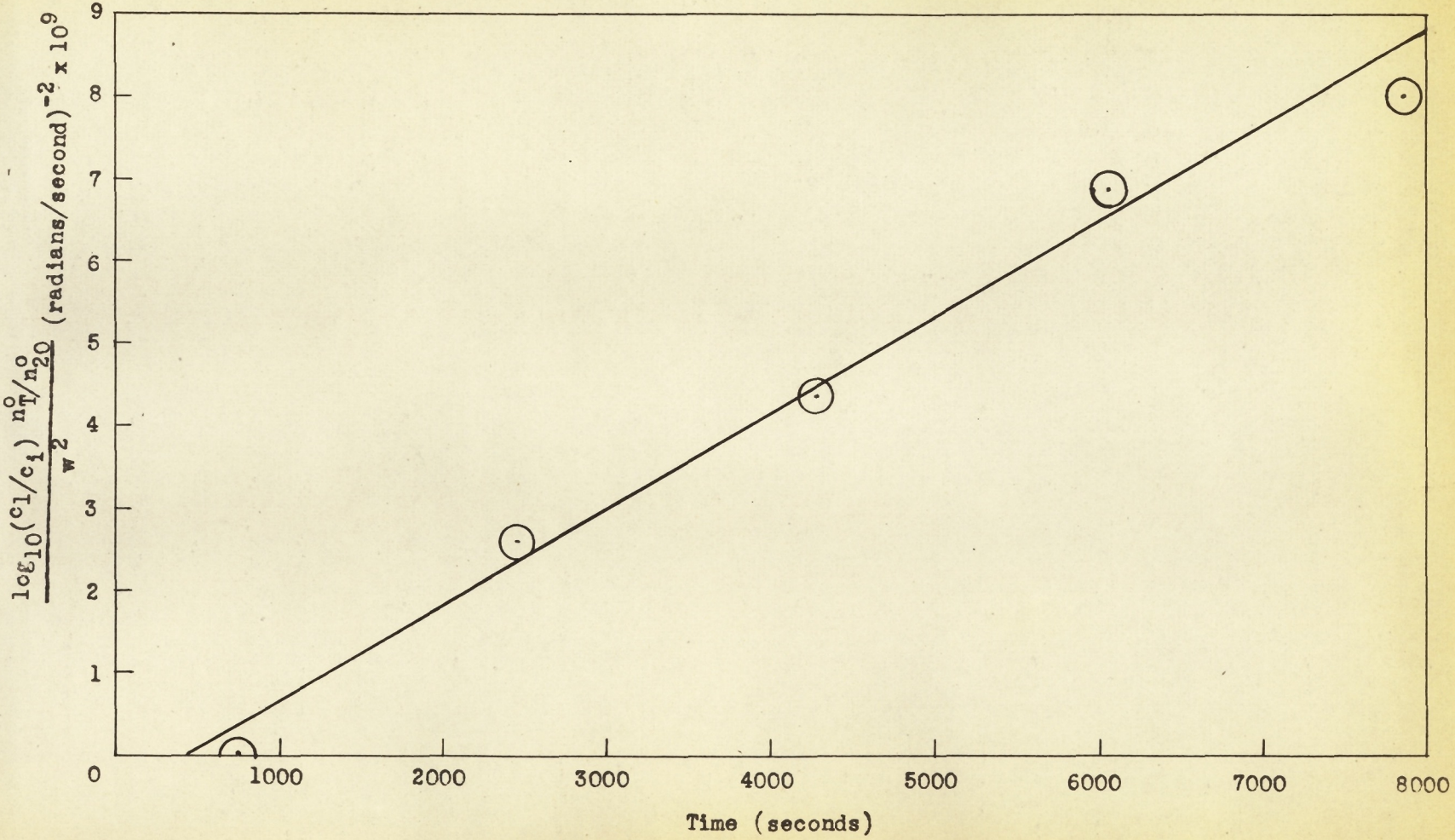


FIG. 24

Serum albumin irradiated two minutes.

2576 Å

Fig. 25

Electron Micrograph of
Unirradiated Urease.

x 17,000 diameters

Fig. 26

Electron Micrograph of
Serum Albumin Irradiated 5 min.

x 17,000 diameters

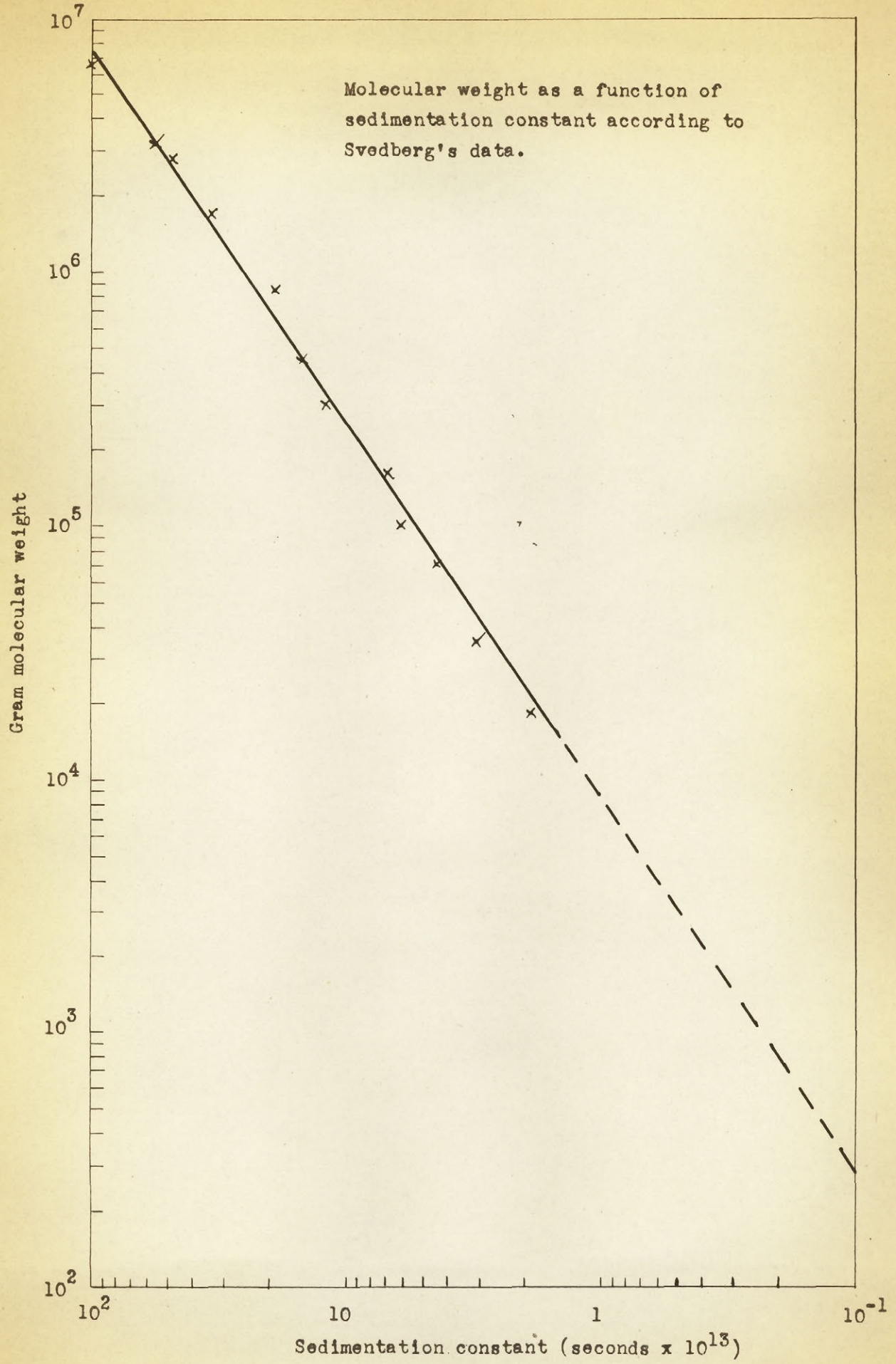

Molecular weight as a function of sedimentation constant according to Svedberg's data.
Gram molecular weight
10^7
10^6
10^5
10^4
10^3
10^2
10^2
10
1
10^{-1}
Sedimentation constant (seconds x 10^{13})

FIG. 27

ii) Very large particles with diameters at least twice that of the urease molecules shown in Fig. 25 are apparent. Since urease molecules have a gram molecular weight of 480,000, the molecular weight of these particles is approximately 4×10^6. The corresponding sedimentation constant, 7×10^{-12} seconds (from Fig. 27) is so high that these particles would sediment to the bottom of the cell before the centrifuge rotor reached its full speed of 730 r.p.s.

iii) Particles of size intermediate between these extremes can also be seen. Their diameters are comparable in size to that of the urease molecules of Fig. 25, giving them a gram molecular weight of approximately 500,000. This is in good agreement with the gram molecular weight (420,000) estimated from the sedimentation constant measured by rate of decrease in concentration at the periphery of the cell.

After eight minutes' irradiation the sedimentation constant, measured by the rate of decrease in concentration at the periphery of the cell, dropped from the value of 14×10^{-13} seconds after four minutes' irradiation, to 6×10^{-13} seconds. The total change in relative concentration was only 9% different from that of normal serum albumin. It is probable that in this run very few aggregation products were present.

Table VII shows the various breakdown products as percentages by weight of serum albumin irradiated two and four minutes. In making these calculations Fig. 10 has been used to find the number of molecules in which a primary ionization occurred. Of these, 20% were assumed to be soluble.

For calculations on fragments other than the insoluble products the concentrations measured at 2576 Å, from Table VII, were used. No calculation could be made of the fraction of material in solution which had approximately the same size as unaffected protein. It is estimated that these would amount to five or ten percent of the irradiated material.

TABLE VIII

	2 minutes	4 minutes
Insoluble material	52%	72%
Unaffected protein	34%	11%
Non-sedimenting fragments	5%	9%
Slowly sedimenting fragments	3%	
Aggregation products	8%	7%
Total	102%	99%

3. Haemoglobin

Results of sedimentation experiments on haemoglobin irradiated 2, 4 and 8 minutes, and absorption experiments for haemoglobin irradiated 0, 1/4, 1, 2, 4 and 8 minutes, are shown in Table IX. The column headings in this table are the same as in Table VII. The effect of proton irradiation on this protein was altogether different from its effect on serum albumin.

i) Irradiation had no effect on the solubility of the protein for irradiation times up to eight minutes.

ii) No aggregation products were observed until after eight minutes' irradiation. This was confirmed by electron microscope examination of haemoglobin irradiated five minutes. No sign of aggregation products was found.

iii) Although the light absorption at 2576 Å and 2920 Å increased as did the absorption of serum albumin when corrected for solubility, the absorption at 3906 Å decreased.

No non-sedimenting material was found at 3906 Å . Non-sedimenting material found at 2920 Å and 2576 Å was found in relative concentrations that did not follow a consistent pattern.

iv) The effect of impurities on the average sedimentation constants measured by the rate of movement of the diffusion boundary at 2576 Å and 2920 Å is unknown. They remained fairly constant, however, for two and four minutes at their respective values of 4.0×10^{-13} sec. and 3.7×10^{-13} seconds, after eight minutes' irradiation. The sedimentation constant measured at 3906 Å rose slightly with time of irradiation from 3.3×10^{-13} seconds for the unirradiated protein, to 4.0×10^{-13} sec. for protein irradiated eight minutes. The particles sedimenting on this line were slightly less polydisperse, as measured by the probable error, than on the other lines.

Since this molecule has the same molecular weight as serum albumin, the analysis of the number of molecules affected by the irradiation should be the same. This was confirmed by the changes in the absorption constants shown in Fig. 15 and Fig. 16.

TABLE IX

Results of sedimentation and absorption experiments on haemoglobin.

Irrad. Time	k	S_{20} Diffusion Boundary	S_{20} Aggregate	Rel. Conc. Aggregate	Rel. Conc. Non-sed. Material
min.	$(mg/cc)^{-1}$	sec x 10^{13}	sec x 10^{13}	%	%
		Line 2576 Å			
0	1.14				
0.25	1.43				
1	1.62				
2	1.69	4.2± 1.0	4.8	0	10
4	1.47	4.3± 0.8	6.1	0	0
8	1.91	3.1± 0.8	16	17	10
		Line 2920 Å			
0	0.99				
0.25	1.06				
1	1.04				
2	1.39	3.8 ± 0.6	9.2	5	0
4	1.25	4.0 ± 1.3	8.1	5	20
8	1.54	2.7 ± 0.3	14	17	0
		Line 3906 Å			
0	3.45	3.3 ± 0.4	6.9		
0.25	2.85				
1	2.43				
2	2.68	3.6 ± 0.5	8.7	5	0
4	1.92	4.5 ± 0.7	8.8	5	0
8	2.43	4.3 ± 0.4	18	17	0

CHAPTER VI - CONCLUSIONS

I IRRADIATED SERUM ALBUMIN

i) The dosage rate of 50 Mev protons with which serum albumin and haemoglobin were irradiated was $6.1 \pm 2.7 \times 10^5$ rep/second.

ii) The ultra-violet absorption constants of serum albumin and haemoglobin changed rapidly with irradiation times up to five minutes. After this the percentage changes remained nearly constant. Calculations from the measured dosage rate show that at this irradiation time a primary ionization had occurred in 93% of the irradiated molecules.

iii) In sedimentation experiments on serum albumin irradiated for times up to three minutes, small fragments were found with a sedimentation constant of 2.5×10^{-14} seconds and an estimated gram molecular weight of 1000. These did not absorb in the ultra-violet at 2920 Å.

These particles were most evident for serum albumin irradiated two minutes. For irradiation times less than this their concentration was not high enough to allow a measurement of the sedimentation constant. For longer irradiation times a continuum of fragments of comparable size was found.

From Fig. 10 it is seen that for the measured dosage rate, the effects of a single primary ionization on molecules of serum albumin should be most evident after irradiation for 111 seconds. At this time a maximum number of molecules have received one, and only one, primary ionization. It is concluded that bovine serum albumin molecules in which only a single primary ionization has occurred break

up to give fragments of gram molecular weight 1000 preferentially. These particles are probably peptides, which do not contain the amino acid tryptophane, which absorbs very strongly at 2920 Å. The relative concentration of these fragments, as measured by their absorption at 2576 Å, was only about 6%. However, their relative number might be considerably higher than this if absorption increases with the size of the molecules.

iv) As the number of primary ionizations occurring in the serum albumin molecules increased, large numbers of small non-sedimenting fragments were formed. The average size of the sedimenting particles decreased until a continuum of sizes, including non-sedimenting fragments and normal serum albumin molecules, was present.

v) For serum albumin irradiated under eight minutes, high concentrations of aggregation products were found. Their average gram molecular weight was estimated at 500,000. Using the electron microscope still larger particles, with a gram molecular weight estimated at 4,000,000, were seen. After irradiation for eight minutes no aggregation products were found.

II IRRADIATED HAEMOGLOBIN

i) Haemoglobin was found to be much more stable under proton irradiation than was serum albumin. No effects were found in haemoglobin which could be attributed to a single ionization per molecule. With increasing dosages of protons the material became increasingly polydisperse, with an average size similar to that of normal haemoglobin. No large aggregates were formed until after eight minutes of irradiation. These had a gram molecular weight of about 500,000. Their

absence for two and four minute irradiations indicates that at least three ionizations per molecule are required before they form in appreciable quantity.

The decrease in absorption at wavelength 3906 Å is attributed to a rearrangement of chemical bonds, rather than to preferential destruction of the haem group. Total preferential destruction of the haem group would lead to zero absorption at this wavelength after five minutes' irradiation. Similarly, changes in chemical bonds in other groups lead to the increase in absorption at 2576 Å and 2920 Å.

III THE EXPERIMENTAL TECHNIQUE

1. Ultracentrifuge Measurements

It has been shown that the simultaneous measurement of sedimentation in different regions of the absorption spectrum is a very powerful technique in making ultracentrifuge measurements on polydisperse systems. In the present work the technique was limited by the small number of mercury lines in which measurements could be made. A light source giving a continuous spectrum of uniform intensity from 2200 Å to 5000 Å would be preferable to the mercury arc. A hydrogen arc would be excellent for this purpose.

The use of the same technique in the infra-red region, where absorption can be associated more closely with constituents of the molecules, might prove fruitful.

2. Irradiation of the Proteins

The irradiations suffer from the fact that the beam current, and hence the dosage of protons hitting the protein target, cannot be

measured accurately. For such measurements the beam should be focussed on the target, mounted in a Faraday cup to collect the protons. This would in practice necessitate that the beam be extracted from the cyclotron tank.

3. The Apparatus

Operation of the centrifuge at 100 r.p.s. would increase the radial acceleration from 125,000 g, achieved at 730 r.p.s., to 234,000 g. To attain 1000 r.p.s. a new rotor which has a stronger beam holding the cell in place, is required. At this higher speed it would no doubt be possible to resolve more fragments than was possible in the present work.

Wear in the centrifuge bearings is caused chiefly by resonances in the rotating system in acceleration and deceleration. The newly designed bearings were found to be very satisfactory, lasting for at least thirty high-speed runs, each of several hours' duration.

BIBLIOGRAPHY

(1) Parker, H.M. Advances in Biological and Medical Physics, Vol. I. Edited by J.H. Lawrence and J.G. Hamilton, Academic Press Inc. (1948).

(2) Lea, D.E. Action of Radiation on Living Cells. Cambridge University Press. (1946).

(3) Pollard, E. American Scientist, 39, 99, (1951).

(4) Pollard, E. and Dimond, A.E. Physical Review 82, 302, (1951).

(5) Setlow, R.B. Physical Review, 82, 302, (1951).

(6) Appleyard, R.K. Physical Review, 82, 302, (1951).

(7) McNulty, W.P. and Hutchinson, F. Physical Review 82, 303. (1951).

(8) Adams, W.P. and Pollard, E. Physical Review 83, 230. (1951.)

(9) Pollard, E. and Dimond, A.E. Physical Review 86, 612. (1952).

(10) Svedberg, T. and Brohult, S. Nature, 143, 938. (1939).

(11) Carruthers, J.A. Ph.D. Thesis, McGill University, (1949).

(12) Friche, H. Nature, 169, 966, (1952).

(13) Svedberg, T. and Pedersen, K.O. The Ultracentrifuge. Clarendon Press, (1940).

(14) Beams, J.W. Review of Modern Physics, 10, 245, (1938).

(15) Gunton, R.C. Ph.D. Thesis, McGill University, (1947).

(16) Aron, W.A., Hoffman, B.G., Williams, F.C. Private communication to Dr. J.S. Foster from Dr. B.G. Hoffman.

(17) Skarstrom, C. and Beams, J.W., Review of Scientific Instruments 11, 398, (1940).

(18) Rotenberg, A.B. Ph.D. Thesis, McGill University, (1941).

(19) Henry, W.H. Ph.D. Thesis, McGill University, (1951).

(20) James, T.H. and Higgins, G.C. Fundamentals of Photographic Theory. John Riley & Sons, Inc. (1948).

(21) Zworykin, V.K., Morton, G.A., Ramberg, E.G., Hillier, J. and Vance, A.W. Electron Optics and the Electron Microscope. John Wiley & Sons, Inc. (1945).

(22) Cohn, E.J., Hughes, W.L., and Wease, J.A. The American Chemical Society Journal 69, 1753, (1947).

(23) Sumner, J.B., Gralen, N., Eriksson-Quensel, I.B. Jour. Biol. Chem. 125, 37, (1938).

(24) Duggar, B.M. Biological Effects of Radiation, Vol. I. McGraw Hill Book Co. (1936).

(25) Setlow, R.B. Archives of Biochemistry and Biophysics 34, 396, (1951).