

"DETERMINATION OF PHOSPHOROUS IN SOME BIOLOGICAL SYSTEMS
USING KINETICS AND SPECTROPHOTOMETRY METHODS"

A Thesis presented to

AHMADU BELLO UNIVERSITY

For the Degree of

MASTER OF SCIENCE (ANALYTICAL CHEM.)

BY

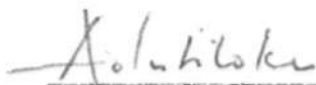
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DECLARATION

This is to state that this work is solely the candidate's own and has not been part of any presentation for any other qualification.



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May God be with you all.

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A B S T R A C T

Analyses for phosphorous in the seeds, back and pulpy part of five different species of pumpkin **was** carried out using the conventional spectrophotometric method and the less common, recent rapid kinetics method, the basis of which is the fast reaction between phosphate and MO(VI) in acid medium. The level of phosphate was determined in the various species and a number of useful indications revealed viz (i) phosphorous content appeared to vary with the pumpkin species being most in the species called *curcubita moschata* (ii) for a particular species of pumpkin, the phosphorous content is most in the seeds and least in the pulpy parts.

The rapid kinetics method of analysing phosphorous by converting phosphorous to phosphate using the 'digestion mixture' consisting of concentrated sulphuric acid, copper-sulphate, potassium hydrogen sulphate and little powdered selenium Jones, Lee, Peacocke (1951) and reacting with acid MO(VI) in a stopped-flow spectrophotometer proved viable for the pulpy parts of the various species but failed for the seeds and the back.

Analysis for phosphorous in the blood serum of two sets of guinea fowls fed separately on guinea corn and Livestock feed for a number of weeks was also attempted both spectrophotometrically and kinetically.

The spectrophotometry and kinetics results for blood serum showed the expected variation of phosphorous in the two sets of fowls based on the phosphorous content of the commercial feeds. Also there seems to be a somehow better agreement between the spectrophotometric and kinetics results for the blood serum than for the pumpkin species. The results for pumpkin species showed marked differences and possible reasons for discrepancies outlined in the body of the text (see chapter 5)

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Chapter 1

INTRODUCTION AND JUSTIFICATION OF RESEARCH PROJECT

Phosphorus is a vital element in the composition of all living matter. In the soft tissues of the body and in the cells of plants, phosphorus exists as phosphate groups in the free state and in organic combination. One of the principal functions of these phosphate groups is to provide a means for storing metabolite energy in the cells, the control and distribution of energy in the living cells being mediated by the energy-rich compounds of phosphorus of which the most indispensable is Adenosine triphosphate (ATP). These energy-rich compounds of phosphorus on hydrolysis yield a large amount of energy which then becomes utilisable for the mechanical work of muscular contraction. In addition, the compounds may be utilised by the cells to carry out specific physiological activities such as movement, growth, secretion etc. They play an essential part in coupling the production of energy from the fermentation or oxidation of food materials during cellular operation to the utilisation of that energy by the cell for its multifold activities.

In view of the biological importance of phosphorus as a source of metabolite energy, it was deemed a worthwhile exercise to determine the level of phosphate in some biological materials using the more

conventional spectrophotometric methods and the less common, rapid kinetics method. Analysis for phosphorus was carried out on pumpkin fruits of various species (including the seeds and the back) and in the blood-serum of adult guinea fowls fed separately on guinea-corn and the Pfizer Livestock feed respectively.

All chemical methods (alkalimetric, gravimetric, spectrophotometric) that have been proposed for the determination of the element phosphorus in organic materials have been based on the destruction of the organic matter by oxidation and the final conversion of phosphorus to orthophosphoric acid.

The early methods for the determination of phosphorus in organic compounds were based on the wet-combustion method of Carius [Carius (1865)] first published in 1865 which uses nitric acid in a sealed tube at elevated temperature. The use of the Carius method lasted for at least 60 years and had survived in modified form up to the present date.

The wet-oxidation process of Kjeldahl [Kjeldahl (1883)] was originally designed for the determination of nitrogen in organic compounds but on account of its simplicity compared with the Carius method, it found favour particularly in the field of food analysis as a general method for the destruction of organic matter. Gradually, it replaced the Carius method and in one form or another, has formed the basis of most of the wet-oxidation methods for the determination of phosphorus in organic substances. The early Kjeldahl

wet-combustion used sulphuric acid with potassium permanganate as oxidants. Later modification introduced various catalysts and the use of other acids, most particularly nitric acid. The introduction of perchloric acid, which became commercially available some 30 years ago, brought with it, the possibility of determining phosphorus on a microscale, as the final test solution no longer contained large amounts of sulphuric acid that could interfere with some of the methods used for small scale measurements of phosphorus. At the same time as wet-combustion procedures were developing, other methods were coming up into use based on dry ashing of the sample with or without the addition of alkali.

After the destruction of the organic substance using any of the methods described above, the resulting solution is then subjected to any of the chemical methods (alkalimetric, gravimetric and spectrophotometric) of analysing phosphorus. The spectrophotometric methods are however the most frequently used.

For the various species of pumpkin fruits and the commercial feeds, the standard spectrophotometric vanadophosphomolybdate method of the official Association of Analytical Chemists (Methods of Analysis - A.O.A.C. pages 11-13) was used. It involved the formation of pink complex (phosphomolybdovanadate) between inorganic phosphorus and molybdovanadate reagent which was monitored spectrophotometrically at a wavelength of 400nm. Mission [(Mission (1908))] first suggested the use of this reaction for the determination of phosphorus in steel

in 1908. The reaction was first applied to the determination of phosphorus in Biological materials by Koeing and Johnson (1942) who stated that the acidity should be 0.5N. Though not as sensitive as the molybdenum blue method (see subsequent pages); the vanadophosphomolybdate procedure is a very satisfactory process and is free from difficulties inherent in the molybdenum blue method. Whether this is wholly on account of the intrinsic stability of the vanadomolybdate complex or whether in part it is due to the fact that it is not too sensitive is a difficult matter to decide. Koeing and Johnson stated that the complex is very stable, showing an increase in absorption of 2% in two weeks. They found that if the reaction is carried out at an acidity less than 0.2N, an orange-yellow ^{complex} is formed that interferes with the measurement of absorption due to the vanadophosphomolybdate. Above 1.6M the colour is slow in developing. For accurate work, it is therefore necessary to work between the limits. The optimum acidity is considered to be 0.5N. Kitson and Melon stated that the reagents must be added in a specified order (see 2.2 for preparation of solutions).

For analyses in the blood serum of the guinea-fowls, Briggs method [Briggs (1922)] which is a modified form of the Bell-Doisy technique [Bell, R.D. and Doisy, E. (1920)] of analysing phosphorus in blood serum was used. The colorimetric phosphate method of Bell and Doisy, if followed carefully gives results which check gravimetric determinations but this method has one objection, namely, that the

alkaline blue colour which is used for comparison in the colorimeter fades rather rapidly. In the first stage of colour production, a stable green is produced in acid solution which is proportional to the phosphorous content. This colour was not used by Bell and Doisy because with either urine or trichloroacetic acid blood filterates there is an occasional turbidity produced when the acid molybdate is added which interferes with the colour determination due to a precipitate of undetermined nature. In attempting to overcome this difficulty it was found that by a small modification during the trichloroacetic acid preparation of blood serum, the turbidity can be avoided thus allowing the use of acid solutions for colour determination. When blood serum is diluted with 3 volumes of water and 1 volume of 20 percent trichloroacetic acid in a Erlenmeyer flask, shaken for about 10 minutes before filtering, the filterates give with acid molybdate and hydroquinone perfectly clear green colours. The supposition is that when the blood is diluted in a volumetric flask and mixed by merely inverting a few times a small amount of protein gets through into the filterates and this forms precipitate with the molybdic acid. The green colour is however considerably less intense and the determination of low serum phosphorus difficult but the stability of colour offsets this advantage.

In the modified technique introduced by Briggs, it was observed that when a little sodium-sulfite is added to an acid solution

containing phosphate and molybdate that the subsequent addition of hydroquinone causes the formation of a blue instead of green colour and intensity considerably greater than that of green. This colour does not depend on the reduction of the molybdic acid by sulphur-dioxide since sodium sulfite, hydroquinone and acid molybdate solutions when mixed give no colour. The use of these modifications give a clear, blue, non-fading colour the proportionality of which is exact over a wide range. The intensity of the colour allows the determination of phosphate in 0.3 ml_g of serum (as used in this project).

From the various analyses carried out on the pumpkin species and blood serum of guinea fowls using the above colorimetric methods, a number of useful indications became apparent; although further experimentation would be needed to really establish the authenticity of some of the assertions made in the different areas. The short time at the disposal of the project posed a serious handicap in the defining of conditions for the experiments.

Other spectrophotometric methods of analysing phosphate in materials have been reported. The molybdenum blue method which is based on the formation of 12-molybdophosphoric acid from phosphate and molybdate in acid solution and subsequent reduction to blue heteropoly compound [Lindberg, O. and Ernster, L. (1956)]. The method suffers from susceptibility to interferences, instability of the blue product and long measurement times. These problems are especially

significant in biological materials where hydrolysis of phosphate esters, catalysed by molybdate, can occur over the long analysis periods. In addition, turbidity of deprotenised samples can lead to large errors in spectrophotometric results.

The colorimetric method of Holman and Pollard [W. M. Holman and A.G. Pollard (1937)] which is a modification of Denige's method is also adaptable to the determination of phosphate. The blue colours in this technique are produced by the reduction of phosphomolybdic acid with freshly-prepared stannous - chloride solution. It is probably the most sensitive method free from interference by other substances. The colour intensity however depends on the amount of added reagents, temperature and time of reaction. Besides, free mineral acids and alkalis depress the colour. Organic acids such as citric acid, oxalic acid and tartaric acid inhibit the development of the blue colour and substantial amounts of organic matter also interfere. Ferric iron must not be present in the final solution in greater proportion than 1 ppm. hence it should be reduced to the ferrous state, for which purpose a reductor is recommended, Arsenate interferes and must be reduced to arsenite by means of hydrogen sulphide in acid solution. Finally the blue colour in the test is produced by orthophosphates; meta- and pyrophosphates must therefore be completely hydrolysed.

In another spectrophotometric technique for determining phosphate which is practically insensitive to silica and which is a modification of the method advocated by Ernst and Emilio Tschopp [Ernst and Emilio Tschopp (1932)] the colour production is due to the reduction of phosphomolybdic acid with p-methyl-amino-phenol sulphate (metol). Here it is essential that the solutions to be examined for phosphate should be neutral since uniform colour production depends on the final concentration of free sulphuric acid in the solution. Arsenites react with phosphates to give a similar colour and should be removed by precipitation with hydrogen sulphide followed by boiling and filtration. Sugars, lactates, citrates, tartrates, oxalates and other organic salts depress the intensity of the colour produced by phosphates and these compounds if present should be removed.

From the foregoing, it can clearly be seen that the colorimetric method of analysing phosphate have a number of shortcomings which may be summarised thus viz (i) they are highly susceptible to external interferences (the two most troublesome being arsenate and silicate) (ii) they involve long measurement times (iii) instability of the colour used for determination is observed in some cases (iv) turbidity of the sample solution in some cases leads to large errors in the colorimetric results.

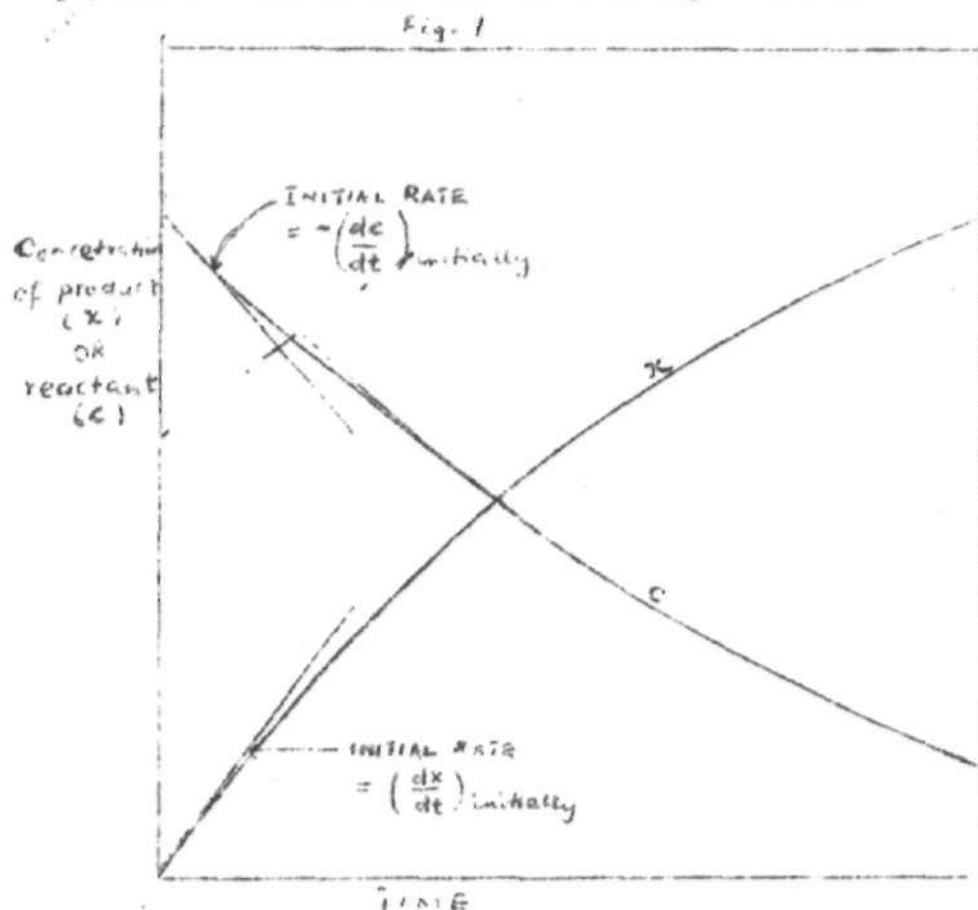
In view of the facts clearly stated above, it would undoubtedly be a laudable adventure and worthwhile contribution to knowledge if

a method can be sought which remedies some (if not all) of these shortcomings that plague the colorimetric methods of phosphate determination.

In this research project, efforts have been directed towards applying kinetics to the determination of phosphate in some biological materials (Pumpkin of various species and blood serum of guinea fowls). This, then ultimately extended the aim of the project to that of comparing the colorimetric and kinetic methods of analysing phosphate.

The subject of chemical kinetics is concerned with the rates of chemical reactions and with the factors upon which the rates depend. The most important of these factors are concentration (activity), temperature and hydrostatic pressure. Because the rate of a chemical reaction is dependent on the concentration of the compounds (or in particular cases, the concentration of the catalyst), it may be used for analytical purposes. The quantitative analytical methods which have as their basis the measurement of reaction rate are more usually referred to as "kinetic methods of analysis". Reactions can either be fast, very fast or slow. The method adopted in studying the kinetics of a particular reaction depends on the rate at which it occurs. Slow reactions (having half-lives of half an hour or more) are usually studied by using the conventional methods of causing the reaction to occur in a reaction vessel and analysing at constant temperature for the concentration of one of the reactant or products at various suitable times during the progress of the reaction using chemical methods.

In this way the extent of the reaction at various times can be determined and concentration measured as a function of time. A plot of the concentration of either reactant or product at various times against time gives the rate curve from which the rate of the reaction at any time can be determined by drawing a line tangential to the curve at that time and determining the slope. The rate determined in this way at the very beginning of the reaction is called the initial rate of the reaction. The shapes of the rate curves when a reaction is followed by analysing for the concentration of either reactant or product at various time are shown in fig. 1 below.



The rate curves show a decrease in the concentration of the reactant with time since it is being used up in the reaction and an increase in the concentration of the product with time since it is being formed continuously as the reaction proceeds.

Some reactions, are however so fast that special techniques have to be employed. Such techniques are of two main types. Those of the first type employ essentially the same principles as used for slow reactions, but the methods are modified so as to be suitable for more rapid reactions. Those of the second type are of a different character and utilise special principles. The main reasons why conventional techniques lead to difficulties for very rapid reactions are: (i) the usual time that is taken to mix reactants or to bring them to a specified temperature may be significant in comparison with the half-life of the reaction (ii) the time that it takes to make a measurement of concentration is significant compared with the half-life. The first difficulty can be surmounted by using special techniques for bringing the reactants very rapidly into the reaction vessel and for mixing them very rapidly. Normally using conventional techniques, it takes from several seconds to a minute to bring a mixture of gases, liquids or solutions into a reaction vessel and to have them completely mixed. This time can be reduced markedly by using a rapid flow and flow techniques are therefore frequently used for rapid reactions. One particular modification of these methods is the 'stopped - flow techniques'. The second difficulty referred to above ~~may~~ be resolved

by employing techniques which allow properties to be determined instantaneously. For reactions in solution, spectrophotometric methods are commonly employed. If the products of the reaction absorb differently from the reactants at a particular wavelength it is possible to pass monochromatic light of this wavelength through the reaction vessel; using photoelectric device with suitable electronic circuits the output can be led to a recorder or oscilloscope.

Since the basis of the kinetics approach to the determination of phosphate in this research project is the very fast reaction between phosphate and MO(VI) in acid solution, then rapid kinetics applies. At low acid concentration and excess MO(IV) the initial rate of formation of 12-molybdophosphoric acid is directly proportional to the phosphate concentration. The basic considerations in the choice of these experimental parameters result from the mechanistic investigation of the rate of formation of 12-MPA from phosphate and MO(IV) in acid medium. In their application of the method to the determination of phosphate in blood serum, Javier, Crouch and Malmstadt of the University of Illinois [Javier, Crouch and Malmstadt (1969)] utilised a completely automated stopped-flow spectrophotometer to measure the initial rate of formation of 12-molybdophosphoric acid from phosphate and MO(IV) in acid medium. This rapid reaction yields digital rate data proportional to the phosphate concentration about 100 milliseconds after the reactants have been automatically mixed. The stopped-flow system used for this research project, however, had no digital

read-out system incorporated with it, hence the tedious method of having to determine initial rate from measurement of the slope ($\frac{\Delta A}{\Delta t}$) of the linear traces displayed on the oscilloscope was adopted. Besides, as a result of the non-automatic nature of the Durrum - Gibson model of the stopped flow system used, the introduction as well as the mixing of the reactant solutions had to be done manually. In determining the initial rate of formation of 12-MPA (12-molybdophosphoric acid) from the slope of the linear traces, the method of 'close approximation' was used which would inevitably check on the accuracy of the results.

Fig. 1-A shows a block diagram of the stopped-flow system used for the project. Figs. 1-B and 1-C show respectively oscilloscope traces for complete reaction and at the very beginning of the reaction, the slope of which is proportional to the initial rate of the formation of 12-MPA.

The kinetics method, has several potential advantages over the usual conventional methods of analysing phosphate. The selectivity of the kinetics method is often superior, because the analyst can take advantage of the different rates of reaction of different members of a multi-component solution. Besides, the technique of rapid kinetics extends the time domain of kinetics investigations down to milliseconds or even microseconds. Hence quantitative initial rate measurements can be obtained in a matter of seconds leading to shorter

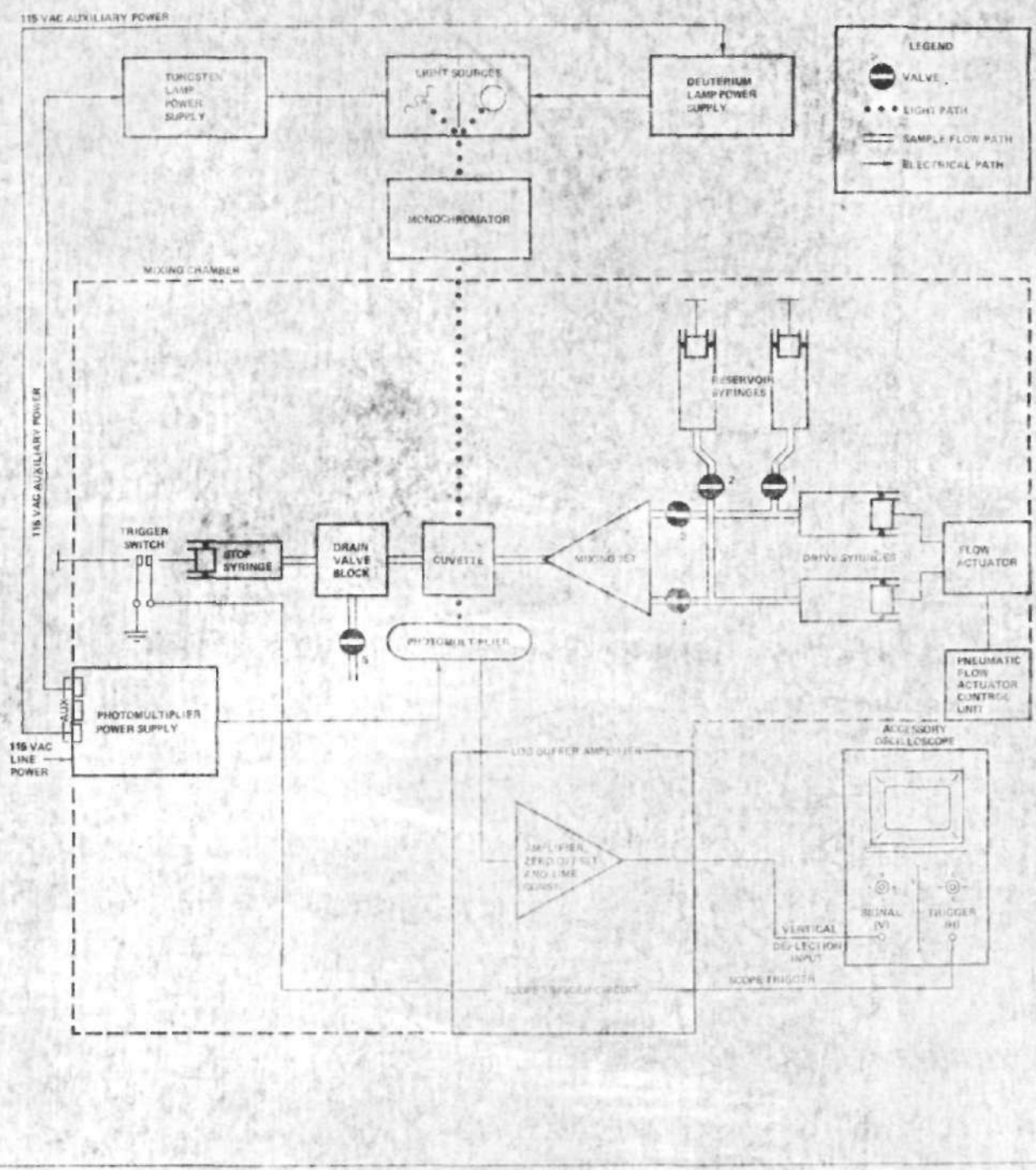
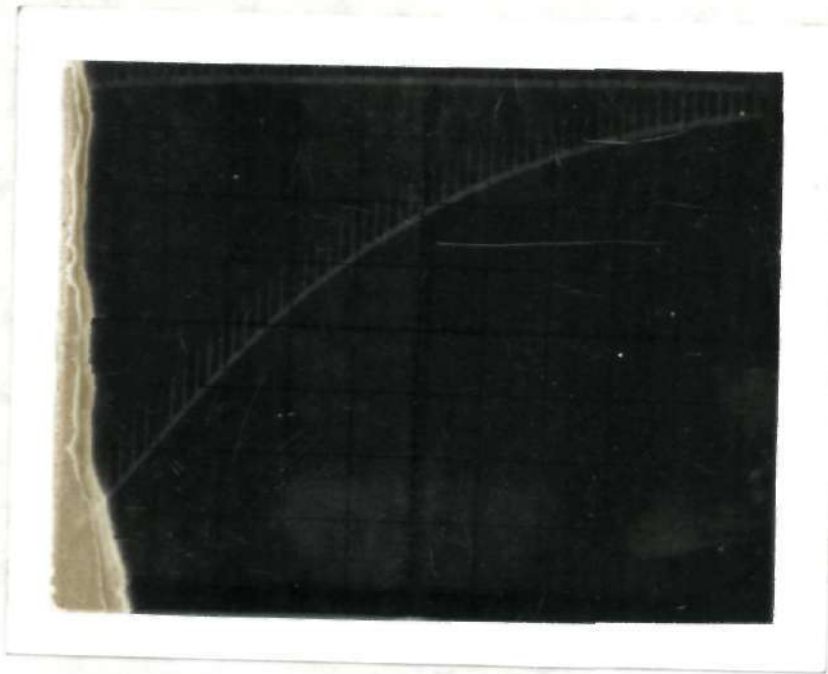


Figure 1-A Functional Block Diagram of Stopped-Flow Spectrophotometer System.

Fig. 1-B



Time base = 1 second

Fig. 1-C



Time base = 0.1 sec.

analysis times. Initial rate measurements can be made very quickly after the reaction is started before slowly interfering reactions have begun thus eliminating interferences due to slowly reacting species. One of the most important characteristics of the reaction rate technique is that, it involves relative measurements. The absolute value of the parameters (absorbance, cell-potential etc.) chosen to monitor the reaction does not need to be accurately known. It is only necessary to measure the rate of change of the parameter with time accurately. Hence even for very rapid reactions, the reaction rate method can offer freedom from interferences which contribute to the absolute value of the parameter (turbidity, dirty cells, junction potential) but which do not enter the reaction and do not contribute to the rate of change of the parameter.

Chapter 2

SPECTROPHOTOMETRIC (COLORIMETRIC) PHOSPHOROUS ANALYSIS IN PUMPKIN SPECIES

Five different species of pumpkin were analysed for the phosphorous content in the seeds, the back and the pulpy part to see the trend of variations of phosphorous in the different parts and for comparison with the results of kinetic analysis. For a given species of pumpkin, the sampling procedure consisted of peeling parts from three different segments of the pumpkin material. Three independent determinations were then attempted on the peeled parts and the results showed no significant variation in the phosphorous content for the different segments. Two different determinations were also carried out on the seeds of a particular species.

The standard spectrophotometric (colorimetric) vanadophosphomolybdate method of the official Association of Analytical Chemists was used for analysis. The method involved the formation of a pink complex (phosphomolybdovanadate complex) between inorganic phosphorous and molybdovanadate reagent which was then monitored spectrophotometrically (colorimetrically) at a wavelength of 400nm. Method was found to be most suitable for macrodetermination of phosphorous and

would not be applicable to materials yielding coloured solutions or solutions containing ions other than orthophosphate which form coloured complexes with molybdovanadate.

Instrument: Bausch and Lomb spectronic -- 20 colorimeter was used for all colour measurements.

Reagents: All reagents (Chemicals) used for the preparation of solutions were ^{AR} products of the BDH Chemicals Limited, England.

2.1 Method:

Appropriate parts of the different species of pumpkin were peeled with the aid of a sharp knife (or removed with hand in the case of seeds) and dried in the oven at a temperature of between 80° - 90°C for about 24 hours. This was properly ^uground to obtain a fine powdery mass and 1.0 gm of the dried, ^uground part, in each case, was weighed into an ignition crucible using the Mettler 8H balance and ashed in the muffle furnace at 600°C until carbon-free to get rid of the organic part. The resulting ash containing inorganic phosphorus as P₂O₅ was cooled in a dessicator and washed into 250 ml beaker with 13 mls of 1:3 hydrochloric acid added in small portions. About 3-5 drops of concentrated nitric acid were added and the solution heated to boiling (to completely decompose all organic matter) cooled and transferred into a 250 ml volumetric flask and diluted to volume with distilled water. Resulting solution was partly filtered into a boiling tube and 10 mls pipetted into a 100 ml volumetric flask

followed by the addition of 10 mls of molybdovanadate reagent and finally diluted to volume. After mixing well, the solution was allowed to stand for 10 minutes for maximum colour development and the absorbance/transmittance determined against a blank (10 mls of molybdovanadate reagent diluted to volume in a 100 ml volumetric flask) at a wavelength of 400nm.

2.2 Preparation of Solutions:

Molybdovanadate reagent: This was prepared by weighing out 40 gms. of ammonium - molybdate, dissolved in 400 mls of water and cooled.

2.0 gms. of ammonium metavanadate was dissolved in 250 mls of water (hot), cooled and 313 mls of 60% perchloric acid added.

The molybdate solution was gradually added to the metavanadate solution with stirring and diluted to 2 L.

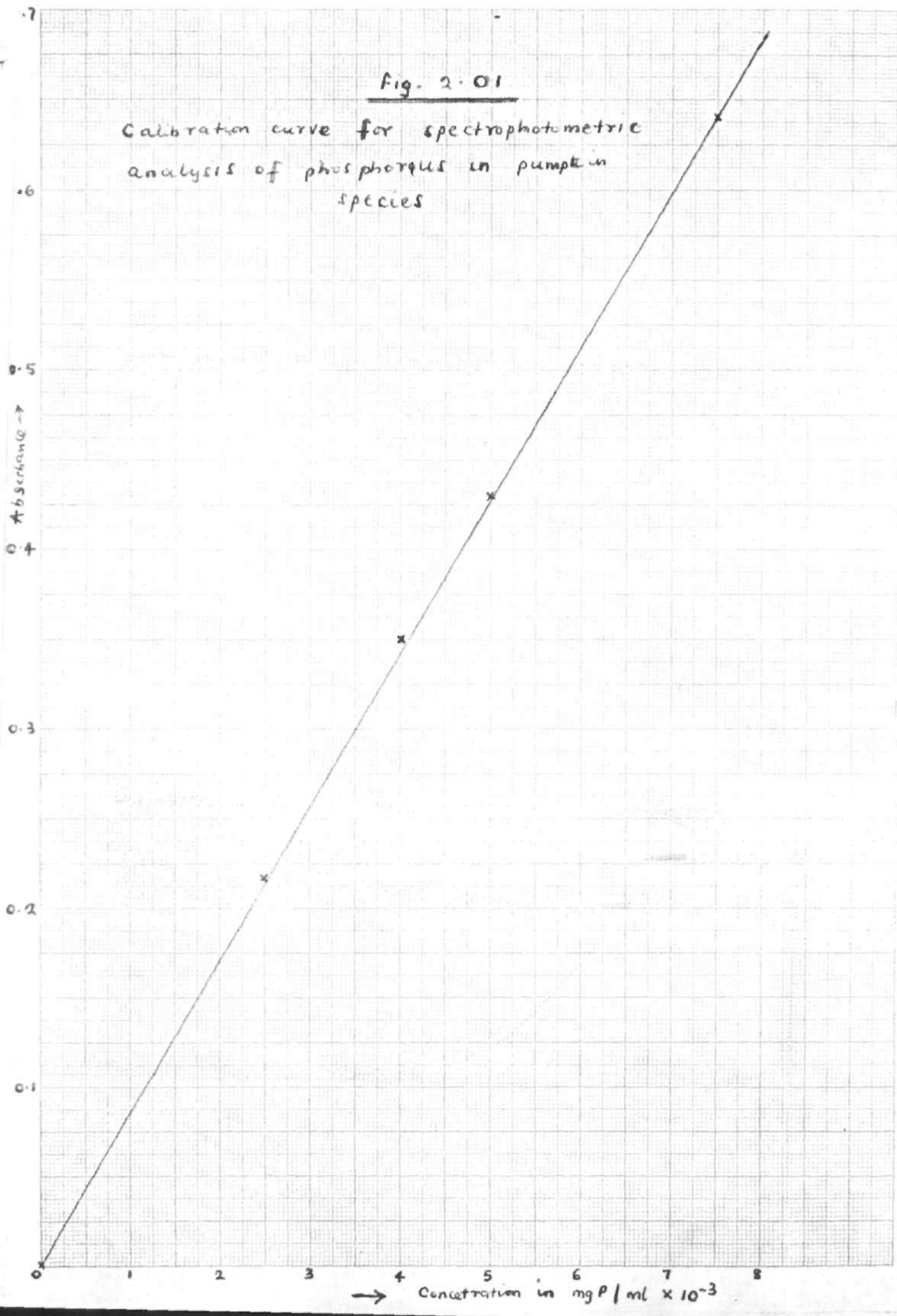
Phosphorous working standard:

2 mg P/ml stock solution was prepared by accurately weighing out 8.788 gms of potassium dihydrogen ortho-phosphate (previously dried for 2 hours at a temperature of 105°C) dissolved in water and diluted to one litre in a standard volumetric flask.

Working standard solution of concentration 0.1 mg/ml was prepared from the stock solution by diluting 50 mls of the stock solution to one litre in a volumetric flask.

Fig. 2.01

Calibration curve for spectrophotometric analysis of phosphorus in pumpkin species



Instrumental results (i.e. absorbance values) for various parts of the five different species of pumpkin are shown in tables II-VI:

Table II: Pumpkin A (Curcubita moschata)

Part of Pumpkin	Absorbance			Corresponding % Transm.		
	1st determ.	2nd determ.	3rd determ.	1st determ.	2nd determ.	3rd determ.
Pulpy part	0.29	0.280	0.280	51.0	53.0	53.0
	0.29	0.285	0.285	51.0	53.3	53.2
	0.28	0.290	0.280	50.0	51.0	53.0
Back	0.345	0.35	0.345	46.0	45.0	46.0
	0.350	0.35	0.350	45.0	45.0	45.0
	0.345	0.35	0.350	46.0	45.0	45.0
Seeds	0.39	0.39	0.40	41.0	41.0	40.0
	0.40	0.40	0.39	40.0	40.0	41.0
	0.395	0.40	0.39	40.5	40.0	41.0

Table III: Pumpkin B (Curcubita melopepo)

Part of Pumpkin	Absorbance			Correxponding % Transm.		
	1st determ.	2nd determ.	3rd determ.	1st determ.	2nd determ.	3rd determ.
Pulpy part	0.152	0.13	0.13	70.2	73.0	72.5
	0.152	0.14	0.14	70.2	72.0	72.0
	0.155	0.14	0.13	69.5	72.0	73.0
Back	0.24	0.250	0.250	57.0	56.0	56.0
	0.25	0.248	0.245	56.0	56.2	56.5
	0.25	0.248	0.245	56.0	56.2	56.5
Seeds	0.35	0.35	0.34	44.0	44.0	45.0
	0.34	0.35	0.35	45.0	44.0	44.0
	0.34	0.35	0.35	45.0	44.0	44.0

Table IV: Pumpkin C (Curcubita maxima)

Part of Pumpkin	Absorbance			Corresponding % Transm.		
	1st determ.	2nd determ.	3rd determ.	1st determ.	2nd determ.	3rd determ.
Pulpy part	0.10	0.90	0.90	79.0	81.5	81.5
	0.95	0.95	0.95	79.8	79.5	79.5
	0.97	0.95	0.95	79.5	79.5	79.5
Bark	0.17	0.130	0.130	67.0	73.0	73.0
	0.16	0.125	0.125	68.0	73.5	73.5
	0.16	0.125	0.130	68.0	73.5	73.0
Seeds	0.23	0.23	0.22	58.0	58.0	59.0
	0.22	0.22	0.22	59.0	59.0	59.0
	0.22	0.22	0.22	59.0	59.0	59.0

Table V: Pumpkin D (Curcubita pepo)

Part of Pumpkin	Absorbance			Corresponding % Transm.		
	1st determ.	2nd determ.	3rd determ.	1st determ.	2nd determ.	3rd determ.
Pulpy part	0.155	0.155	0.150	69.0	69.0	70.0
	0.150	0.155	0.145	69.0	69.0	70.9
	0.155	0.150	0.150	70.0	70.0	70.0
Bark	0.30	0.31	0.30	50.0	50.1	50.0
	0.30	0.30	0.31	50.1	50.0	50.1
	0.31	0.32	0.30	50.0	50.0	50.0

Table VI: Pumpkin E (Benin case *cevifera* var *ovifera*)

Part of Pumpkin	Absorbance			Corresponding % Transm.		
	1st determ.*	2nd determ.	3rd determ.	1st determ.	2nd determ.	3rd determ.
Pulpy parts	0.10	0.10	0.10	79.0	79.0	79.0
	0.10	0.11	0.11	79.0	78.5	78.5
	0.10	0.11	0.10	79.0	79.0	79.0
Back	0.14	0.14	0.15	72.5	72.5	71.0
	0.14	0.14	0.15	72.5	72.5	71.0
	0.15	0.15	0.15	71.0	71.0	72.5
Seeds	0.21	0.210	0.205	62.0	62.0	62.5
	0.21	0.205	0.210	62.0	62.5	62.0
	0.21	0.210	0.205	62.0	62.0	62.5

*determ = determination; a determination consists of 3 trials.

Concentrations in, mg P/ml, corresponding to the different absorbance values for different parts of the various pumpkin species were read on the Calibration curve and Calculations made of the phosphorus content in mg/100 gm of the dried part.

Table VII shows phosphorus content in mg/100 gm of dried part for different parts of five species of pumpkin. Results labelled 1, 2, 3 correspond to three different segments of a particular species of pumpkin.

Table VII

Species of Pumpkin	Phosphorus content in mg/100 gm. of dried part			Mean phosphorus in mg/100 gm.
	1	2	3	
<u>Pulpy parts</u>				
A	837.5	812.5	825.0	825.0
B	450.0	445.0	450.0	447.5
C	287.5	270.0	268.8	275.4
D	450.0	437.5	437.5	443.7
E	287.5	338.7	287.5	313.0
<u>Back</u>				
A	1000.0	1012.5	1012.5	1006.0
B	725.0	700.0	725.0	712.5
C	493.8	462.5	493.8	478.2
D	875.0	900.0	875.0	887.5
E	406.3	437.5	437.5	421.9
<u>Seeds</u>				
A	1162.5	1131.3	-	1146.9
B	1137.5	987.5	-	1062.5
C	662.5	637.5	-	650.0
E	612.5	593.8	-	603.1

No analysis on the seeds of species D of pumpkin because at the time of the experiment, none was available.

2.4 Discussion on the Phosphorus content of the different parts of the various Species of Pumpkin:

Table VII shows no significant variation in the distribution of phosphorus in the bulk of a given species of pumpkin. This is evident from the almost constant, not too different results, obtained for parts removed from three different segments of a species.

The table also shows a clearly distinct variation in the phosphorus content of the pulpy parts of the different species of pumpkin; being maximum in species A (*Curcubita moschata*) and least in species C (*Curcubita maxima*). It must be emphasized that these statements are only indicative and not conclusive in themselves. Ideally, one would have needed to carry out analyses on many pumpkin fruits of a particular species and compare results with those obtained on fruits of other pumpkin species to arrive at a justifiable and more definite conclusion. However, time element was a limiting factor.

Apart from serving as a source of metabolite energy in the cells, phosphorus is not all that indispensable food value-wise when compared with other nutrient elements like calcium, magnesium etc.; hence it may be impossible to relate the phosphorus content of a particular species of pumpkin to its degree of utility as a food substance. However analysis for the pythin (calcium magnesium salt of inositolhexaphosphoric acid) content of the various species, if present, would indicate which species is the most indispensable

medicinally. It has long been recognised that a large proportion of the total phosphorus in cereals, vegetable products and some fruits (those containing a large number of edible seeds e.g. Figs) may be present in the form of pythin. This compound which is insoluble is commercially manufacturable and is widely recommended as tonic [Ihm (1929), Paulson (1929)].

Finally, table VII shows the presence of phosphorus maximally in seeds of a particular species of pumpkin and least in the pulpy parts. One may in the light of this indication discourage the practice of throwing away seeds of this material which may probably turn out to be useful if, for example, analysed for the pythin content. Further research into this area by food experts would perhaps resolve the issue.

3.1 SPECTROPHOTOMETRIC (COLORIMETRIC) ANALYSIS OF
PHOSPHORUS IN THE COMMERCIAL FEEDS AND
BLOOD - SERUM OF ADULT GUINEA-FOWLS

The A. O. A. C. spectrophotometric vanadophosphomolybdate method was again used to analyse for phosphorus in the feeds (i.e. Guinea corn and Pfizer Livestock feed) of the adult guinea-fowls. The sampling of the feeds to obtain a representative sample on which three different determinations were attempted was done according to the 'Coning and Quaterly' method [Maxwell, J. A., Rock and mineral analysis page 51.]

Instrument: The Pye Unicam SP.600 Spectrophotometer was used for all colour measurements.

Reagents: All reagents (Chemicals) were also of the analar grade and all solutions were prepared in the same way as for analyses on the pumpkin species.

3.2 Method:

3.0 gms. each of the livestock feed and Guinea corn was weighed out in an ignition crucible and muffled in the furnace at a temperature of 600°C until carbon-free. The resulting residue was picked up in acid solutions and raised to boiling to decompose all organic matter (see analysis of pumpkin species). 20 mls of the molybdovanadate reagent was however used to complex with phosphorus in 10 mls of the

the acid solution in a 100 ml volumetric flask and made up to the mark. The coloured solution was mixed well allowed to stand for 10 minutes and the absorbance/transmittance determined against a blank at a wavelength of 400nm. Three determinations were carried out on the representative sample of a given feed.

3.3 Preparation of Solutions:

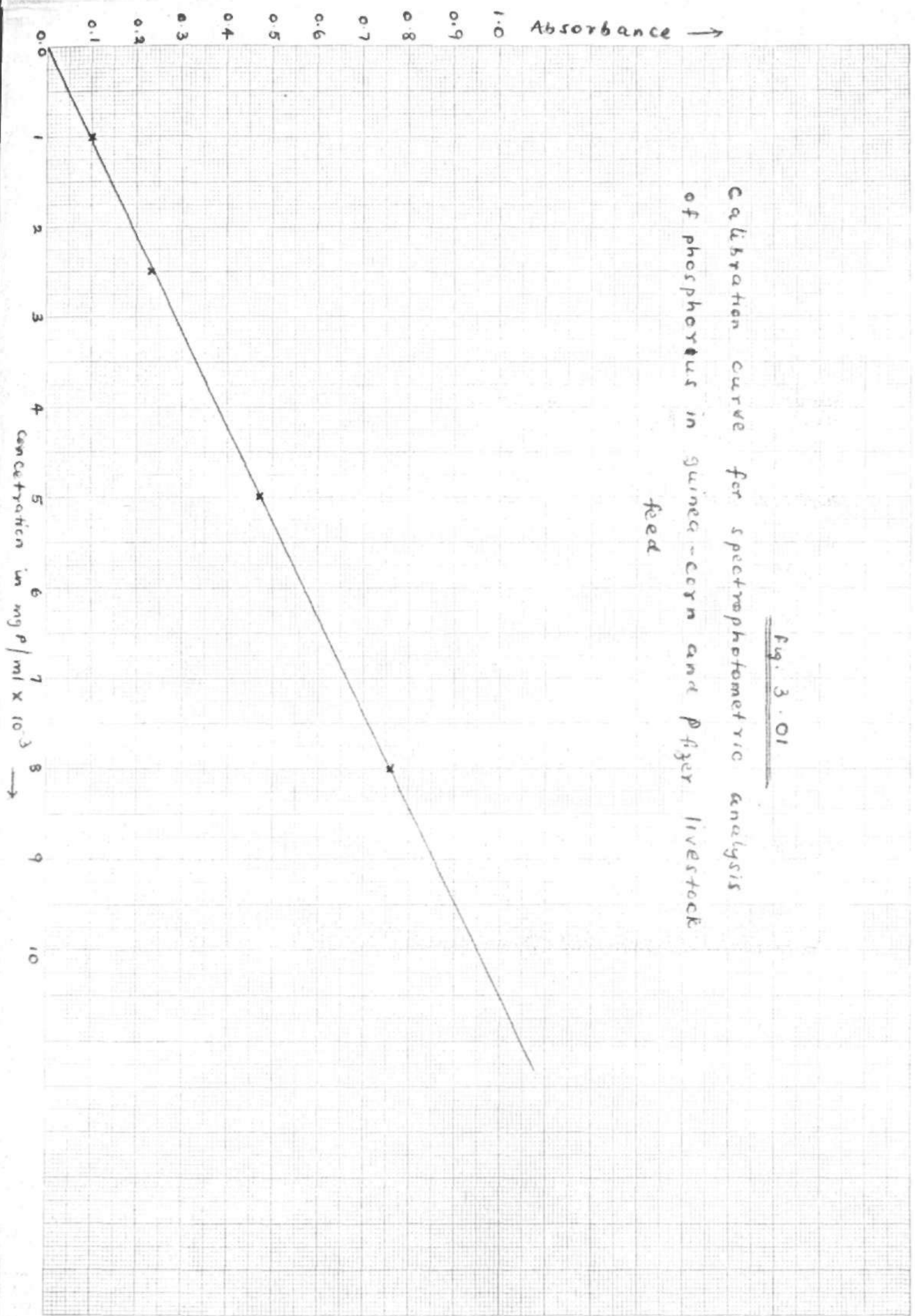
All solution preparations were done in the same way as for analyses on the various pumpkin species.

3.4 Preparation of Calibration Curve:

A new calibration curve was prepared by pipetting 1.0, 2.5, 5.0, 8.0 mls of the phosphorus working standard (0.1 mg/ml) into separate 100 ml volumetric flask. Each was treated in turn with 20 mls of the molybdovanadate reagent and made up to the mark with distilled water. Each solution was mixed well, allowed to stand for 10 minutes and the colour determined against a blank using the Unicam SP.600 spectrophotometer.

Calibration curve was prepared by making a plot of mg P of the standard solutions against the corresponding absorbance/transmittance value (see fig. 3.01).

Instrumental results for calibration are shown in table VIII.



Calibration curve for spectrophotometric analysis of phosphorus in guinea-corn and Piger livestock feed

Fig. 3.01

Table VIII

Concentration of phosphorus in mg/ml x 10 ⁻³	Absorbance	Corresponding % Transmittance
1.0	0.100	79.5
2.5	0.235	58.0
5.0	0.475	33.5
8.0	0.760	17.4

Instrumental results (i.e. absorbance/transmittance values) for the two feeds are shown in table IX below.

Table IX

Type of feed	Absorbance			Corresponding % Transmittance		
	1st determ.*	2nd determ.	3rd determ.	1st determ.	2nd determ.	3rd determ.
Guinea corn	0.220	0.220	0.215	60.2	60.2	61.0
	0.215	0.210	0.220	61.0	61.8	60.2
	0.225	0.225	0.225	59.5	59.5	59.5
Livestock feed	0.485	0.480	0.485	32.8	33.0	32.8
	0.480	0.480	0.480	33.0	33.0	33.0
	0.490	0.485	0.480	32.2	32.8	33.0

*determ. = determination; a determination consists of 3 trials.

On addition of 20 mls of molybdovanadate reagent to 10 mls of the acid solution of the residue (resulting from muffling in the furnace) of the Livestock feed in a 100 ml flask and determining spectrophotometrically after making up to the mark, a high value of absorbance (0.99) was obtained. Since such a high value is usually not within the range of validity of Beer-Lambert law, a two-fold dilution (25.0 mls diluted to 50 mls) of the original coloured solution was done and the values of absorbance/% Transmittance reported in table IX are for the diluted solution. The dilution is however taken care of, in the calculation for phosphorus content in the feed.

Concentrations, in mg P/ml, corresponding to the various absorbance values for a given feed were read on the calibration curve and calculations made of the mean phosphorus content in mg/100 gms. of particular feed.

Table X below shows the phosphorus content in mg/100 gms. of feed for the two feeds. Results labelled 1, 2, 3 correspond to absorbance values for the same feed.

Table X

Type of feed	Phosphorus content in mg/100 gms of feed			Mean value in mg/100 gm. of feed
	1	2	3	
Guinea corn	191.67	187.50	195.83	191.67
Livestock feed	841.3	833.3	850.0	841.53

3.1.1 Spectrophotometric Phosphorus Analysis on the Blood-Serum of Adult Guinea Fowls:

As usual analysis was attempted on the blood serum of two sets (A, B, C, D); (X, Y, Z, J) of guinea fowls fed on the Pfizer Livestock feed and Guinea corn respectively for a number of weeks to see the trend of variation of phosphorus in the ^{serum of the} two sets and compare the results of the colorimetric and the kinetic methods (Chapter 4) of analyses.

The Briggs method (see introduction) which is a modified form of the Bell-Doisy colorimetric method was used.

Instrument: Pye Unicam SP.600 spectrophotometer was used for all colour measurements.

Reagents: All reagents used were of the analar grade and were products of the BDH Chemicals Limited, England.

3.1.2 Method:

The blood serum of each guinea fowl was prepared by withdrawing about 4 mls of whole blood from a vein located around the neck using a sharply pointed disposable pyrex syringe (after sterilisation using alcohol-soaked cotton wool) into a medium sized centrifuge tube and allowed to clot when a clear, yellow supernatant liquid separated.

0.3 ml of the blood serum in each case was diluted with 0.9 ml of distilled water and 0.3 mls of 20% trichloroacetic acid (to

deprotenise) added in a small erlenmeyer flask. The flask was stoppered and shaken vigorously for a few seconds and after standing for about 10 minutes, the contents were transferred to a dry ashless filter-paper to obtain a clear, colourless filtrate containing the inorganic phosphorus.

1.0 ml of the trichloroacetic acid filtrate was treated in turn with 1.0 ml of acid molybdate solution, 0.5 mls of sodium-sulfite solution, 0.5 mls of hydroquinone solution in a 5 ml volumetric flask and diluted to the mark with distilled water. This was allowed to stand for about 30 minutes for colour production and colour measurements carried out at a wavelength of 620nm using the Unicam SP.600 spectrophotometer.

The maximum wavelength of absorption of the blue compound was not indicated in the literature and this was found to be about 620nm by manual operation of the SP.1800 spectrophotometer and Unicam SP.500 spectrophotometer.

3.1.3 Preparation of Solutions:

Phosphorus working standard: A stock solution of phosphorus of concentration 0.1 mg/ml was prepared by weighing 0.4394 gm. of dry KH_2PO_4 and dissolved in 1 litre of distilled water.

The phosphorus working standard (0.0125 mg/ml) was prepared from the stock solution by pipetting 25 mls of the stock and diluting to 200 mls in a standard volumetric flask. All solutions were

preserved with chloroform.

Ammonium - molybdate solution: 25 gms. of ammonium - molybdate were dissolved in 300 mls of water. To this were added 200 mls of water containing 75 mls of concentrated sulphuric acid. The molybdate reagent was stored in polyethylene bottle to prevent leaching of silicon from the volumetric glassware.

Hydroquinone solution: 0.5 gm. of hydroquinone was dissolved in 100 mls of water and a drop of concentrated sulphuric acid was added to retard oxidation.

Sulfite solution: The solution contained 20% (w/v) of sodium-sulfite i.e. 20 gms. of sulfite dissolved in 100 mls of water. The solution was kept well stoppered.

3.1.4 Preparation of Calibration Curves:

1.0, 2.0, 3.0, 4.0 and 5.0 mls of the phosphorus working standard were pipetted into separate 10-ml volumetric flasks and treated in turn with 1.0 ml of the acid molybdate solution, 0.5 ml of sodium-sulfite solution, 0.5 ml of hydroquinone solution and diluted to the mark with distilled water. They were allowed to stand for 30 minutes for maximum colour production and colour measurements done at 620nm using the Unicam SP.600 spectrophotometer. The calibration curve was prepared by making a plot of mg P of the standard solution (diluted) against the corresponding absorbance value (see fig. 3.02).

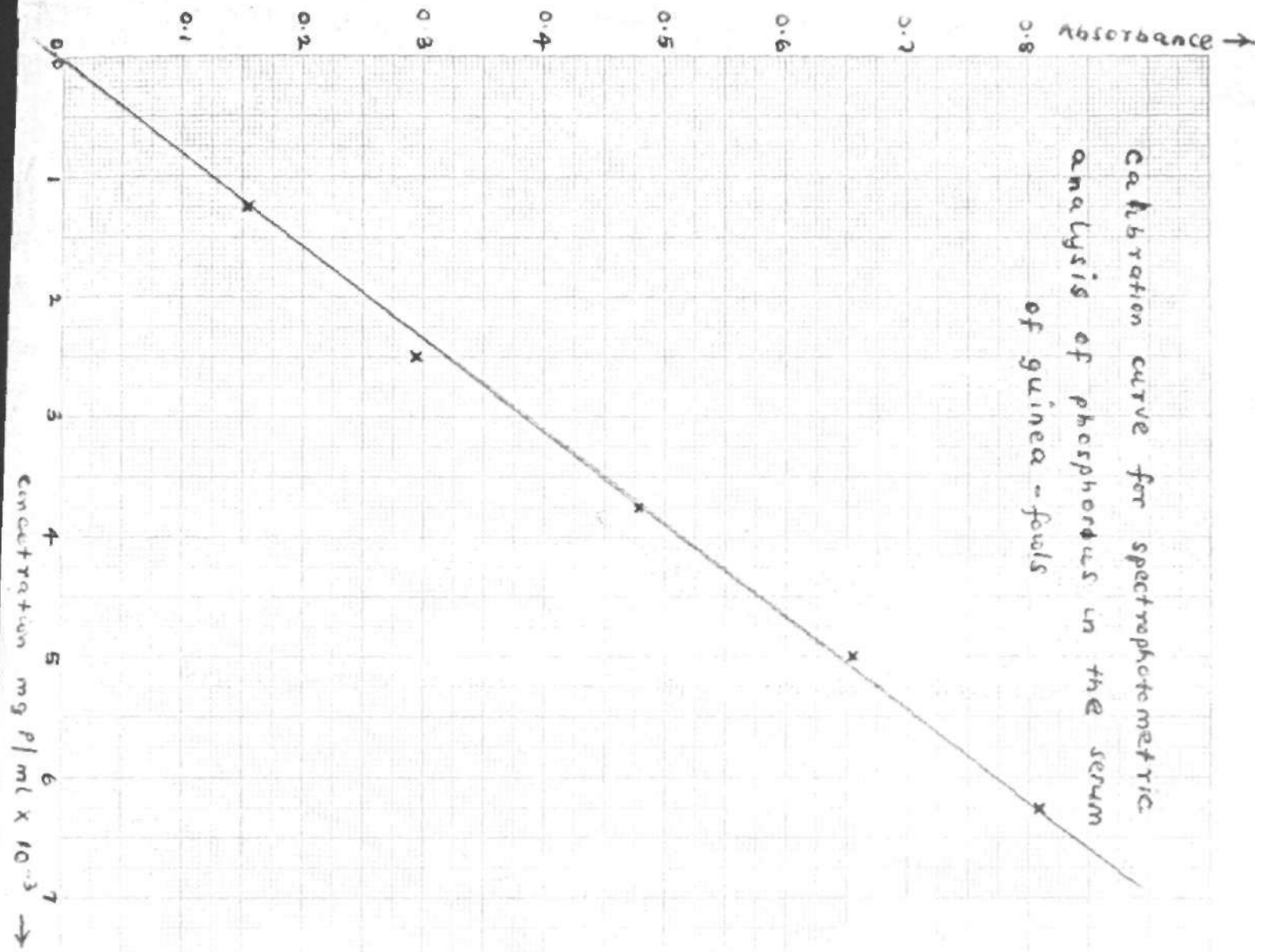


Fig. 3.02

Instrumental results for calibration are shown in table XI below.

Table XI

Concentration of phosphorus in mg/ml $\times 10^{-3}$	Absorbance	Corresponding % Transmittance
1.25	0.155	70.0
2.50	0.297	50.8
3.75	0.480	33.0
5.00	0.655	22.0
6.25	0.810	15.5

Instrumental results for the blood serum of the eight adult guinea-fowls

A, B, C, D, X, Y, Z, J are shown in table XII below.

Table XII

Blood serum of Guinea Fowl	Absorbance		Corresponding % Transm.	
	1st determ.	2nd determ.	1st determ.	2nd determ.
A	0.425	0.445	37.5	36.0
	0.430	0.410	37.0	43.0
	0.448	0.368	35.7	43.5
B	0.465	0.370	34.2	42.6
	0.470	0.345	33.8	45.0
	0.355	0.350	44.0	44.2
C	0.305	0.380	49.5	41.6
	0.304	0.385	49.6	41.2
	0.300	0.385	50.0	41.0
D	0.430	0.435	37.1	36.6
	0.407	0.440	39.0	36.2
	0.415	0.410	38.5	38.8
X	0.278	0.285	52.6	51.8
	0.234	0.275	58.2	53.0
	0.238	0.235	52.8	58.0
Y	0.186	0.190	65.1	64.5
	0.195	0.176	63.8	66.6
	0.190	0.180	64.5	66.0
Z	0.342	0.340	45.6	45.7
	0.340	0.350	45.7	44.6
	0.340	0.310	45.7	49.0
J	0.220	0.230	60.2	58.8
	0.235	0.240	58.2	57.3
	0.240	0.238	57.5	57.8

Concentrations, in mgP/ml, corresponding to various absorbance values for the blood serum of a particular guinea fowl were read on the calibration curve and calculations made of the mean phosphorus content in 100 mls of each blood serum.

Table XIII shows mean values of the phosphorus content in mg P/100 mls of serum for serum of the eight adult guinea fowls.

Table XIII

Blood serum of Guinea fowl	Mean phosphorus content in mg P/100 mls of serum
A	5.20
B	5.37
C	4.45
D	4.95
X	3.67
Y	2.46
Z	4.41
J	3.51

3.1.5 Discussion on the Phosphorus content of the Serum of the Guinea Fowls:

The results in table XIII above seemed to have indicated the presence of a slightly higher amount of phosphorus in the blood serum of Guinea fowls fed on the Livestock feeds than in those of their counterparts fed on guinea corn. This may be explained in terms of

the higher phosphorus content in the livestock feed than in guinea corn as can be seen from the analytical results in table X. In view of the much disparity between the phosphorus content of the two feeds (one being about 4 to 5 times the other) one would have expected a larger difference in the phosphorus content of the two sets of Guinea fowls. In this respect, it seems probable that there is a 'threshold value' of phosphorus in the blood serum beyond which phosphorus is excreted as a waste product by the birds.

The slightly higher results obtained for the blood serum of the livestock fed guinea fowls is also understandable since the birds fed on this diet were supposed to be egg layers and since they were not laying eggs at the time of the experiment; phosphorus which is one of the essential components of the constitution of the egg shell would not have been used up.

Within a set of guinea fowls fed on the same diet, one does not observe a constancy in the values of the phosphorus content, rather there are slight differences from one bird to another. If other factors such as body size (surface area, weight), age, sex, rate of feeding, climatic condition (environmental temperature), remain constant for a set of guinea fowls on the same diet, one would have expected an almost constant value for the phosphorus content since these are factors affecting the basal metabolic activity of any living organism. However, the slight differences in result observed probably reflect variation in one or more of the above parameters for a given set of guinea fowls on the same diet.

Chapter 4

KINETICS ANALYSIS OF PHOSPHOROUS IN THE PULPY PARTS OF PUMPKIN SPECIES AND SERUM OF GUINEA-FOWLS

4.1 Analysis was attempted on the pulpy parts of four different species A, B, C, E of pumpkin (same as those used for the spectrophotometric analysis), the seeds and the back. Destruction of the organic material was effected through the use of the digestion mixture suggested by Jones, Lee and Peacocke [Jones, S.A., Lee, W.A., Peacocke, A.R. (1951)]. This consists of a mixture of concentrated sulphuric acid, copper-sulphate, potassium-hydrogen sulphate and little powdered selenium.

It is pertinent to note that with pulpy parts of the various pumpkin species, colourless digests were obtained using the Jones et al digestion mixture but with the seeds and the back (under the conditions that prevailed in the digestion procedure) gave no colourless digests but intensely coloured digests. Since the basis of the kinetics method of analysing phosphorous is the very fast reaction between $MO(VI)$ and phosphate in acid medium to give the yellow complex 12-molybdophosphoric acid, the rate of formation of which is then monitored spectrophotometrically at 400nm, the kinetics method was limited to the pulpy parts of the various species.

Other digestive agents for phosphorus in organic materials like perchloric acid, [Fiske and Subbarow (1925)] nitric acid, Carius (1865), mixture of sulphuric acid and nitric acid perchloric acid, [Kjeldahl (1883)] etc would have been tried on the back and the seeds of the various species but for the short time at the disposal of the project. However, the non-viability of the Jones et al digestion mixture for the seeds and the back of the species is probably indicative of some possible differences in the composition of the pulpy parts, the seeds and the back of the pumpkin species.

4.2 Method of Digestion:

1.0 gm. of the pulpy part of each species was weighed and placed in a micro-kjeldahl flask 0.4 mls of concentrated sulphuric acid, 0.6 mls of copper-sulphate solution (10 gm/litre), potassium hydrogen sulphate (120 gm/litre) and a few crystals of selenium were added in turn and heated on a Kjeldahl heating mantle at 40° - 50° C for about 1 hour. Further heating for about 30 minutes resulted in a colourless digest.

Test for Phosphate:

A digest of the pulpy part of each species was prepared as described above, cooled, diluted and a portion of it used to test for the presence of phosphate. A yellowish colouration which was observed on addition of ammonium molybdate solution indicated and confirmed the presence of phosphate.

4.3 Kinetic Measurements:

Instrument: Durrum-Gibson Model D-110 stopped-flow spectrophotometer.

When the colourless digest of phosphorus as the orthophosphate had been obtained as described above for a species. It is crucially important to bring the pH of the solution to about the same value as that of the phosphorous working standards. This is **necessary** since the fast reaction between phosphate and MO(VI)



which is the basis for the kinetic measurements is very sensitive to pH. The effect of acid on the reaction is very complex influencing not only the amount of 12-MPA formed but also the rate of its formation. Control of acidity of the digest was effected through the use of the Griffin pH-meter and concentrated solution of sodium hydroxide. The digest in each case was found to be highly acidic (as indicated by a pH value of between 1.2-1.8) and by addition of concentrated solution of NaOH in drops in a beaker, the pH was adjusted to that of the phosphorous working standard (4.9-5.0).

The digest was then transferred (with washing) into a 100 ml volumetric flask and made up to the mark with water. Determinations were carried out by reacting portions of the phosphate solution at a time with MO(VI) in the Durrum-Gibson Model D-110 stopped-flow spectrophotometer. Two runs, each, for the phosphate digest of a

particular species of pumpkin were carried out at a wavelength of 400nm in the spectrophotometer and the slopes of the linear traces (for the various species) displayed on the oscilloscope calculated (see introduction - fig. 1-B).

4.4 Preparation of Solutions:

Reagents: All reagents used for the preparation of solutions were of the analytical grade and were products of the BDH Chemicals Limited, England.

Sodium molybdate solution: A 0.1M aqueous stock solution of MO(VI) was prepared by weighing out 24.196 gms. of sodium-molybdate dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$) to make up one litre of solution. The stock solution was stable over months.

A working standard solution of $5.0 \times 10^{-2}\text{M}$ MO(VI) was prepared from the stock by pipetting 50 mls of the stock solutions and making up to 100 mls with 0.4M nitric acid.

Both the stock and the working solutions were stored in polyethylene bottles to prevent leaching from silicon of the volumetric glassware.

Potassium dihydrogen orthophosphate (KH_2PO_4) Solutions:

Solution of KH_2PO_4 (10^{-3}M) was prepared by weighing accurately using the Mettler 8H balance of KH_2PO_4 0.136 gm and making up to one litre of solution.

4.5 Preparation of Calibration Curve:

3.0, 12.5, 25.0 and 50.0 mls of the phosphorus stock solution ($10^{-3}M$) were pipetted into separate 100 ml volumetric flasks and made up to the mark. Each standard solution was reacted in turn with MO(VI) in the stopped-flow spectrophotometer (see 4.6 for description and mode of operation). The stopped-flow system was always properly flushed with distilled water to ensure cleanliness of the relevant parts so that all determinations (runs) will be made at minimum input voltage. Two runs were attempted for each solution.

First a time base was chosen on the oscilloscope for which the reaction between MO(VI) and a given standard phosphorous solution went to completion when an oscilloscope display of the type shown in fig. 1-B would be obtained (see introduction). Since it is desired to study the MO(VI)/ PO_4 reaction within the first 10% of the total reaction to enable determine the initial rate of formation of 12-MPA for a given standard solution of phosphorus; runs were ultimately done by selecting a time base 10% of that required for the total reaction when linear oscilloscope trace of the type shown in fig. 1-C would be obtained and snapped photographically.

The gain (in millivolts or volts) and the base-time in (milli-seconds or seconds) for each linear trace corresponding to a particular concentration of phosphate solution were recorded and calculations made of the slope (gradient) in each case. Linear traces with different

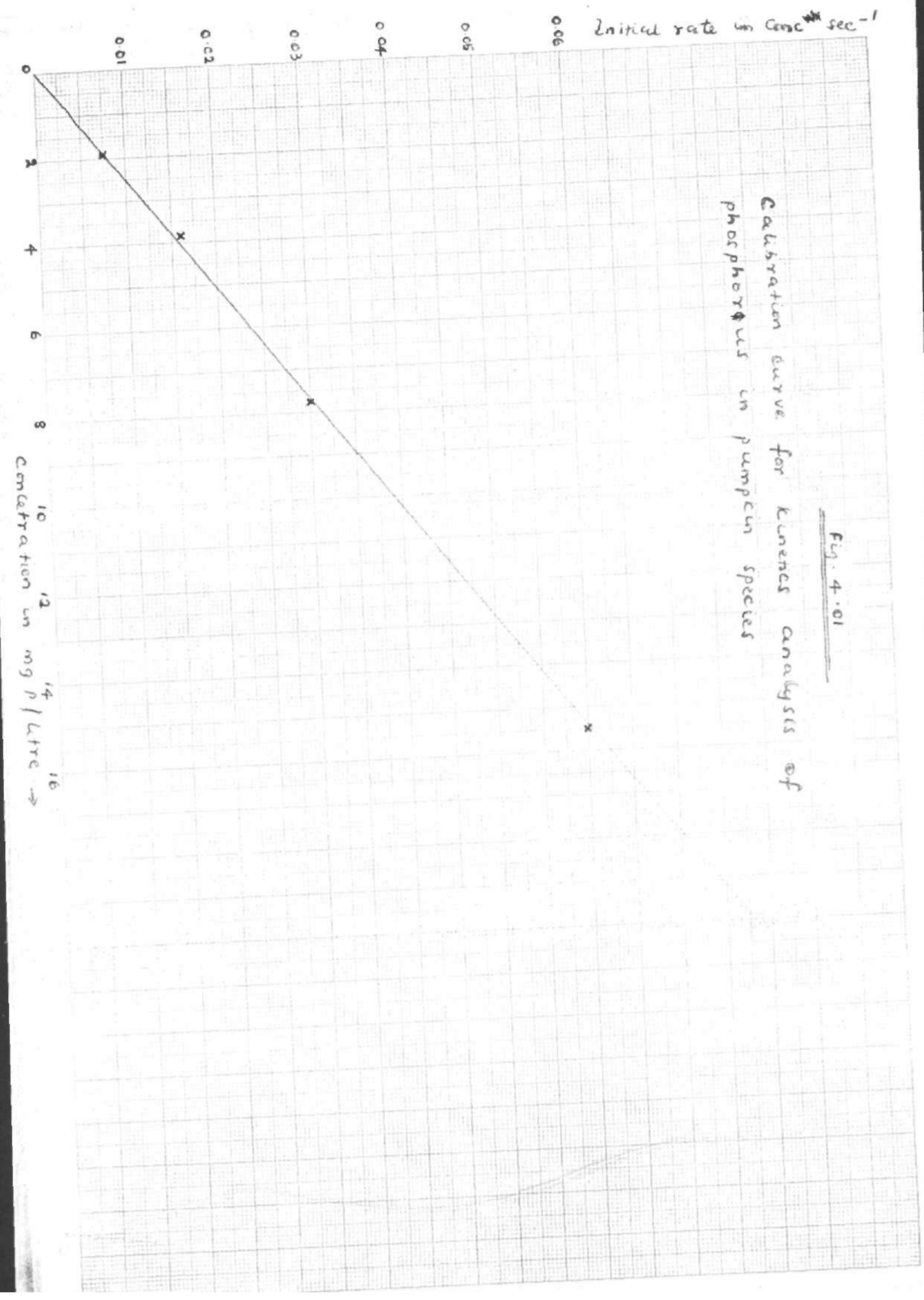
slopes were obtained for different phosphate solutions. Since a linear trace represents an automatic plot of absorbance (proportional to concentration) against time, the slope in each case would be proportional to the initial rate of formation of 12-MPA. The calibration curve was prepared by making a plot of slope against the corresponding phosphate concentration either in molarity or weight of phosphate/litre (see fig. 4.01).

Results for calibration are shown in Table XIV below.

Table XIV

Concentration of KH_2PO_4 in Molarity $\times 10^{-4}$	Corresponding weight of phosphorus/litre in mg.	Initial Rate α Slope in conc sec^{-1}
0.625	1.938	0.0075
1.250	3.875	0.016
2.500	7.750	0.030
5.000	15.500	0.060

Slopes of the linear traces for the species A, B, C, E of pumpkin as displayed on the oscilloscope were found respectively to be 0.024, 0.0095, 0.0076, 0.005.



Calibration curve for kinetics analysis of phosphorus in pumpkin species

Fig. 4.01

Values of the slopes for the various species were read on the calibration curve and the corresponding concentration of phosphorus also read. Calculations were ultimately made of the phosphorus content in mg/100 gm. of species for the different species and shown in table XV below.

Table XV

Pumpkin Species (Pulpy part)	Phosphorus content in mg P/100 gm. of species $\times 10^2$
A	6.10
B	2.35
C	1.90
E	1.24

4.6 Description of the Stopped Flow System:

A block diagram of the spectrophotometric stopped-flow system used is shown in fig. 1-A (see introduction). The Durrum-Gibson Model D-110 stopped flow spectrophotometer is a complete system for rapidly mixing two liquid reactant solutions and measuring their change in optical transmission or absorbance as a function of time. The system derives its name from the fact that the flow of sample is stopped immediately after mixing to permit observation of changes in optical characteristics without interference from turbulence of flow artifacts. The reaction of the solution is observed by photometrically

monitoring the transmission of light through a port in the mixing chamber cuvette. The output level sensed by the photometer is applied to a Cathode ray oscilloscope where it is displayed against a time base. Monochromatic light is used in the photometer system so that each reaction can be studied at its optimum absorption peak.

The stopped flow spectrophotometer comprises five functional systems:

- (1) the sample flow system (completely contained in the mixing chamber)
- (2) the flow-actuating system (initiates the sample flow for each measurement)
- (3) the optical system (supplies monochromatic light by means of a light source and monochromator, and routes it through the measurement cuvette and into the photomultiplier tube contained in the mixing chamber)
- (4) the electronics (which provide power for all electrically operated components and calibrated readout/display processing for the measurement signal from the photomultiplier)
- (5) the temperature control system (circulates a liquid that maintains all parts of the flow system at a constant temperature to avoid temperature-gradient refraction artifacts that could make the display meaningless).

The system operates as follows: With appropriate valve settings, the drive syringes are filled from the reservoir syringes. Then with valves reset for making a determination, the operator pushes the ACTUATE button, thus causing the flow actuator to force the drive syringe-plunger in slightly. This forces equal volumes of the two reactants to mix in the mixing jet and flow through the cuvette until the stop syringe is filled to the point where its plunger hits the mechanical stop. Flow is abruptly halted at that point so that the reaction takes place in the cuvette with a minimum of turbulence interference. Meanwhile, the trigger switch, which is also actuated by the movement of the stop syringe plunger, has initiated the horizontal time base sweep for the oscilloscope display. The monochromatic light from the monochromator passes through the mixed solutions in the cuvette observation chamber during the reaction and the resultant varying intensity light is projected onto the photomultiplier tube. The photomultiplier output, proportional to the light intensity transmitted by the reacting solution is processed by a photometric log buffer amplifier and drives the vertical axis of the oscilloscope. The resultant oscilloscope display indicates absorbance vs time, starting just before the reaction begins and ending sometime after the reaction has completed.

4.1.1 Kinetic analysis of Phosphorous in the Blood serum of Guinean Fowls:

Analysis was attempted on the blood-serum of guinea fowls A, B, D, X, J, Z (same as those used for the spectrophotometric analysis).

Method:

The experimental procedure adopted was the same as that used by Javier, Crouch and Malmerstadt (see introduction).

The blood serum of each guinea fowl was prepared in the same way as for the spectrophotometric analysis (see 3.1.2).

0.4 mls of the blood serum in each case was treated with 3.6 mls of 9% (w/v) trichloroacetic acid (to deprotenise) in a medium-sized centrifuge tube and centrifuged to obtain a clear, supernatant liquid. This was then brought to the pH of the phosphorous working standard with 1N sodium hydroxide prepared from the acculate reagent.

The trichloroacetic acid filtrate of each blood serum was finally reacted with MO(VI) in the stopped flow system at 400nm to obtain as usual linear traces, the slopes of which were computed in manner similar to that for analyses on the pumpkin species.

4.1.2 Preparation of Solutions:

Phosphorous working standard: This was of concentration 0.0125 mg/ml and was prepared in the same way as for the spectrophotometric analysis (see 3.1.3).

Sodium molybdate solution: A stock solution of concentration $10^{-1}M$ was prepared by weighing out 24.196 grams of sodium-molybdate dihydrate ($Na_2MoO_4 \cdot 2H_2O$) to make up 1 litre of solution.

A working solution of $5 \times 10^{-2}M$ MO(VI) in $0.4HNO_3$ was also prepared.

4.1.3 Preparation of Calibration Curve:

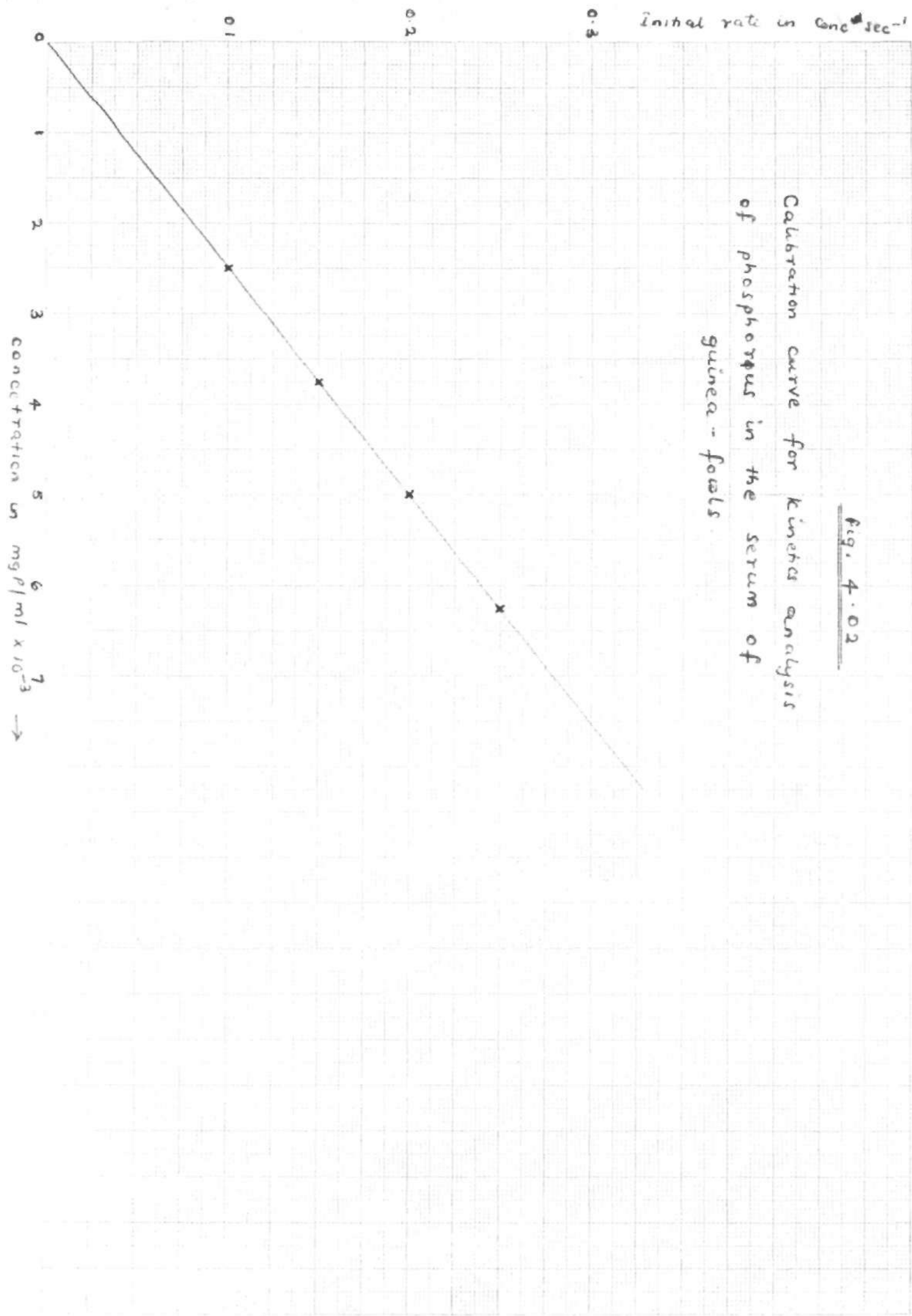
2.0, 3.0, 4.0, 5.0 mls of the phosphorus working solution (0.0125 mg/ml) were pipetted into separate 10 ml volumetric flasks and made up to the mark in each case. Each of the standard solutions was then reacted with MO(VI) in the stopped-flow system at 400nm and slopes of the linear traces obtained calculated.

Calibration curve was prepared by making a plot of slope or gradient against the corresponding concentration in mg/ml of phosphorus (see fig. 4.02).

Results for calibration are shown in table XVI below.

Table XVI

Concentration of standard in mg/ml $\times 10^{-3}$	Slope & Initial rate in conc. unit sec^{-1}
2.50	0.10
3.75	0.15
5.00	0.20
6.25	0.25



The slope of the linear traces displayed corresponding to the blood serum of the Guinea fowls A, B, D, X, J, Z, were found respectively to be ^{0.204}0.2, 0.2, 0.183, 0.15, 0.175.

Concentration of phosphorus corresponding to each of this was read on the calibration curve and calculations made of the phosphorus content in 100 mls of blood serum in each case.

Table XVII below shows results of such calculation.

Table XVII

Blood serum of guinea fowl	Phosphorus content in mg/100 mls of serum
A	3.170
B	3.130
D	3.125
X	2.830
J	2.340
Z	2.710

COMPARISON OF THE SPECTROPHOTOMETRIC AND KINETICS
RESULTS OBTAINED ON THE ANALYSIS OF
PHOSPHOROUS IN PUMPKIN SPECIES AND
BLOOD SERUM OF GUINEA FOWLS

Both results as obtained on the different biological materials are presented in the tables below.

Table XVIII: Pulpy parts of Pumpkin Species

Pulpy part of Pumpkin Species	Phosphorous content in mg/100 gm. of dried part KINETICS METHOD	Phosphorous content in mg/100 gm. of part (raw) SPECTROPHOT. METHOD
A	610	825
B	235	447
C	190	275
E	124	313

Table XIX: The Blood serum of Guinea Fowls

Blood serum of Guinea Fowl	Phosphorous content in mg/100 ml. of serum KINETICS METHOD	Phosphorous content in mg/100 ml. of serum SPECTROPHOT. METHOD
A	3.170	5.20
B	3.130	5.37
D	3.125	4.95
X	2.830	3.67
J	2.340	3.51
Z	2.710	4.41

All results reported above are mean values of three determinations in case.

From table XVIII it can be seen that the kinetics method of analysing phosphorus in the various species of pumpkin indicates the same general direction of variation of phosphorus in the species, being maximum in species A and least in E. Also table XIX indicates non-constancy in the phosphorus content of the blood serum of guinea fowls fed on the same diet, and slightly higher values in the serum of those fed on the Pfizer Livestock diet. All these observations are in alignment with the spectrophotometric ones, and in these respects both methods are similar.

Results for the kinetics analysis, however, were always found to be less than the spectrophotometric ones. The large differences in results observed for analyses on the pumpkin species may be traced to one or more of the following causes (i) the 'close approximation' method used for the computation of the slopes of the linear oscilloscope traces which would inevitably check on the accuracy of the results (ii) the strongly pH sensitive nature of the $\text{MO(VI)}/\text{PO}_4$ reaction which is the basis of the kinetics method may be a factor. The $\text{MO(VI)}/\text{PO}_4$ reaction to give 12-MPA is strongly influenced by acidity affecting not only the amount of 12-MPA formed but also the initial rate of formation 12-MPA from the reactant and hence the slopes of the linear traces which are proportional to the initial rate. Although adequate measures were taken in the procedure to effect control of acidity, one cannot totally rule out the possibility of acid effect on the reaction (iii) the nature of the digestion mixture may also

be a factor since the efficiency of a particular digestion mixture would be measured by how much of phosphorus is released into solution as the phosphate for subsequent spectrophotometric analysis. Parallel to this, the nature of phosphorus in the biological material should be understood, if much of the phosphorus is bound, then not all may be released into solution by a particular digestion mixture, which then leads to obtaining lower results. In short, suitable digestion mixture must be sought for appropriate biological material. In this respect, other phosphorus extractants such as nitric acid alone; mixture of nitric acid and sulphuric acid, mixture of nitric acid, sulphuric acid and perchloric acid; mixture of nitric acid, sulphuric acid and hydrogen-peroxide etc. are recommended. (iv) the kinetics method used the raw pulpy part of each species whilst the spectrophotometric method used the same weight of the dried part. Correction should therefore be made for the slight difference in weight observed when the raw material is dried under the same conditions as the dried part. Ideally, both methods should have been applied on the pulpy part of the species in the same state to make for easy comparison. For example, the digest of the pulpy part of a particular species would have been prepared in duplicate and one used for the colorimetric analysis rather than using the ashing procedure on the dried part as was done for the colorimetric analysis. This however, did not immediately occur to the mind of the experimenter until later in the course of the project (when it was already too late).

Table XIX shows somehow a better agreement between the kinetics and the spectrophotometric methods for analysis in the blood serum of the fowls than in the case of the pumpkin species. In both methods, phosphorus was determined in the same form in the serum of all the fowls.

Much as the kinetics method was to be remedial to the shortcomings of the spectrophotometric method of analysis, the complications peculiar to this method as indicated in the early part of this discussion would not make for a better evaluation of the two procedures. Unless the kinetics procedure is carefully carried out by the analyst (by, for example, controlling the acidity of the samples) and using a more completely automated stopped flow spectrophotometer (unlike the non-automatic one used for this research project) so that the samples can be introduced automatically; and a digital-read-out system incorporated (none for the project) so that the phosphate concentrations can be presented automatically in the digital form (thus eliminating the tedious procedure of having to calculate slopes of linear oscilloscope traces which, in any case, cannot be done accurately and reading slopes on the calibration curve) the kinetics method may not be seen as a better method of analysing phosphate (in terms of accuracy and precision) in spite of its elegancies e.g. eliminating the usual interferences in the colorimetric procedure, short analysis times etc.

The results of the blood serum and pumpkin analyses indicate the suitability of the kinetics procedure to the analysis of biological samples. In addition, the method should be applicable to other phosphate - containing samples.

Conclusion:

The rapid kinetics method utilising the initial reaction rate measurements in the millisecond range can be adopted for the determination of phosphate in biological materials. The method can avoid complications due to unfavourable equilibria and interferences. Also the short analysis time (if the completely automated stopped-flow system is used) makes it an excellent procedure for routine and continuous analyses.

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