STUDIES ON THE INACTIVATION OF INDOLEACETIC ACID BY ROOTS

by

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A thesis submitted for the degree of Doctor of Philosophy in the Faculty of Science in the University of London.

November, 1961.
January, 1962.

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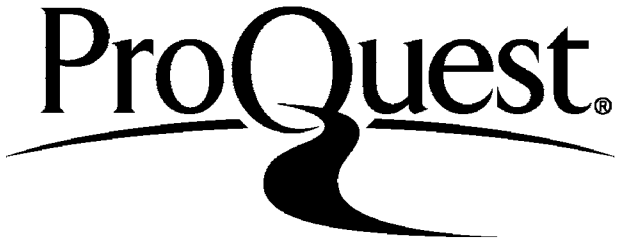


ABSTRACT

The enzymatic breakdown of indoleacetic acid in the presence of aqueous pea root extracts containing an "indoleacetic acid-oxidase" system was studied. A method based on the "Salkowski" colour reaction was used for the estimation of residual indoleacetic acid.

A comparison was made between the "indoleacetic acid-oxidase" activity of extracts made from the roots of plants grown in water and the activity of extracts made from the roots of plants grown in a solution of indoleacetic acid. It was found that the activity of the extracts from the "indoleacetic acid-grown" roots was higher than that of the extracts from the "water-grown" roots. Thus the "maximum rate attained in the reaction" was greater, and the "length of the lag period" shorter, when extracts of the "indoleacetic acid-grown" roots were used. Further, the relationship between the length of the lag period and the maximum rate attained appeared to be slightly different for reaction mixtures containing the two types of extract.

The inhibitory activity of solutions prepared from the "water-grown" root extracts and the "indoleacetic acid-grown" root extracts by i. boiling, ii. seitz filtration, or iii. ultrafiltration, was determined. The inhibitor solutions prepared from the "indoleacetic acid-grown" root extracts were always found to be less active than the corresponding inhibitor solutions prepared from the "water-grown" root extracts. It was thought that this difference in inhibitor level may have accounted for the different level of "enzyme activity" demonstrated for the extracts of the "water-grown" and "indoleacetic acid-grown" roots.

The significance of the enhanced "indoleacetic acid-oxidase" activity of "indoleacetic acid-grown" root extracts was discussed in relation to the hypothesis of adaptive enzyme formation. It was concluded that the enzyme was not formed adaptively in the pea root tissues in response to the applied indoleacetic acid.

ACKNOWLEDGEMENTS.

I should like to express my sincere thanks to Professor L.J. Audus for suggesting this problem and for his interest and advice during the course of the work.

I am grateful also to Mr. C.H. Funnel of the Botany Department, Bedford College, for taking the photographs of figures 1 - 3, and 14 - 18, and to Mrs. I. Cowie who prepared the typescript.

Finally I wish to acknowledge the receipt of a Research Studentship awarded by the Department of Scientific and Industrial Research.

CONTENTS

Chapter 1. INTRODUCTION.

The subject of this investigation was the inactivation of the plant growth hormone indoleacetic acid by the enzyme "indoleacetic acid-oxidase" present in the roots of Pisum sativum L. The starting point for the work was an idea put forward by Galston and Dalberg (1954). It was suggested that the "indoleacetic acid-oxidase" enzyme found in higher plants was an "adaptive enzyme" of the type which has been shown to occur in microorganisms. The implications of the term "adaptive enzyme" were discussed by Spiegelman (1950). It has been applied to certain enzymes produced by bacteria and yeasts which were shown to arise only when the substrates for which they were specific were present in the culture media. In some cases - for example that of a galactozymase enzyme of yeast - it was demonstrated that the appearance of enzyme activity was accompanied by the formation of the actual protein (apo - enzyme) component of the enzyme. Thus Galston and Dalberg thought that it was possible that the indoleacetic acid destroying enzyme might be formed in plant tissues only in response to the presence there of endogenous indoleacetic acid. They reported that treatment of pea tissues with a solution of synthetic indoleacetic acid caused an enhancement of the "indoleacetic acid-oxidase" activities of extracts made from them. The present work represented an attempt to substantiate this finding using pea roots.

Historical survey of work on the biochemistry of "indoleacetic acid-oxidase".

In 1940 Larsen demonstrated that the juice of epicotyls of etiolated seedlings of Phaseolus multiflorus contained a heat-labile substance which could bring about the inactivation of indoleacetic acid. Tang and Bonner (1947) made a detailed study of an enzyme system present in an aqueous extract from etiolated pea epicotyls, and followed the disappearance of indoleacetic acid using both a bioassay technique - the Went Avena coleoptile curvature test, and also a colorimetric method based on the Salkowski reaction. They demonstrated conclusively that an oxidation was involved in the reaction. Manometric experiments showed that approximately 1 mole of oxygen was consumed and 1 mole of carbon dioxide was liberated for each mole of indoleacetic acid destroyed. The reaction was prevented when the Warburg flasks were either evacuated or filled with argon. The enzyme was called therefore, "indoleacetic acid-oxidase". The pH optimum lay in the region of 6.2 to 6.7 in phosphate-citrate buffer. Inhibition of activity by low concentrations of potassium cyanide indicated the presence of a heavy metal, while light-reversible carbon monoxide inhibition suggested that this metal was iron rather than copper. In a second paper, (Tang and Bonner, 1948), the existence of an endogenous inhibitor of the indoleacetic acid destroying activity was reported. First it had been discovered that the activity of an aqueous extract of etiolated pea epicotyls was increased by about 20 per cent by acetone precipitation. Later it was shown also that boiled aqueous extracts from the same tissues contained a thermostable substance/

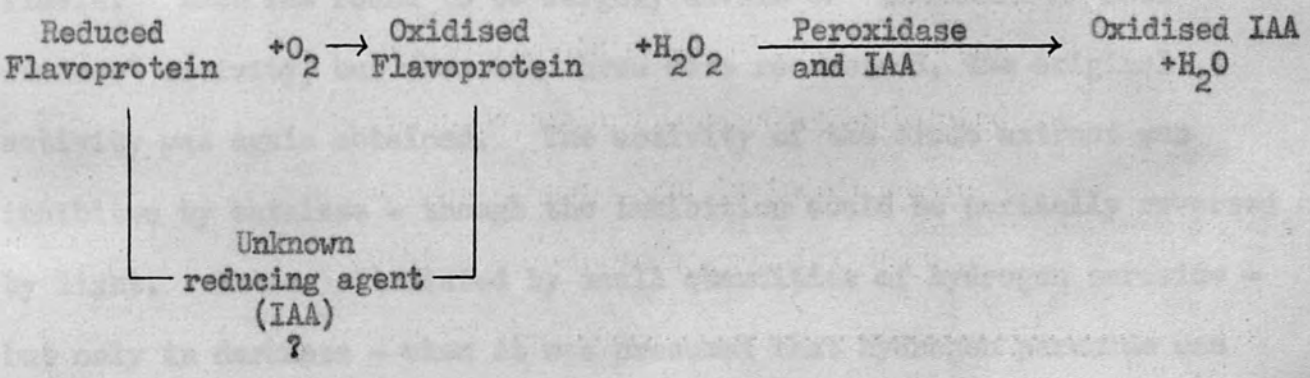
substance which inhibited the activity of the acetone-precipitated enzyme extracts. When the results of one experiment were plotted in the manner suggested by Lineweaver and Burk (1934), the inhibition was found to be non-competitive.

Wagenknecht and Burris (1950) studied "indoleacetic acid-oxidase" preparations from yellow wax bean roots and etiolated pea epicotyls. Their pH optima and responses to heavy metal inhibitors such as cyanide were as described by Tang and Bonner (1947). However these authors were not able to obtain any inhibition with carbon monoxide and were of the opinion that the carbon monoxide inhibition reported by Tang and Bonner (1947) was probably due merely to the lower oxygen content of the gas mixtures. On the basis of inhibitions obtained using sodium diethyl-dithiocarbamate, potassium ethyl xanthate and thiourea, they concluded that the heavy metal component of the enzyme was copper and not iron. The activity of the extracts from both sources was stimulated by low concentrations of manganese (Mn^{++} ion). Furthermore, whereas each extract was found to lose about one third of its activity on dialysis, this could be restored to its original level by the addition of $MnCl_2$.

One of the earliest schemes for the mechanism of the enzymatic breakdown of indoleacetic acid was that of Galston et al. (Galston, Bonner and Baker, 1950, 1953), who postulated a coupled system involving peroxidase and a hydrogen peroxide - producing flavoprotein enzyme. As shown in the diagram below, a direct peroxidation of indoleacetic acid was thought to take place, which resulted in the formation of "oxidised indoleacetic/

indoleacetic acid" and water. The method of regeneration of the reduced flavoprotein - on which the continued production of hydrogen peroxide would depend - was unknown, though it was suggested that indoleacetic acid itself might act as the reducing agent.

Hypothetical scheme for the enzymatic oxidation of indoleacetic acid. (After Galston, Bonner and Baker (1953)).



(IAA = indoleacetic acid).

The idea that a flavoprotein enzyme might be involved originated from the observation made by Galston and Baker (1949) that the oxidation of indoleacetic acid by an aqueous extract of etiolated pea epicotyl tissues was light stimulated, and that the action spectrum for this light stimulation corresponded with the absorption spectrum for riboflavin. Later it was alleged by Galston, Bonner and Baker (1950, 1953) that this same indoleacetic acid oxidising system had been separated into three components - peroxidase, flavoprotein enzyme (apoenzyme) and riboflavin. Each was found to be largely devoid of "indoleacetic acid - oxidase" activity, but when the three were recombined, the original activity was again obtained. The activity of the crude extract was inhibited by catalase - though the inhibition could be partially reversed by light. It was stimulated by small quantities of hydrogen peroxide - but only in darkness - when it was presumed that hydrogen peroxide was limiting. In support of the proposition that the heavy-metal containing enzyme present in the system was peroxidase, Galston, Bonner and Baker found that horseradish root peroxidase could bring about the oxidation of indoleacetic acid in the presence of added hydrogen peroxide. As final "confirmation" of their scheme, Galston, Bonner and Baker demonstrated that an artificially constituted system containing peroxidase, flavoprotein enzyme and flavoprotein substrate could destroy indoleacetic acid.

Many of the ideas formulated by Galston, Bonner and Baker (1950, 1953) have persisted. The participation of peroxidase for example has/

has remained undoubted. All the "indoleacetic acid-oxidase" containing extracts derived from higher plants have also possessed peroxidase activity. Important indirect evidence for a peroxidatic reaction was obtained at about the same time by Goldacre, Galston and Weintraub (1953). They discovered that a large number of monophenols - all potential peroxidase substrates - caused marked increases in the "indoleacetic acid -oxidase" activity of a crude pea epicotyl extract. Of the evidence put forward in support of the role of hydrogen peroxide, although the enhancement of activity brought about by the addition of hydrogen peroxide has appeared to be a general phenomenon, the inhibition of the reaction by catalase has since been shown (Waygood, Oaks and Maclachlan, 1956b) to have been caused by the presence in the type of catalase preparation used of a photolabile, dialysable inhibitor of the indoleacetic acid oxidation. In addition it was reported (Waygood and Maclachlan, 1956) that catalase could actually replace peroxidase in the reaction mixture. Under the conditions used, low hydrogen peroxide concentration and high catalase concentration - it was presumed that the catalase was acting peroxidatically. A light-reversible inhibition of a crude horseradish root extract by Mn^{++} (Galston, Bonner and Baker, 1953) - although enigmatic - probably did not result from a catalytic decomposition of hydrogen peroxide by the Mn^{++} as originally suggested.

In contrast, no further evidence at all has ever been forthcoming in support of the idea that hydrogen peroxide was formed by a second enzyme - that is by an aerobic dehydrogenase. Light stimulation of "indoleacetic/

"indoleacetic acid-oxidase" systems has been reported from time to time but these have remained difficult to interpret, particularly as Goldacre (1954) showed that a large number of substances, including riboflavin - the supposed prosthetic group of the hypothetical flavo-protein enzyme - are capable of photosensitising a non-enzymatic destruction of indoleacetic acid. The experiment in which the prosthetic group was apparently separated from the flavoprotein enzyme has never been confirmed. No substrate for the flavoprotein enzyme appeared to be present in the pea epicotyl extracts, although - as suggested - indoleacetic acid itself might have filled this role. Finally certain systems have been described in which breakdown occurred in the absence of hydrogen peroxide - unless it was assumed that in these cases the peroxide was formed not merely during, but probably also as a result of, the indoleacetic acid oxidation. In this connection it is of interest to point out that Galston, Bonner and Baker (1953) themselves reported the remarkable discovery that a relatively pure sample of horseradish root peroxidase could-with no additions - begin to destroy indoleacetic acid after a suitable period of incubation with the substrate.

The interpretation of the reaction mechanism put forward by Kenten (1955) was based on similarities which he observed between an indoleacetic acid oxidising system from waxpod bean roots and three systems in which manganese oxidation was demonstrated. Basically these were constituted as follows:-

1. Mn^{++} + Horseradish root extract (containing peroxidase) + Hydrogen peroxide. (Kenten and Mann, 1949).
2. Mn^{++} + Pea epicotyl extract (containing peroxidase and a hydrogen peroxide - producing diamine oxidase). (Kenten and Mann, 1952).
3. Mn^{++} + Certain dicarboxylic acids. (Kenten and Mann, 1953).

Kenten and Mann (1949) showed that the oxidation of Mn^{++} in system 1. above was dependent on the presence of certain peroxidase substrates - which could be supplied to an inactive sample of crude extract, or to a purified horseradish peroxidase preparation by adding either a boiled active extract, or the monophenol p-cresol. Next, (Kenten and Mann, 1950), using a purified horseradish peroxidase preparation plus Mn^{++} and hydrogen peroxide, they demonstrated that whereas several monophenols were active, diphenols (with the exception of resorcinol), and triphenols were inactive and were inhibitory. Hence in the system given under 2. above, it was thought (Kenten and Mann, 1952), that Mn^{++} oxidation was being mediated by naturally occurring peroxidase substrates. Kenten and Mann (1949), suggested that the actual oxidation of Mn^{++} arose indirectly by a reaction between Mn^{++} and an intermediate product of phenol oxidation. When the phenols in question were incubated with peroxidase and hydrogen peroxide in the absence of Mn^{++} - the normal coloured products of phenol oxidation were produced, whereas when Mn^{++} was present, these were not found. Itwas thought (Kenten and Mann, 1950) that during Mn^{++} oxidation, the monophenols were not being oxidised to diphenols but that some intermediate oxidation product was being reduced back to the monophenol.

These authors showed further (Kenten and Mann, 1953) that, in certain cases, the oxidation of Mn^{++} in the presence of dicarboxylic acids (system 3. above) was once again dependent on the presence of a peroxidase substrate. Thus, for example, Mn^{++} was oxidised and oxalic acid decomposed in the presence, but not in the absence, of p-cresol. It was thought that here also the peroxidase substrate was required for the oxidation of Mn^{++} exactly as described for the previous systems. Besides being independent of added hydrogen peroxide, the reaction in the systems containing dicarboxylic acids was always preceded by a lag period. Since this could be removed by the addition of hydrogen peroxide, Kenten and Mann postulated that the oxidised manganese stimulated hydrogen peroxide production. With oxalate for example they presumed that manganous oxalate became autoxidised extremely slowly with the formation of hydrogen peroxide and that once a small amount of hydrogen peroxide had been produced, manganic manganese was formed and hence by a method described by Launer (1933) more hydrogen peroxide. The equations given by this last author are reproduced below.

$$Mn^{+++} + C_2O_4^{--} = Mn^{++} + CO_2 + CO_2^-$$

$$CO_2^- + O_2 = O_2CO_2^-$$

$$O_2CO_2^- + Mn^{++} + 2H^+ = Mn^{+++} + CO_2 + H_2O_2$$

Kenten (1955) examined an indoleacetic acid oxidising system containing waxpod bean root sap. This could be fractionated by ultrafiltration into a thermolabile fraction with peroxidase activity and a thermostable fraction. In view of the fact that the "Kenten and Mann/

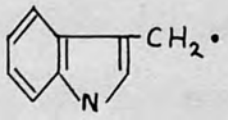
Mann peroxidase substrates" - that is those compounds active in the manganese oxidising systems described above - had already been shown to activate the oxidation of indoleacetic acid by pea epicotyl extracts (Goldacre, Galston and Weintraub, 1953), the question at once arose as to whether the thermostable fraction of the waxpod bean root system contained peroxidase substrates. This was confirmed by showing that pre-treatment of the ultrafiltrate with peroxidase and hydrogen peroxide caused a decrease in its activity. Pre-treatment of certain of the active synthetic phenols - such as 2:4:6 trichlorophenol for example - also resulted in a considerable, but not complete, destruction of activity. The possible participation of Mn^{++} in the activating effect of the ultrafiltrate was also considered, but since the effect of ultrafiltrate was much higher than that of added Mn^{++}, it was concluded that the peroxidase substrates were the important constituents. Indirect evidence was however obtained for the presence of Mn^{++}. Thus inhibitions of indoleacetic acid oxidation by the addition of pyrophosphate were obtained - though admittedly only when high concentrations of ultrafiltrate were used. (Pyrophosphate was known to inhibit "indoleacetic acid-oxidase" systems - in the presence - but not in the absence - of Mn^{++}).

Kenten was thus of the opinion that the oxidation of indoleacetic acid was brought about by the reaction of this compound with intermediate phenol oxidation products - the indoleacetic acid acting as an electron donor instead of the Mn^{++}. Since, like the oxidations of the dicarboxylic acids the indoleacetic acid oxidation was independent of extraneous/

extraneously supplied hydrogen peroxide, he assumed that the source of the hydrogen peroxide was again the substrate. If autoxidation of indoleacetic acid proceeded at all, even at a very low rate, trace amounts of hydrogen peroxide would be produced which could be used by the peroxidase for the peroxidation of the phenol. Further quantities of hydrogen peroxide might then be formed by the autoxidation of intermediates formed from the substrate (indoleacetic acid).

A rather different view concerning the mechanism of indoleacetic acid oxidation was taken by Waygood et al. They examined in great detail the properties of a system containing a well dialysed aqueous extract from wheat leaves. The preparation showed negligible "indoleacetic acid - oxidase" activity when tried alone and was activated to some extent by the addition either of Mn^{++} as $MnCl_2$, or of any member of the "Kenten and Mann phenols". However the addition of these two simultaneously produced an enormous increase in activity - which was measured by following the oxygen uptake manometrically, (Waygood, Oaks and Maclachlan, 1956a, 1956b). Added hydrogen peroxide was again not required. These authors concluded (Waygood, Oaks and Maclachlan, 1956a) that the manganese was being oxidised - presumably by the mechanism described by Kenten and Mann (1949), that is by reaction with intermediate oxidation products of the phenol. However, unlike Kenten (1955), Waygood et al. decided that this oxidation of manganese was an essential feature of the indoleacetic acid destroying system. Thus they suggested that the primary attack on the indoleacetic acid molecule was the removal of a hydrogen atom from the carboxyl group by the trivalent manganese. They considered/

considered that this would then be followed by spontaneous decarboxylation and the formation of a free (skatole) radical with the formula:-



In addition they suggested that the intermediate oxidation product formed during the reversible oxidation-reduction reaction of the phenol was also a free radical - the semiquinol or aryl radical.

The evidence from the behaviour of the enzymatic system for the participation of Mn^{+++} was chiefly as follows. As already mentioned, the indoleacetic acid oxidation was activiated by the addition of Mn^{++} plus a "Kenten and Mann phenol" (resorcinol was used extensively by these authors). This activization did not take place in the presence of chelating buffers such as citrate or pyrophosphate. Other phenols such as catechol and pyrogallol inhibited the reaction - as they had the oxidation of manganese by peroxidase and hydrogen peroxide in the systems described by Kenten and Mann (1950). Waygood, Oaks and Maclachlan (1956b) assumed that these compounds were inhibiting by reacting with oxidised manganese. In support of this explanation, they were able to show that such phenols were active in decolourising manganiversene (a complex formed by manganic ions and ethylenediaminetetracetate), while in contrast the "Kenten and Mann phenols" showed little or no activity. The fact that the active "Kenten and Mann phenols" were found to exhibit optimum concentrations in the "indoleacetic acid-oxidase" system - above which/

which further increases in concentration caused the activity to decline, was also assumed to be caused by a tendency even for these phenols to react with oxidised manganese when present in high concentrations. A lag period was found to precede the oxidation of indoleacetic acid in all the reaction mixtures, and was most pronounced in those containing natural or artificial inhibitors. This was interpreted by Waygood, Oaks and Maclachlan (1956a) as representing the period during which the concentration of Mn^{+++} ions was building up to a "threshold concentration". It was claimed that in general the inhibitors of the indoleacetic acid oxidation could be classified into two groups according to their effect on the course of the reaction. Thus the polyphenol catechol for example was found to prolong the lag phase, but to have no effect on the subsequent oxidation rate. Presumably this reacted with the Mn^{+++} formed initially, but after being itself oxidised in this way, then had no further effect on the reaction. The second category recognised by Waygood, Oaks and Maclachlan (1956b) were those - such as riboflavin and hydroquinone for example - which had no effect on the lag phase but retarded the oxidation from its onset. It was suggested that here again the inhibitors were competing for the oxidised manganese, but that this time the products of the oxidation were capable of giving rise to a further production of manganic ions indirectly. Any factors which were found to overcome the lag period were thought to be acting by causing a more rapid production of Mn^{+++} ions. These included oxygen - thought to promote a "resorcinol oxidase" reaction - referred to again later, manganese dioxide/

dioxide - which presumably acted as an oxidant of the Mn^{++} ions, and hydrogen peroxide. The assumption that the "Kenten and Mann phenols" were operating in crude plant extracts - though according to Waygood et al. serving solely as mediators in the oxidation of the Mn^{++} - was again strengthened by the isolation, by Waygood, Oaks and Maclachlan (1956b) of an ether soluble natural activator from the dialysate. This preparation was not pure but contained also a number of inhibitors - at least some of which were phenolic in nature - as shown by their colour reaction on chromatograms with a ferric chloride spray.

Perhaps the most compelling evidence put forward in support of the proposed role of manganese however was the discovery of Maclachlan and Waygood (1956a) that indoleacetic acid would itself react with manganiversene. At pH 5.0 or lower, a voluminous purple-black precipitate resulted, while at a pH above 5.0 there was no precipitation and the solution became yellow. In the second case oxygen was consumed and it was found that one mole of oxygen was taken up and one mole of carbon dioxide was released for every mole of indoleacetic acid oxidised. This behaviour could be explained by assuming that a free radical was formed from indoleacetic acid - as already described - and that this either polymerised (purple-black precipitate) or reacted with oxygen (yellow solution). Furthermore these authors stated that the soluble yellow product produced non-enzymatically from indoleacetic acid was identical with the product of the enzymatic oxidation - though no details have in fact been profferred.

So far no mention has been made of a source of peroxide for the

peroxidase/

peroxidase. Waygood et al considered that during the reaction, an organic peroxide was formed by reaction of the skatole radical with atmospheric oxygen to give oxidised skatole radical or indole peroxide. This was then used by the peroxidase for the peroxidation of the phenol - the other product of this reaction being the final oxidation product of indoleacetic acid. It remained then to explain how the reaction was initiated - assuming that a supply of Mn^{+++} ions was not present initially in the reaction mixture. Maclachlan and Waygood (1956b) suggested that small quantities of Mn^{+++} ions would be produced by a "resorcinol oxidase" system involving peroxidase, Mn^{++} and atmospheric oxygen.

Maclachlan and Waygood observed, as had also Kenten (1955) that except in the case of small amounts of indoleacetic acid, the oxidation did not proceed to completion. This appeared to be a direct result of the inactivation of the peroxidase component of the system during the oxidation. They suggested that this resulted from the reaction of the oxidised skatole radical with the enzyme. Other "termination reactions" were also proposed : the reaction of the phenol with oxidised manganese (the reverse of the reaction by which manganic manganese was thought to be formed), and the polymerisation of aryl radicals to give unknown phenolic oxidation products.

The scheme formulated by Waygood et al. is given below. The reaction was described as "a chain autoxidation initiated and propagated by two enzyme controlled peroxidations".

Proposed scheme for the oxidation of indoleacetic acid in a standard reaction mixture containing wheat leaf peroxidase, manganous ions, resorcinol and indoleacetic acid. (Maclachlan and Waygood, 1956b).

Initiation.

$$Mn^{++} \xrightarrow[+ ROH]{\text{peroxidase or catalase}} Mn^{+++}$$

Propagation.

$$Mn^{+++} + S - COOH \longrightarrow Mn^{++} + H^{+} + S - COO$$

$$S - COO \longrightarrow S^{\bullet} + CO_2$$

$$S\bullet + O_2 \longrightarrow SO_2\bullet$$

$$SO_2\bullet + ROH \xrightarrow{\text{peroxidase or catalase}} RO\bullet + SO_2H$$

$$RO\bullet + H^{+} + Mn^{++} \longrightarrow ROH + Mn^{+++}$$

Termination.

$$SO_2\bullet + \text{enzyme} \longrightarrow (SO_2 - \text{enzyme})\bullet$$

$$Mn^{+++} + ROH \longrightarrow RO\bullet + H^{+} + Mn^{++}$$

$$2RO\bullet \longrightarrow \text{products}$$

(S - COOH = indoleacetic acid.

S• = skatole radical.

SO_2• = oxidised skatole radical or indole peroxide.

SO_2H = final reaction product.

ROH = phenolic cofactor i.e. resorcinol.

RO• = semiquinol or aryl radical.)

Although an attractive idea in many ways, the participation of manganese as a major reactant has been doubted. At the same time it had been necessary for Waygood et al. to suggest that the activity of systems to which Mn^{++} had not been added was due to the presence of small quantities of Mn^{++} in them - presumably derived from the plant extract. Also, the activating effect produced by adding phenol alone was explained by supposing that the phenol had a capacity to "co-ordinate the non-dialysable manganese" present in the enzyme preparations - a state of affairs which Ray (1958) has considered unlikely. Citrate and pyrophosphate inhibited the "indoleacetic acid-oxidase" reaction only in systems containing added manganese. An alternative suggestion for the inhibitory action of phenols such as catechol was put forward by Kenten (1955), namely that they competed for the available peroxide. Also, the inhibitory effect of high concentrations of the "Kenten and Mann phenols" might have been caused by an inhibitory effect of the high phenol concentration on the peroxidase enzyme. The lag phase need not necessarily have been due to the build up of a concentration of Mn^{+++} ions. The formulation of the peroxidatic reaction between the oxidised skatole radical and the phenol to give the final oxidation product has been severely criticised by Ray (1958). This author pointed out that the supposed product of this reaction (SO_2H) is of a type of compound which would be likely itself to act as a peroxidase substrate, and also that there is no reason to suppose that this reaction would occur in preference to the analogous one proposed for the non-enzymatic system - that is the/

the reaction between the oxidised skatole radical and the substrate - shown below:-

$$SO_2\cdot + S - COOH \longrightarrow SO_2H + S\cdot + CO_2$$

Nevertheless it has recently been recognised that free radicals are important intermediates in certain peroxidase-catalysed reactions. The lag phase which appeared to be inherent in the indoleacetic acid oxidation - though under certain conditions very short - was typical of the type of autocatalytic reaction which free radicals have been shown to take part in. The inhibition by polyphenols even, could also be cited as an indication that free radicals were being formed.

A free radical mechanism for the oxidation of indoleacetic acid by peroxidase was also postulated by Yamazaki and Souzu (1960). They showed for the first time that indoleacetic acid could be oxidised by peroxidase in an anaerobic system in which oxygen was replaced by inorganic iron (Fe^{+++} ions) as hydrogen acceptor. The system used contained highly purified turnip peroxidase and added hydrogen peroxide. It was thought that the reactions occurring were those given under (1) to (4) below:-

$$TP + H_2O_2 \longrightarrow \text{Complex I} \quad (1)$$

$$\text{Complex I} + IAA \longrightarrow \text{Complex II} + IAA^{I} \quad (2)$$

$$\text{Complex II} + IAA \longrightarrow TP + IAA^{I} \quad (3)$$

$$IAA^{I} + Fe^{+++} \longrightarrow IAA^{II} + Fe^{++} \quad (4)$$

(TP = turnip peroxidase.
IAA = indoleacetic acid.
IAA^{I} = one equivalent oxidised form of IAA.
IAA^{II} = two equivalents oxidised form of IAA.)

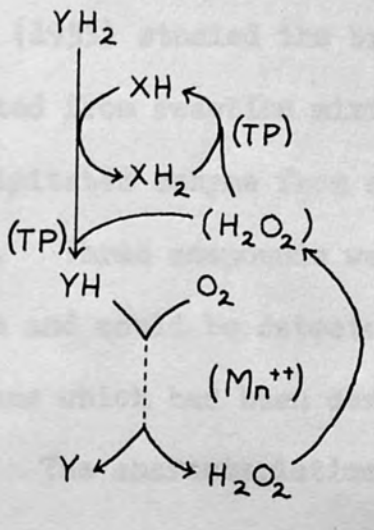
First a peroxidase - hydrogen peroxide complex (Complex I) was formed. This was then presumed to react with one molecule of the substrate (indoleacetic acid) with the formation of Complex II and a free radical IAA^{1} ("one equivalent oxidised form of indoleacetic acid"). The next reaction was between Complex II and a second indoleacetic acid molecule to give another IAA^{1} radical and free peroxidase. The IAA^{1} radicals so produced then presumably reacted with Fe^{+++} to give Fe^{++} and IAA^{11} radicals ("two equivalents oxidised form of indoleacetic acid"). The formation of Complex II and its subsequent reaction with indoleacetic acid was demonstrated by following changes in the absorbancy of the reaction mixture at 424 mμ. Yamazaki and Souzu pointed out that the strict requirement for Fe^{+++} (or other H - acceptor) in the anaerobic system was probably a consequence of its ability to remove the IAA^{1} radicals - which would otherwise react with the enzyme and so inactivate it.

In the aerobic oxidation of indoleacetic acid, Yamazaki and Souzu again suggested that the reactions between peroxidase, hydrogen peroxide and indoleacetic acid were exactly as described for the anaerobic system (reactions (1) to (3)). Here however the enzyme was thought to be "protected from inactivation" by the reaction of the radicals derived from the substrate with atmospheric oxygen. This reaction would also lead to the formation of the hydrogen peroxide utilised by the peroxidase. (The aerobic oxidation was not dependent on added hydrogen peroxide). Yamazaki and Souzu, using their purified turnip peroxidase, found/

found that there was a short "induction phase" before the aerobic indoleacetic acid breakdown commenced - and by following the change in optical density at 424 mμ, they further showed that this induction phase corresponded with the initial increase in density due to the formation of peroxidase Complex II. A second, more gradual increase in the optical density at 424 mμ was attributed to the accumulation of the brownish oxidation products. They assumed that the reaction was initiated during the induction phase by trace amounts of hydrogen peroxide which were either present in the mixture, or arose by the autoxidation of the substrate.

Phenols such as p-cresol were found to increase the rate of oxidation both in the presence and the absence of oxygen. This was explained by assuming that the phenol was peroxidised by peroxidase and hydrogen peroxide to give phenol radicals which then reacted with the substrate to give IAA^1 radicals, and also that the rate of formation of the IAA^1 radicals in this reaction wasfaster than in the "normal" direct peroxidation of indoleacetic acid. Mn^{++} ions were found to increase the rate of indoleacetic acid oxidation only in the aerobic system and only in the absence of added hydrogen peroxide. Since manganese was not effective in the anaerobic system, Yamazaki and Souzu concluded that it could not be influencing reactions (1) to (3) in the aerobic system. They suggested instead that it promoted the reaction between the IAA^1 radical and oxygen in which hydrogen peroxide was produced.

Hypothetical scheme for the aerobic oxidation of indoleacetic acid by peroxidase. (After Yamazaki and Souzu, 1960).



(TP = turnip peroxidase.

YH_2 = substrate (indoleacetic acid).

XH_2 = phenol.

(H_2O_2) = trace amount of H_2O_2.)

This scheme appeared to fit all the facts, except that only 0.5 mole of oxygen would be consumed per mole of indoleacetic acid oxidised and not 1 mole - as has been found experimentally by the majority of workers. However Yamazaki and Souzu pointed out that the reaction between IAA^{1} and oxygen might occur in some other way, perhaps as shown in the equation below for example,

$$IAA^{1} + O_2 \longrightarrow IAA^{11}O + \tfrac{1}{2}H_2O_2 ,$$

thus explaining the observed oxygen uptake.

The pathway of the enzymatic oxidation of indoleacetic acid and the products of the reaction.

Manning and Galston (1955) studied the breakdown products of indoleacetic acid extracted from reaction mixtures containing an ammonium sulphate - precipitated enzyme from etiolated pea epicotyls and 2,4 - dichlorophenol. Three compounds were detected. Two were extracted into chloroform and could be detected by spraying Ehrlich reagent on to chromatograms which had been developed in Isopropanol : Ammonia : Water (2:1:1). The characteristics of these are shown in the table below.

Rf of compound	pH at which extracted into chloroform	Nature of compound	Colour with Ehrlich reagent
0.91	3.0	Acidic	Yellow → red
0.94	3.0 and 9.0	Neutral	Yellow → orange

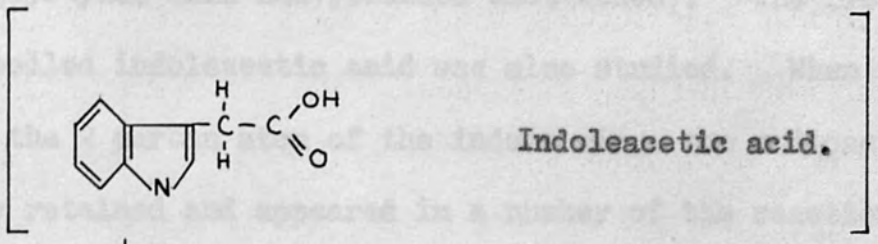
A third compound, which did not appear on the chromatograms, was extracted into chlorofrom at pH 9.0, and gave a positive Hopkins - Cole (indole) test. The compounds running at Rf 0.91 and 0.94 - particularly the former - were shown to accumulate with time. It was found that indolealdehyde did not give rise to the above products when added to the enzyme system. (Indolealdehyde was not detected among the reaction products but evidence for its formation had been obtained earlier by Racusen(1955)).

Stutz (1957) used a highly purified enzyme from Lupinus seedlings to/

to which both Mn^{++} and 2,4 - dichlorophenol were added. The products were extracted with ether and chromatograms run using Isopropanol : Ammonia : Water (10:1:1). The papers were sprayed with Salkowski reagent and examined under ultraviolet light. The six compounds detected are shown below. The non-indolyl ones were detectable only by their fluorescence behaviour.

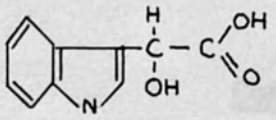
Rf of spot.	Extracted from reaction mixture without adjustment to acid or alkaline pH.	Extracted from reaction mixture at pH :	
		10.0	3.0
0.3	Indoleacetic acid		Indoleacetic acid
0.45 (#1)	Indolyl		Indolyl
0.6 (#2)	Non-indolyl		Non-indolyl
0.7 (#3)	Non-indolyl	Non-indolyl	Non-indolyl
0.8 (#4)	Indolyl	Indolyl	Non-indolyl
0.9 (#5)	Indolyl	Indolyl	

Later, (Stutz 1958), indolealdehyde was detected as a UV-fluorescent spot between spots #4 and #5. (Indolealdehyde reacts only weakly with Salkowski reagent). The fate of this compound when added to the enzyme system was again studied - also that of indoleglycolic acid, indoleglyoxylic acid, and indolecarboxylic acid. The formulas and possible relationships between these compounds are shown below.



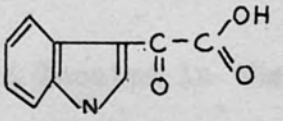
Indoleacetic acid.

+ O



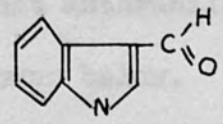
Indoleglycolic acid.

- 2H



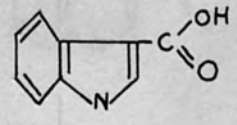
Indoleglyoxylic acid.

$- CO_2$



Indolealdehyde.

+ O



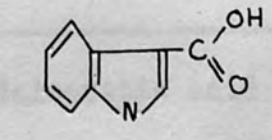
Indolecarboxylic acid.

According to Stutz, indoleglyoxylic acid, indolealdehyde and indolecarboxylic acid were not utilised by the enzyme. Indoleglycolic acid on the other hand gave rise to indolealdehyde - presumably as a result of simultaneous dehydrogenation and decarboxylation (since indoleglyoxylic/

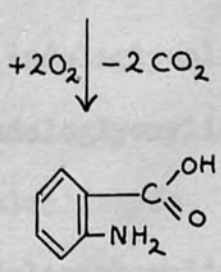
indoleglyoxylic acid had remained unattacked). The breakdown of C^{14}-labelled indoleacetic acid was also studied. When the label was in the 2 carbon atom of the indole ring. the radioactivity was largely retained and appeared in a number of the reaction products depending to some extent on the nature of the buffer used. These are shown in the table below.

Buffer	Compounds containing C^{14}
TRIS.	(Indoleacetic acid), # 2, # 4, Indolealdehyde.
Phosphate.	(Indoleacetic acid), # 4, Indolealdehyde, # 5.

The spot # 4 located in these experiments with 2-C^{14}-labelled indoleacetic acid was eluted and found to contain three or four components, one of which appeared to be an anthranilate. It is possible that anthranilic acid was derived from indolecarboxylic acid as shown below.



Indolecarboxylic acid.



Anthranilic acid.

Another compound was also present which was only located in the tracer experiments, This was strongly acidic and had an Rf lower than indoleacetic/

indoleacetic acid. The only groups detected by infra-red spectroscopy were the methylene and carboxyl groups - indicating an aliphatic nature. Finally, Stutz was also responsible for demonstrating that when the label was in the carbon atom of the carboxyl group, the tracer was almost completely lost, except that radioactivity was still present in the non-indolyl spot #2.

Melchior (1958) added indoleacetic acid to an acetone precipitated enzyme from cabbage leaves suspended in phosphate buffer pH 6.6. The products were extracted with ether and chromatographed using Isopropanol: Ammonia : Water (80:2:15). An attempt was made - by measuring spot size - to estimate the quantities of certain of the compounds present at various stages during the oxidation. The results of an experiment of this type-which lasted for twenty-four hours - are given in the table below. As a control indoleacetic acid was incubated in the absence of enzyme.

Time in hours.	0 (control)	2	4	8	12	24	24 (control)
		Planimeter units					
IES = indoleacetic acid	212	178	100	15	-	-	208
	Not	Planimeter units					Not
IGS(?) = indoleglycolic acid (?)	measure-able	28	46	-	-	-	measure-able.
		Micrograms					
IGyS = indoleglyoxylic acid.	-	6	8-10	-	-	-	-
		Micrograms					
IA = indolealdehyde	-	1	2	3	5	8-10	-
		Micrograms					
ICS = indolecarboxylic acid	-	?	?	?	1	2	-
"W ?" = unidentified compound probably identical with one detected in the photo destruction experiments described by Melchior (1957), i.e. "W"	-	+	+	++	++	+++	-

A complete list of products for the two hour incubation period was published - as shown below. Those not included in the first table (above) were present only in very small amounts.

Rf	Colour with Salper reagent	Colour with van Eck reagent	Identity
0.26	Orange - bright red	-	ICS
0.37	Pink (Masked by IES)	Yellow	IGyS
0.55 *	Red - violet *	-	IGS ?
0.56	Dirty Yellow	-	?
0.75	Violet	-	?
0.89	Bright brown	Yellow	IA
0.93	Red	-	"W ?"

* Data from Melchior (1957).

The Rf of indoleacetic acid was not given for this particular experiment but it was presumably slightly lower than that of indoleglyoxylic acid. After twenty-four hours incubation two further products were found in the reaction mixture. These were "O" which ran at the same Rf as indolealdehyde and "q" (Rf 0.85). "O" gave a yellow colour both with Ehrlich and van Eck reagents while "q" gave only a blue-green colour with Ehrlich reagent. The "W?" of these experiments was thought to be identical with the indole compound extracted into chloroform at pH 9.0 by Manning and Galston (1955). The compound IGS (?) was at first considered to be indoleglycolic acid, but as pointed out by Melchior (1957)/

(1957), this was not so, since Greenberg, Galston, Shaw and Armstrong (1957) showed that pure synthetic indoleglycolic acid was unstable in the alkaline chromatographic solvent used. Melchior (1958) also examined the products formed when indoleglyoxylic acid (IGyS), indolealdehyde (IA) and indolecarboxylic acid (ICS) were incubated with the enzyme. Unlike Stutz (1958), he found that all three were utilised - and gave rise to the products shown below.

Substrates:	IGyS	IA	ICS
Products:	IA ICS "W ?"	ICS "W ?"	"W ?"

Evidence for the enzymatic breakdown of indoleacetic acid "in vivo".

Tang and Bonner (1948) demonstrated the disappearance of indoleacetic acid from etiolated pea plants which had been infiltrated with a solution of synthetic indoleacetic acid. By infiltrating the seedlings with Salkowski reagent immediately after indoleacetic acid infiltration they obtained the pink Salkowski colour "in situ". When this was tried after a period of incubation however, little or no colour developed - showing that the indoleacetic acid had been destroyed. The same result was obtained when the indoleacetic acid contents of the tissues were determined using the Went Avena coleoptile curvature test. Fang and Butts (1957) injected

carboxyl/

carboxyl - C^{14} - labelled indoleacetic acid into the leaves of pea, bean and corn plants and were able to show that radioactive $C^{14}O_2$ was given off. In this case however it appeared at least possible, that in the dark the indoleacetic acid was being broken down in injured tissues, and also that in the light a non-enzymatic photo-oxidation was taking place. Andreae and van Ysselstein (1956) fed indoleacetic acid to pea stem sections. At concentrations below 10^{-4} M, no free indoleacetic acid could be detected in the tissues - while only one fifth of it could be found in the form of indoleaspartic acid. Hence it followed that the other four fifths had been destroyed - presumably by the "indoleacetic acid-oxidase" system. At concentrations above 10^{-4} M however, free indoleacetic acid was located, which, on transfer of the tissues to a moistchamber, was found to be almost quantitatively converted to indoleaspartic acid. This led tothe conclusion that certain tissues - the parenchyma of the pith and cortex - were devoid of indoleacetic acid-destroying activity, and that it was here that conjugation had taken place. They suggested that the degradation of indoleacetic acid was restricted to the epidermis and vascular tissues - that is to those tissues mentioned by van Fleet (1952) as containing high peroxidase activity. This idea was supported by the observation of Ebert (1955) that no Salkowski positive substance accumulated in the epidermis and root cap (another region with high peroxidase activity) when root tissues were incubated with indoleacetic acid.

The only evidence available for the supposition that endogenous indoleacetic acid was similarly broken down was necessarily very indirect. Pilet (1957), for example, found a correlation between the chloroform extractable endogenous auxin content of different zones of Lens roots and the "indoleacetic acid - oxidase" activities of extracts made from the corresponding tissues. Enzyme activity was relatively high and auxin content relatively low in the root cap and in the zone of elongation, while the enzyme activity was low and the auxin content high in the region of the apical meristem. (The inactivation of naturally occurring growth substance on incubation with plant extracts was demonstrated very early on - first by Thimann (1934) and also by Larsen (1936)).

The possible role of "indoleacetic acid - oxidase" in controlling extension growth.

Several authors have suggested that "indoleacetic acid - oxidase" was responsible for "dwarfism" in plants. Van Overbeek (1935) showed that extracts of dwarf corn plants destroyed auxin faster than those of non-dwarf ones - and also that the auxin contents of the tissues of the former were less. A similar higher auxin-destroying activity was claimed by Galston (1957) for dwarf peas - though von Abrams (1953) could demonstrate no significant difference. Secondly it was thought that the high auxin-destroying activity of etiolated shoots (as opposed to that of green ones) had some significance in protecting the tissues from/

from the effects of "excess" auxin. Thus, although etiolated pea epicotyls in fact contained more auxin than green ones, and showed - presumably as a consequence - the phenomenon of "hyper-elongation", Tang and Bonner (1948) suggested that, if it were not for the high "indoleacetic acid - oxidase" activity of the etiolated tissues, the concentration of auxin in them would be even higher. Again, Tang and Bonner suggested that the high oxidising activity which they found in roots - compared with that in shoots - was effective in protecting the roots from the inhibitory effect of auxin. (An inhibitory effect of "applied auxin" on roots was demonstrated by Kögl, Haagen-Smit and Erxleben (1934).) A more recent idea was that of Galston and Dalberg (1954) who suggested that in the development of all plant cells, the change over from elongation to maturation, was controlled by the disappearance of auxin. This disappearance they attributed to an enhanced "indoleacetic acid - oxidase" activity in the tissue. In support of this they showed that as one passed backwards from the root or shoot meristem of peas, into the elongating zone, an increase in "indoleacetic acid - oxidase" activity was encountered (when the activity was expressed "per unit protein nitrogen"). A similar gradient of activity was observed in Lens roots by Pilet and Galston (1955).

A variety of explanations were offered to account for the differences in auxin-destroying activity in the examples just described. The extracts of dwarf pea plants were shown to have a higher peroxidase activity (van Overbeek, 1935), and a lower inhibitor content (Galston, 1957/

1957), than those of non-dwarf ones. The low "indoleacetic acid - oxidase" activity of green tissues appeared to be fairly definitely related to the formation of large amounts of an inhibitor in light. However Tang and Bonner (1948) carried out an experiment in which it was shown that the ammunt of enzyme extractable with acetone from etiolated tissues decreased slightly when these were exposed to light. (More recently Hillman and Galston (1957) showed that when etiolated pea epicotyls were treated with red light for a period of only one hour, the "indoleacetic acid - oxidase" activity of extracts made from the buds, was very much decreased - owing to the formation of a powerful inhibitor of the enzyme. The red light treatmentwas effective, even when carried out sixteen hours before harvest, yet it could be reversed by a subsequent four minute treatment with infra-red light - provided that this was given immediately after the red light treatment). The observed gradient in auxin-destroying activity extending from the meristem into the elongating zone has been attributed both to a gradient in inhibitor concentration (Galston, 1957) and also to a gradient in activator level (Pilet and Galston, 1955).

The "adaptive formation" of "indoleacetic acid - oxidase".

In addition to assuming that the cessation of elongation and the onset of maturation in developing cells was the result of an increase in "indoleacetic acid - oxidase" activity, Galston andDalberg (1954) also proposed that this increase was caused by the "adaptive formation" of/

of the enzyme "indoleacetic acid - oxidase" in response tothe continued supply of indoleacetic acid transported to the tissue from the apex. Once sufficient "indoleacetic acid - oxidase" had been formed, the cell would then be insensitive to the growth promoting activity of the auxin reaching it.

These authors stated (Galston and Dalberg, 1954) that, "In order to establish the adaptive nature of a particular enzyme, at least two criteria must be fulfilled:-

a) It must be shown that the removal of the substrate from the medium proceeds slowly at first; then as the enzyme for this substrate is formed, its disappearance becomes rapid.

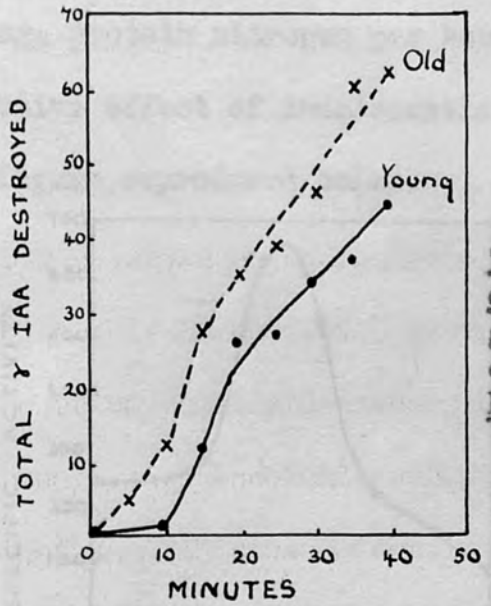
b) It must be shown, in vivo, and if possible in vitro, that enzymatic activity is absent or low in the absence of the substrate and higher in thepresence of the substrate. "

They claimed also tohave established both these for the "indoleacetic acid - oxidase" enzyme in pea tissue.

The evidence for a) above was as follows:-

500 mg. of tissue composed of sections taken from the growing zone of the 3rd internode of 8-day old etiolated pea seedlingswere incubated in each of 8 beakers containing 3 ml. of a solution containing indoleacetic acid (2×10^{-4} M) and dichlorophenol (2×10^{-5} M) and M/60 phosphate buffer pH 6.1. At "zero time" and at 5 minute intervals thereafter, 2 ml. aliquots were removed from one of the beakers/

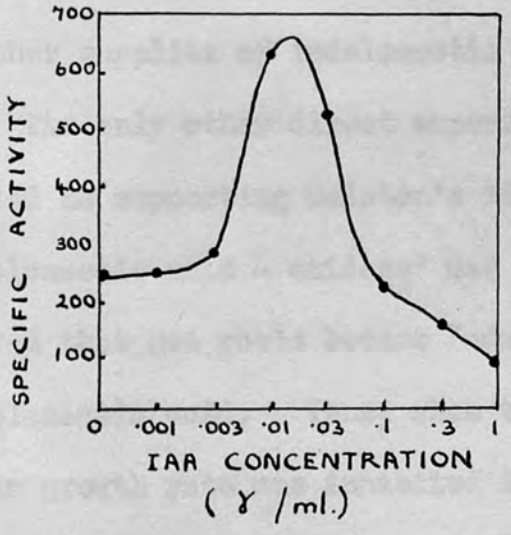
beakers and residual indoleacetic acid determined using the Salkowski colorimetric method. A lag period of 10 minutes was observed before the onset of rapid breakdown. When the same procedure was carried out using sections taken from the non-growing region of the internode however, no lag period was observed. The figure published by Galston and Dalberg is reproduced below.



The kinetics of indoleacetic acid destruction in vivo by young, rapidly growing and by older, slower growing epicotyl sections. (Galston and Dalberg, 1954).

The experimental evidence put forward by Galston and Dalberg for b) was that after "root, stem and terminal bud tissues of the etiolated pea seedlings" had been incubated with 10^{-7} M indoleacetic acid solution for a period of 1 hour, the "indoleacetic acid - oxidase" activity of brei made from these tissues was higher than that made from control tissues. The only actual results quoted were those obtained using terminal bud tissues and a wide range of "inducing" indoleacetic acid concentrations. 300 mg. replicates of tissue were incubated in 10 ml. M/60 /

M/60 pH 6.1 phosphate buffer containing the appropriate concentration of indoleacetic acid. After 1 hour the tissues were removed from the solutions and extracts made from them. "Indoleacetic acid - oxidase" activities were determined, and also protein nitrogen contents. The "indoleacetic acid-oxidase" activity of each extract was then expressed in terms of "specific activity", that is, "µg indoleacetic acid destroyed per mg. protein nitrogen per hour". An "optimum concentration" for the inductive effect of indoleacetic acid was thereby found - as shown in the figure reproduced below.



The relation between indoleacetic acid concentration supplied to tissue and the subsequent indoleacetic acid - oxidase activity. (Galston and Dalberg, 1954).

Galston and Dalberg stated that, "Protein levels remained constant, so that the altered activities shown in the figure (see above) must represent the formation of enzymatically active protein". They also stated that the inductive effect of indoleacetic acid was demonstrated also "in vivo" - but no details were given.

On the assumption that endogenous indoleacetic acid also produced enzyme activity adaptively - a premise which was incapable of direct proof/

proof or disproof - Galston and Dalberg extended their ideas on the subject of the physiological role of "indoleacetic acid - oxidase" to include not only the phenomenon of aging (that is "decreased sensitivity to applied auxin"), but alsothe phenomena of apical dominance and endogenous rhythms. In apical dominance for example, they suggested that the growth of lateral buds was inhibited by the indoleacetic acid passed back to them by the apical bud. This auxin was thought to cause the adaptive formation of "indoleacetic acid - oxidase" activity in the buds, thus rendering them insensitive to the growth-promoting action of further supplies of indoleacetic acid.

The only other direct experimental evidence which might be interpreted as supporting Galston's idea of the adaptive formation of indoleacetic acid - oxidase" was that of Bakhsh (1956). This author showed that pea roots became "adapted" to growing in solutions of indoleacetic acid. Thus, when transferred to indoleacetic acid solution, their growth rate was inhibited initially but later recovered. Similar observations were made by Burström (1957). Bakhsh then grew pea seedlings in water and in indoleacetic acid solution and demonstrated that the extracts of the "indoleacetic acid - grown" roots possessed significantly increased indoleacetic acid destroying activities.

The aim of the present investigation.

The present work was carried out with a view to clarifying the position asregards the possible adaptive formation of the enzyme "indoleacetic acid - oxidase" in pea roots. An attempt was made to confirm/

confirm the reports of Galston and Dalberg (1954) and Bakhsh (1956) that the incubation of tissues in the presence of low concentrations of indoleacetic acid, caused an increase in the "indoleacetic acid - oxidase" activities of the extracts subsequently made from them. The material and procedures used were almost identical with those of Bakhsh (1956). Thus etiolated pea plants were grown in water and in indoleacetic acid solution 10^{-7} g./ml. for two days. Aqueous extracts of the roots were then prepared, and the "indoleacetic acid - oxidase" activities of these extracts determined in reaction mixtures containing - in addition to the enzyme extract and indoleacetic acid - only phosphate - citrate buffer and the activator 2,4 - dichlorophenol. A few experiments were also done to compare the so-called "in vivo" activity of "indoleacetic acid - grown" and "water - grown" pea roots. The activity of "ambient" solutions - from which the tissues had been removed, was also tested.

Since the activity of the extracts prepared from the "indoleacetic acid - pretreated" tissues was found to be higher than that of the "non-treated controls", an explanation of this difference was sought by comparing the inhibitor levels of the two types of extract. It was recognised that at least three other factors might have been involved, namely,

1. a difference in peroxidase content,
2. a difference in activator level,
3. a difference in endogenous manganese concentrations.

Nevertheless there was already at least some precedent for the idea that inhibitor/

inhibitor levels were particularly important in pea tissues. Thus, as mentioned previously, the presence of the inhibitor has frequently been demonstrated in crude extracts (for example Tang and Bonner 1948; Galston 1957) while variations in inhibitor level in epicotyl tissues have been found to take place in response to light treatments (Tang and Bonner, 1948; Hillman and Galston, 1957).

Chapter 2. METHODS

I. Cultivation of plants.

Seeds of Pisum sativum var. Meteor were soaked in water for 24 hours in a soaker of the type described by Audus (1956) and germinated for 48 hours in earthenware seed pans containing moist, sterile sand. The seedlings were transferred to stone tanks containing either a) tap water or b) indoleacetic acid solution 10^{-7} g./ml. (made up in tap water). The seeds were supported just above the level of the culture medium by perforated bakalite sheets through which the roots were threaded. A drawing of these tanks wasgiven by Bakhsh (1956). A further growth period of 48 hours at 25 ± 0.5°C in the dark was then allowed. The water and indoleacetic acid solution was renewed after 12, 24 and 36 hours.

The transfer of seedlings from seed pans to tanks, the renewing of the culture solutions and harvesting were carried out using only a weak green light - produced by using a 15 watt bulb and a Kodak "Wratten Series" OA filter.

Photographs of the roots at three stages in their development are shown in figures 1 - 3. The germinated seedlings are shown in figure 1. The plants in figure 2 had been grown in water (figure 2a) or in indoleacetic acid solution (figure 2b) for one day. The roots of the latter were easily recognisable since they were shorter and slightly broader. They also showed a characteristic swelling just behind the apex. The plants in figure 3 had been grown in water (figure 3a) or in indoleacetic acid/

acid solution (figure 3b) for two days. The roots of both types of plant had increased considerably in length, while the differences mentioned above were again very striking. The swelling shown by the roots grown in indoleacetic acid solution was however located at a greater distance from the apex than in the one day old roots.

Figure 1. Photograph of pea seedlings germinated for 48 hours in moist sand. (Approximately natural size. Length of scale = 5 cm.).

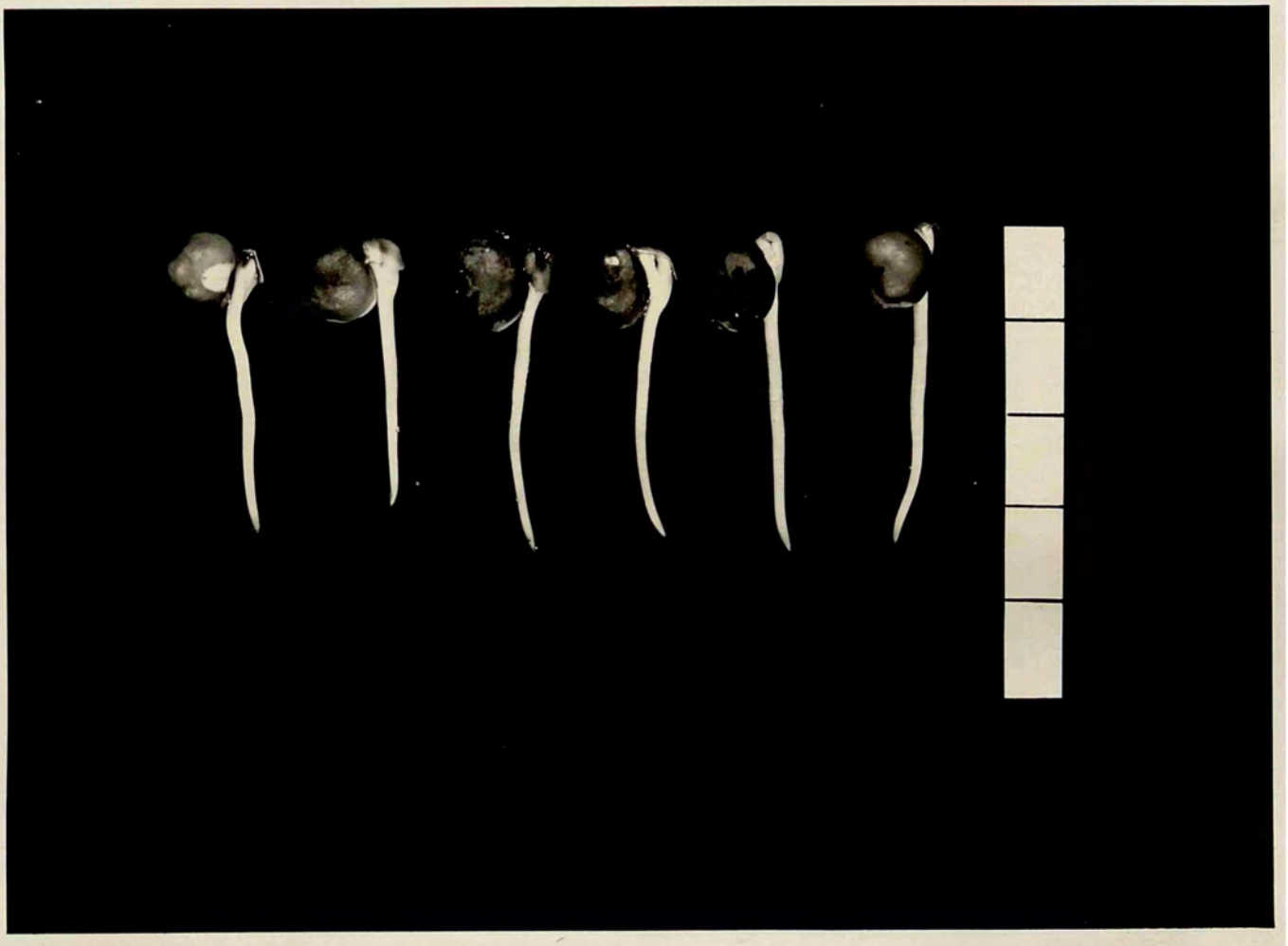
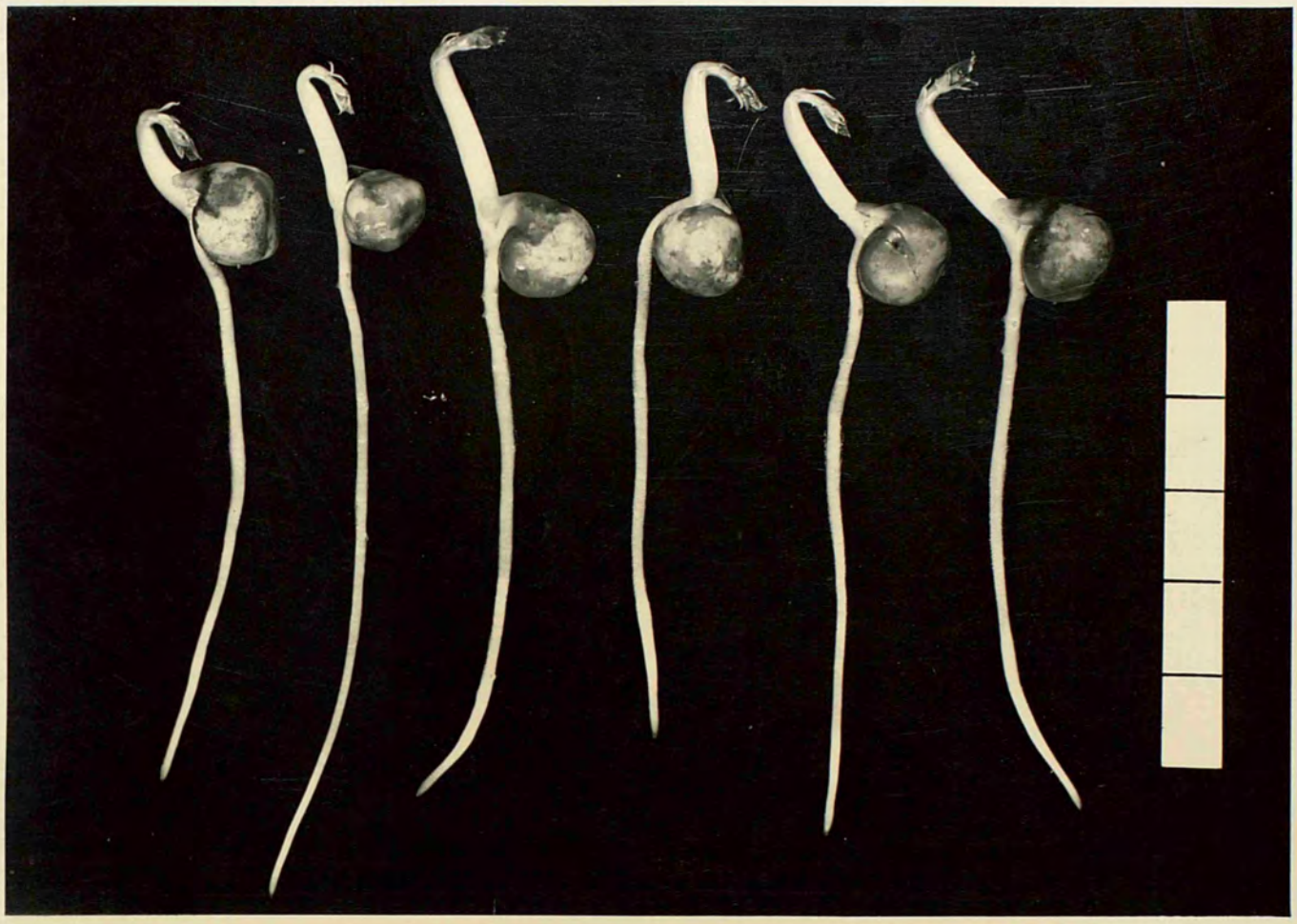


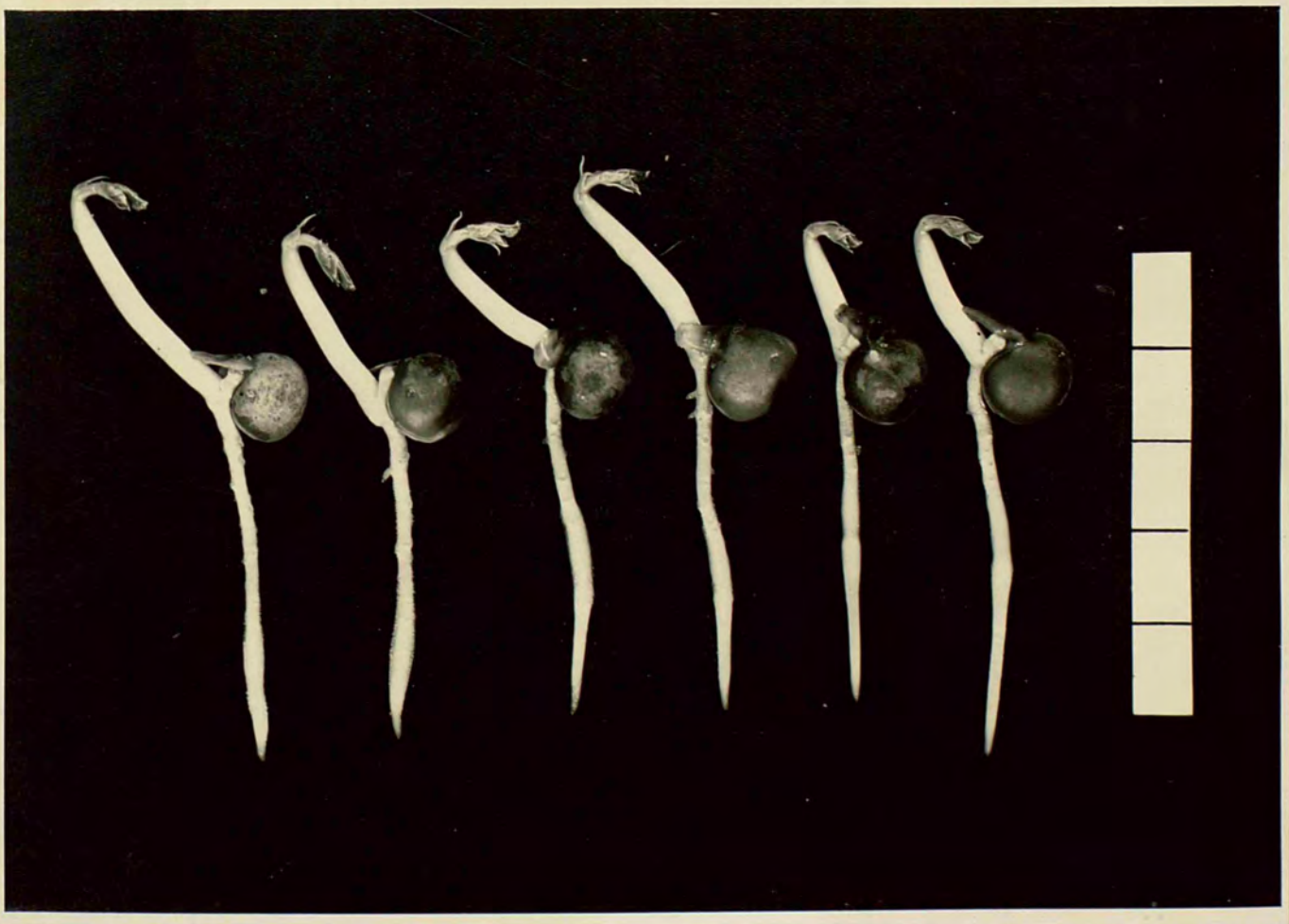
Figure 2a. Photograph of pea seedlings germinated for 48 hours in moist sand and then grown for a further period of 24 hours in water - in darkness. (Approximately natural size).

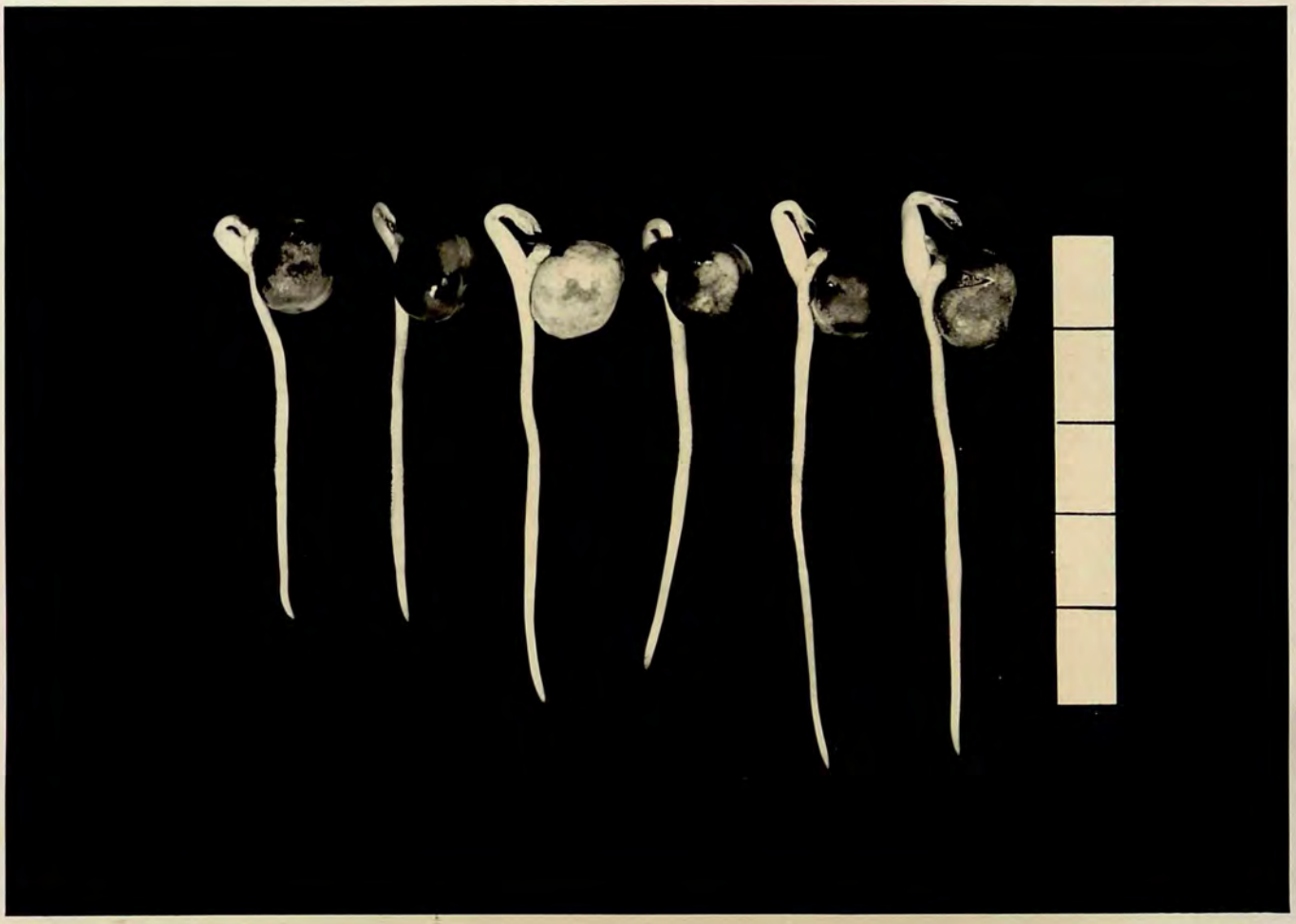
Figure 2b. Photograph of pea seedlings germinated for 48 hours in moist sand and then grown for a further period of 24 hours in indoleacetic acid solution 10^{-7} g./ml. - in darkness. (Approximately natural size).

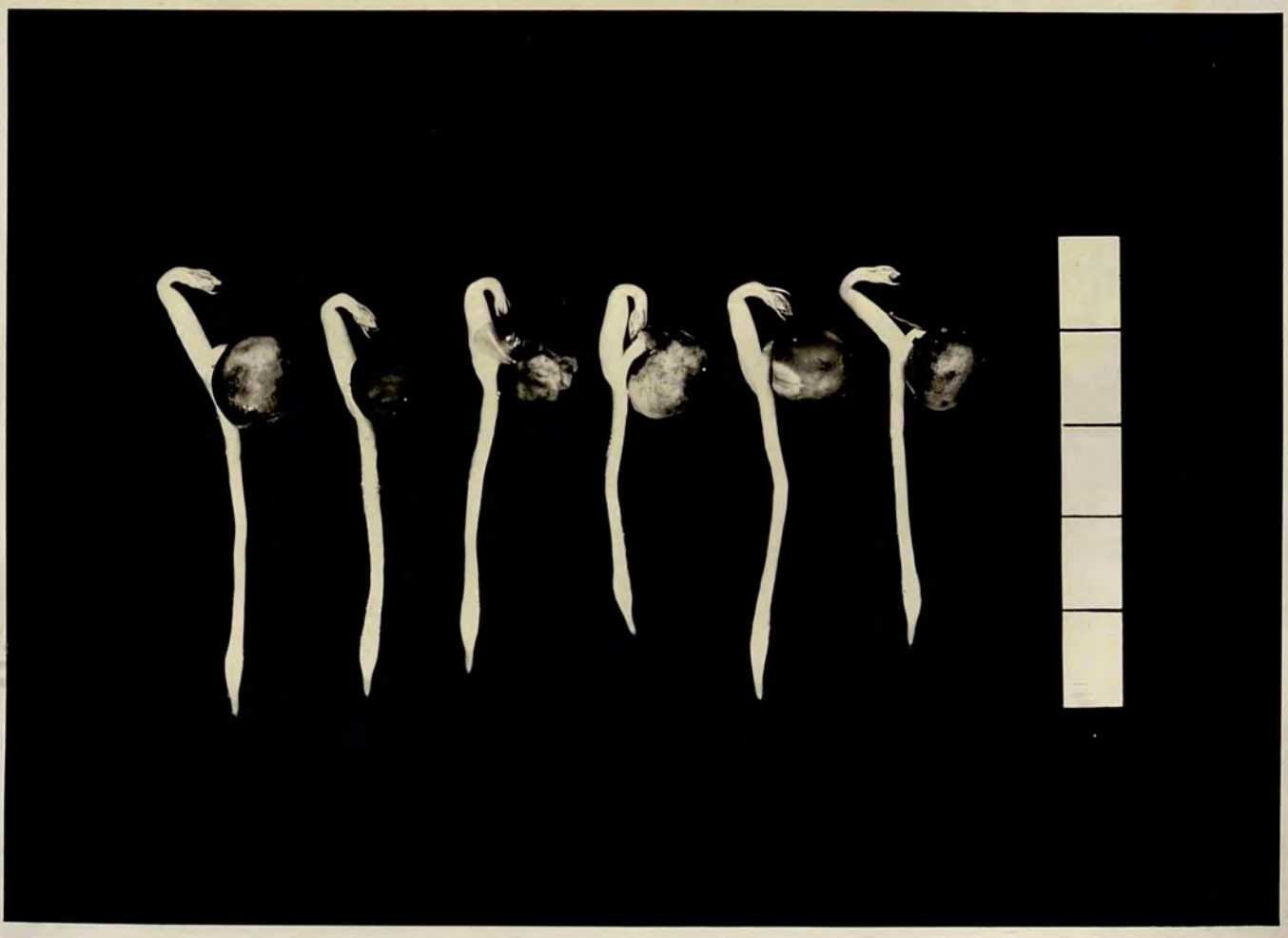
Figure 3a. Photograph of pea seedlings germinated for 48 hours in moist sand and then grown for a further period of 48 hours in water - in darkness. (Approximately natural size).

Figure 3b. Photograph of pea seedlings germinated for 48 hours in moist sand and then grown for a further period of 48 hours in indoleacetic acid solution 10^{-7} g./ml. - in darkness. (Approximately natural size).









II. Preparation of enzyme extracts.

Aqueous extracts were prepared from the roots of plants grown in a) water or b) indoleacetic acid solution (see above) by the following method:-

Whole roots were harvested, rinsed with distilled water and blotted dry. One g. samples were ground for 1 minute in a mortar (previously chilled in the deep freeze for 5 minutes) with 2 ml. distilled water. The extract was passed through 2 layers of cheese - cloth with 2 water rinses, centrifuged at 2,500 r.p.m. for 30 minutes and made up to 150 ml. with distilled water. The extract was then stored in a refrigerator at + 2°C.

III. Quantitative estimation of indoleacetic acid.

1. Colorimetric method.

The method was based on a reaction first reported by Salkowski (1885). He discovered that when ferric iron and a mineral acid (HCl) were added to a solution of indoleacetic acid, a pink colour was produced. Since a linear relationship has been shown to exist between the intensity of this pink colour and the concentration of the indole-acetic acid solution - over a limited concentration range - the reaction may be used for the quantitative estimation of indoleacetic acid.

A reagent containing ferric chloride and concentrated sulphuric acid (Salkowski reagent) was used extensively by Tang and Bonner (1947, 1948) for the determination of residual indoleacetic acid in measurements of/

of the "indoleacetic acid - oxidase" activity of aqueous extracts of pea tissues and an identical method was used by Bakhsh (1956). However in this investigation a modified reagent containing perchloric acid was used. This is frequently referred to as "Salper reagent" and has, according to Gordon and Weber (1951), the advantages of increased sensitivity and specificity. The proportions recommended by these authors were as follows:-

2 ml. Salper reagent (containing 3 ml. of 0.5M. $FeCl_3$ in 50 ml. 35% $HClO_4$)	:	1 ml. of a solution containing 0 - 45 µg. indoleacetic acid.

In the present investigation, however, a diluted reagent was prepared by adding distilled water to the Salper reagent in the proportions of 1 part distilled water to 4 parts Salper reagent. To 2.5 ml. of the resulting solution (which will be referred to as "diluted Salper reagent") was added 0.5 ml. of a solution containing from o - 25 µg. of indoleacetic acid. The reason for this change was that the initial concentration of indoleacetic acid in the reaction mixtures was usually 50 µg. indoleacetic acid / ml. whereas the calibration curve for indoleacetic acid is linear only up to about 30 µg. indoleacetic acid per sample.

The experimental procedure in the routine determination of indoleacetic acid concentration is given below:-

To 2.5 ml. of diluted Salper reagent (Undiluted Salper reagent containing 3 ml. 0.5M. $FeCl_3$ in 50 ml. 35% perchloric acid 4 parts : distilled water 1 part) in a 2" x 1" glass specimen tube was added 0.5 ml. of an aqueous solution containing up to 25 µg. indoleacetic acid/

acid. The mixture was shaken immediately after the addition and the colour allowed to develop at room temperature in diffuse daylight (the colour was found to fade rapidly in bright sunlight) for a minimum period of 1 hour (and a maximum of 2½ hours), and the absorbancy read at 525 mμ using a "Unicam S.P. 350 Diffraction Grating Spectrophotometer". A "blank" prepared by adding 0.5 ml. distilled water to 2.5 ml. diluted Salper reagent was used as a standard for each reading.

"Wavelength-absorbancy" curves, data for the rate of colour development and a calibration curve for synthetic indoleacetic acid are given under "Results", Section I.

2. Chromatographic method.

20 ml. of reaction mixture (for details of the composition of the reaction mixtures and conditions of incubation see "Results", Section I) was acidified to pH 3.0 by the addition of 0.2 ml. NHCl. It was then shaken 3 times for 10 minutes with 40 ml. diethyl ether. The extract was dried overnight over solid sodium sulphate at -14°C.

The ether extract was evaporated to dryness under reduced pressure. The residue was taken up in 0.5 ml. methanol and spotted on 41.5 cm. x 46 cm. sheets of Whatman No. 1 filter paper - using fine glass capillary tubes. "Marker spots" of synthetic indoleacetic acid dissolved in methanol were applied similarly.

30 minutes was allowed for equilibration and the chromatograms were developed in one direction by the descending technique using the alkaline solvent:-

isopropanol	80	)	
		)	
water	15	)	for 16 hours
		)	
ammonia	2	)	

(The neutral solvent:-

isobutanol	80	)	
		)	
methanol	5	)	was also used in qualitative
		)	
water	15	)	

experiments. The development time was again 16 Hours).

When dry the chromatograms were sprayed with

Salper reagent. 1. ml. 0.5 M. $FeCl_3$ in 50 ml. 35% $HClO_4$.

(In qualitative experiments,

Ehrlich reagent. 1% p-dimethyl-amino-benzaldehyde in N. HCl.

and Van Eck reagent. 5% benzidine in acetic acid were also used).

The quantities of IAA present were determined by measuring the area of the IAA spots using a planimeter.

IV. 1. Determination of the "indoleacetic acid - oxidase" activity of enzyme extracts.

The "indoleacetic acid - oxidase" activity of the enzyme extracts was determined in reaction mixtures which contained - in addition to enzyme and substrate - buffer solution and 2,4-dichlorophenol. Details of the composition of the reaction mixtures are given under "Results", Sections I, II, III and VI:

The reaction was carried out in boiling tubes held in a water bath maintained/

maintained at 25°C. Enzyme extract and buffer were incubated in the water bath for a period of 15 minutes before the addition of the 2,4-dichlorophenol and indoleacetic acid at "zero time". 2,4-dichlorophenol was not incubated with the enzyme on account of the report of Furuya and Galston (1959) that prior incubation of the enzyme with 2,4-dichlorophenol, at least under certain conditions, led to an inhibition of the indoleacetic acid oxidation. The 2,4 - dichlorophenol and indoleacetic acid solutions were prepared separately and not as one solution (cf. Bakhsh, 1956).

Reaction mixtures were sampled at "zero time" and at various intervals thereafter (for sampling intervals and periods of incubation see "Results", Sections I, II, III and VI.) The concentration of indoleacetic acid in the samples was determined by a colorimetric method (for which the volume of the sample taken was 0.5 ml.) or a chromatographic method (volume of sample 20 ml.), (See "Methods", Sections III-1 and III-2)

2. Determination of the "indoleacetic acid - oxidase" activity of whole roots.

The "indoleacetic acid - oxidase" activity of whole roots which had been harvested from etiolated pea seedlings grown as described under "Methods", Section I, was determined. In addition the roots of plants which had been grown for 1 day and 3 days were also used (See "Results", Section IV).

12 roots of any one type were harvested and suspended in a 20 ml. quantity of a mixture which contained:-

15 ml. phosphate - citrate buffer pH 4.0.

2.5 ml. indoleacetic acid solution 4×10^{-4} g./ml.

2.5 ml. 2,4-dichlorophenol solution 4×10^{-4} g./ml.

The only difference between this mixture and the reaction mixture used in the determination of the indoleacetic acid oxidase activity of the enzyme extracts was that here 15 ml. buffer was present instead of 5 ml. buffer and 10 ml. enzyme extract. The incubation was carried out at laboratory temperature in 2" x 1" glass specimen tubes. The roots were threaded on to a thin wire about 4.5" long. (The wire was pushed through the ends furthest from the root apices). The wire with the attached roots was bent into the form of a loop and the free ends hinged on to the edge of the specimen tube so that the roots were supported and immersed in the solution up to a level just below the wire. Care was taken to see that the cut ends of the roots did not come into contact with the solution. At "zero time" and at intervals thereafter (for sampling intervals and periods of incubation see "Results", Section IV), 0.5 ml. samples of the solution were removed and the indoleacetic acid concentrations determined colorimetrically (See "Methods, Section III-1).

3. Determination of the "indoleacetic acid - oxidase" activity of "ambient solutions" - solutions in which whole roots had been incubated.

The "indoleacetic acid - oxidase" activities of "ambient solutions" - prepared by incubating the roots of etiolated pea plants grown as described/

described under "Methods", Section I, in a mixture of the following composition[#] :-

(15 ml. phosphate - citrate buffer pH 4.0.
(
(2.5 ml. 2,4-dichlorophenol 4×10^{-4} g./ml.

was determined. Twelve roots of any one type were suspended in the mixture as described under "Methods", Section IV -2. After periods of 1 hour, 3 hours and 24 hours, 4.4 ml. quantities of the "ambient solution" were removed and 0.6 ml. indoleacetic acid solution 4×10^{-4} g./ml. added to each. (This gave a reaction mixture in which the concentrations of buffer, 2,4-dichlorophenol and indoleacetic acid were approximately the same as in the reaction mixture used in the experiments described in the previous subsection ("Methods", Section IV - 2). At "zero time" - that is immediately following the addition of the indoleacetic acid solution - and at intervals thereafter (for sampling intervals and periods of incubation see "Results", Section V), 0.5 ml. samples of the solution were removed and the indoleacetic acid concentrations determined colorimetrically (See"Methods", Section III-1).

(# In a further experiment - in which only roots grown in water were used, the effect of

1/ decreasing the number of roots to 6 per specimen tube and

2/ increasing the 2,4-dichlorophenol concentration to 10^{-3}g./ml.

was determined. The activity of the "ambient solutions" was determined after a period of 24 hours, as described above.)

V. 1. Treatment of the enzyme extracts by high-speed centrifuging.

Samples of enzyme extract were centrifuged at 10,000 r.p.m. for 20 minutes at 10°C in a refrigerated centrifuge.

2. Treatment of the enzyme extracts by dialysis.

Samples of enzyme extract were dialysed in dialysis sacs made from 10 - 15 cm. lengths of Visking seamless cellulose tubing ¼" in diameter. The tubing was thoroughly wetted with distilled water before use and the two ends of each sac were tied with cotton. The sacs were immersed in water in beakers of 1 litre capacity, which were placed in a refrigerator at +2°C for 3 days.

VI.1. Preparation of inhibitor solutions from the enzyme extracts by boiling.

25 ml. quantities of the enzyme extracts were placed in 50 ml. beakers and boiled for a period of 5 minutes. The boiled extracts were then allowed to cool down to laboratory temperature and made up to volume (25 ml.) with distilled water. These were then used as inhibitor preparations.

2. Preparation of inhibitor solutions from the enzyme extracts by seitz filtration.

25 ml. quantities of the enzyme extracts were passed through a seitz filter pad in order to remove any bacteria present. The filtrates obtained werefound to be devoid of enzyme activity, andwere used as inhibitor preparations.

3. Preparation of inhibitor solutions from the enzyme extracts by ultrafiltration.

The technique used for ultrafiltration was the one described by Smith (1958). The enzyme extracts were placed in "thimbles" made from Visking seamless cellulose tubing and the ultrafiltrate obtained by sucking the water and dissolved substances of small molecular weight through the membrane by reducing the pressure around the thimble. This wasdone by attaching an evacuated desiccator to the system.

The apparatus used is shown in figure 4. A piece of the cellulose tubing, (approximately 10 cm. long and $\frac{1}{4}$" in diameter), moistened with distilled water, was sealed at one end by a knot. The other end was then slipped over the end of a piece of glass tubing which passed through a rubber bung into the glass "evaporating tube" with the side arm. It was secured in position with a length of cotton. The enzyme extract was then placed in the cellulose tubing using a pipette and the vacuum turned on momentarily between each small addition - thus inflating the membrane. When the thimble was full, the end of the glass "filling tube" was connected, either by means of rubber tubing or of polythene tubing, to the small glass tube containingwater. The vacuum was then turned on, and, provided that there were no leaks in the system (the latter indicated by a flow of air bubbles through the water in the small tube), the apparatus was left for several hours - the vacuum in the desiccator being renewed from time to time. About 0.5 to 1.0 ml. of ultrafiltrate was thus obtained after about 3 hours.

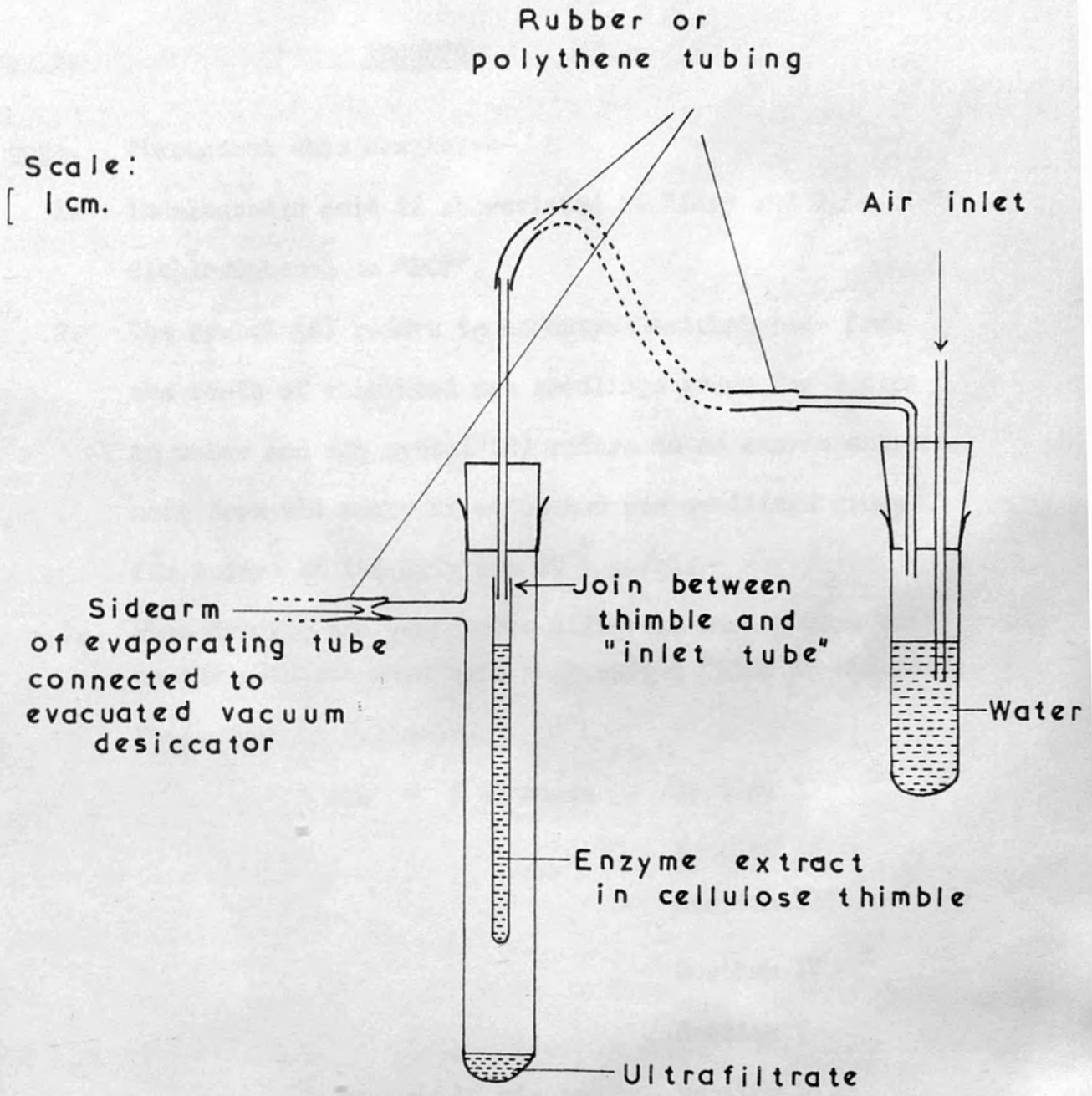


FIG. 4 ULTRAFILTRATION APPARATUS.

Chapter 3. RESULTS

Note. Throughout this chapter:-

1. Indoleacetic acid is abbreviated to "IAA" and 2,4-dichlorophenol to "DCP".
2. The symbol (A) refers to an enzyme extract made from the roots of etiolated pea seedlings grown for 2 days in water and the symbol (B) refers to an enzyme extract made from the roots of etiolated pea seedlings grown for 2 days in IAA solution 10^{-7} g./ml.
3. When drawing the graphs two different scales were used on the abscissa when this represented "time in minutes". These were as follows:-

1 cm. = 5 minutes.	Section I
	Section II
	Section III
	Section IV
	Section V
1 cm. = 10 minutes.	Section VI.

The scale on the ordinate when this represented "absorbancy at 525 mμ" was not varied.

I. A COMPARISON OF THE "IAA-OXIDASE" ACTIVITIES OF EXTRACT (A) MADE FROM THE ROOTS OF ETIOLATED PEA SEEDLINGS GROWN FOR 2 DAYS IN WATER AND EXTRACT (B) MADE FROM THE ROOTS OF ETIOLATED PEA SEEDLINGS GROWN FOR 2 DAYS IN IAA SOLUTION 10^{-7} G./Ml.

1. Comparison of the "IAA-oxidase" activities of extracts (A) and (B) using a colorimetric method.

Extracts (A) and (B) were prepared as described under "Methods", Section II, and their "IAA-oxidase" activities compared at pH 4.0 in the presence of 2,4-dichlorophenol as described under "Methods", Sections IV-1. and III-1. In the first instance, reaction mixtures identical with those of Bakhsh (1956) were used. However in addition two slightly different reaction mixtures were prepared in which the enzyme extract had been diluted further with distilled water before use. Before describing this experiment in detail, the results of three preliminary experiments will be given.

1st Preliminary experiment. Wavelength-absorbancy curves for IAA.

Solutions of IAA were prepared containing 10, 30 and 50 µg. IAA/ml. 0.5 ml. of each was added to 2.5 ml. diluted Salper reagent and the pink colours allowed to develop for 1 hour. The absorbancy of the solutions was then measured over the widest possible wavelength range in the visible spectrum, readings being taken at 10 mµ. or 5 mµ. intervals. The results over the wavelength range 440-590 mµ. are shown in figure 5. The curves indicated that with increase in wavelength the absorbancy rose to a maximum which fell in the region between 510 and 530 mµ. In all subsequent work absorbancy readings were made at 525 mµ.

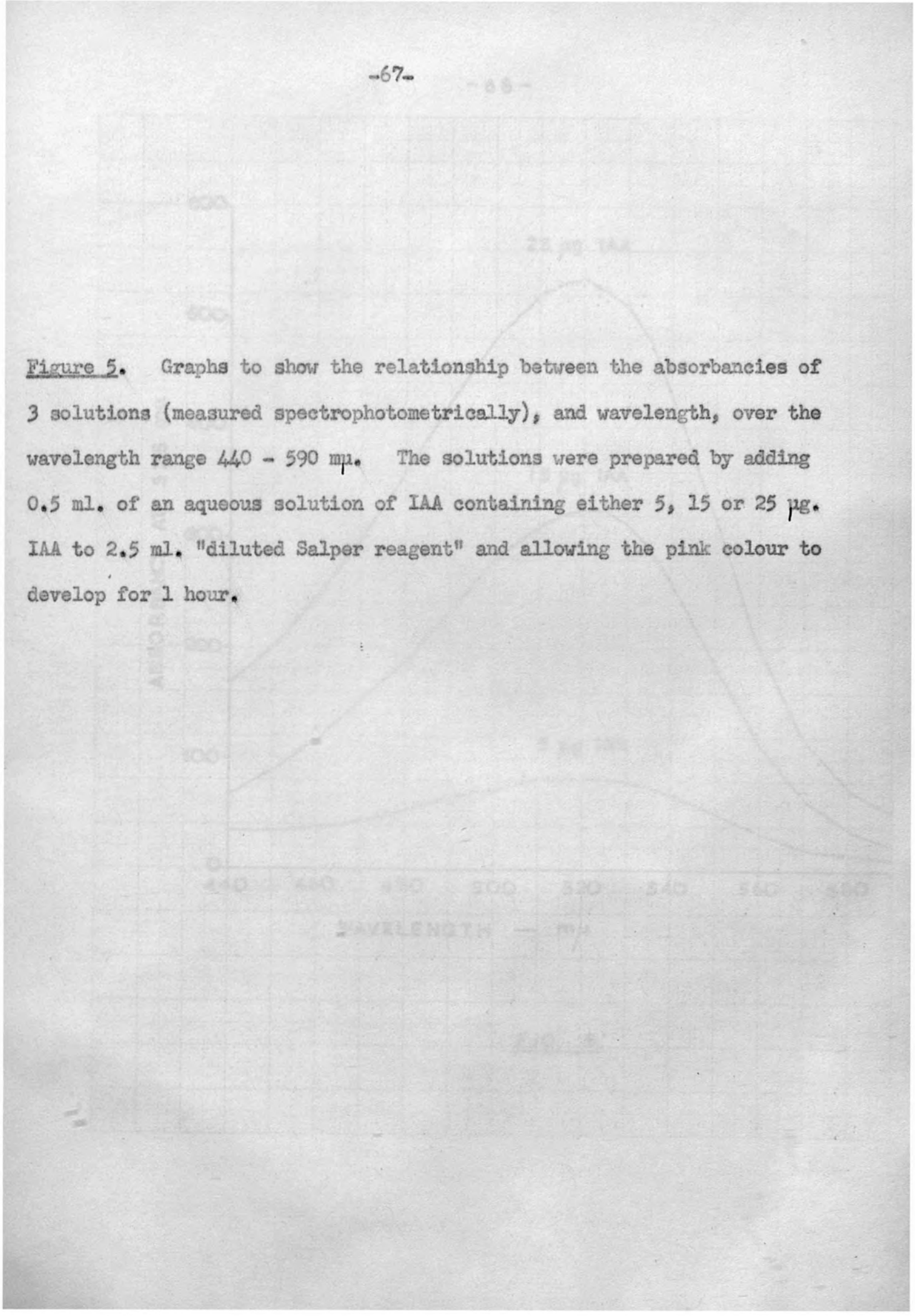


Figure 5. Graphs to show the relationship between the absorbancies of 3 solutions (measured spectrophotometrically), and wavelength, over the wavelength range 440 - 590 mμ. The solutions were prepared by adding 0.5 ml. of an aqueous solution of IAA containing either 5, 15 or 25 μg. IAA to 2.5 ml. "diluted Salper reagent" and allowing the pink colour to develop for 1 hour.

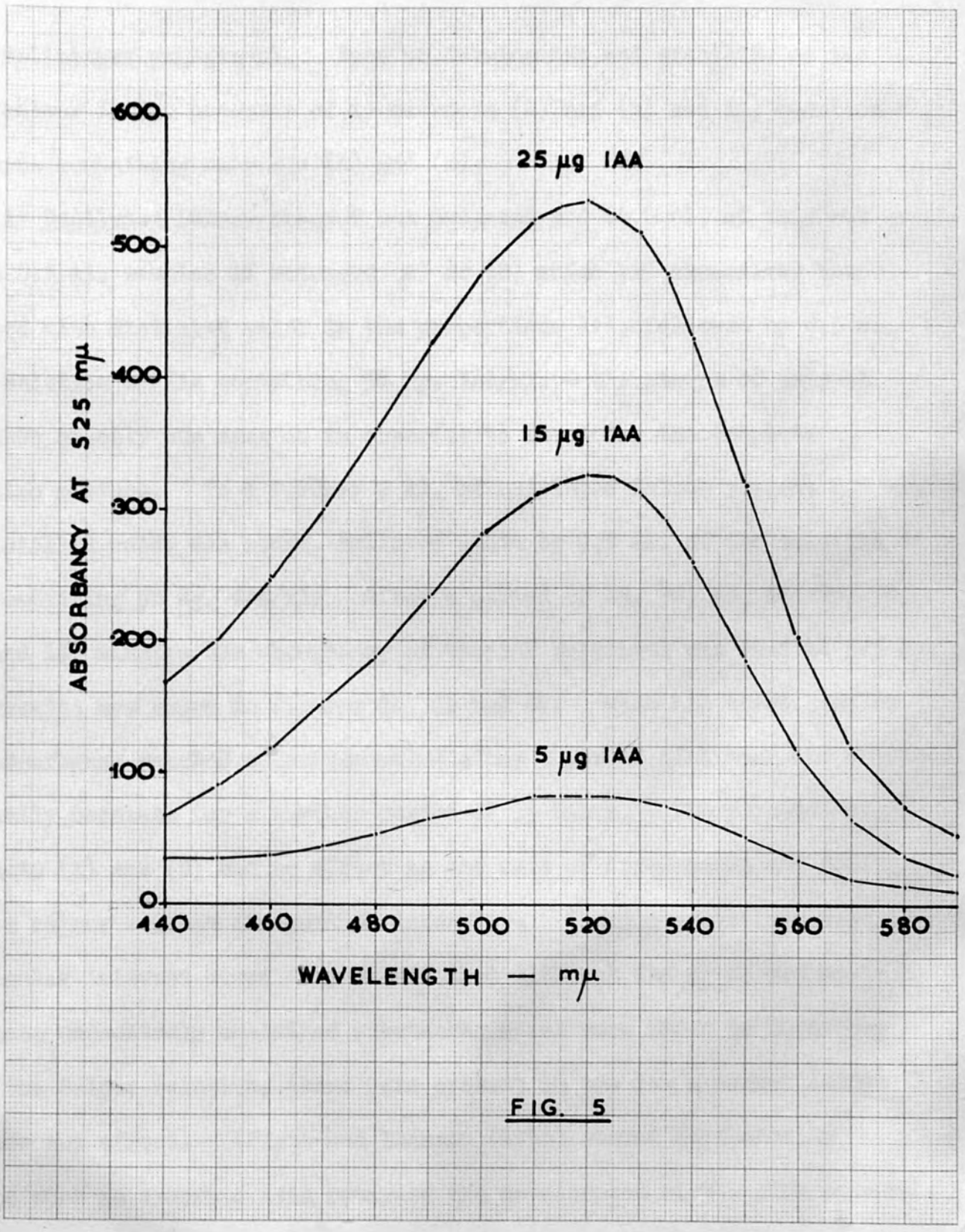

25 µg IAA
15 µg IAA
5 µg IAA
ABSORBANCY AT 525 mµ
600
500
400
300
200
100
0
440
460
480
500
520
540
560
580
WAVELENGTH — mµ

FIG. 5

2nd Preliminary experiment. Rate of development and stability of the pink colour in the presence of i) extracts (A) and (B) and ii) reaction mixtures containing extracts (A) and (B).

i) Undiluted Salper reagent was prepared and to 2 ml. of this was added 0.5 ml. samples of extracts (A) or (B) which had themselves been diluted with distilled water in the proportions 1:1, followed by 0.5 ml. of a solution of IAA containing 50 µg. IAA/ml. - the amount of extract was thus exactly the same as in experiments involving the complete reaction mixture. To a further 2 ml. of undiluted Salper reagent 0.5 ml. of distilled water was added, followed again by 0.5 ml. of the solution of IAA containing 50 µg. IAA/ml. After a period of 15, 30, 45, 60, 75, 90, 120 and 150 minutes the absorbancy of thepink solutions was read at 525 mµ. The results are shown in figures 6a, 6b and 6c. With all three samples the absorbancy reached a maximum value after a period of 1 hour and remained virtually constant for a further period of 1½ hours. The presence of extracts (A) and (B) had no effect on the rate of development and fading of the colour - or on its maximum intensity. These observations were of particular interest since they showed that although the crude pea root extracts undoubtedly contained substances which were known to interfere with the Salper reaction, these were present in too low a concentration to have any effect. (Platt and Thimann (1956) showed that reducing agents such as ascorbic acid retarded the development of the pink colour, while certain phenols such as catechol and resorcinol affected not the rate of colour development, but the final absorbancy value. On the basis/

basis of these results they stated that, "Reducing agents and polyphenols are so widespread in plants, and so drastic in their Salkowski interference, that the Salkowski assay, either in solution or on chromatograms, should be considered uncertain when one is dealing with any but the most highly purified of plant extracts".)

Figure 6a. Graph to show the rate of development (measured as an increase in absorbancy at 525 mμ.) of the pink colour produced in the Salper reaction by 25 μg. IAA - in the presence of enzyme extract (A).

Figure 6b. Graph to show the rate of development (measured as an increase in absorbancy at 525 mμ.) of the pink colour produced in the Salper reaction by 25 μg. IAA - in the presence of enzyme extract (B).

Figure 6c. Graph to show the rate of development (measured as an increase in absorbancy at 525 mμ.) of the pink colour produced in the Salper reaction by 25 μg. IAA.

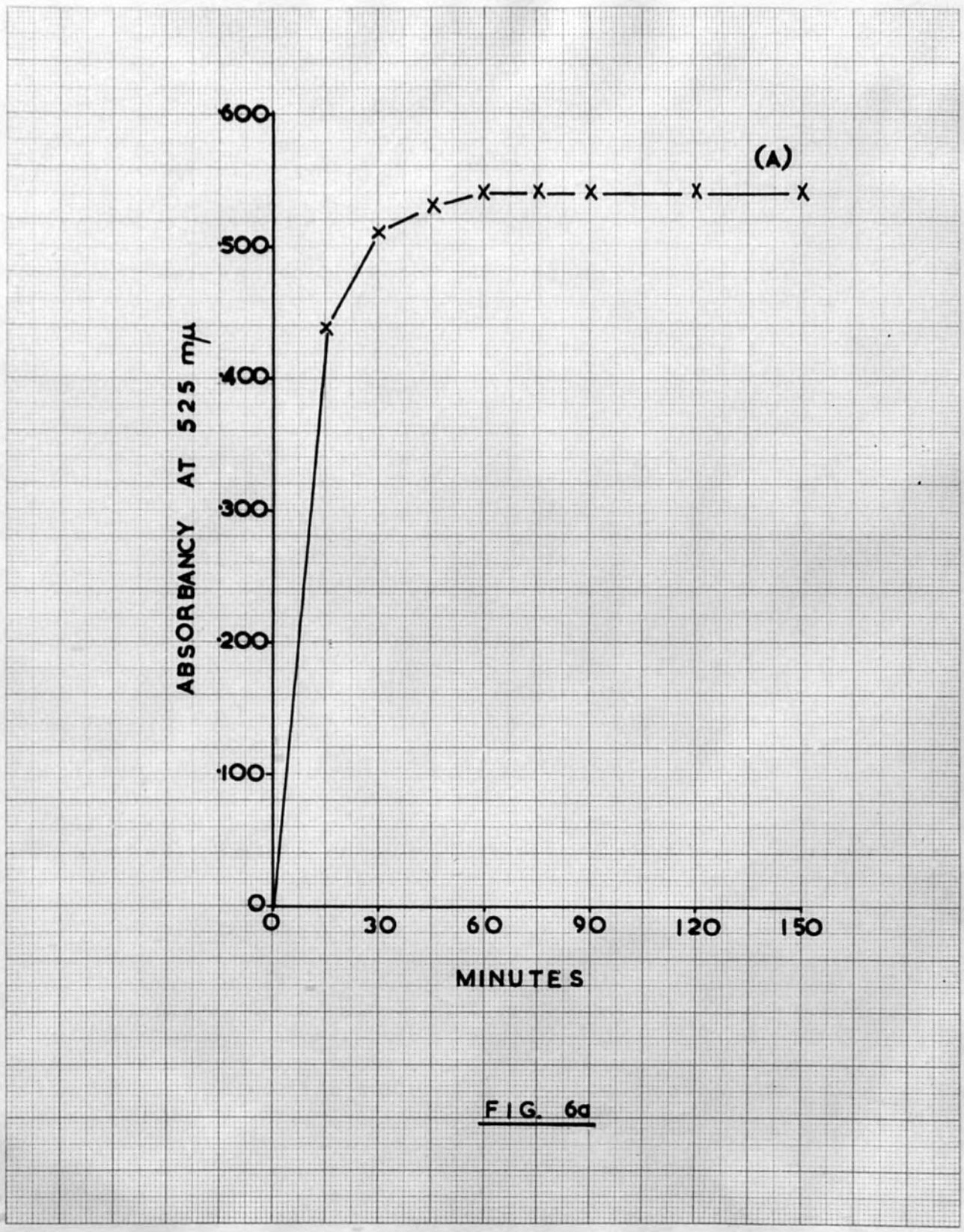

600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
(A)
0
30
60
90
120
150
MINUTES

FIG. 6a

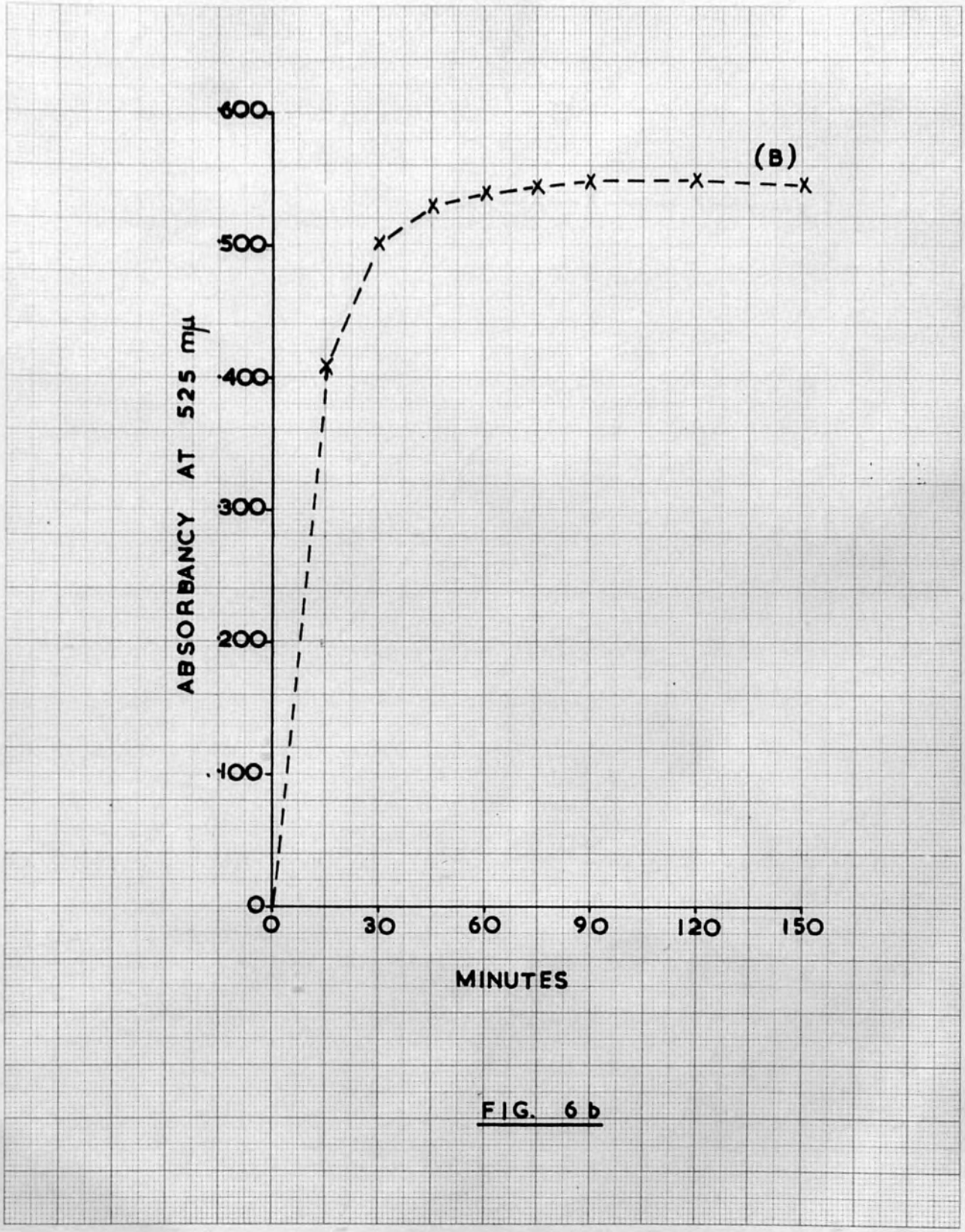

600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
(B)
0
30
60
90
120
150
MINUTES

FIG. 6 b

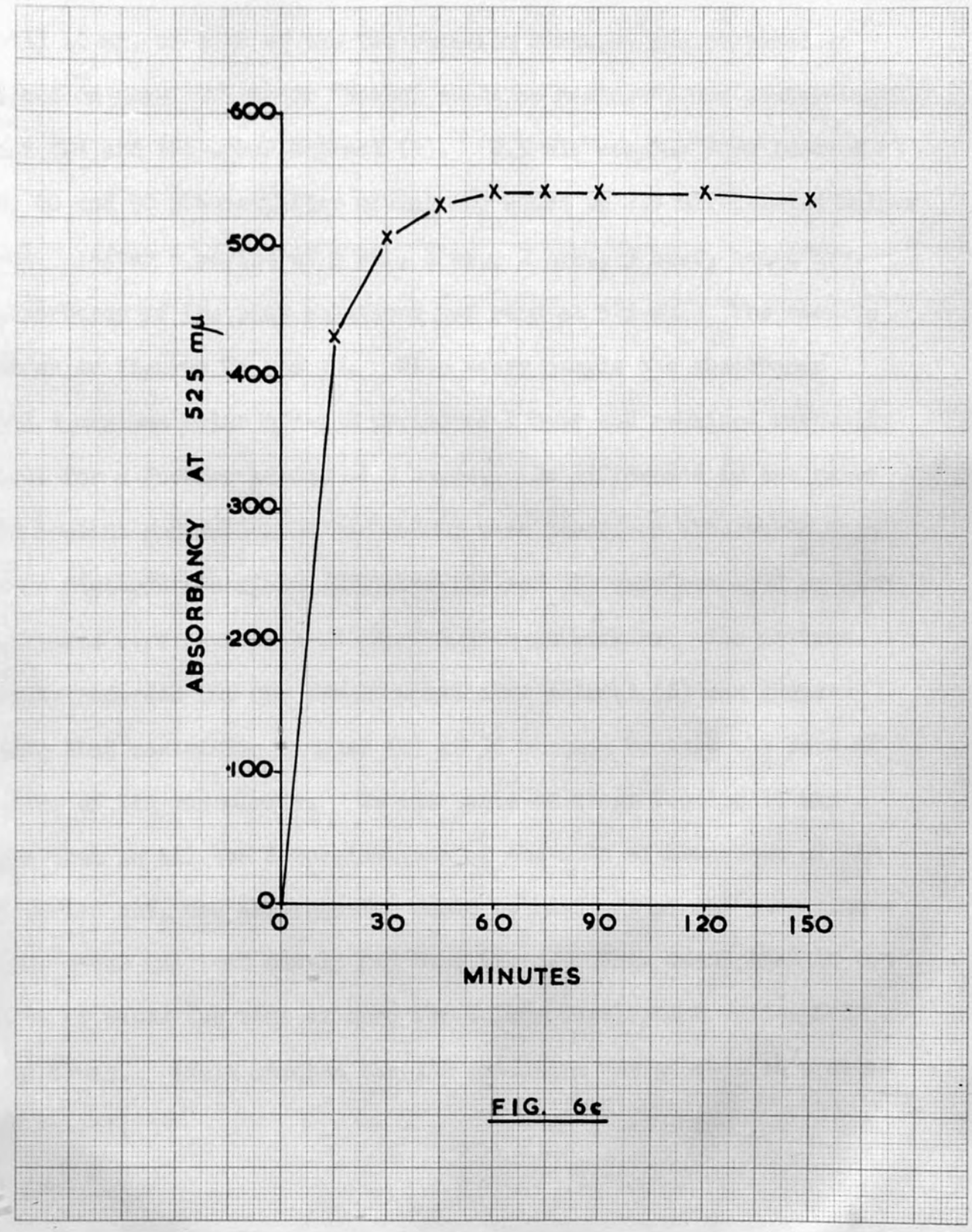

600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
0
30
60
90
120
150
MINUTES

FIG. 6c

ii) 10 ml. of each of the two reaction mixtures was prepared as described on page 82, under "Bakhsh reaction mixture", one containing extract (A) and the other extract (B). 0.5 ml. samples were removed at 0, 30, 60 and 90 minutes after mixing and added to 2.5 ml. diluted Salper reagent. After a period of ½ hr., 1 hr., 2 hrs., 3 hrs., and 4 hrs. the absorbancy of the pink solutions was read at 525 mμ. The results are shown in figures 7a and 7b. With every sample the absorbancy reached a maximum value after a period of 1 hour and remained virtually constant for a further period of 3 hours. No difference in the rates of development and fading of the colour were therefore observable when reaction mixtures containing extracts (A) and (B) were compared or when the mixtures were sampled at different time intervals. The maximum intensity recorded for the sample containing extract (A) was greater than for that containing extract (B) at 30 minutes because the rate of breakdown of IAA was slower. On the basis of these results it was decided that in all the determinations of the rate of breakdown of IAA here carried out, the absorbancies could be read off at the same time - one hour after the last sample had been taken. This meant that in the majority of cases the time allowed for colour development varied from 1 to 2½ hours. (This was when the IAA oxidation was allowed to proceed for 1½ hours).

Figure 7a. Graphs to show the rate of development (measured as an increase in absorbancy at 525 mμ.) of the pink colour produced in the Salper reaction by 4 different concentrations of IAA - in the presence of a reaction mixture containing enzyme extract (A).

The largest quantity of IAA (that present initially) was 25 μg. The remaining 3 quantities were unknown, and were dependent on the amount of IAA breakdown which had taken place in the reaction mixture after 30, 60 and 90 minutes.

Figure 7b. As figure 7a, for a reaction mixture containing enzyme extract (B).

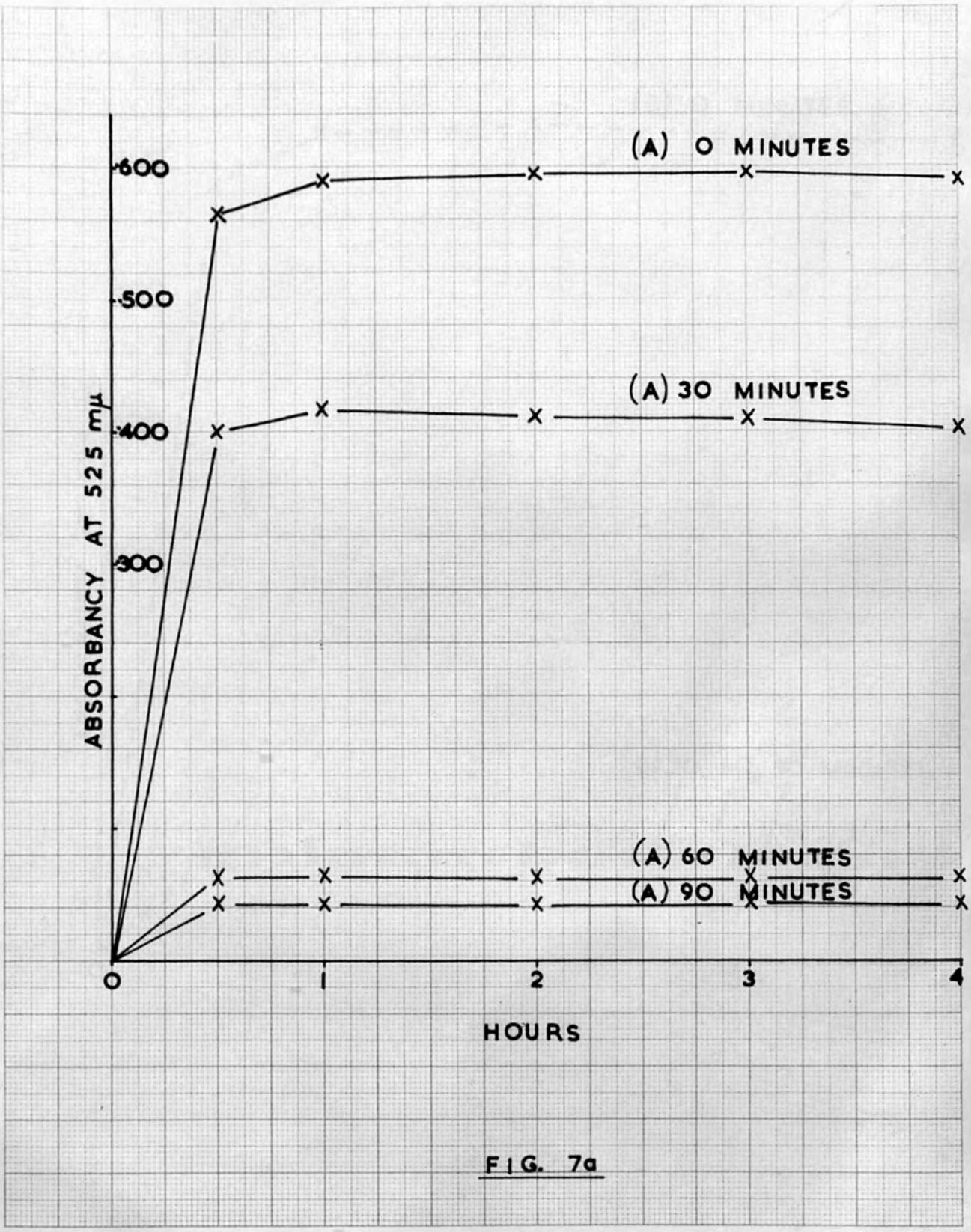

(A) 0 MINUTES
(A) 30 MINUTES
(A) 60 MINUTES
(A) 90 MINUTES
600
500
400
300
ABSORBANCY AT 525 mμ
0
1
2
3
4
HOURS

FIG. 7a

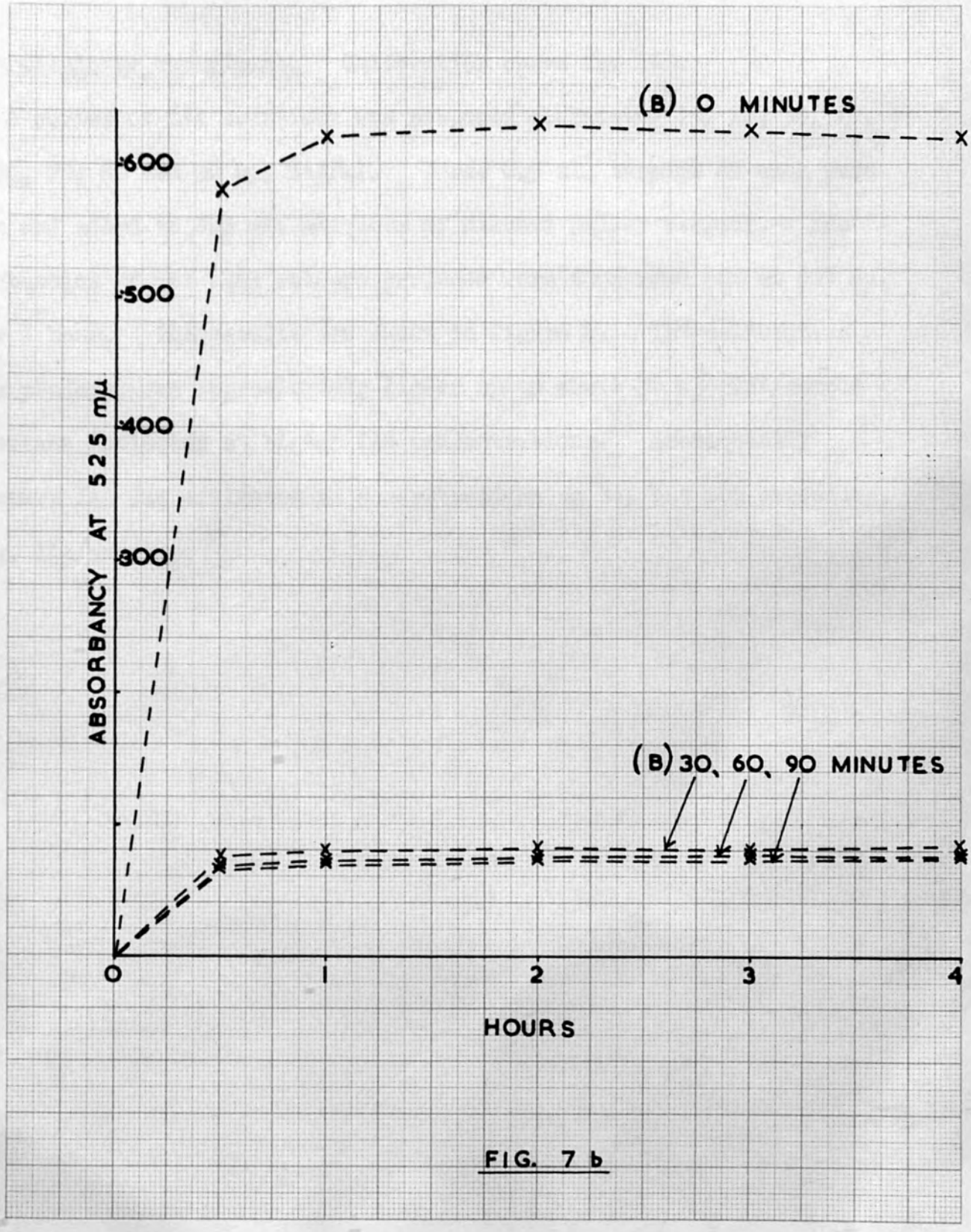

(B) O MINUTES
600
500
400
300
ABSORBANCY AT 525 mμ
(B) 30, 60, 90 MINUTES
0
1
2
3
4
HOURS

FIG. 7 b

3rd Preliminary experiment. Calibration curve for IAA.

A series of IAA solutions was prepared containing 10, 20, 30, 40, 50, 60, 70, 80 and 90 µg. IAA/ml. Three 0.5 ml. samples of each were taken and added to 2.5 ml. aliquots of diluted Salper reagent. The absorbancies of the pink colours produced were then read off at 525 mµ. after 1 hour. The results are shown in figure 8. The calibration curve produced was approximately linear up to about 30 µg. IAA/sample but became inflected at higher IAA concentrations. Subsequently therefore IAA was estimated in concentrations up to, but not exceeding, 25 µg. IAA/sample.

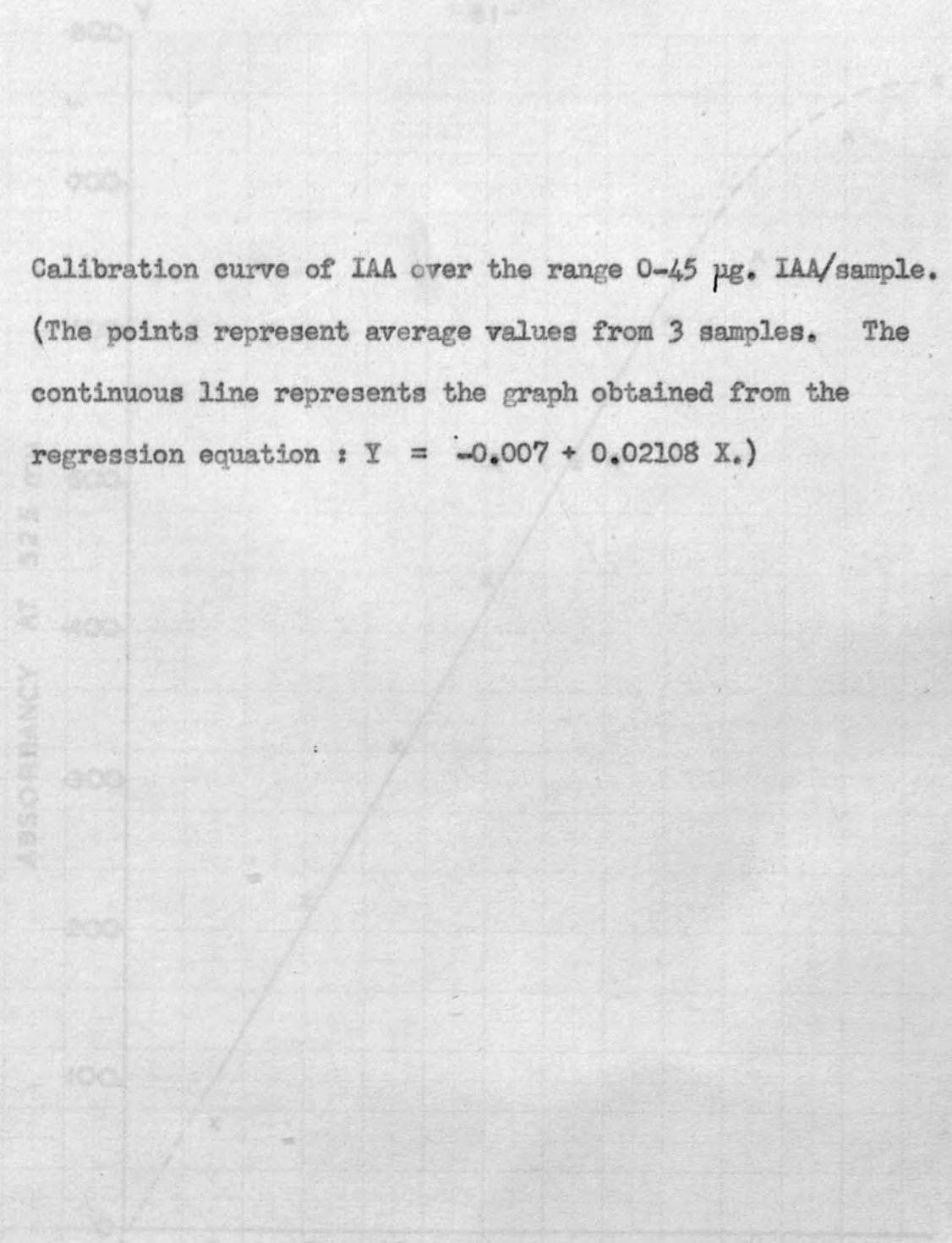


Figure 8. Calibration curve of IAA over the range 0-45 µg. IAA/sample. (The points represent average values from 3 samples. The continuous line represents the graph obtained from the regression equation : Y = -0.007 + 0.02108 X.)

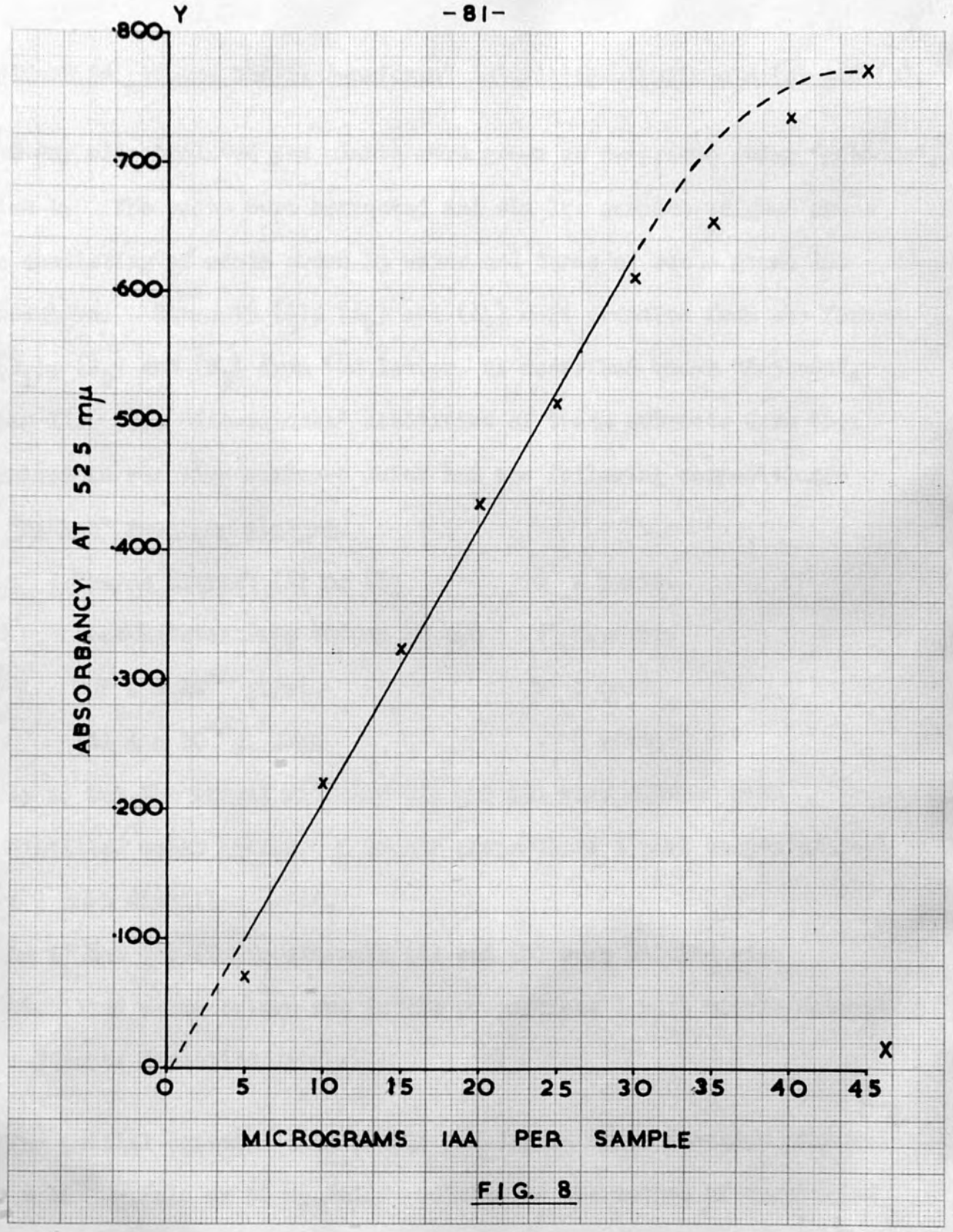

Y
800
700
600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
X
0
5
10
15
20
25
30
35
40
45
MICROGRAMS IAA PER SAMPLE

FIG. 8

Experiment to compare the "IAA-oxidase" activities of extracts (A) and (B).

2-day oldeetiolated pea plants were grown as described under "Methods", Section I. The roots were harvested and six 1g. samples weighed out - three consisting of roots grown in water and three of roots grown in IAA solution. Extracts (A_1), (A_2) and (A_3) were prepared from the former and (B_1), (B_2) and (B_3) from the latter, as described under "Methods", Section II. The "IAA-oxidase" activities of these extracts were then determined in reaction mixtures which had the following composition:-

i) "Bakhsh" reaction mixture.

Enzyme extract (A) or (B)	: 4 parts.
Phosphate-citrate buffer pH 4.0	: 2 parts.
DCP 4×10^{-4} g./ml.	: 1 part.
IAA 4×10^{-4}g. /ml.	: 1 part.

ii) As i) but the enzyme extracts (A) and (B) were diluted with distilled water before use in the proportions 1 part enzyme extract : 1 part distilled water.

iii) As i) but the enzyme extracts (A) and (B) were diluted with distilled water before use in the proportions 1 part enzyme extract : 3 parts distilled water.

The initial concentration of DCP and IAA in these reaction mixtures was 5×10^{-5} g./ml. or 50 µg./ml. Details of the method of incubation of the reaction mixtures and colorimetric determination of residual IAA concentration are given under "Methods", Sections IV-1 and III-1.

i) 10 ml. amounts of 6 reaction mixtures were made up, each containing one of the 6 different extracts, and the rate of IAA destruction in each determined. The reaction mixtures were sampled at "zero time" and at 10 minute intervals thereafter for a period of 1 hour. The results are shown in figures 9a, 9b, and 9c, in which absorbancy has been plotted directly against time. The values for an extract of the "A" group have been plotted alongside those of an extract of the "B" group for the sake of convenience in demonstrating the differing rates of reaction in the two groups - though any member of the "A" group could just as well have been plotted alongside any one of the three in the "B" group.

All the graphs showed that the breakdown of IAA was slow at first and increased until a maximum rate was attained. Breakdown was then linear with time - until the end of the reaction - which did not appear to go to completion. In some graphs a slowing down towards the end of the reaction was indicated. However this was not to be observed in the graphs for (B_1) or (B_2) and it seemed likely that this effect was apparent rather than real - resulting from the fact that residual IAA was determined only at 10 minute intervals.

A comparison of the graphs in the "A" series with those in the "B" series revealed certain consistent differences. Thus the time taken for the reaction to "reach completion" was lower in the "B" series than in the "A" series. Arbitrary estimates of this time were made as follows:-

Method for determining values for the "time taken for the reaction to reach completion".

The line on the graph whose slope represented the maximum rate of the reaction was extended forwards towards the abscissa. A line parallel to the abscissa was then drawn at the level of absorbancy obtained at the last sampling interval (here the 60 minute interval). The point of intersection of these lines was found, and the coordinate of this point on the time axis read off - in minutes.

The results are shown in table 1 below.

Table 1. Values for the "time taken for the reaction to reach completion" estimated from figures 9a, 9b, and 9c.

Extract Present	Time taken for reaction to reach completion (minutes)	Extract Present	Time taken for reaction to reach completion (minutes)
(A_1)	35 . 25	(B_1)	30 . 50
(A_2)	34 . 75	(B_2)	28 . 50
(A_3)	34 . 75	(B_3)	31 . 00

A "t" test indicated that the difference observed between the values in the "A" group (mean 34.92) and those in the "B" group (mean 30.00) was statistically significant. ($t = 6.302$ with 4 degrees of freedom. Cf. 1% value of $t = 4.604$).

In order to determine whether this difference between the "times taken for the reaction to reach completion" in the two groups was due to a difference in the length of the lag period, or to a difference in the/

the maximum rate attained, or both, estimates were obtained for these also as described below.

Method for determining values for the length of the lag period. (Here taken to mean the time interval which elapsed before the maximum rate of IAA destruction wasattained).

The line on the graph whose slope represented the maximum rate of the reaction was extended backwards towards the ordinate. A line parallel to the abscissa was then drawn at the level of absorbancy obtained at the first sampling interval ("zero time"). The point of intersection of these two lines was found, and the coordinate of this point on the time axis read off - in minutes.

Method for determining values for the "maximum rate attained" in the reaction.

Values for the decrease in absorbancy over each successive time interval were obtained from the original absorbancy values by subtraction, and the largest figure taken as the value for the maximum rate (expressed as change in absorbancy/10 minutes).

The results obtained are shown in tables 2 and 3 below.

Table 2. Values for the length of the lag period estimated from figures 9a, 9b and 9c.

Extract present	Length of lag period (minutes)	Extract Present	Length of lag period (minutes)
(A_1)	15 . 5	(B_1)	10 . 0
(A_2)	15 . 0	(B_2)	8 . 5
(A_3)	14 . 5	(B_3)	12 . 0

Table 3. Values for the "maximum rate attained" in the reaction obtained from the data of figures 9a, 9b and 9c (change in absorbancy/10 minutes).

Extract present	Maximum rate	Extract present	Maximum rate
(A_1)	. 240	(B_1)	. 241
(A_2)	. 240	(B_2)	. 250
(A_3)	. 252	(B_3)	. 265

A "t" test on the data of table 2 showed that the difference observed between the values for the length of the lag period in the "A" group (mean 15.0) and those in the "B" group (mean 10.2) was statistically significant. ($t = 4.548$ with 4 degrees of freedom. Cf. 5% value of $t = 2.776$). A "t" test on the data of table 3 however/

however showed that the difference observed between the values for the "maximum rate attained" in the "A" group (mean .244) and those in the "B" group (mean .252) was not statistically significant (t = 0.992 with 4 degrees of freedom. Cf. 5% value of t = 2.776). It was concluded therefore that the difference between the "times taken for the reaction to reach completion" in the two groups was due largely to a difference in the length of the lag period. (See however the analysis of figures 10a, 10b and 10c which follows and also the additional data given on page 175).

Note. More reliable information about the length of the lag period and the maximum rate attained - for the reaction mixtures containing the (B) extracts - could have been obtained if the samples had been taken at 5 minute intervals. Thus,

1. An examination of figure 9b showed that the estimated value for the maximum rate for extract (B_2) was obviously an underestimate of the actual maximum rate. The latter probably occurred over a 10 minute interval midway between 10 and 30 minutes and not over either the 10-20 or 20-30 minute interval.

2. Neither the maximum rate nor the length of the lag period could be obtained with any degree of certainty for any of the 3 mixtures containing extracts of the "B" group. This was because the 10, 20 and 30 minute absorbancy readings all tended to be on a straight line, while the shape of the graph over these intervals and the slope of the line representing the/

the maximum rate might have been found to be different if the 15 and 25 minute absorbancy values had been available.

Figure 9a. Graphs to show the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A_1) and (B_1) Enzyme extracts undiluted.

Figure 9b. As figure 9a. Enzyme extracts (A_2) and (B_2).

Figure 9c. As figure 9a. Enzyme extracts (A_3) and (B_3).

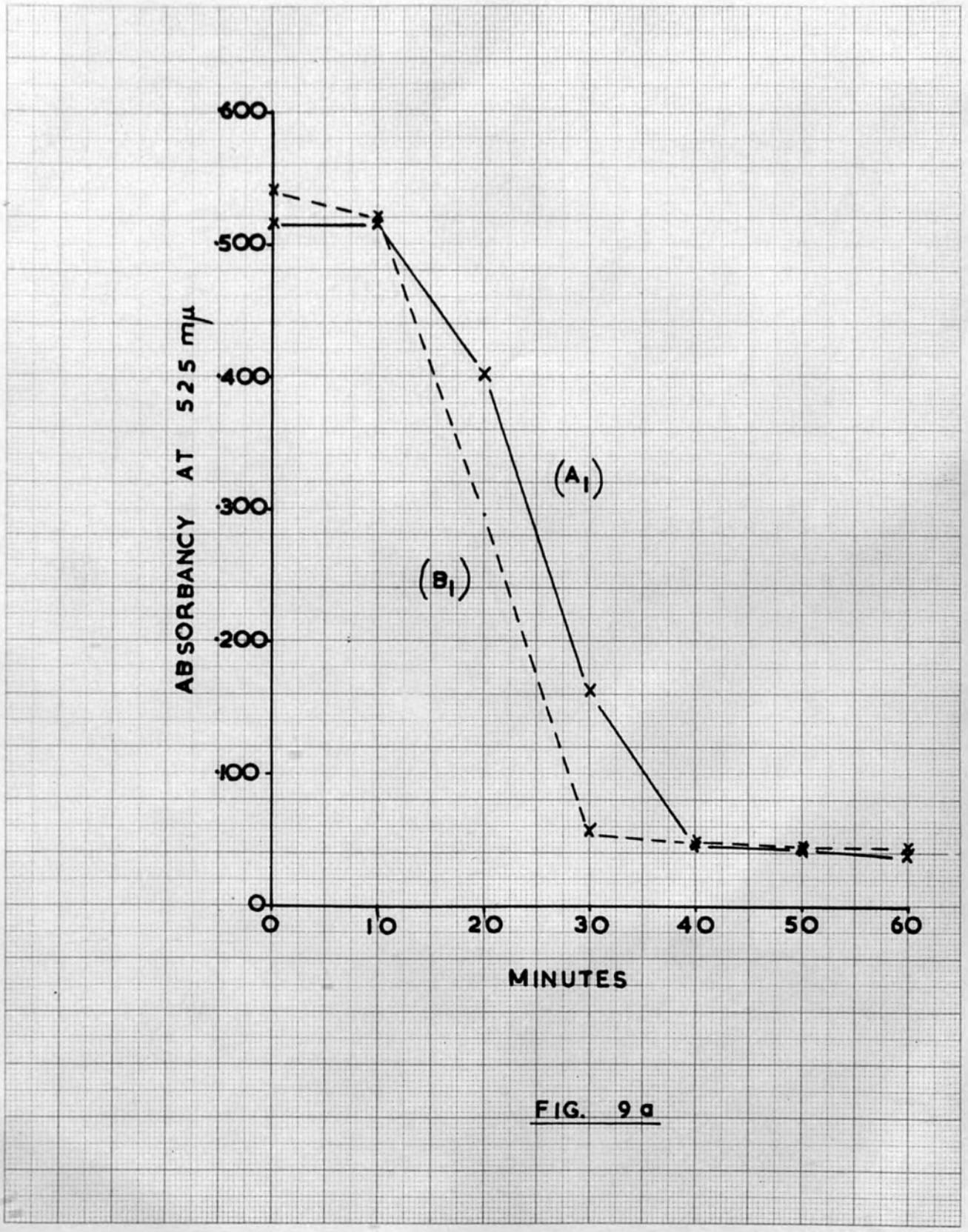

600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
(A1)
(B1)
0
10
20
30
40
50
60
MINUTES

FIG. 9 a

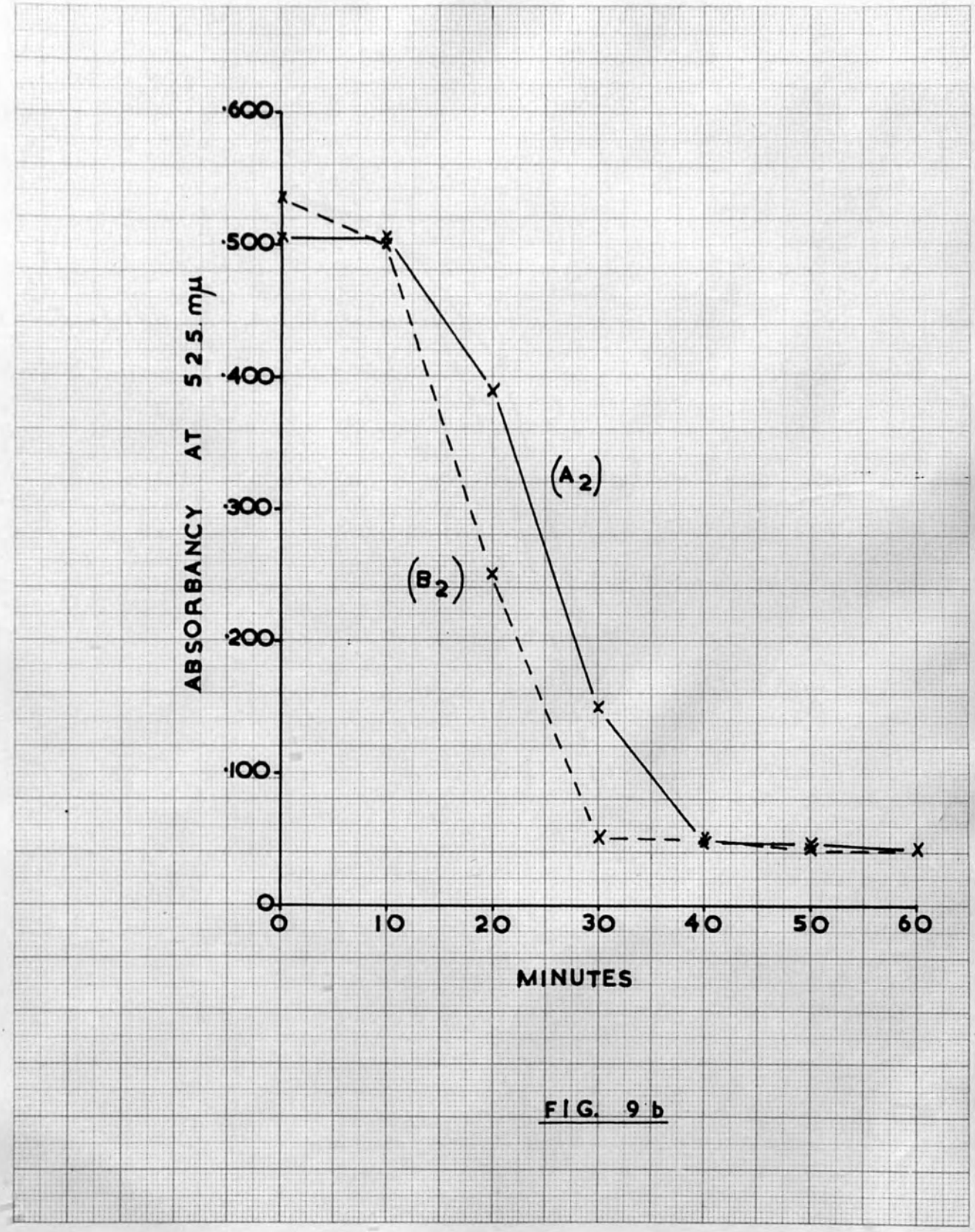

ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
MINUTES
(A2)
(B2)

FIG. 9 b

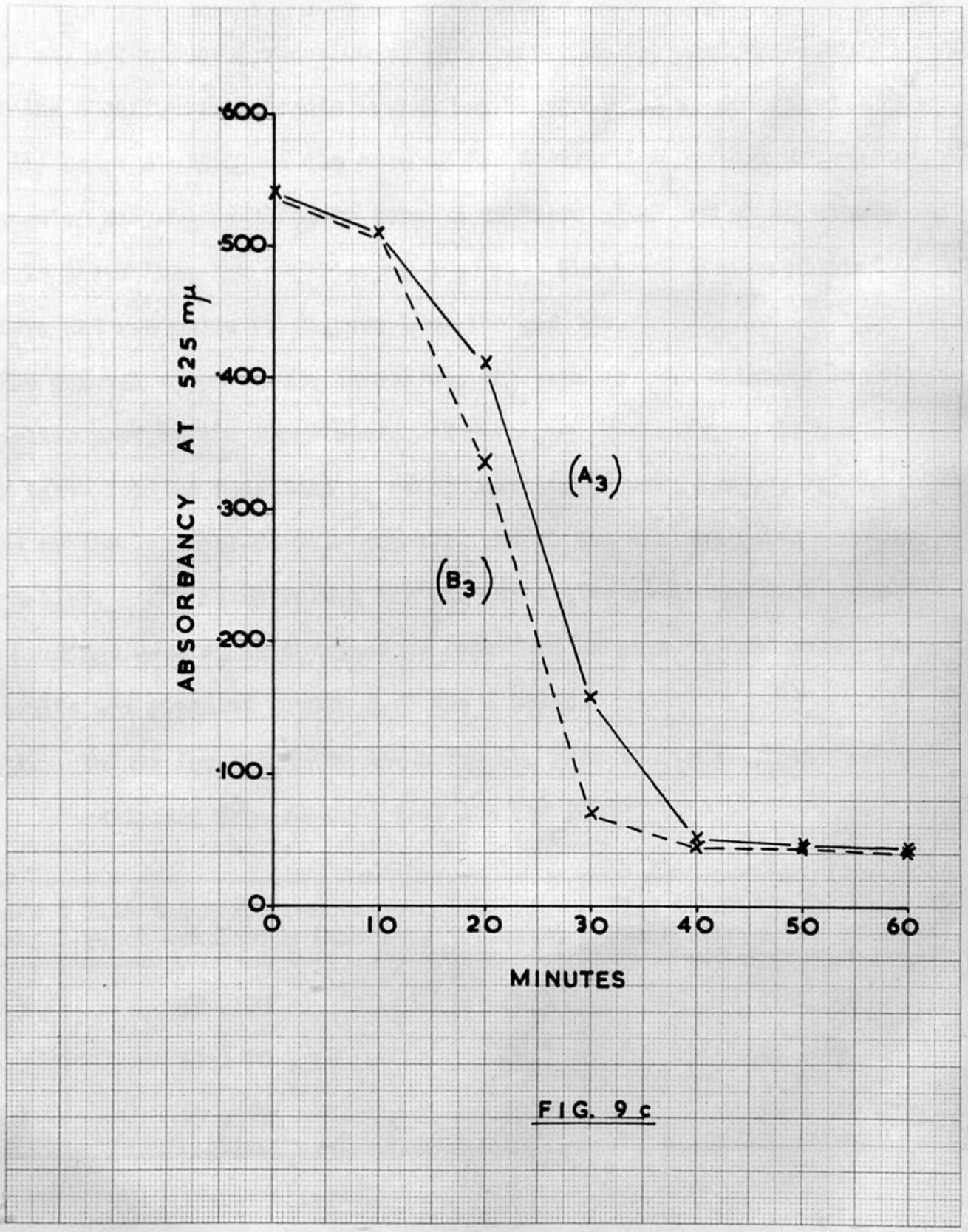

600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
(A3)
(B3)
0
10
20
30
40
50
60
MINUTES

FIG. 9 c

ii) 10 ml. amounts of 6 reaction mixtures were made up, each containing one of the 6 different extracts (which had been diluted as described under ii) on page 82), and the rate of IAA destruction in each determined. The reaction mixtures were again sampled at "zero time" and at 10 minute intervals thereafter for a period of 1 hour. The results were plotted as before and are shown in figures 10a, 10b and 10c.

The general shape of the curves was the same as that obtained in the earlier part of the experiment. However, w'out exception, 1. the "times taken for the reactions to reach completion" were longer, 2. the lengths of the lag periods were greater, and 3. the maximum rates attained were smaller. Estimated values for the quantities mentioned under these three headings were again obtained as described on page 84 and page 285. The results are given in tables 4, 5 and 6 below.

Table 4. Values for the "time taken for the reaction to reach completion" estimated from figures 10a, 10b and 10c.

Extract present	Time taken for reaction to reach completion (minutes)	Extract present	Time taken for reaction to reach completion (minutes)
(A_1)	57 . 00	(B_1)	47 . 50
(A_2)	63 . 00	(B_2)	45 . 75
(A_3)	59 . 75	(B_3)	52 . 00

Table 5. Values for the length of the lag period estimated from figures 10a, 10b and 10c.

Extract present	Length of lag period (minutes)	Extract Present	Length of lag period (minutes)
(A_1)	24 . 0	(B_1)	20 . 0
(A_2)	23 . 5	(B_2)	18 . 0
(A_3)	21 . 5	(B_3)	19 . 5

Table 6. Values for the "maximum rate attained" in the reaction obtained from the data of figures 10a, 10b and 10c. (change in absorbancy/10 minutes).

Extract present	Maximum rate	Extract Present	Maximum rate
(A_1)	. 148	(B_1)	. 172
(A_2)	. 124	(B_2)	. 183
(A_3)	. 128	(B_3)	. 160

A "t" test on the data of table 4 again indicated that the difference observed between the values in the "A" group (mean 59.92) and those in the "B" group (mean 48.42) was statistically significant ($t = 4.520$ with 4 degrees of freedom. Cf. 5% value of $t = 2.776$).

A "t" test on the data of tables 5 and 6 showed however that both the difference observed between the values for the length of the lag period, and that between the values for the "maximum rate attained" for the "A" series (means 23.0 and .133) and "B" series (means 19.2 and .172) were statistically significant. (Thus t = 3.907 with 4 degrees of freedom (data of table 5) and t = 4.287 with 4 degrees of freedom (data of table 6), Cf. 5% value of t = 2.776).

(See also the additional data given on page 175.)

Figure 10a. Graphs to show the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A_1) and (B_1).

Enzyme extracts diluted with distilled water in the proportions 1 : 1.

Figure 10b. As figure 10a. Enzyme extracts (A_2) and (B_2).

Figure 10c. As figure 10a. Enzyme extracts (A_3) and (B_3).

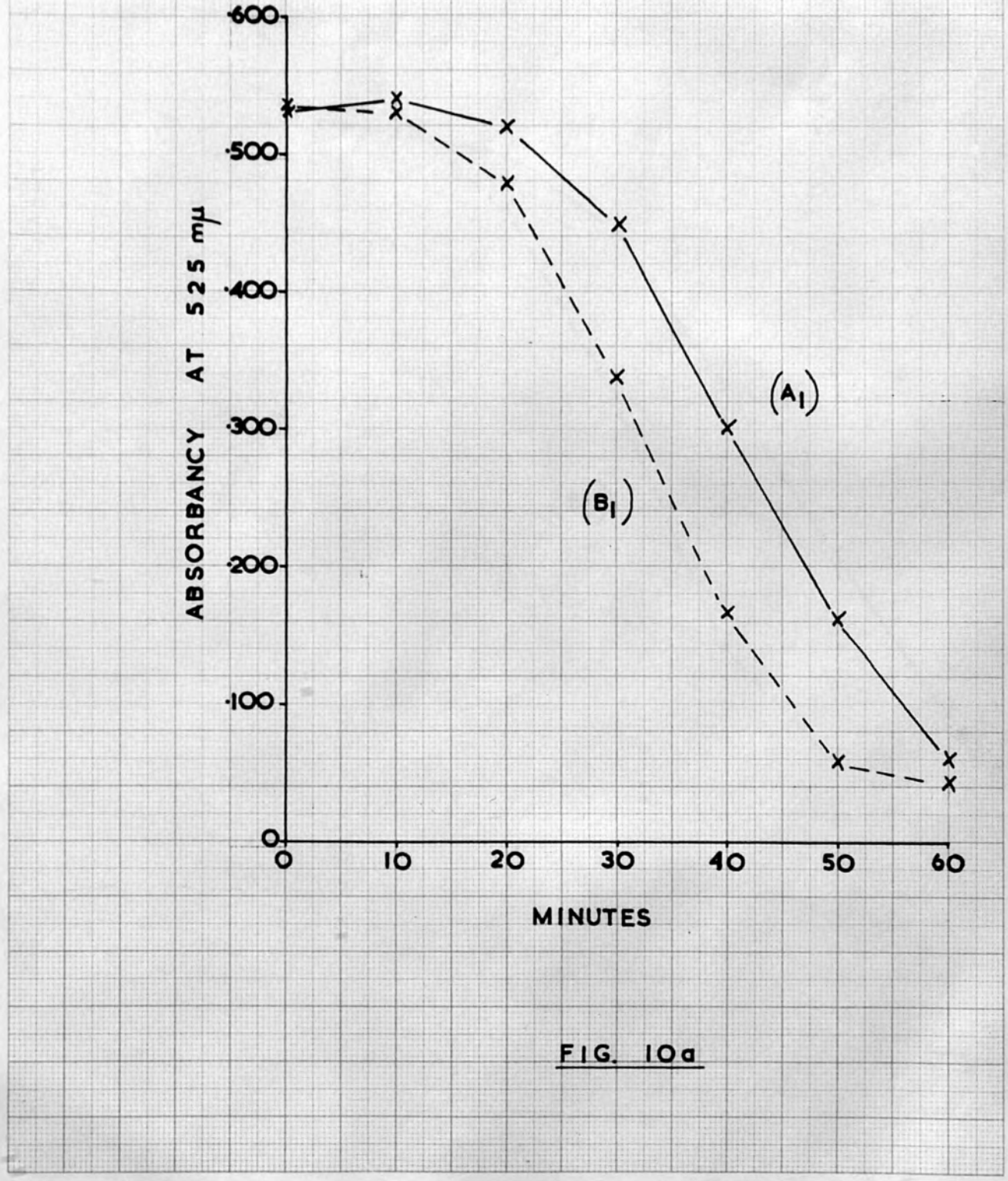

ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
MINUTES
(A1)
(B1)

FIG. 10a

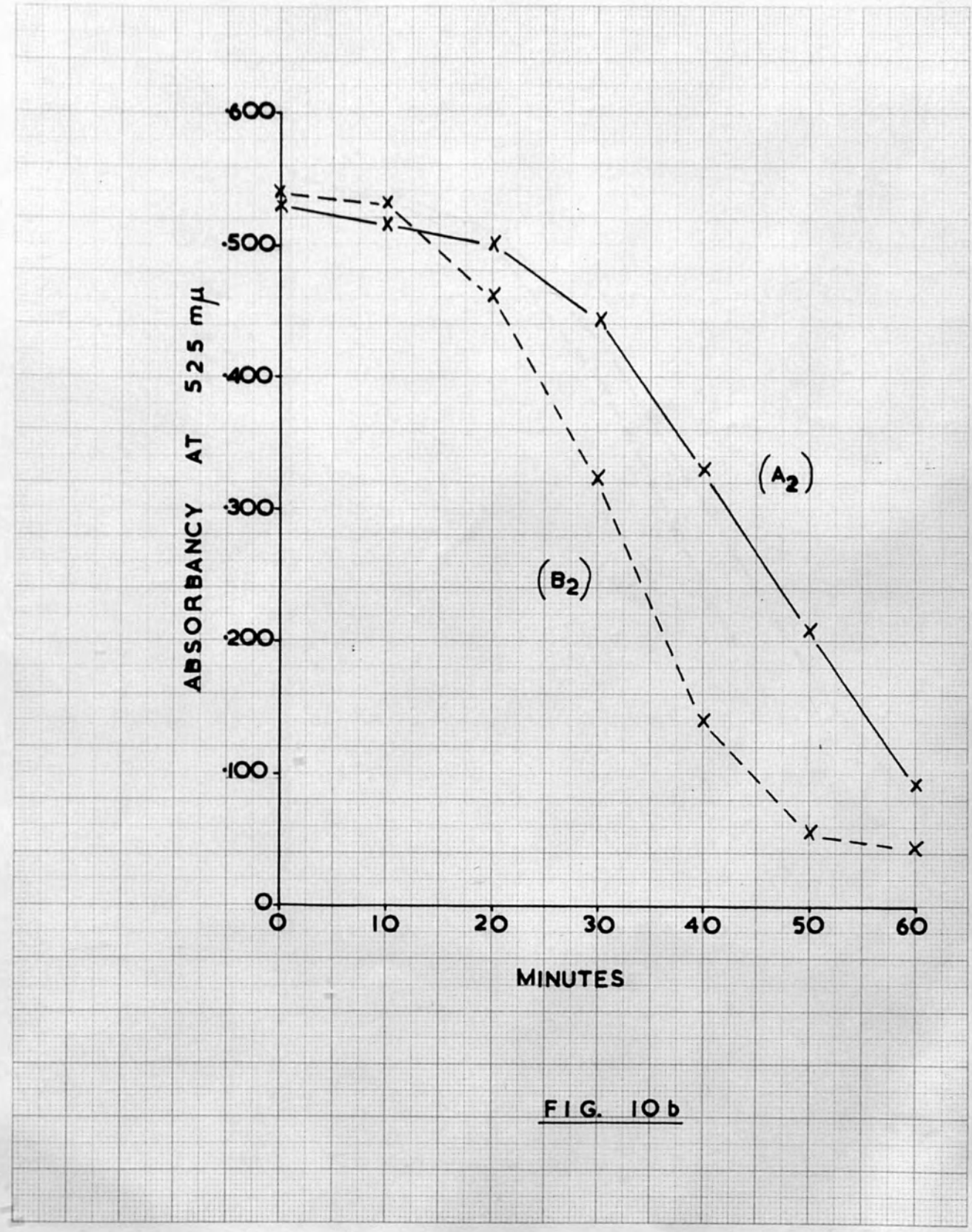

ABSORBANCY AT 525 mμ
600
500
400
300
200
100
0
0
10
20
30
40
50
60
MINUTES
(A2)
(B2)

FIG. 10b

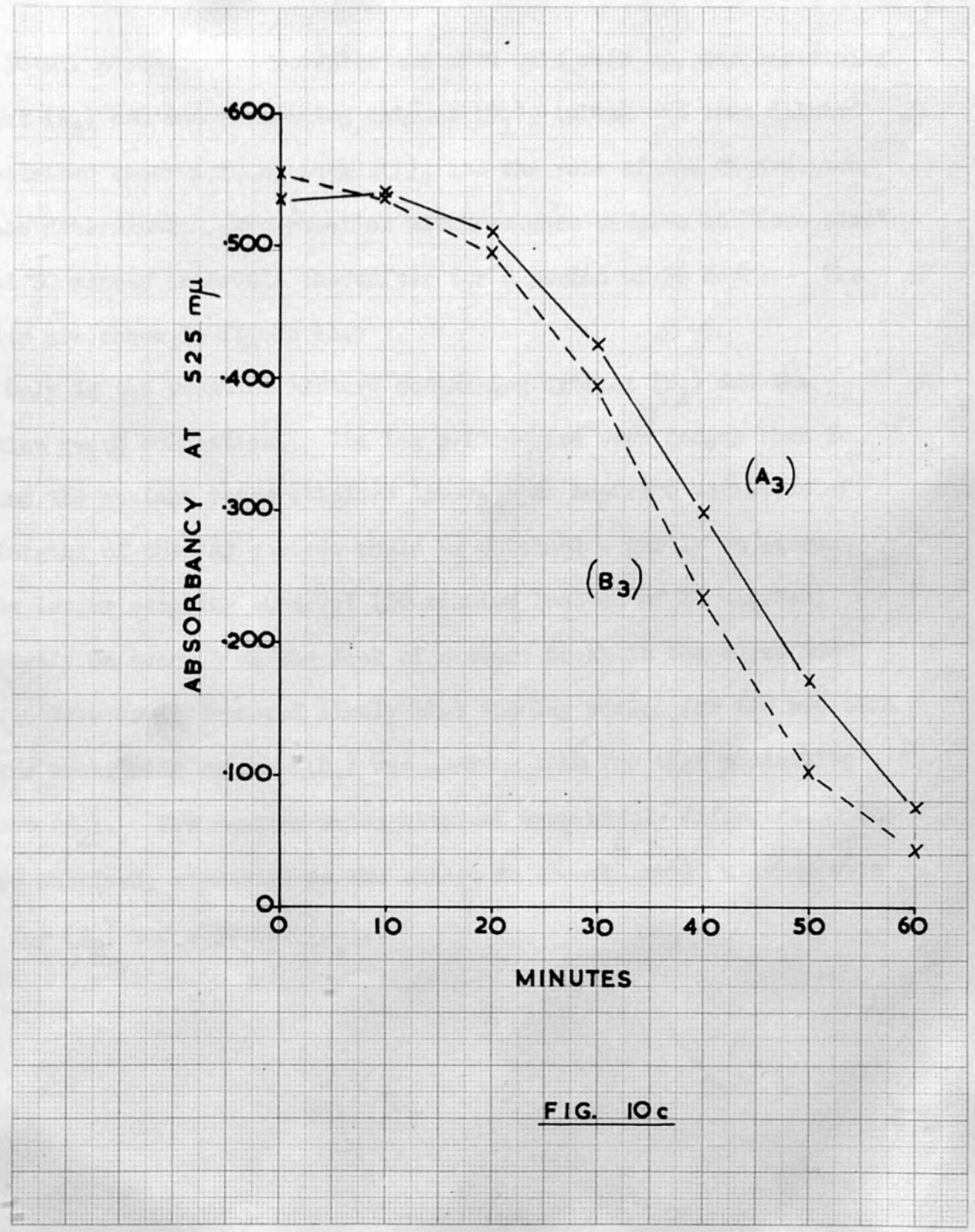

600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
0
10
20
30
40
50
60
MINUTES
(A3)
(B3)

FIG. 10c

iii. 10 ml. amounts of 2 reaction mixtures were made up, one containing extract (A_2) and one containing extract (B_1), (which had been diluted as described under iii. on page 82), and the rate of IAA destruction in each determined. The reaction mixtures were samples at "zero time" and at 30 minute intervals thereafter for a period of 3½ hours. The results are shown in figure 11.

Only in the reaction mixture containing extract (B_1) did the reaction reach completion. The lag periods were even longer than in ii. and the maximum rates attained lower. No accurate estimates of the lengths of the lag periods could be obtained - partly on account of the longer sampling interval (30 minutes instead of 10 minutes) and partly on account of the lack of a sharp break in the curve for (A_2). However it appeared likely that the lag period for the reaction mixture containing extract (B_1) was shorter than for that containing extract (A_2). The maximum rates attained were widely different. The values obtained, expressed as the change in absorbancy/10 minutes were .017 for (A_2) and .052 for (B_1).

Figure 11. Graphs to show the rates of IAA breakdown in reaction mixtures containing extracts (A_2) and (B_1).

Enzyme extracts diluted with distilled water in the proportions 1 part enzyme extract : 3 parts distilled water.

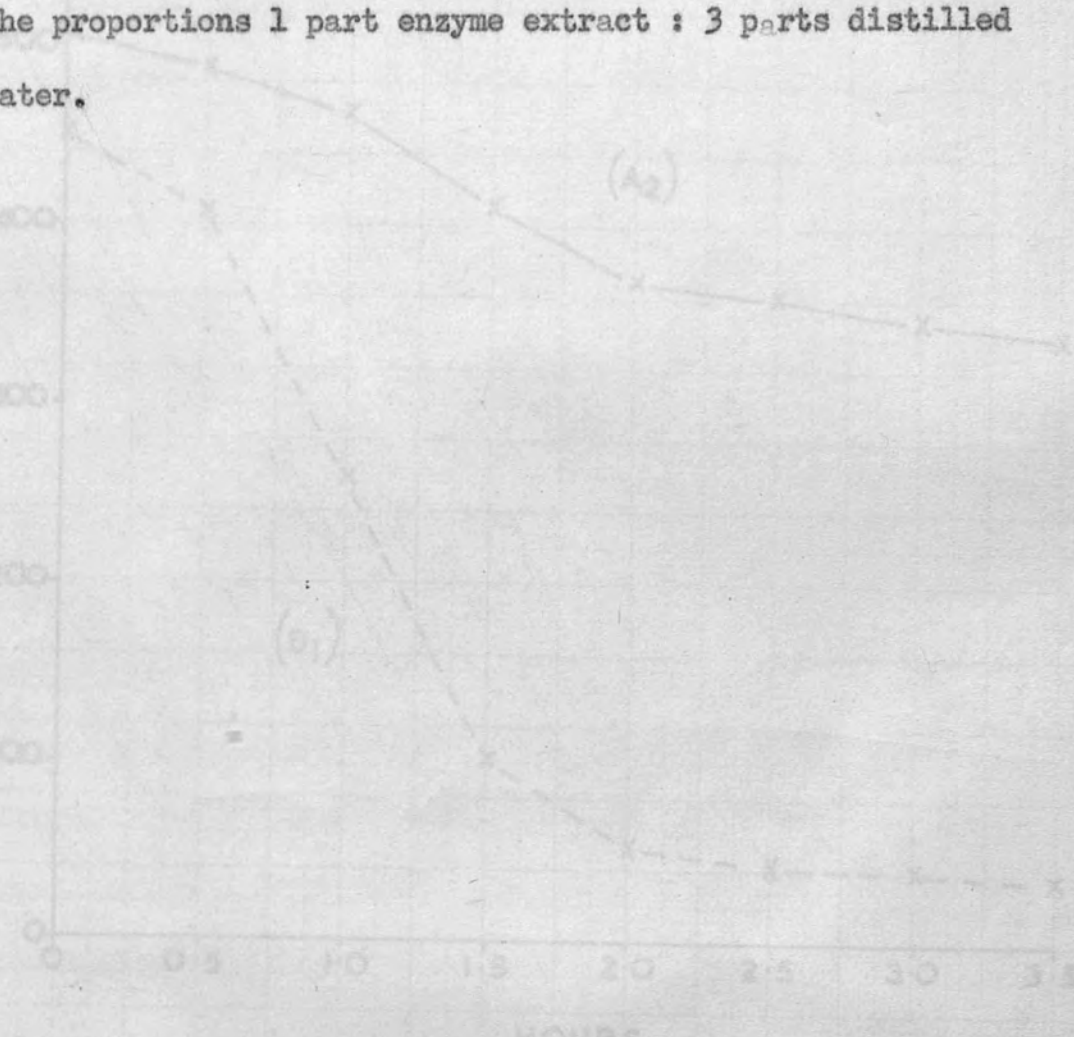


FIG. 11

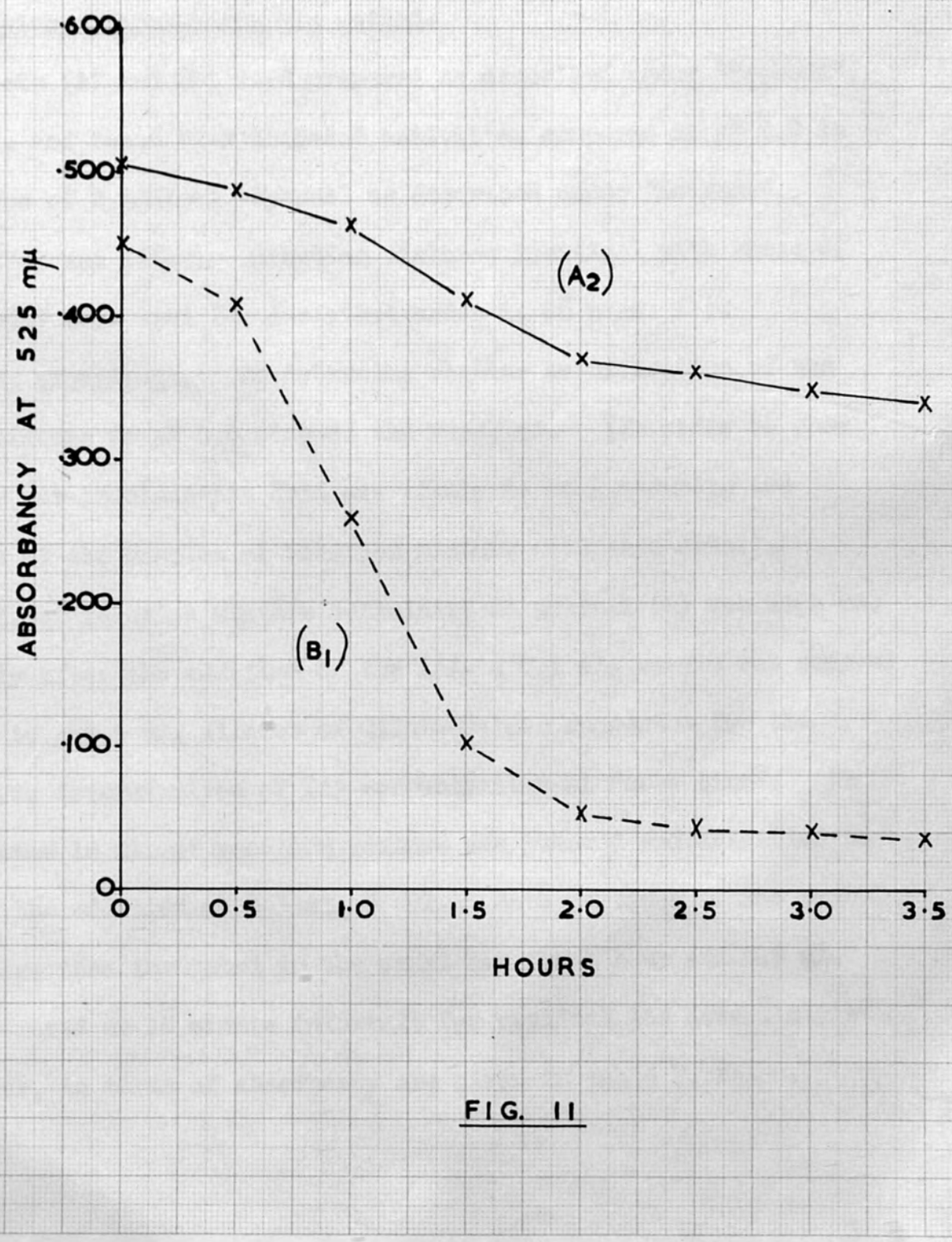

·600
·500
·400
·300
·200
·100
0
ABSORBANCY AT 525 mμ
(A2)
(B1)
0
0·5
1·0
1·5
2·0
2·5
3·0
3·5
HOURS

FIG. 11

2. Comparison of the "IAA-oxidase" activities of extracts (A) and (B) using a chromatographic method.

Extracts (A) and (B) were prepared as described under "Methods", Section II, and their "IAA-oxidase" activities compared at pH 4.0 in the presence of 2,4-dichlorophenol as described under "Methods", Sections IV-1 and III-2. Reaction mixtures identical with those of Bakhsh (1956) were used (As described under i. on page 82).

Preliminary experiment. To determine whether acidification of the reaction mixture to pH 3.0 stopped the reaction. (In order to show whether or not indoleacetic acid was likely to be lostduring the extraction of the samples of reaction mixture with acid ether).

20 ml. of reaction mixture containing an extract (A) was made up. Immediately after the addition of the IAA, a 0.5 ml. sample was removed and added to a 2.5 ml. aliquot of dilutedSalper reagent - for the colorimetric determination of IAA concentration at "zero time". At the same time 10 ml. of reaction mixture was removed and acidified to pH 3.0 by the addition of 0.1 ml. N HCl. Both portions of the reaction mixture were then incubated in the usual way for 1 hour and 0.5 ml. samples removed at 10 minute intervals for residual IAA determination. The results, in terms of absorbancy are given in table 7, below.

Table 7. Breakdown of IAA shown in terms of decrease in absorbancy in the Salper colorimetric test in 2 identical portions of reaction mixture - one at pH 4.0 and the other acidified to pH 3.0 with N HCl.

Interval - minutes	Absorbancy	
	Reaction mixture at pH 4.0	Reaction mixture at pH 3.0
0	.525	-
10	.535	.520
20	.464	.545
30	.243	.545
40	.067	.545
50	.053	.500
60	.051	.515

IAA breakdown appeared to have gone almost to completion in the original reaction mixture, but in the portion acidified to pH 3.0, only a slight decrease in the amount of IAA present occurred.

Experiment to compare the "IAA-oxidase" activities of extracts (A) and (B).

160 ml. amounts of 2 "Bakhsh" reaction mixtures were made up containing either an extract (A) or an extract (B). (For details of the composition of the reaction mixture, see under i. page 82). These were incubated simultaneously, as described under "Methods", Section IV-1. At "zero time" and at intervals of 1 hour two samples were removed from each reaction mixture - one of 0.5 ml. for a colorimetric determination of residual IAA (see "Methods", Section III-1) and 20 ml. for the chromatographic determination of residual IAA (see "Methods", Section III-2).

A "control" experiment was also carried out in which 40 ml. of a mixture was made up containing all the components of the reaction mixture, with the exception of the enzyme extract (which was replaced by distilled water). At "zero time" and after 60 minutes, 0.5 ml. samples were removed for the colorimetric determination of IAA and 19.5 ml. samples for chromatography.

In addition a solution containing 5 mg. of IAA dissolved in 5 ml. of methanol was prepared and 1.0 ml., 0.5 ml. and 0.1 ml. of this were spotted and chromatographed to act as "markers" - though these chromatograms were actually run in a different tank from the chromatograms of the extracted materials. These chromatograms were also sprayed with Salper reagent.

The results of this experiment are given in figures 12 - 15, and tables 8 and 9. Figure 12 is a graph showing the rates of IAA breakdown/

down as determined colorimetrically. Figure 13 is a graph showing the rates of IAA breakdown as determined by the chromatographic method. They have been placed side by side to facilitate comparison and the scale of figure 13 was deliberately chosen so that the graphs correspond approximately in size with those of figure 12. Figures 14a and 14b are diagrams of the chromatograms obtained for the two reaction mixtures containing either extract(A) (figure 14a) or extract (B) (figure 14b). Figure 14c is a diagram of the chromatogram obtained for the "control" sample of reaction mixture, from which the enzyme extract was omitted. Figure 15 is a diagram of the chromatogram of the IAA "marker spots". The average Rf values of the spots occurring on the chromatograms are tabulated in tables 8 (Figures 14a, 14b and 14c) and 9 (Figure 15). The colour reactions of the spots with Salper reagent are also given.

An examination of figure 12 showed that the maximum rates of IAA breakdown in thepresence of extract(A) and of extract (B) occurred over the same time interval - from 20 to 30 minutes. Alsothe values obtained for these maximum rates, expressed as change in absorbancy/10 minutes were similar (.254 for extract (A) and .267 for extract (B)). The length of the lag period in the presence of extract (B) was however obviously shorter than that in the presence of extract (A). These are the results which were expected (this part of the experiment was identical with the experiments carried out under i. in the first part of this section (figures 9a, 9b and 9c).

An examination of figure 13 showed that the rate of IAA breakdown as measured by the chromatographic method corresponded quite well with the rate of IAA breakdown measured colorimetrically. The maximum rates attained however were slightly different, that for the reaction mixture containing extract (A) being slower than that for the one containing extract (B). The length of the lag period in the presence of extract (B) was again shorter than in the presence of extract (A).

The graphs in figure 13 showed also that the reaction (breakdown of IAA) in fact went to completion (this was assuming that small amounts of IAA were not present towards the ends of the reaction and were subsequently lost during chromatography). The graphs in figure 12 on the other hand indicated that a fraction of the IAA remained intact. An examination of the chromatograms from which the data of figure 13 was derived (figures 14a and 14b) showed that the "residual absorbancy" obtained with the Salper reagent in the experiments in which IAA destruction was measured colorimetrically, was not attributable to IAA itself, but to certain reaction products having higher Rf values than IAA. Since it was not possible to assess the contribution made by these, (and by any possible intermediate products also giving a colour reaction with Salper reagent), to the absorbancy reading obtained at the various sampling intervals during the course of the reaction, it was not thought profitable to convert the absorbancy readings obtained in the colorimetric estimation of IAA to absolute amounts.

The/

The readings therefore have been plotted directly throughout.

A discussion of the possible identity of the spots obtained on the chromatograms of figures 14a and 14b is given on page 129, following a further examination of these compounds, described below.

The graphs for the "control" experiments in figures 12 and 13 showed that in the absence of the enzyme extract, IAA was not appreciably broken down. The chromatogram of figure 14c (on which the "control" graph in figure 13 was based) however, also showed a number of coloured spots, in addition tothat of IAA. The chromatogram of the "marker" spots of a solution of IAA in methanol (figure 15), again showed a number of additional spots. The possible identity of these compounds is also given on page 129 of this section.

The origin of the various compounds found on the chromatograms apart from IAA itself (figures 14 - 18) - and their significance with respect to the pathway of the enzymatic oxidation is commented on in the "Discussion", page 246.

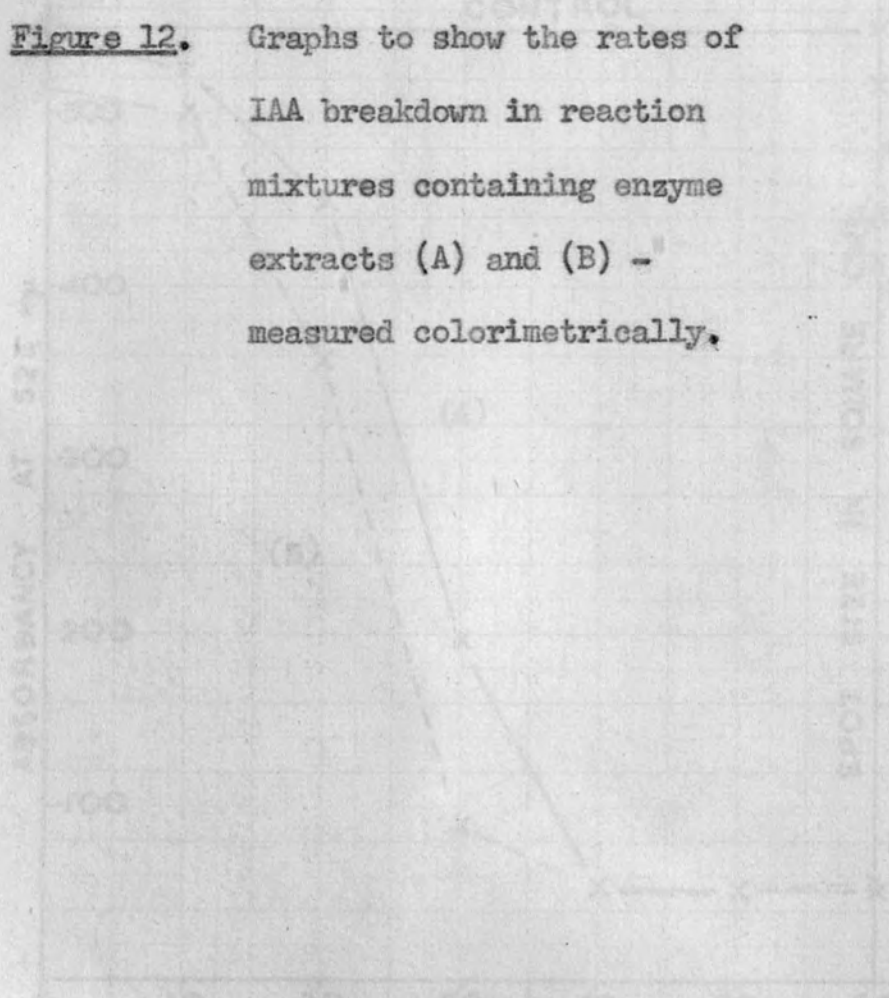


Figure 12. Graphs to show the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A) and (B) - measured colorimetrically.

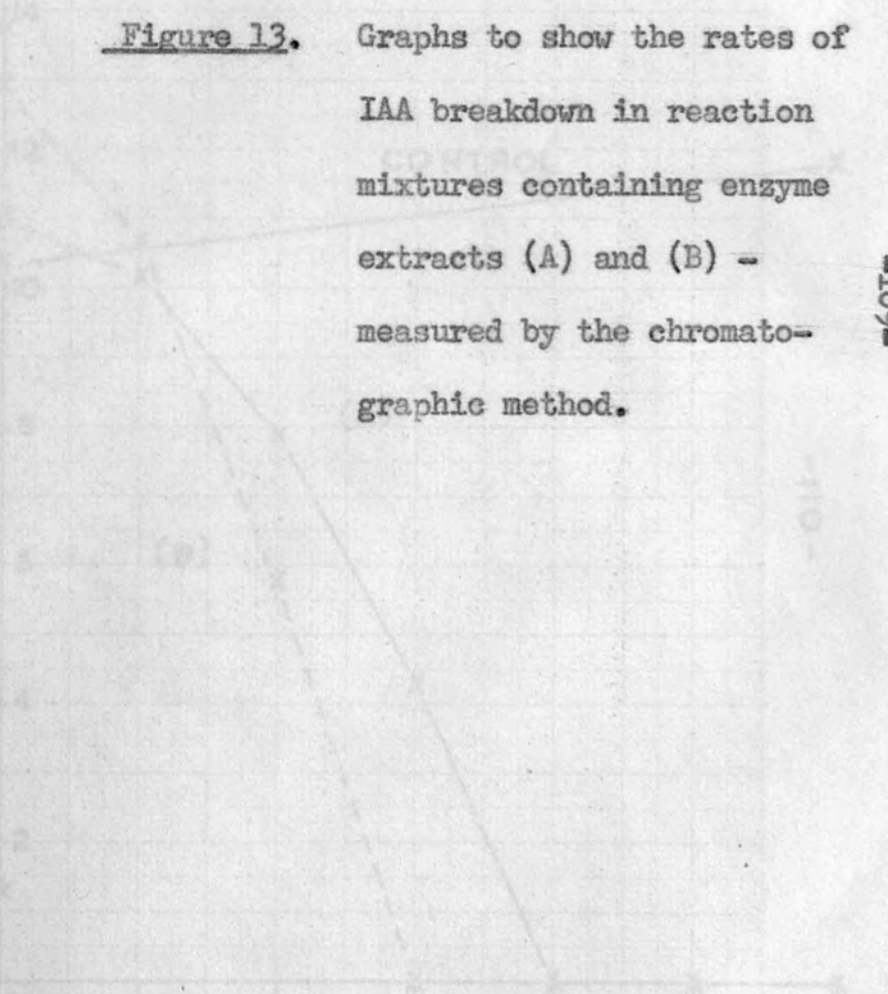


Figure 13. Graphs to show the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A) and (B) - measured by the chromatographic method.

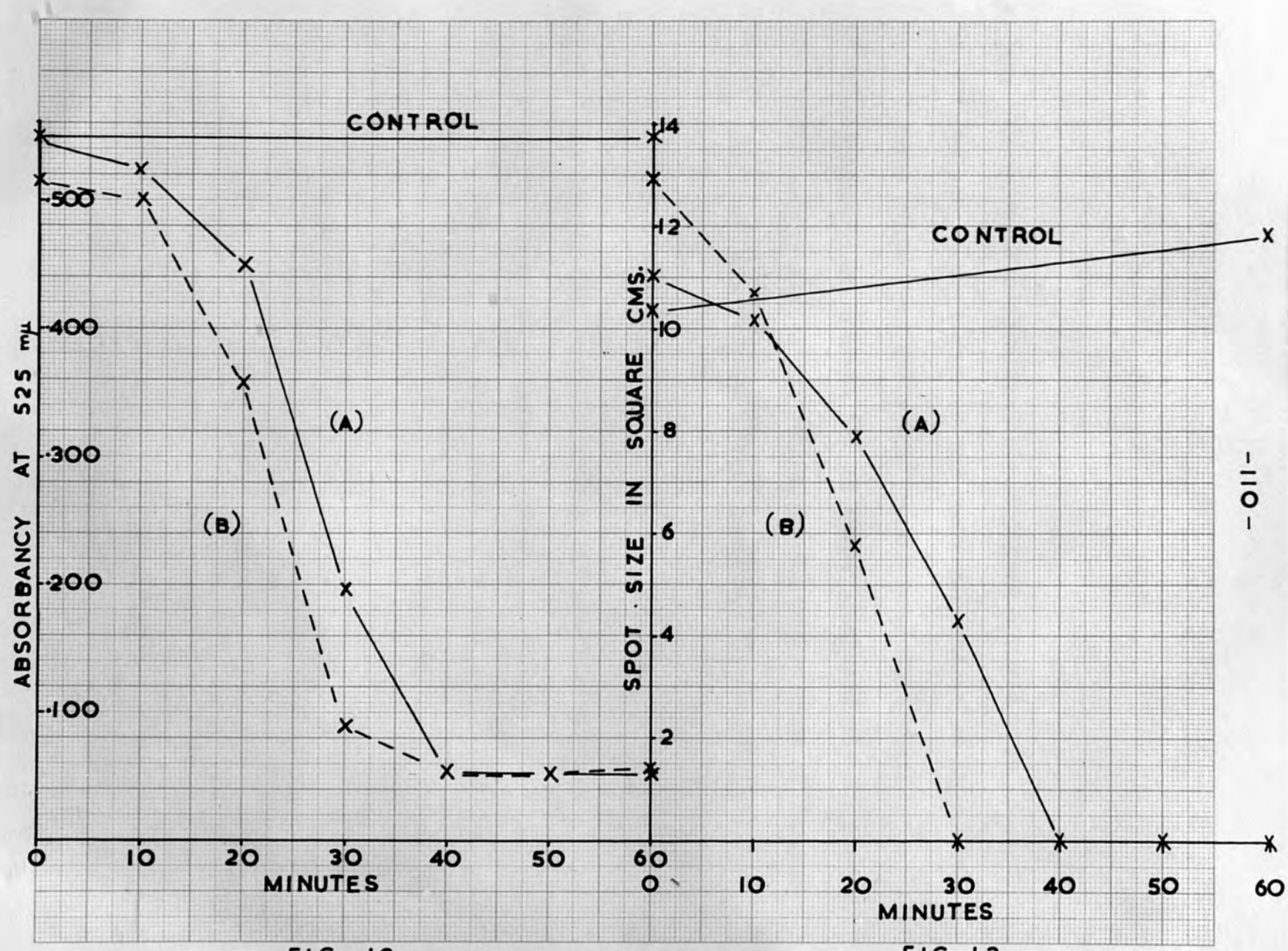

CONTROL
(A)
(B)
ABSORBANCY AT 525 mμ
500
400
300
200
100
0
10
20
30
40
50
60
MINUTES

FIG. 12

SPOT - SIZE IN SQUARE CMS.
14
12
10
8
6
4
2
CONTROL
(A)
(B)
0
10
20
30
40
50
60
MINUTES

FIG. 13

Figure 14a. Diagram of a developed chromatogram sprayed with Salper reagent showing the compounds extracted into acid ether from a "Bakhsh" reaction mixture which contained an extract (A) at "zero time" and at intervals during the course of the destruction of IAA. (Solvent isopropanol (80); ammonia (2); water (15).).

Figure 14b. As figure 14a, but for a "Bakhsh" reaction mixture which contained an extract (B).

FIG. 14b

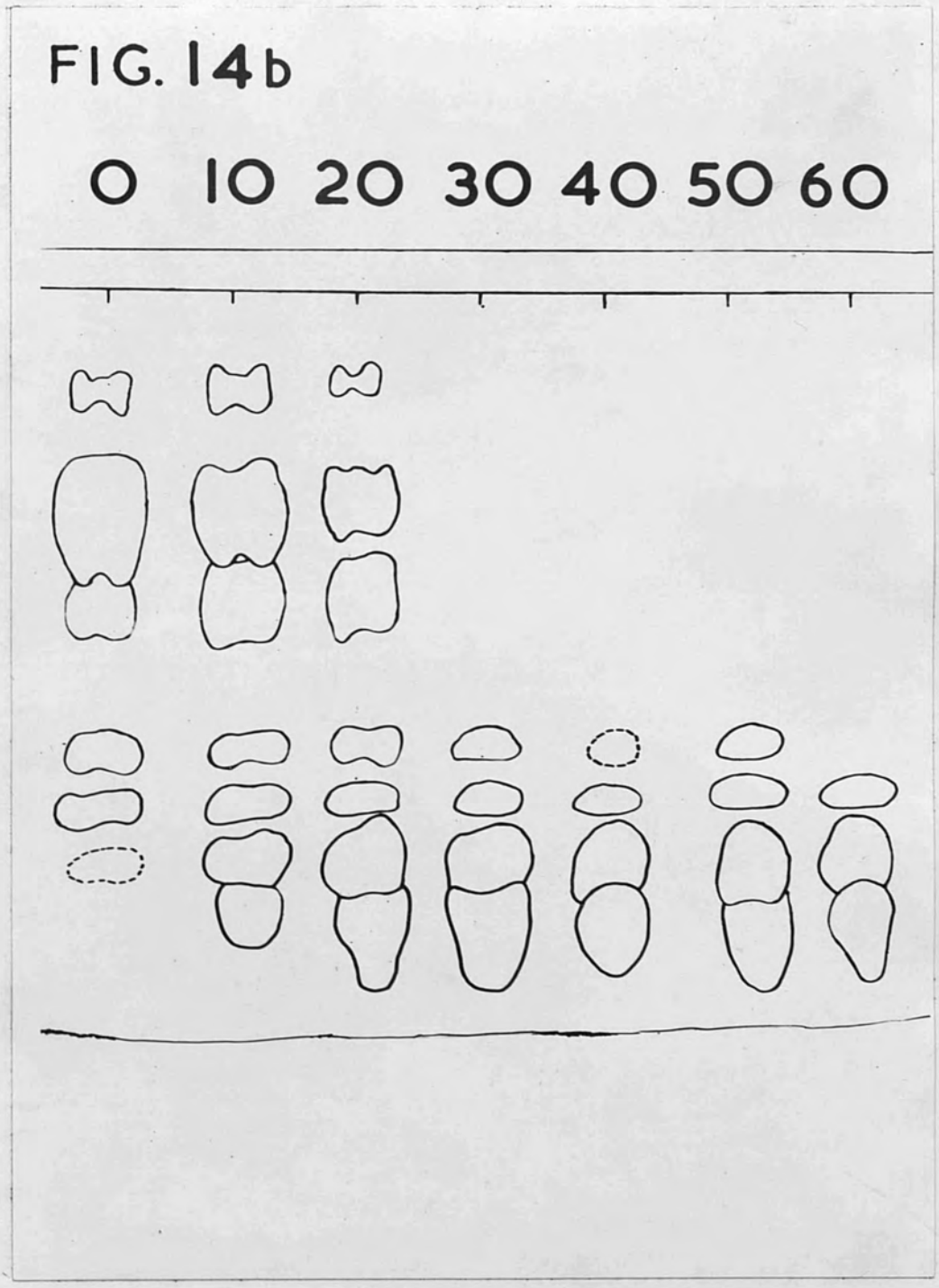


FIG. 14a

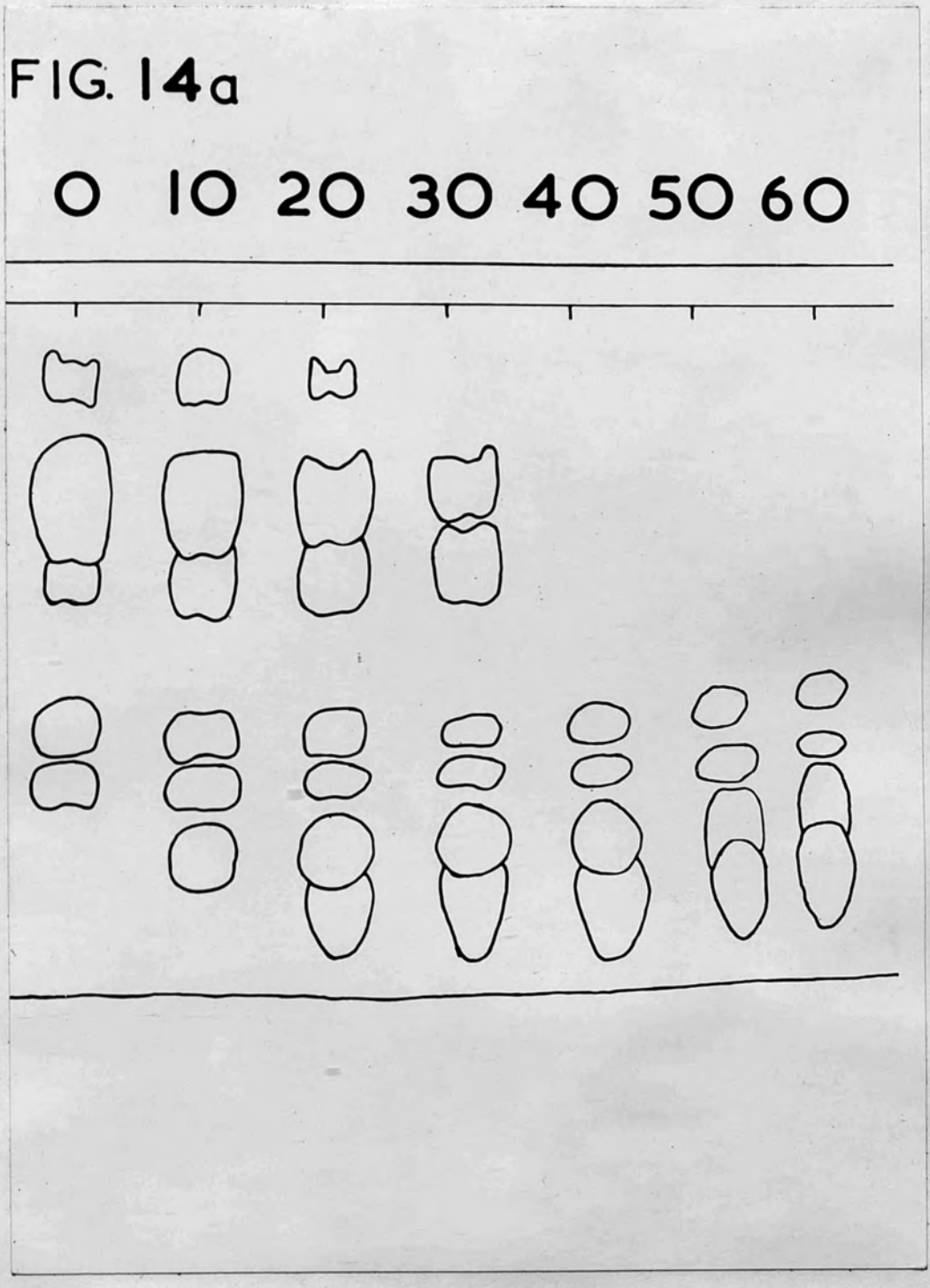


Figure 14c. Diagram of a developed chromatogram sprayed with Salper reagent showing the compounds extracted into acid ether from a "Bakhsh" reaction mixture from which the enzyme extract had been omitted at "zero time" and after 60 minutes incubation.
(Solvent isopropanol (80); ammonia (2); water (15).)

FIG. 14 c

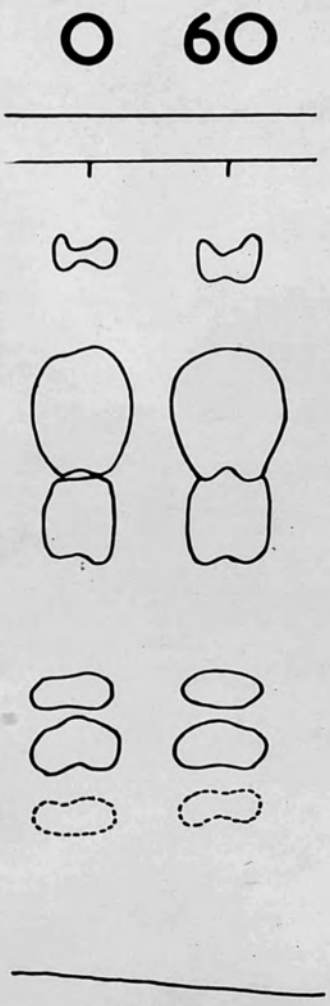


Figure 15. Diagram of a developed chromatogram sprayed with Salper reagent showing the compounds present in 1.0 ml., 0.5 ml. and 0.1 ml. samples of a solution of IAA in methanol containing 1,000 µg. IAA/ml. (Solvent isopropanol (80); ammonia (2); water (15).)

FIG. 15

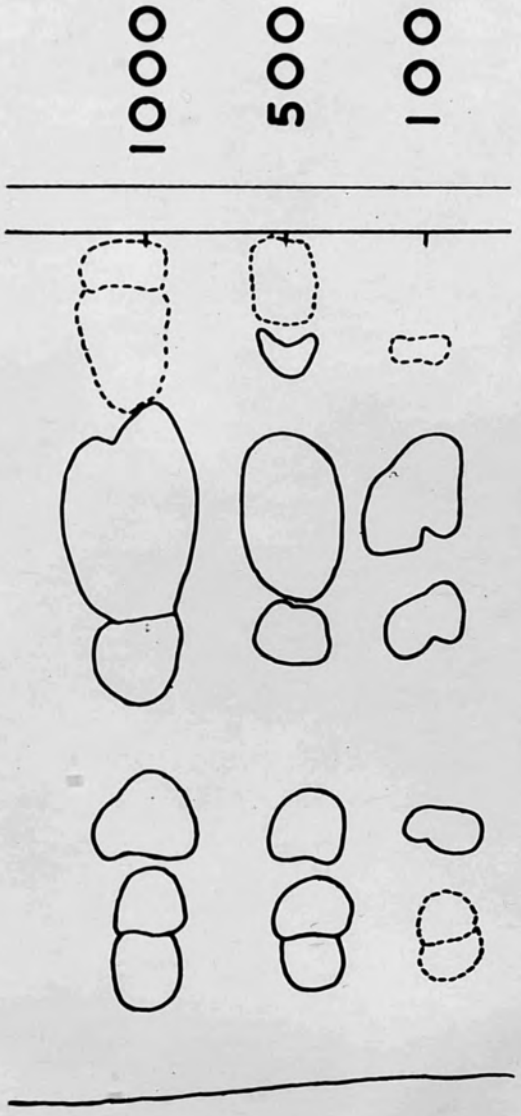


Table 8. Average Rf values of spots on the chromatograms shown in figures 14a, 14b and 14c, together with their colour reactions with Salper reagent.

Rf	Colour with Salper reagent
.12	Pink.
.29	Deep pink - to - purple.
.41	Deep pink.
.61	Pale salmon pink.
.68	Purple.
.77	Orange - brown.
.86	Pink-to-purple.

Table 9. As table 8, for the spots shown in figure 15.

Rf	Colour with Salper reagent
.06	Pale pink.
.14	Pale pink.
.32	Deep purple.
.47	Bright pink.
.70	Blackish brown.
.79	Brownish (faint).
.85	Pink.

A further examination by chromatography of the compounds located on the chromatograms of figures 14a and 14b.

Two further chromatograms were obtained in an identical manner to the one given in figure 14a. However these were not sprayed with Salper reagent. Instead, one was sprayed with Ehrlich reagent and the other with Van Eck reagent. Diagrams of these chromatograms are given in figures 16 and 17, and the average Rf values and colour reactions of the spots are tabulated in tables 10 and 11.

Finally another chromatogram was obtained again in a similar manner to the one given in figure 14a. It differred in that it was developed, not in an alkaline solvent, but in a neutral solvent (isopropanol (80); methanol (5); water (15).). Also in this case the reaction mixture was sampled only at "zero time" and after 30 minutes and 60 minutes incubation. As in the chromatogram of figure 14a, the spray was Salper reagent. A diagram of this chromatogram is given in figure 18 and the average Rf values and colour reactions of the spots are tabulated in Table 12.

Figure 16. Diagram of a developed chromatogram sprayed with Ehrlich reagent showing the compounds extracted into acid ether from a "Bakhsh" reaction mixture which contained an extract (A) at "zero time" and at intervals during the course of the destruction of IAA.
(Solvent isopropanol (80); ammonia (2); water (15).)

Figure 17. As figure 16 but sprayed with Van Eck reagent.

FIG. 16

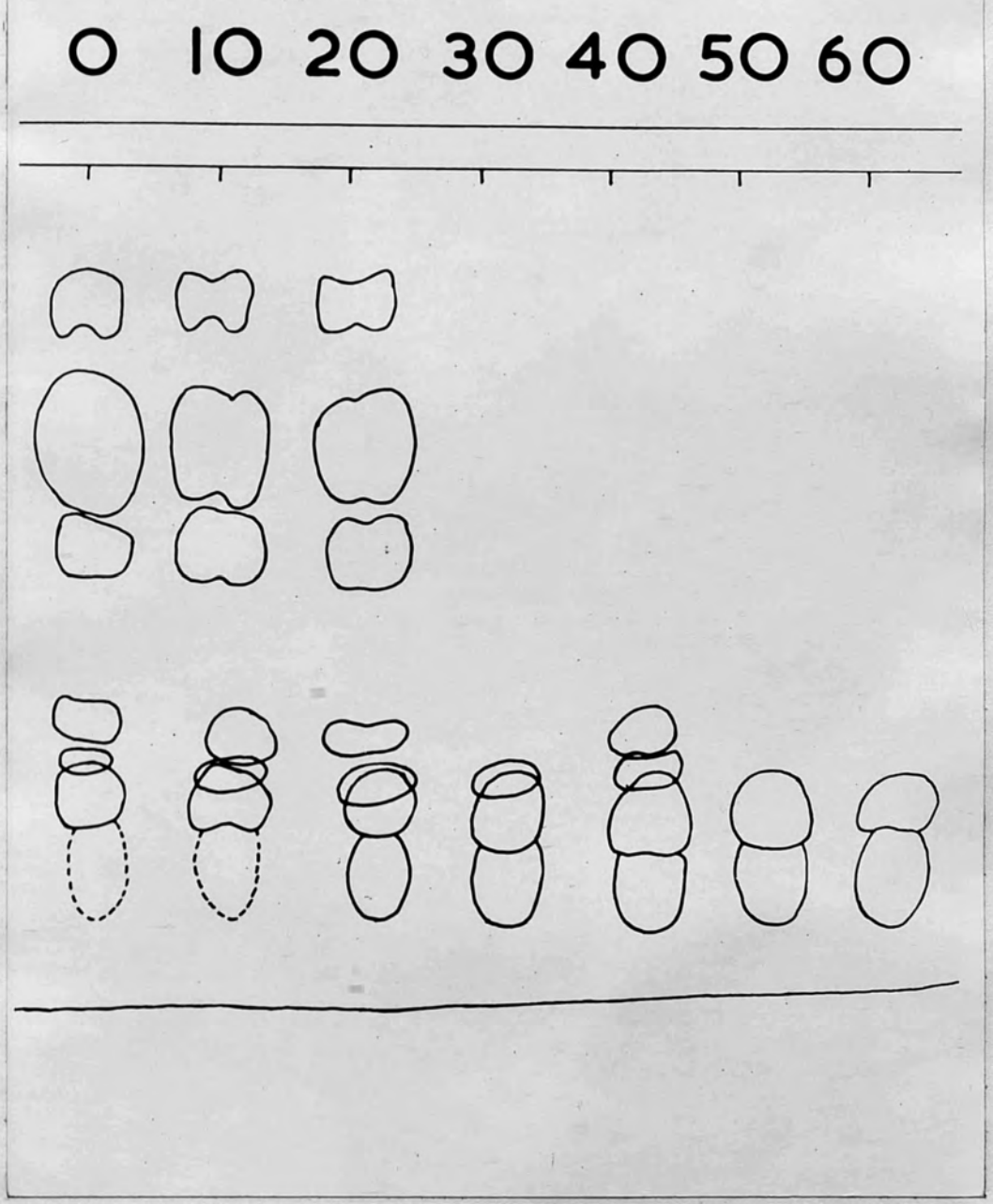


FIG. 17

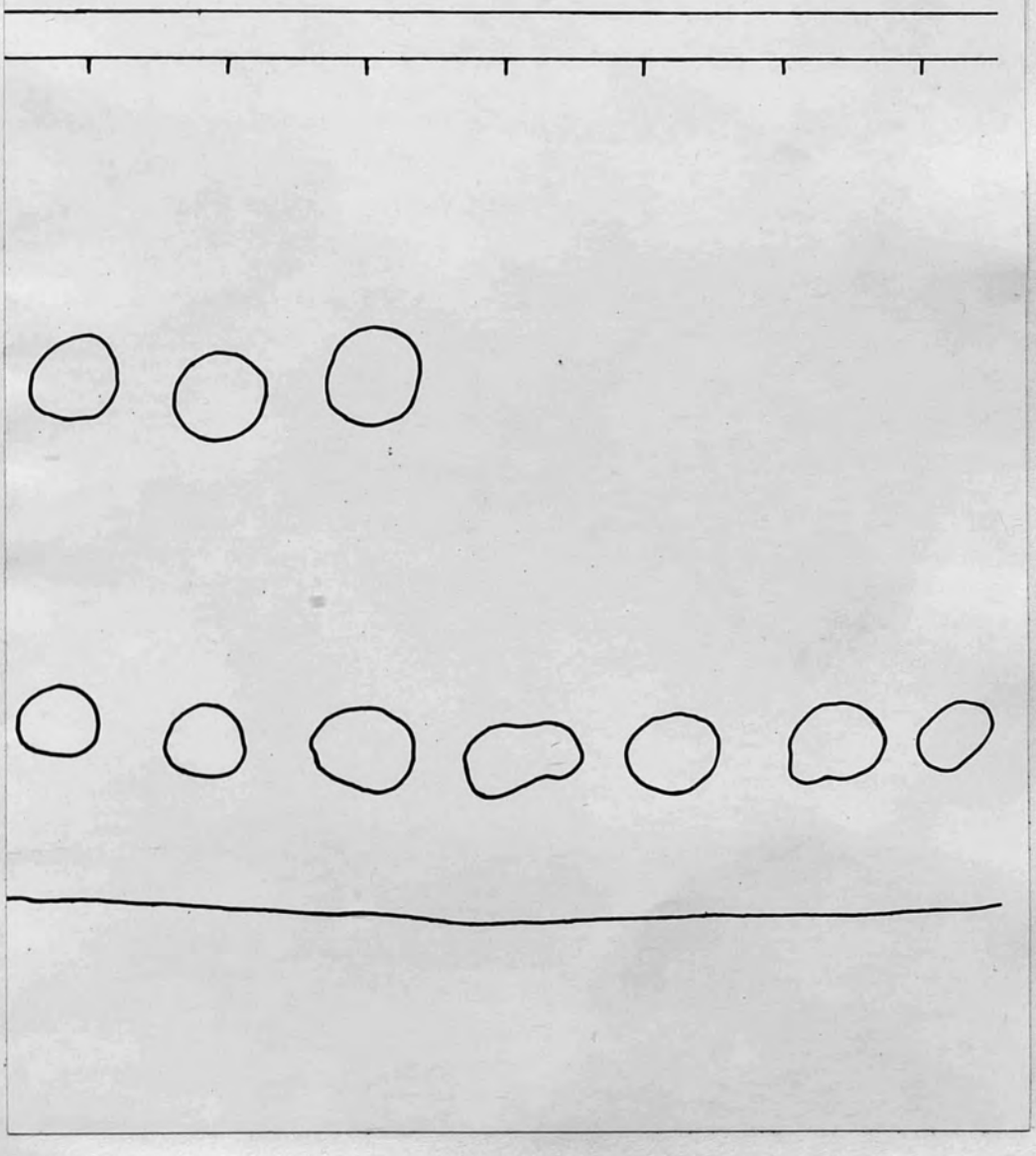


Figure 18. Diagram of a developed chromatogram sprayed with Salper reagent showing the compounds extracted into acid ether from a "Bakhsh" reaction mixture which contained an extract (A) at "zero time" and at intervals during the course of the destruction of IAA.
(Solvent isobutanol (80); methanol (5); water (15).)

FIG. 18

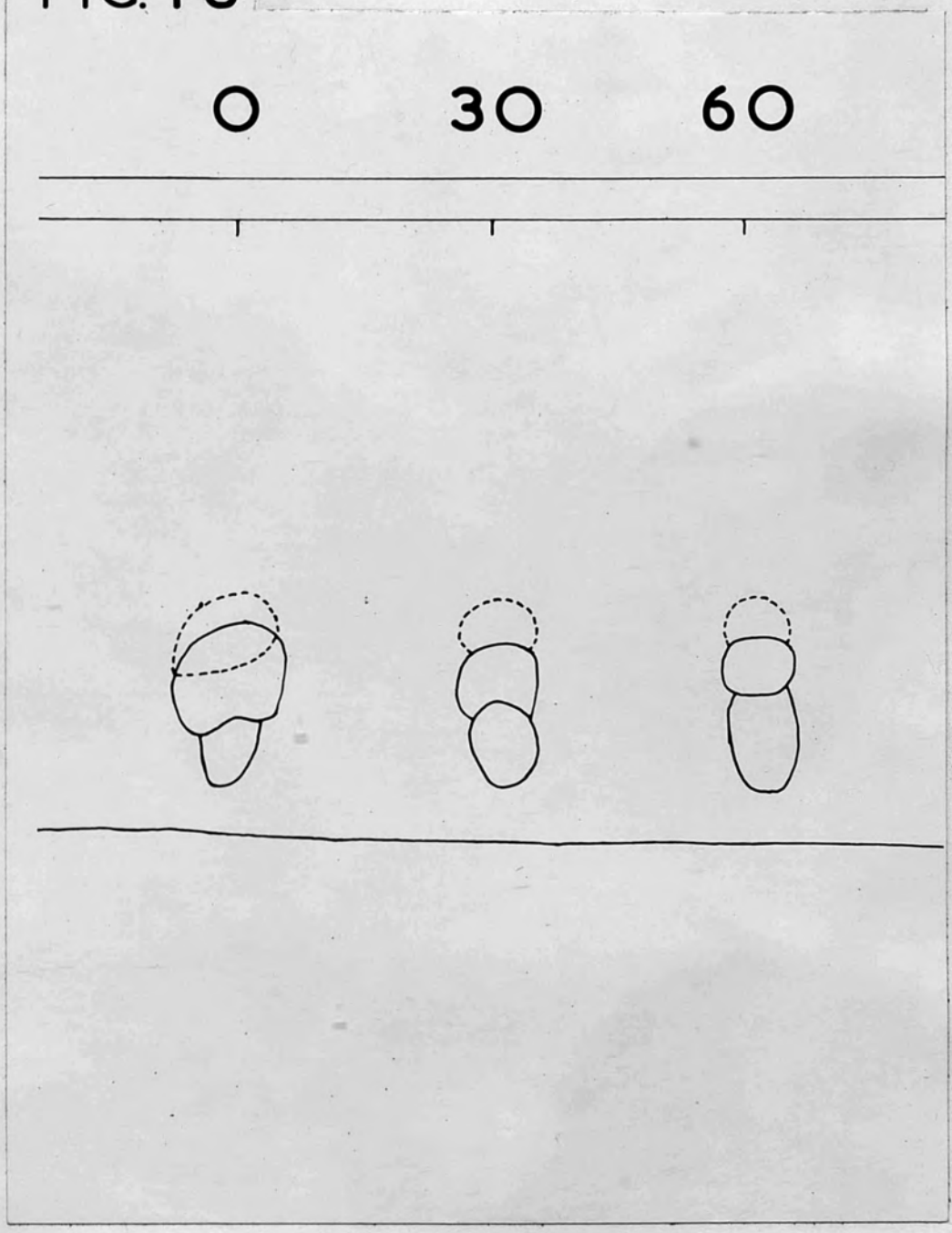


TABLE 10. Average Rf values of spots on the chromatogram shown in figure 16, together with their colour reactions with Ehrlich reagent.

Rf	Colour with Ehrlich reagent.
.15	Pink.
.33	Violet.
.45	Pink.
.67	Purple - black.
.72	Yellowish (several days after spraying)
.76	Yellowish brown.
.85	Pink.

TABLE 11. Average Rf values of spots on the chromatogram shown in figure 17, together with their colour reactions with Van Eck reagent.

Rf	Colour with Van Eck reagent
.39	Yellow.
.80	Yellow.

TABLE 12. Average Rf values of spots on the chromatograms shown in figure 18, together with their colour reactions with Salper reagent.

Rf	Colour with Salper reagent
.66	Brownish.
.72	Brownish-violet.
.83	Violet.

Possible identity of the compounds detected on the chromatograms of figures 14 - 18.

The Rf values and colour reactions of the spots which appeared on the chromatograms developed in the "Isopropanol : Ammonia : Water" solvent (figures 14-17) are summarised below, under three headings.

1. Spots detected on chromatograms of ether extracts of samples of reaction mixtures containing active enzyme preparations.

Spot Number	Rf	Chromatogram on which located	Colour with Salper Reagent	Colour with Ehrlich Reagent	Colour with Van Eck Reagent
No. 1	.12 .15	14a, 14b 16	Pink	Pink	
No. 2	.29 .33	14a, 14b 16	Deep pink-purple	Pink	
No. 3	.39	17			Yellow
No. 4	.41 .45	14a, 14b 16	Deep pink	Pink	
No. 5	.61	14a, 14b	Pale salmon pink		
No. 6	.68 .67	14a, 14b 16	Purple	Purple-black	
No. 7	.72	16		Yellowish	
No. 8	.77 .76	14a, 14b 16	Orange-brown	Yellowish-brown	
No. 9	.80	17			Yellow
No.10	.86 .85	14a, 14b 16	Pink-purple	Pink	

2. Spots detected on the chromatogram of ether extracts of samples of the control reaction mixture - in which the enzyme preparation was replaced by distilled water.

Spot Number	
No. 1	Located on chromatogram 14c. (Rf and colour with Salper reagent asgiven for 14a and 14b, under 1. above.)
No. 2	
No. 4	
No. 6	
No. 8	
No. 10	

(Nos. 3, 7 and 9 were not tested for).

3. Spots detected on the chromatogram of solutions of IAA in methanol.

Spot Number	Rf	Colour with Salper reagent	
No. 0 *	.06	Pale pink.	Located on chromatogram 15.
No. 1	.14	Pale pink.	
No. 2	.32	Deep purple.	
No. 4	.47	Bright pink.	
No. 6	.70	Blackish brown.	
No. 8	.79	Brownish (faint).	
No. 10	.85	Pink.	

* Not present on chromatograms 14a, 14b and 14c.

(Nos. 3, 7 and 9 were not tested for.)

Of the spots listed 0 - 10 above, none were identified with certainty - apart from IAA. However it was thought that certain of them corresponded with those found under similar conditions by Manning and Galston (1955), Stutz (1957, 1958) and Melchior (1958) - as given in the table below. (An account of the results obtained by these workers was given on page 30 of the "Introduction".)

Spot Number	Manning and Galston	Stutz	Melchior
No. 0			
No. 1			ICS
No. 2		IAA	IES (=IAA)
No. 3			IGyS
No. 4		# 1	IGS ?
No. 5			
No. 6			Compound Rf 0.75
No. 7		(Anthranilate found in # 4 after elution)	
No. 8	Compound Rf 0.91	# 4	IA
No. 9		Indolealdehyde	
No. 10	Compound Rf 0.94	# 5	"W ?"

Note 1. At first it was thought that spot No. 3 was indoleglyoxylic acid since it corresponded in position with the spot given the symbol "IGyS" by Melchior. Later however it was realised that a yellow colour with Van Eck reagent at about the same Rf as IAA, occurred most strongly on chromatograms with large IAA spots, and alsothat the density of the colour was increased if the chromatograms were stored before spraying. It was concluded therefore that IAA was being broken down on the paper and hence that the yellow spot was again that of indolealdehyde.

Note 2. According to Melchior the two spots with Rfs .89 and .93 which gave colour reactions with Salper reagent were IA (indolealdehyde) and "W ?" - the indolealdehyde also giving a yellow colour with Van Eck reagent. In the present work however it was found that indolealdehyde (Rf .80) was only detectable by its reaction with Van Eck reagent, and that it was located between the two Salper-reacting spots (Rfs .77 and .86). This corresponded with findings of Stutz.

None of the spots detected on the chromatogram developed in the "Isobutanol : Methanol : Water" solvent were identified, although the spot with an Rf of .72 giving a brownish-violet colour with Salper reagent was presumably attributable, at least in part, to IAA.

Additional observations on the nature of the reaction products.

The oxidation of IAA in the reaction mixtures used in this investigation was always accompanied by the formation of a brownish red colour. This however had no interfering effect in the colorimetric method for IAA/

IAA estimation. Since Yamazaki and Souzu (1960) reported the same phenomenon for a reaction mixture which did not contain a phenolic activator, it was thought to result from an accumulation of the products of the oxidation of IAA itself and not of the phenol (DCP). Nevertheless it must be noted that Leopold and Plummer (1961) claimed that they had detected the formation of coloured complexes of IAA with quinones formed from catechol, chlorogenic acid and caffeic acid during the oxidation of IAA in the presence of polyphenol oxidase. These were insoluble in ether and stable only in neutral chromatographic solvents.

When the ether extract of the reaction products was evaporated down, the concentrated extract frequently appeared reddish in colour - as also did the spot obtained by applying the residue dissolved in methanol, to the chromatography paper. This colouration did not however appear on the developed chromatograms. It may have been caused by the presence of urorosein - a pigment formed by the condensation of two molecules of indolealdehyde in the presence of acid. (Fearon and Boggust, 1950).

II 1. THE EFFECT ON THE "IAA-OXIDASE" ACTIVITIES OF EXTRACTS (A) AND (B) OF VARIATIONS IN THE pH OF THE REACTION MIXTURE.

Extracts (A) and (B) were prepared as described under "Methods", Section II, and their "IAA-oxidase" activities determined as described under "Methods", Sections IV-1 and III-1, in a number of different reaction mixtures of varying pH. The composition of these reaction mixtures was basically that used by Bakhsh (as given under i. on page 82). The composition of the phosphate - citrate buffer however was varied to give reaction mixtures of pH 3, 4,5 and 6. 20 ml. amounts of each were made up and these were sampled at "zero time" and at 10 minute intervals thereafter for a period of 1½ hours. The results are given in figure 19 (in which the absorbancy values were plotted directly) and in figure 20, in which the lengths of the lag periods (in minutes) and the maximum rates attained (expressed as change in abosrbancy / 10 minutes) were plotted against pH. These latter values were obtained over a number of 10 minute intervals, varying from 10 - 20 minutes (pH 4, extracts (A) and (B)) to 70 - 80 minutes (pH 3, extract (A)).

The graphs showed that the pH optimum for the activity of the enzyme extracts (A) and (B) was in the region of 4. Thus the maximum rates were highest and also the lengths of the lag periods shortest at this pH. This corresponded with the value obtained using crude pea extracts by Bakhsh (1956) and by Reinert, Schraudolf and Reinert (1957). A number of workers however have reported that the pH optimum for such extracts was in the region of 6 (Tang and Bonner, 1947; Galston and Dalberg/

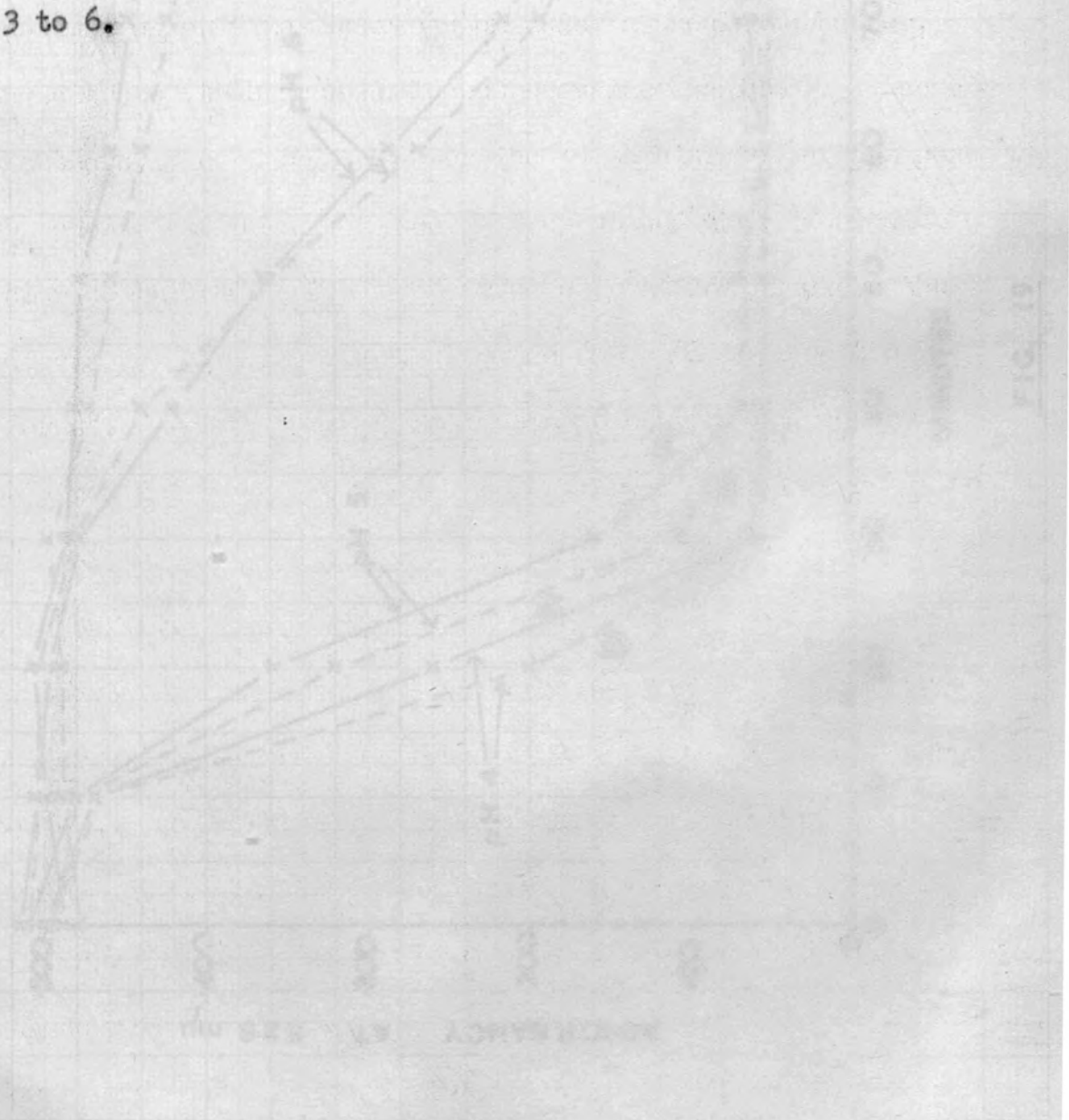
Dalberg, 1954). No explanation of this discrepancy can be offered at present. Mudd, Johnson, Burris and Buchholtz (1959), who worked with purified IAA-oxidase preparations from quack-grass rhizomes, suggested that the acid pH optima obtained by previous workers could be attributed to the fact that phosphate-citrate buffers had been used. They obtained an optimum of pH 5 in their system using a succinate buffer, and of 3 using citrate (this was the lowest pH value examined). According to these authors, the pH optima determined with citrate buffers were invalidated by chelation (of manganese). They stated that "appreciable activity in citrate buffer should be observed only at low pH where the undissociated acid and the monoanion, which do not chelate, are the predominant forms". However the pH optimum of 6 of Tang and Bonner (1947) was obtained using phosphate-citrate buffer, while conversely the pH optimum of 4 of Reinert, Schraudolf and Reinert (1957) was obtained using phosphate buffer.

In order to determine whether the pH optimum occurred at a higher value if a buffer which did not contain citrate was used - on a subsequent occasion - the activity of a reaction mixture containing an extract (A) was determined at pH 4 (phosphate-citrate buffer) and at pH 6 (phosphate buffer). 10 ml. amounts of the two reaction mixtures were made up and these were sampled at "zero time" and at 10 minute intervals thereafter for a period of 1 hour. The results are given in figure 21. The rate of IAA oxidation in phosphate buffer at pH 6 was found to be much slower than in phosphate-citrate buffer at pH 4. It therefore seemed/

seemed unlikely that the pH optimum would have been found to be 6 if the rates had been determined in phosphate buffer over the complete range from pH 3 to pH 6. (Phosphate buffers buffer poorly below pH 6).

An examination of figure 19 showed that the rates of oxidation of IAA in the reaction mixtures containing extract (B) were faster than in the reaction mixtures containing extract(A). Thus the maximum rates attained were, in general, higher and the lengths of the lag periods lower (figure 20) in the presence of extract (B) than in the presence of extract (A).

Figure 19. Graphs to show the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A) and (B) in the presence of phosphate-citrate buffers over the pH range 3 to 6.



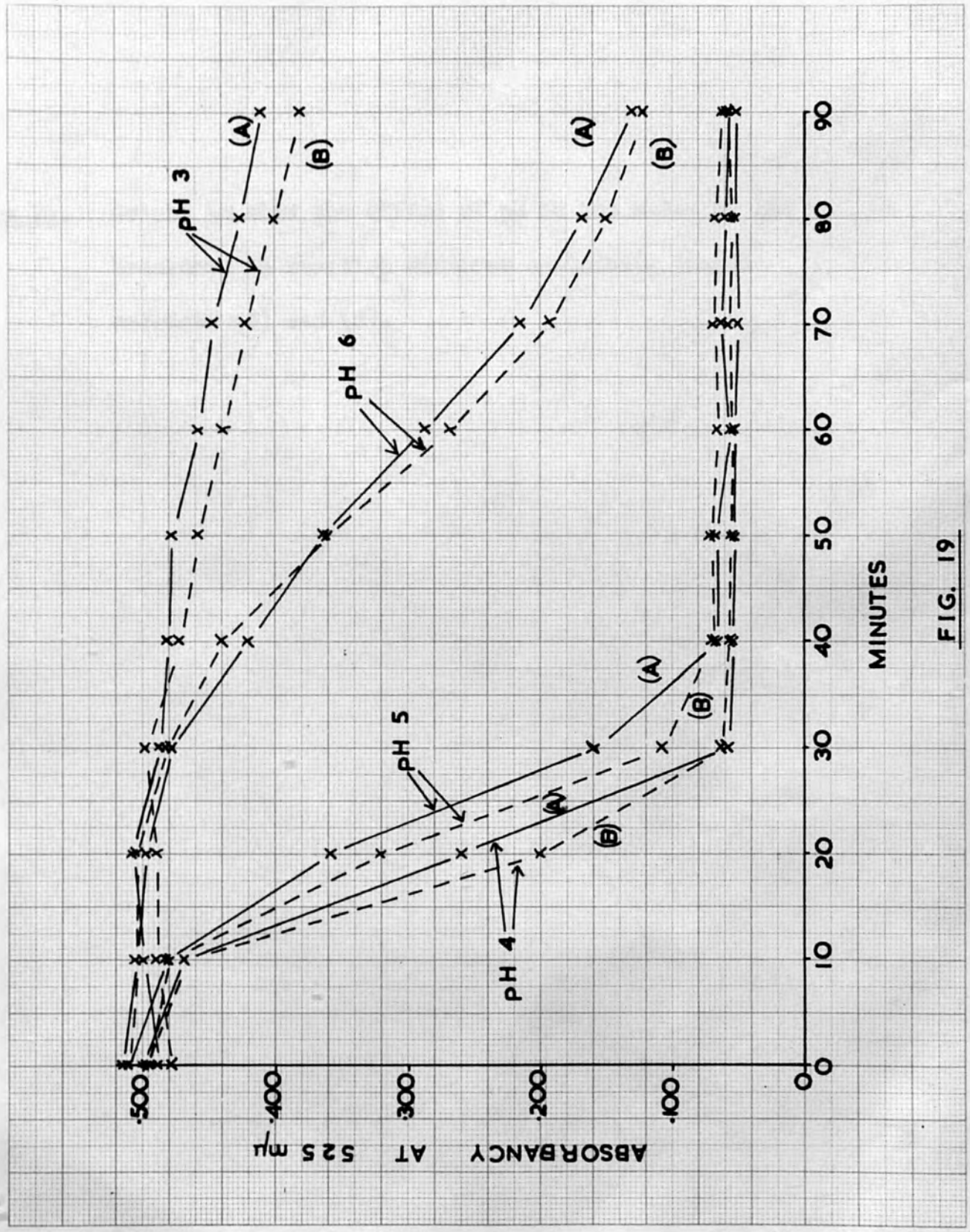

ABSORBANCY AT 525 mμ
.500
.400
.300
.200
.100
0
10
20
30
40
50
60
70
80
90
MINUTES
pH 3
pH 4
pH 5
pH 6
(A)
(B)

FIG. 19

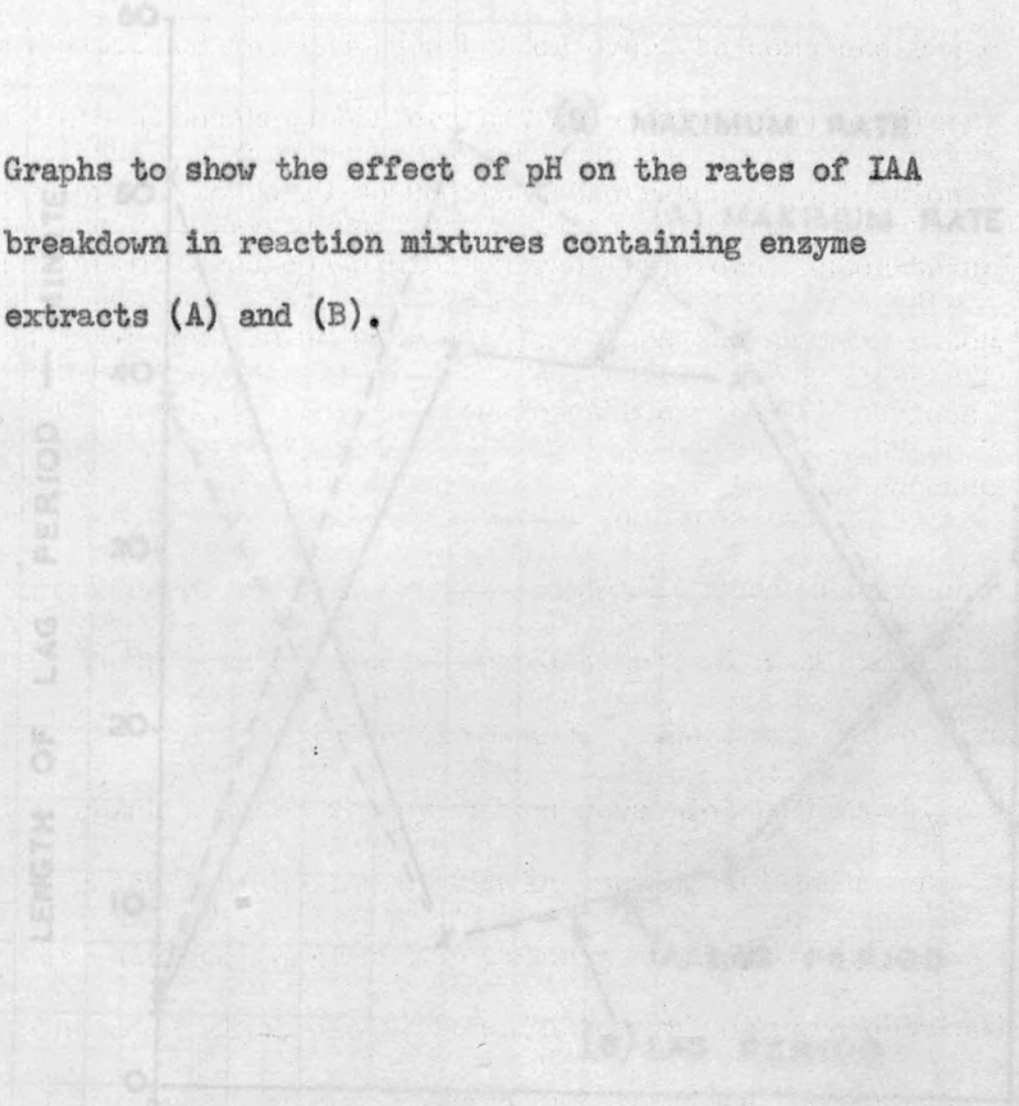


Figure 20. Graphs to show the effect of pH on the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A) and (B).

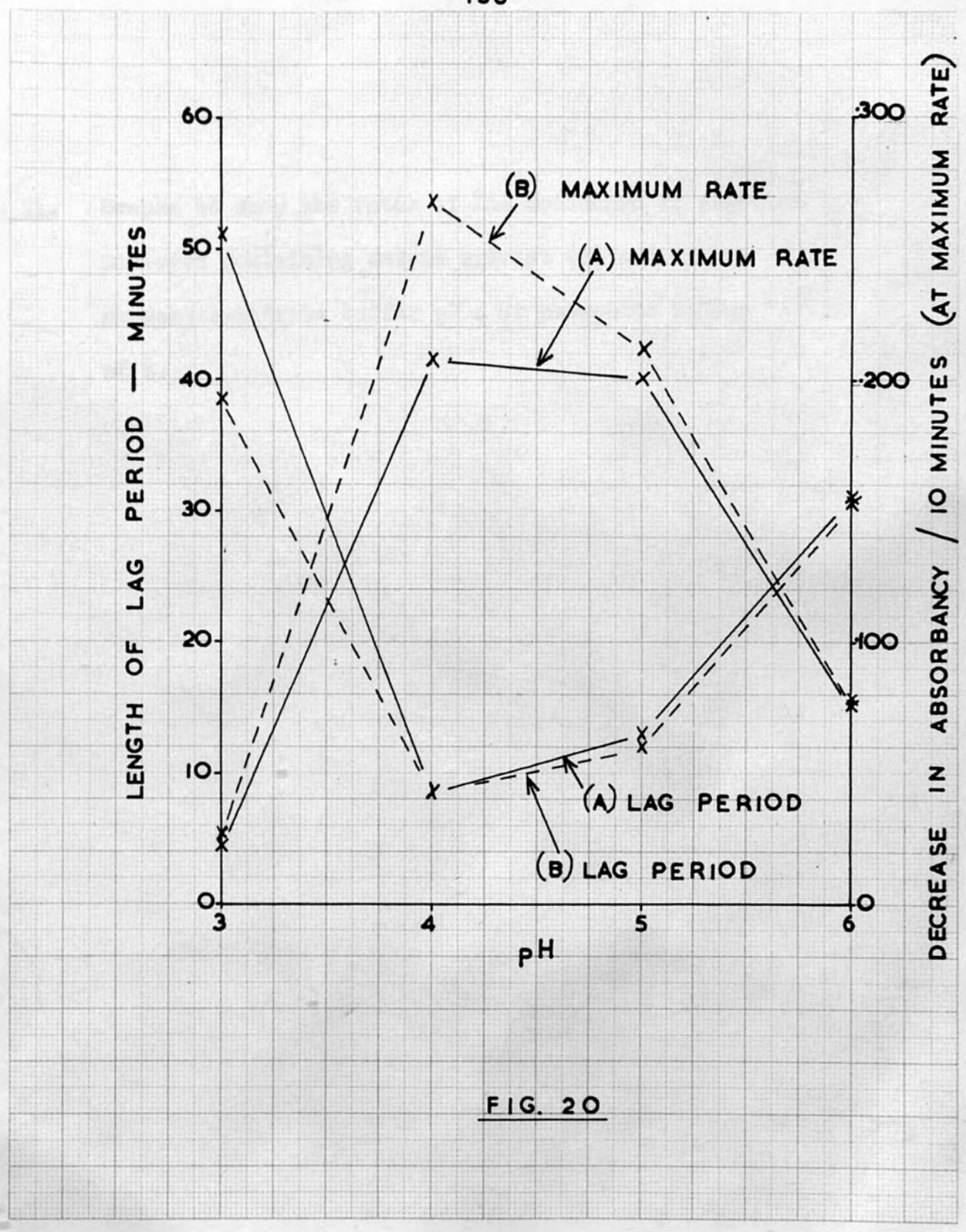

LENGTH OF LAG PERIOD — MINUTES
DECREASE IN ABSORBANCY / 10 MINUTES (AT MAXIMUM RATE)
60
50
40
30
20
10
0
300
200
100
0
3
4
5
6
pH
(B) MAXIMUM RATE
(A) MAXIMUM RATE
(A) LAG PERIOD
(B) LAG PERIOD

FIG. 20

Figure 21. Graphs to show the rates of IAA breakdown in reaction mixtures containing enzyme extract (A) and either phosphate-citrate buffer pH 4 or phosphate buffer pH 6.

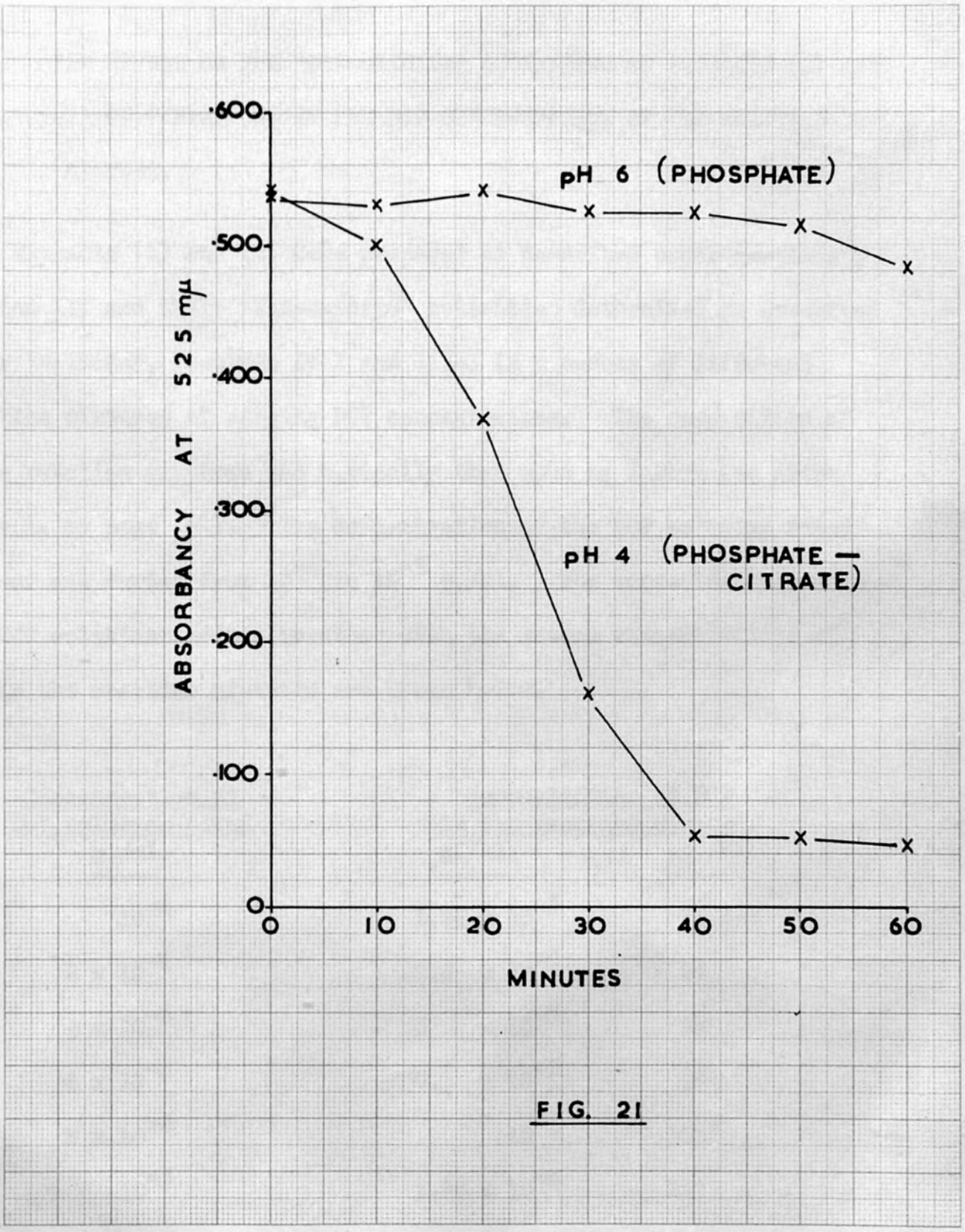

pH 6 (PHOSPHATE)
pH 4 (PHOSPHATE — CITRATE)
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
MINUTES

FIG. 21

II 2. THE EFFECT ON THE "IAA-OXIDASE" ACTIVITIES OF EXTRACTS (A) AND (B) OF VARIATIONS IN THE DCP CONCENTRATION OF THE REACTION MIXTURE.

Extracts (A) and (B) were prepared as described under "Methods", Section II, and their "IAA-oxidase" activities determined as described under "Methods", Sections IV-1 and III-1 in a number of different reaction mixtures of varying DCP concentration. The composition of these reaction mixtures was basically that used by Bakhsh (as given under i. on page 82). The concentration of the DCP solution added however was varied from 10^{-4} to 10^{-3} g./ml. The concentrations of the DCP solutions added, together with the actual concentrations of DCP in the reaction mixtures are shown below.

Concentration of DCP solution added g./ml.	Concentration of DCP in the reaction mixture. g./ml.	µg./ml.
10^{-4}	1.25×10^{-5}	12.5
2×10^{-4}	2.5×10^{-5}	25
4×10^{-4}	5×10^{-5}	50
6×10^{-4}	7.5×10^{-5}	75
8×10^{-4}	10^{-4}	100
10^{-3}	1.25×10^{-4}	125

10 ml. amounts of each reaction mixture were made up and these were sampled at "zero time" and at 10 minute intervals thereafter for a/

a period of 1 hour. The results are given in figures 22a - 22f, in which the absorbancy results were plotted directly against time.

An examination of figures 22a - 22f showed that there appeared to be an increase in "IAA-oxidase" activity followed by a decrease, with increasing concentration of DCP. From each graph an estimate was obtained for the time taken for the reaction to reach completion - as described on page 84. The reciprocals of these values were then calculated to give relative rates (arbitrary values) and these were plotted against DCP concentration (figure 23). From the curves obtained (figure 23), it appeared that the optimum concentration of DCP for both extract (A) and extract (B) lay between 50 µg./ml. and 100 µg./ml. - possibly at 75 µg./ml. The flattening of the curve for the (B) extract at the lower concentrations of DCP may have been an "artefact" - since the graphs given in figures 22a, 22b and 22c may have yielded false values for the rates of IAA destruction - for the reason discussed under "Results", Section I, page 87(2.). The value obtained for the (A) extract at a DCP concentration of 75 µg./ml. was also subject to the same uncertainty. Estimated values for the lengths of the lag periods and the maximum rates attained are given in table 13, page 169. In general the shortest lag periods, and the highest maximum rates were obtained for reaction mixtures in which the "time taken for the reaction to reach completion" were shortest. However the relationship between the three quantities was somewhat irregular, and since the data was obtained from one experiment only it was not thought desirable to attach importance to the individual values.

The conclusion reached therefore was that the optimum concentration was slightly higher than that reported for the same reaction mixture by Bakhsh (1956). The difference however was not great - 75 µg./ml. as opposed to 50 µg./ml. The relationship between the "IAA-oxidase" activities of extracts (A) and (B) was found to be similar to that reported under "Results", Section I - that for extract (B) being higher. It is relevant to emphasise again here however that the optimum concentration of DCP was the same for both types of extract. Thus this might be considered to show that the difference between the (A) and (B) extracts was not one merely of a different level of a DCP-like activator. If this had been so, then it would have been expected that the optimum concentration of DCP for the (A) extract would have been higher than that for the (B) extract (assuming that the level of the endogenous activator was suboptimal in both cases). Similarly if a difference in activator level had been the only difference, the added activator might have been expected to raise the activities of the two extracts to an equal level, whereas figure 23 showed that the activity of the (B) extract was always higher than that of the (A) extract. Finally, in spite of the "flattening" of the curve for the (B) extract (figure 23), it was considered that the amount of activation of the enzyme produced by a given amount of DCP was independent of the "basal activity" of the enzyme extract - so that observed activity was always proportional to this "basal activity" (over the range of DCP concentration studied). This supported the assertion of Galston and Dalberg (1954) that DCP "was/

"was found never to alter the relative activities of the enzymes in different breis, but only to give greater absolute differences in activities in # shorter times".

It might be inferred from this statement that the amount of IAA broken down in two reaction mixtures - one with DCP and one without - would be the same - although in the absence of activator, the breakdown would take longer. However, assuming that the reaction mixtures used in the present investigation were similar tothose of Galston and Dalberg, this was probably not the case - except possibly with relatively low concentrations of IAA. Thus, under conditions in which the rate of IAA breakdown - for a given initial IAA concentration - did not exceed a certain minimum value, the reaction did not proceed to completion. In "Results", Section I, for example, rates of IAA breakdown were given for three reaction mixtures - differing from each other only in enzyme concentration. Whereas at the two higher concentrations of enzyme the IAA was completely destroyed (figures 9 and 10), at the lowest concentration, the reaction proceeded for a time at a very much slower rate, and then virtually ceased (figure 11). This was attributed to the inactivation of the enzyme during the reaction - probably as a result of a reaction with IAA$^{\cdot}$ radicals (as discussed on page 27 of the "Introduction"). Further information on the subject of this phenomenon of enzyme inactivation was obtained in the experiment described below.

Figure 22a. Graphs to show the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A) and (B) in the presence of DCP at a concentration of 12.5 µg./ml.

Figure 22b. As figure 22a. DCP concentration 25 µg./ml.

Figure 22c. As figure 22a. DCP concentration 50 µg./ml.

Figure 22d. As figure 22a. DCP concentration 75 µg./ml.

Figure 22e. As figure 22a. DCP concentration 100 µg./ml.

Figure 22f. As figure 22a. DCP concentration 125 µg./ml.

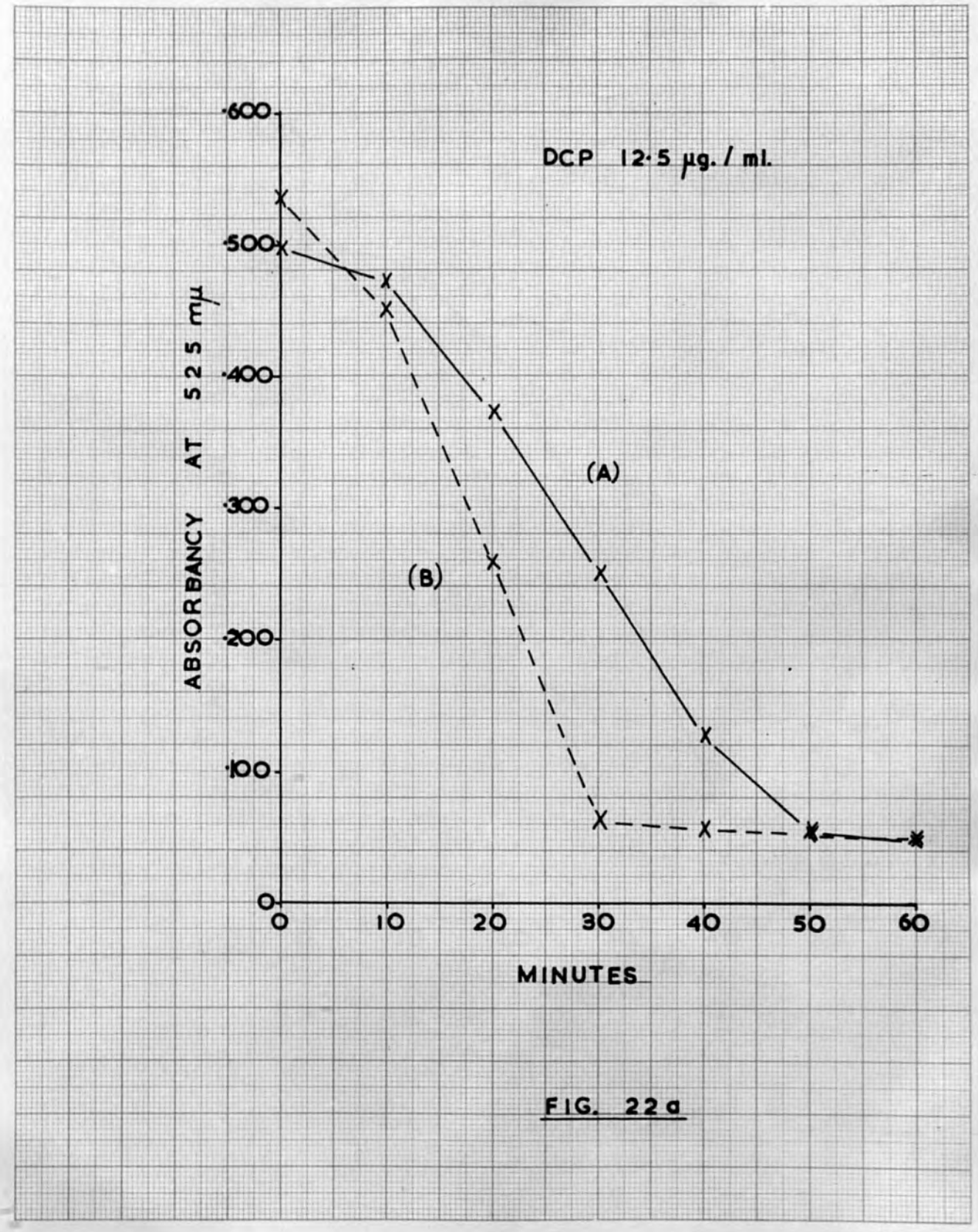

DCP 12·5 µg. / ml.
ABSORBANCY AT 525 mµ
600
500
400
300
200
100
0
(A)
(B)
0
10
20
30
40
50
60
MINUTES

FIG. 22a

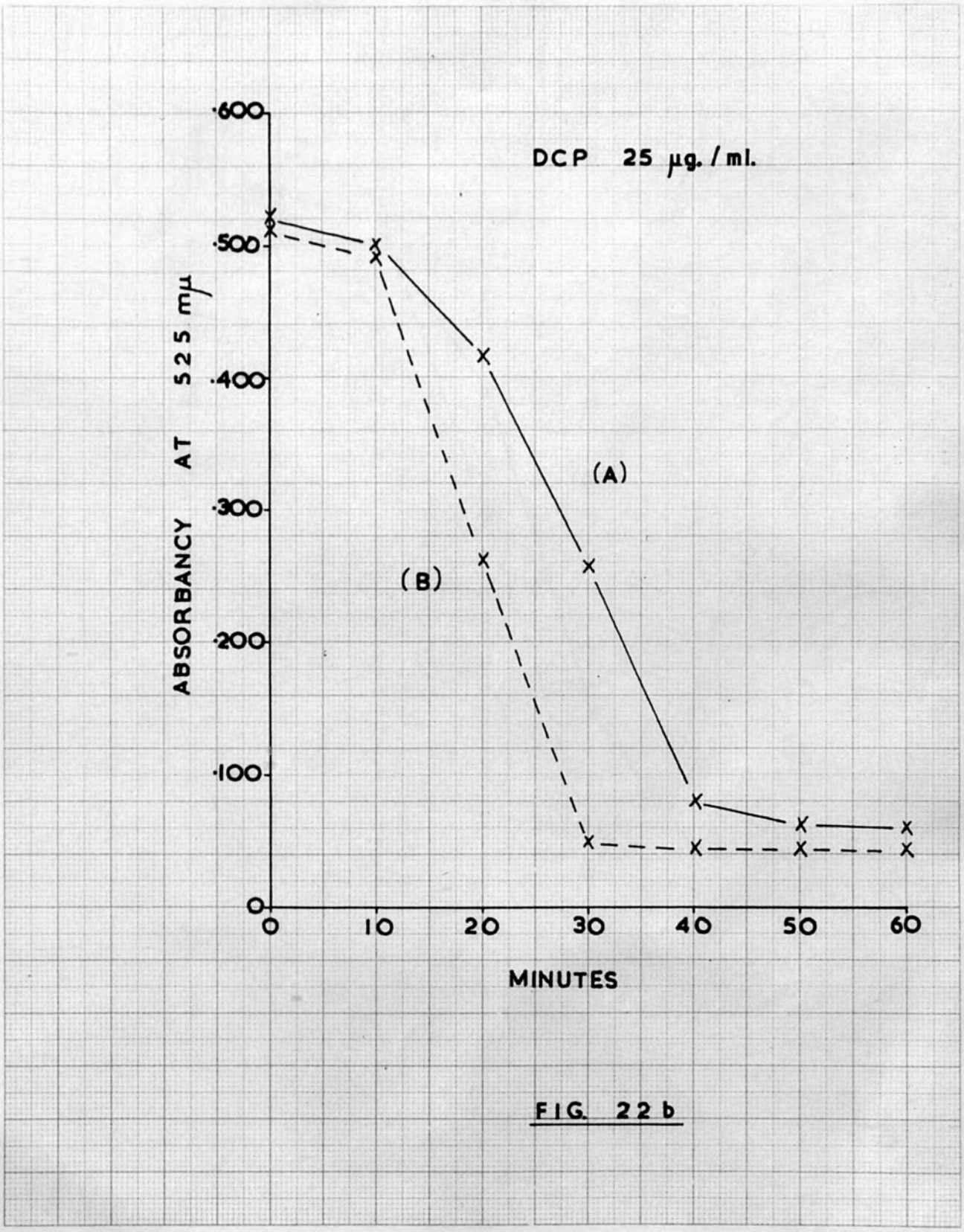

DCP 25 µg./ml.
ABSORBANCY AT 525 mµ
·600
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
MINUTES
(A)
(B)

FIG. 22 b

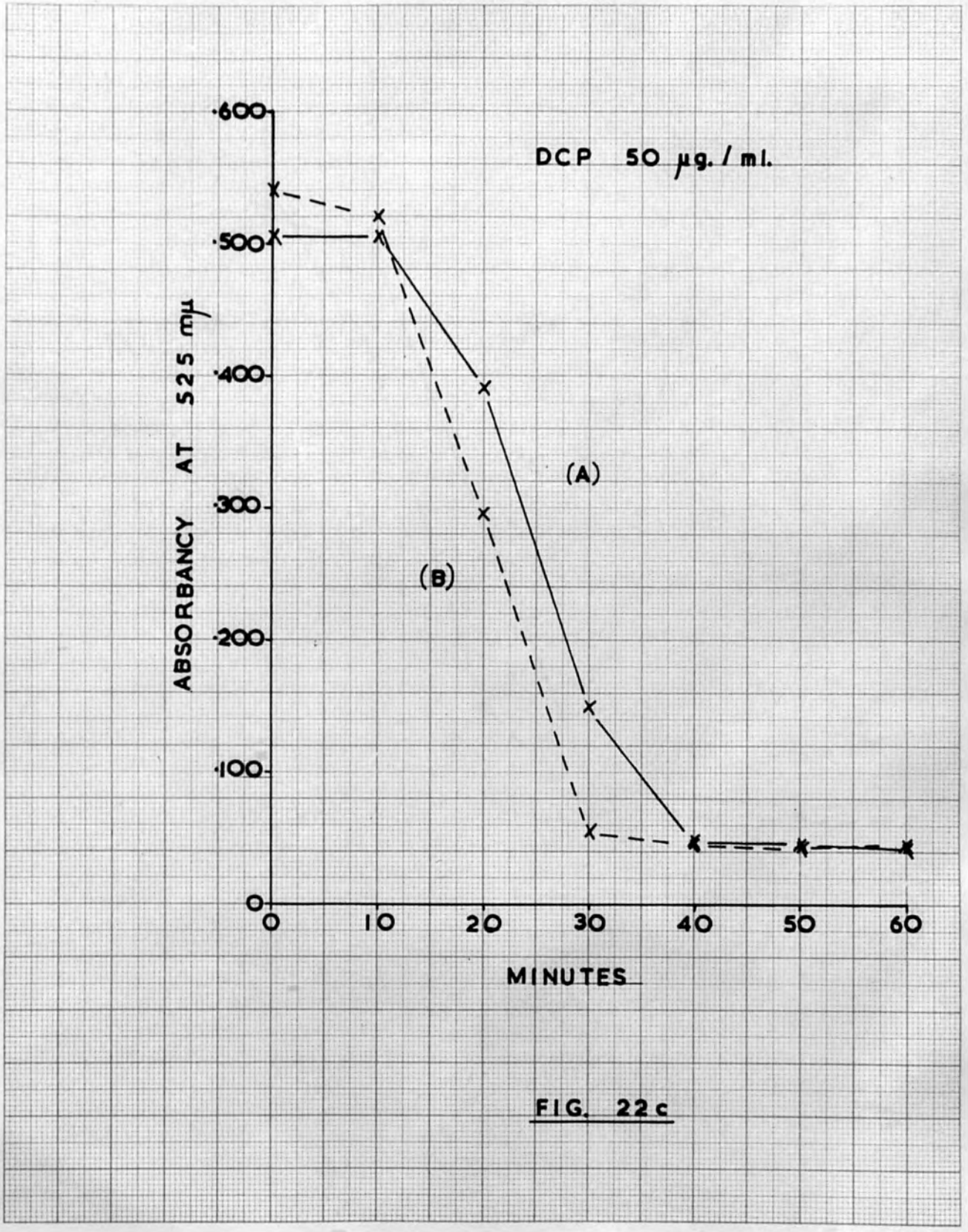

DCP 50 µg. / ml.
ABSORBANCY AT 525 mµ
·600
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
MINUTES
(A)
(B)

FIG. 22c

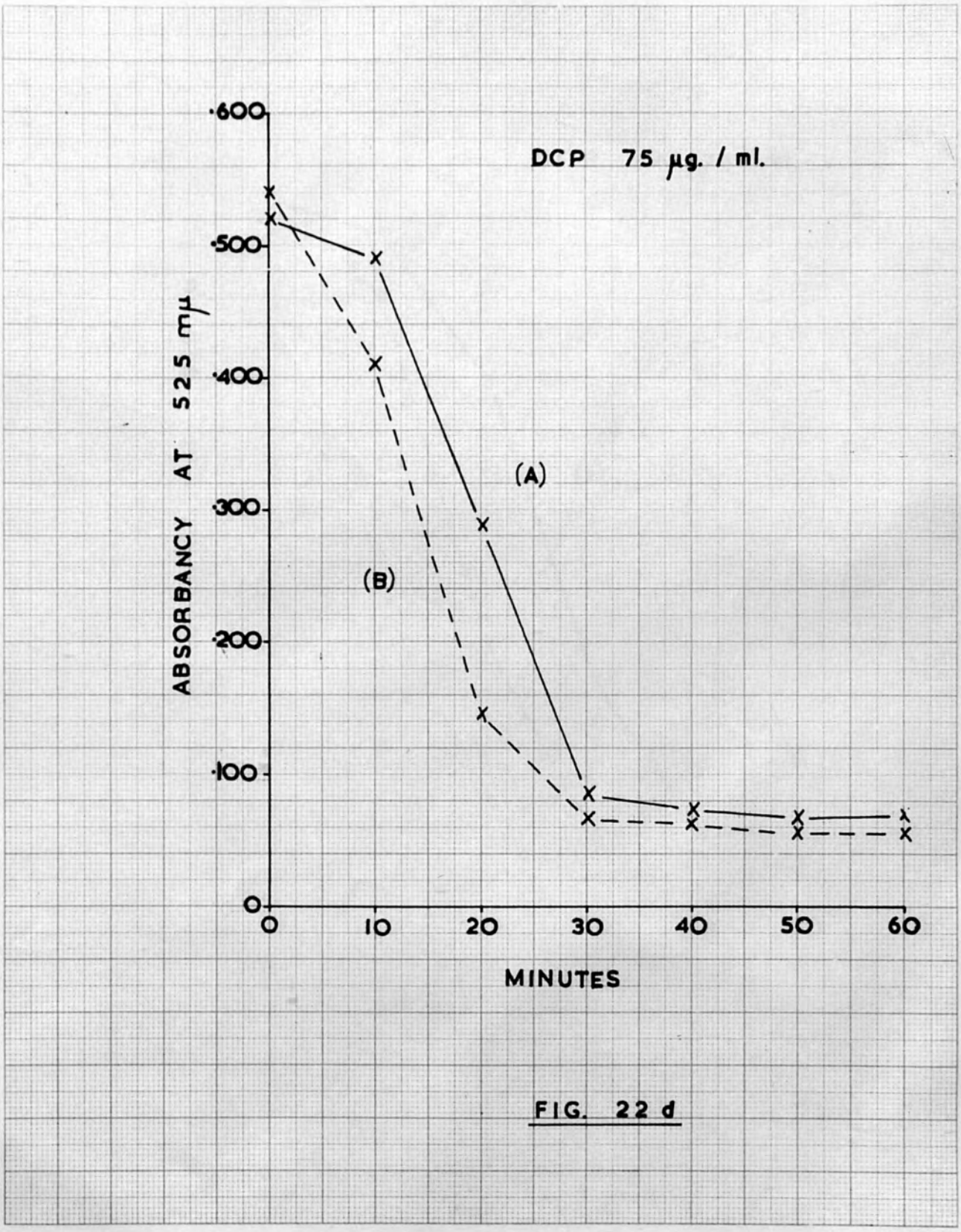

DCP 75 µg. / ml.
ABSORBANCY AT 525 mµ
·600
·500
·400
·300
·200
·100
0
(A)
(B)
0
10
20
30
40
50
60
MINUTES

FIG. 22 d

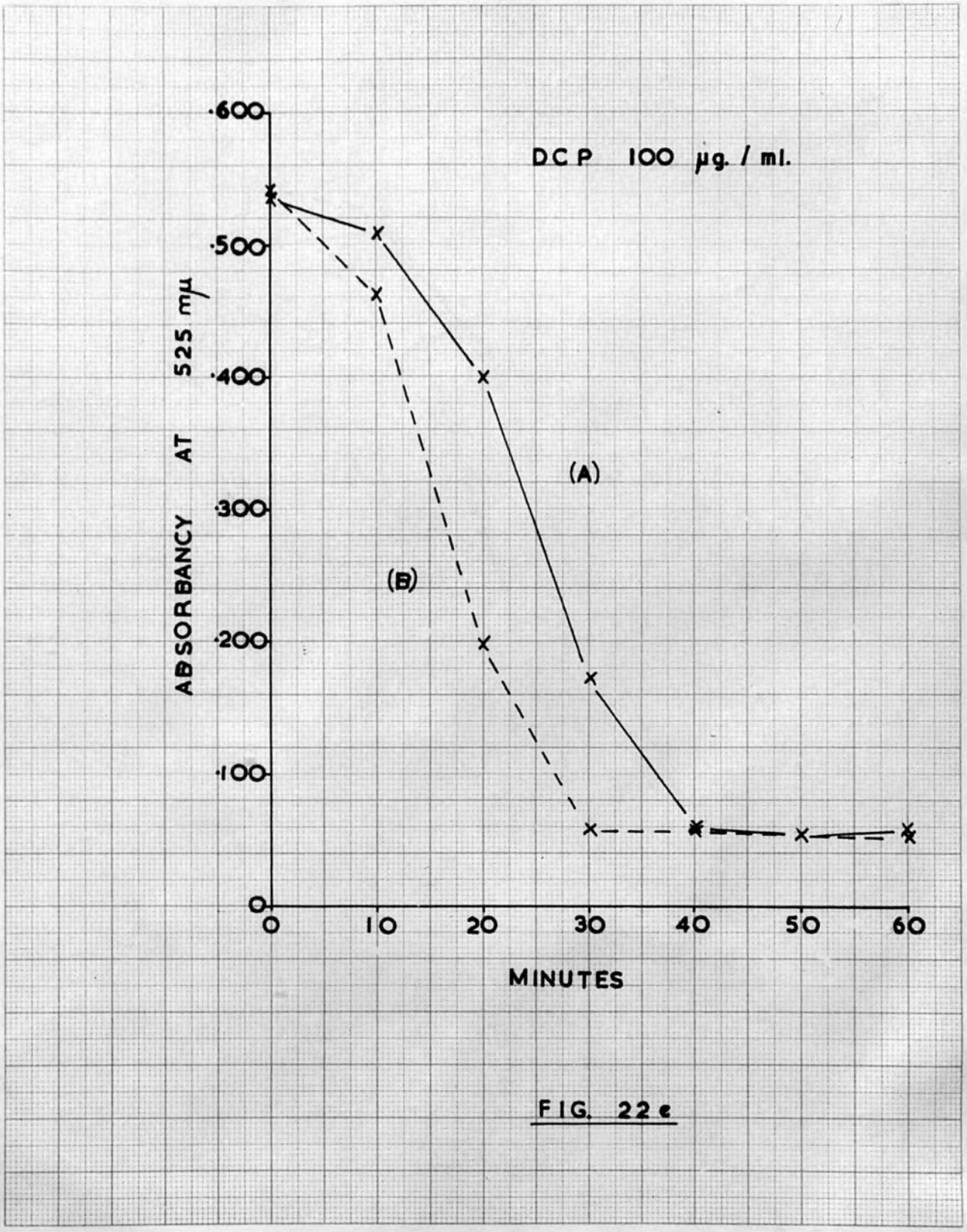

DCP 100 µg. / ml.
ABSORBANCY AT 525 mµ
·600
·500
·400
·300
·200
·100
0
(A)
(B)
0
10
20
30
40
50
60
MINUTES

FIG. 22e

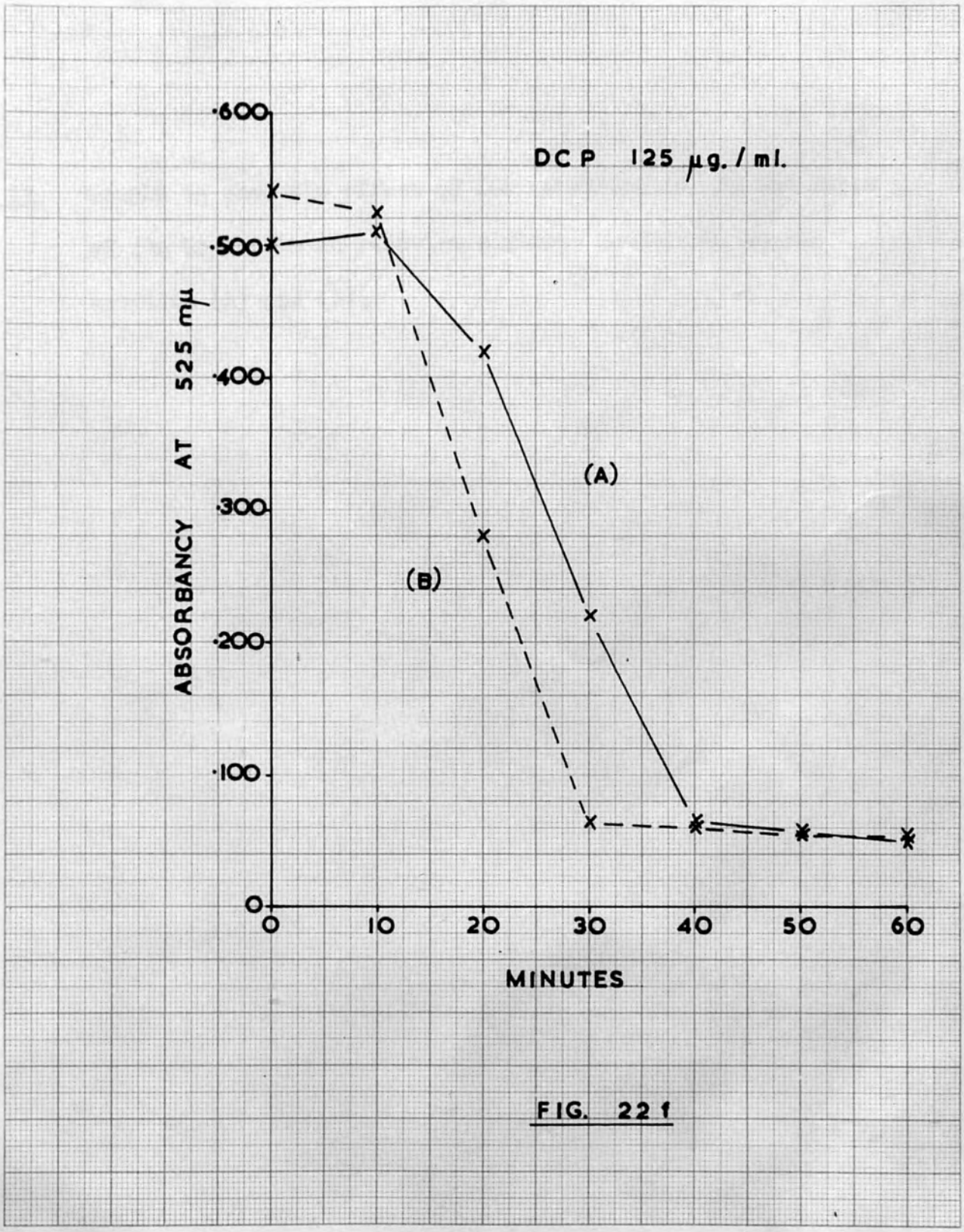
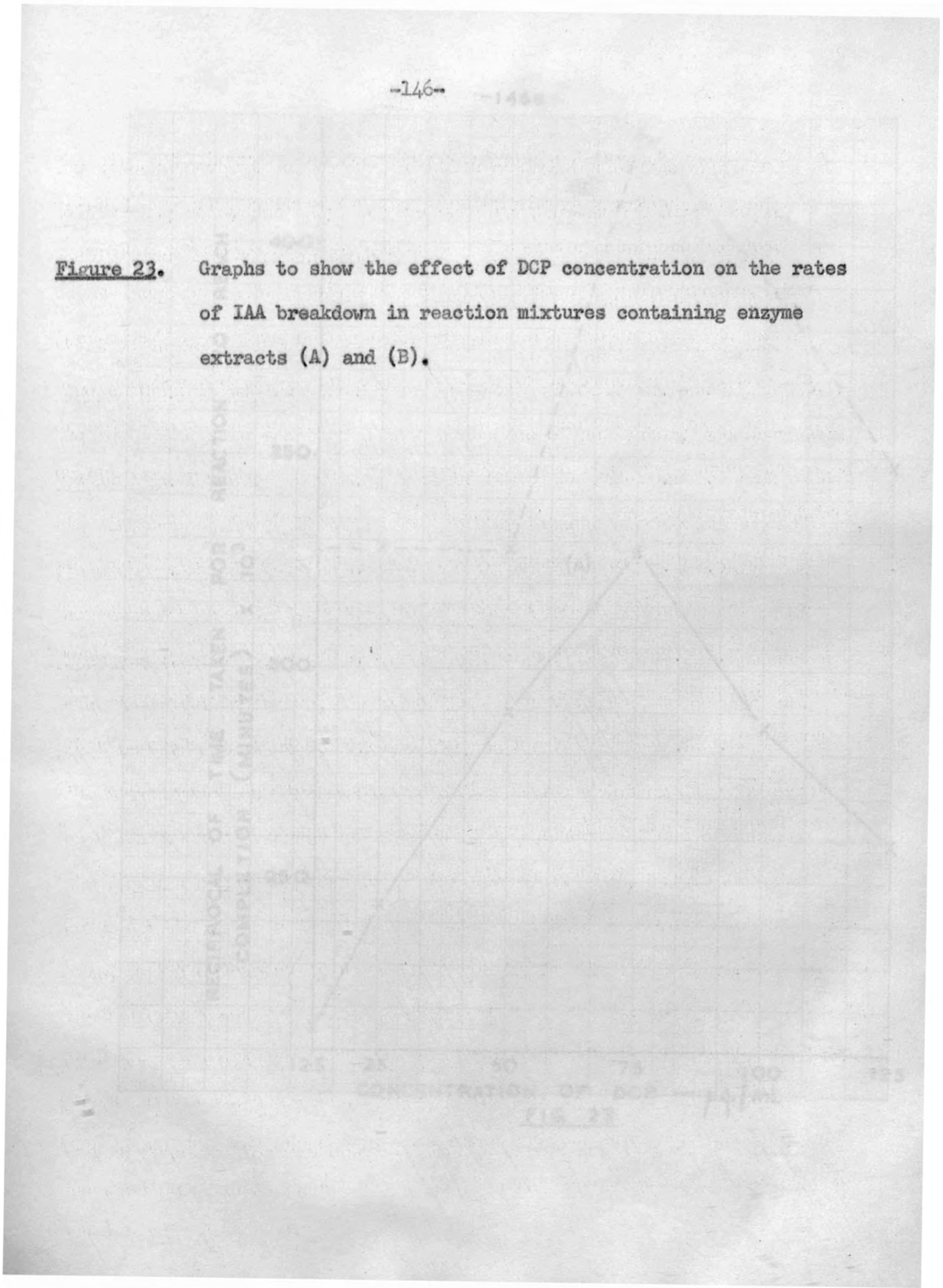

DCP 125 µg./ml.
ABSORBANCY AT 525 mµ
·600
·500
·400
·300
·200
·100
0
(A)
(B)
0
10
20
30
40
50
60
MINUTES

FIG. 22 f

Figure 23. Graphs to show the effect of DCP concentration on the rates of IAA breakdown in reaction mixtures containing enzyme extracts (A) and (B).



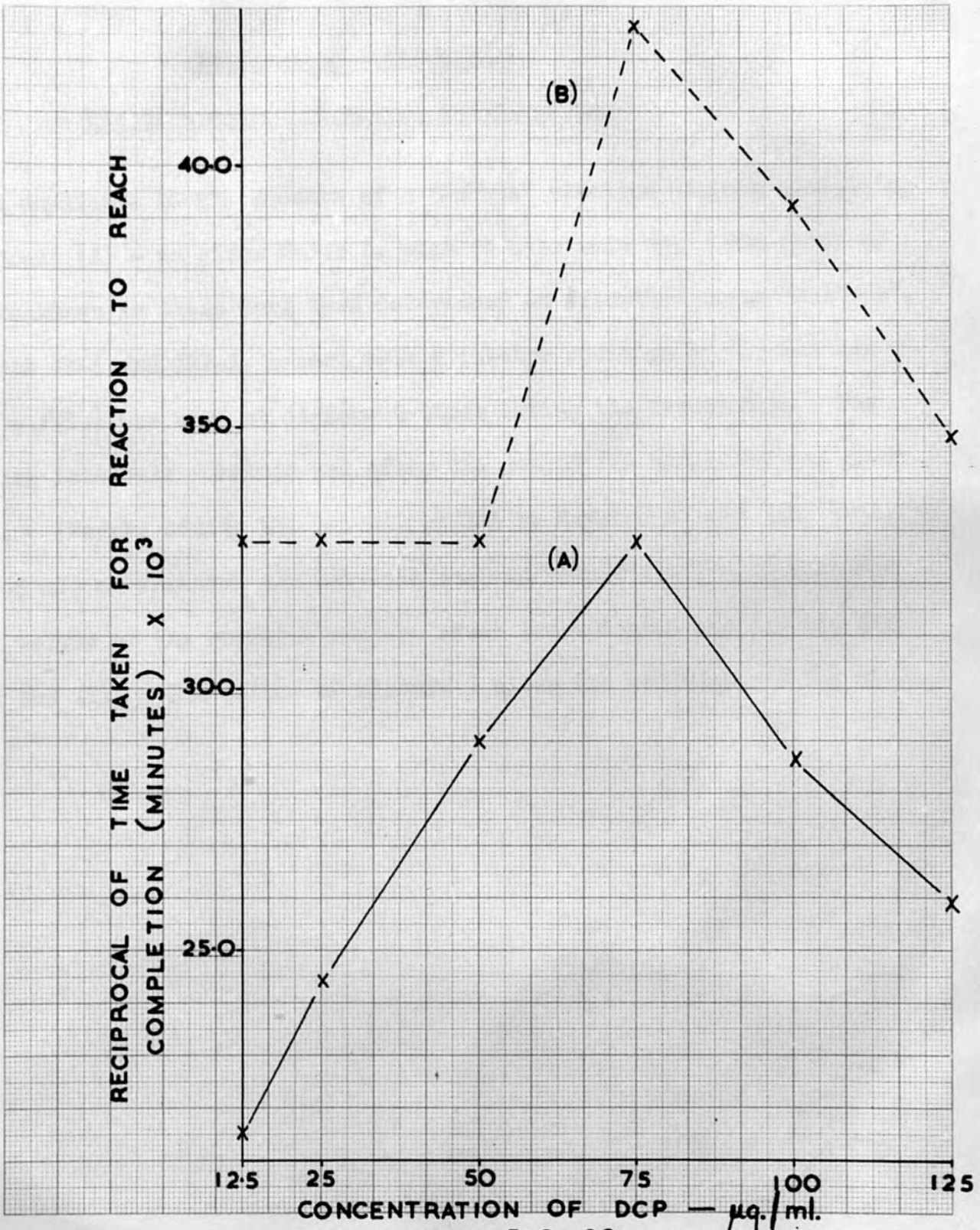

RECIPROCAL OF TIME TAKEN FOR REACTION TO REACH COMPLETION (MINUTES) X 10^3
40·0
35·0
30·0
25·0
(B)
(A)
12·5
25
50
75
100
125
CONCENTRATION OF DCP — µg./ml.

FIG. 23

Addendum to Section II.

The time course of enzyme inactivation.

4 duplicate 10 ml. amounts of a "Bakhsh" reaction mixture containing an extract (A) - as given under i. page 82 were made up. The rates of IAA breakdown in these were then determined as described under "Methods", Sections IV-1 and III-1, except that a further addition of 0.5 ml. IAA (10^{-3} g./ml.) was made at different times during the incubation. The sampling intervals - before and after the second IAA additions are given below. (Before adding the 0.5 ml. of IAA to Samples II and III, 1 ml. and 0.5 ml. of reaction mixture were removed respectively - so that the final volume of the reaction mixture after the addition was 8.5 ml. for I, II and III. The volume of mixture I after the addition was 7.5 ml.).

Sampling intervals:

	Before the 2nd addition of IAA.	After the 2nd addition of IAA.	
Sample I.	"zero time"	"zero time"	(IAA added at 120 minutes)
	10 minutes	60 minutes	
	20 minutes	Next day.	
	30 minutes		
	40 minutes		
	50 minutes		
Sample II.	"zero time"	"zero time"	(IAA added at 10 minutes)
	10 minutes	20 minutes	
		40 minutes	
		60 minutes	
		Next day.	
Sample III.	"Zero time"	"zero time"	(IAA added at 20 minutes)
	10 minutes	20 minutes	
	20 minutes	40 minutes	
		60 minutes	
		Next day.	
Sample IV.	"zero time"	"zero time"	(IAA added at 30 minutes)
	10 minutes	20 minutes	
	20 minutes	40 minutes	
	30 minutes	60 minutes	
		Next day.	

The results are given in figure 24.

In the reaction mixture showing the fastest rate of IAA breakdown (Sample I before the 2nd IAA addition) the reaction went to completion, whereas in the remaining three in which the rates of breakdown were slower (Samples II, III and IV after the 2nd IAA addition) the reaction failed to reach completion (if samples had been taken for a longer a period for residual IAA estimation, instead of only until an hour after the 2nd addition - a "flattening off" of the curves similar tothat observed in figure 11 would probably have been obtained). This result was similar to that obtained in the experiment of "Results", Section I, discussed on page 82 - except that in the present case, the amounts of IAA present in the reaction mixtures were different. Here then the difference in the rate of breakdown could only have been caused by some factor associated either with the difference in IAA concentration (the concentrations in Samples II, III and IV were all greater than that in Sample I) or with the difference in the amount of IAA breakdown which had taken place before the second part of the IAA was added (nil for Sample I, but measurable for Samples II, III and IV). This factor was considered to be the inactivation of the enzyme.

The fact that enzyme inactivation had taken place when the IAA in Sample I had been completely oxidised was evident from an examination of the results of figure 24. Thus when a second addition of IAA was made to Sample I, this was not broken down at all during the first 60 minutes following the addition, and no appreciable breakdown had occurred after a much longer period of incubation (by the next day). This discrepancy between/

between the fate of the 1st addition and the 2nd addition - could not have been due to different rates of enzyme inactivation during the two breakdown reactions (caused by different IAA levels) since the amounts of IAA present at the start of the reactions was approximately the same.

This last observation - that the enzyme had become completely inactivated by the end of the reaction (Sample I) - appeared to support the idea mentioned above - that the larger amounts of IAA (Samples II, III and IV, after the 2nd addition of IAA) were not completely broken down because the enzyme had become partially inactivated before the addition of the second quantity of IAA. However this was probably not so - and it seemed likely that even if both amounts of IAA had been added initially, the reactions would not have gone to completion. One reason for suspecting this was that the phenomenon of enzyme inactivation did not appear to have come into play during the first 20 minutes at least of the reaction - since when the curves for Samples II and III were compared it was seen that the rate of breakdown was faster in Sample III than in Sample II. (The concentration of IAA in the two samples was the same). The rate was faster still in Sample III - though it might be argued here that this was because the concentration of IAA present initially was slightly lower. Thus it seemed that the progressively higher rates observed in Samples II, III and IV might have resulted from the "activation" of the enzyme in some way - by some reaction which took place during the "lag period" - before the 2nd amount of IAA was added. This was possibly the formation of H_2O_2 (as described under "Introduction", page/

page 27). In any case it was concluded that the low rates of reaction (and hence the failure of the reaction to reach completion) observed in Samples II, III and IV, as compared with that in Sample I must have been due to increased rates of enzyme inactivation following the 2nd addition of IAA, and caused by the higher IAA concentrations.

The question remained as to when the inactivation of the enzyme began to take place - in Sample I. If a 2nd addition of IAA had been made to a fifth sample at the 40 minute interval it might have been found that the rate of breakdown observed subsequently was slower than in the Samples II, III and IV, thus showing that enzyme inactivation had set in. On the other hand the process of inactivation might only have taken place after the completion of the reaction - though this seemed to be rather less likely in view of the fact that the inactivation was thought to involve $IAA^{\cdot}$ radicals.

FIGURE 24. Graphs to show the rates of IAA breakdown in a sample of reaction mixture containing extract (A) and in duplicate samples to which supplementary amounts of IAA were added at different times during the course of the oxidation.

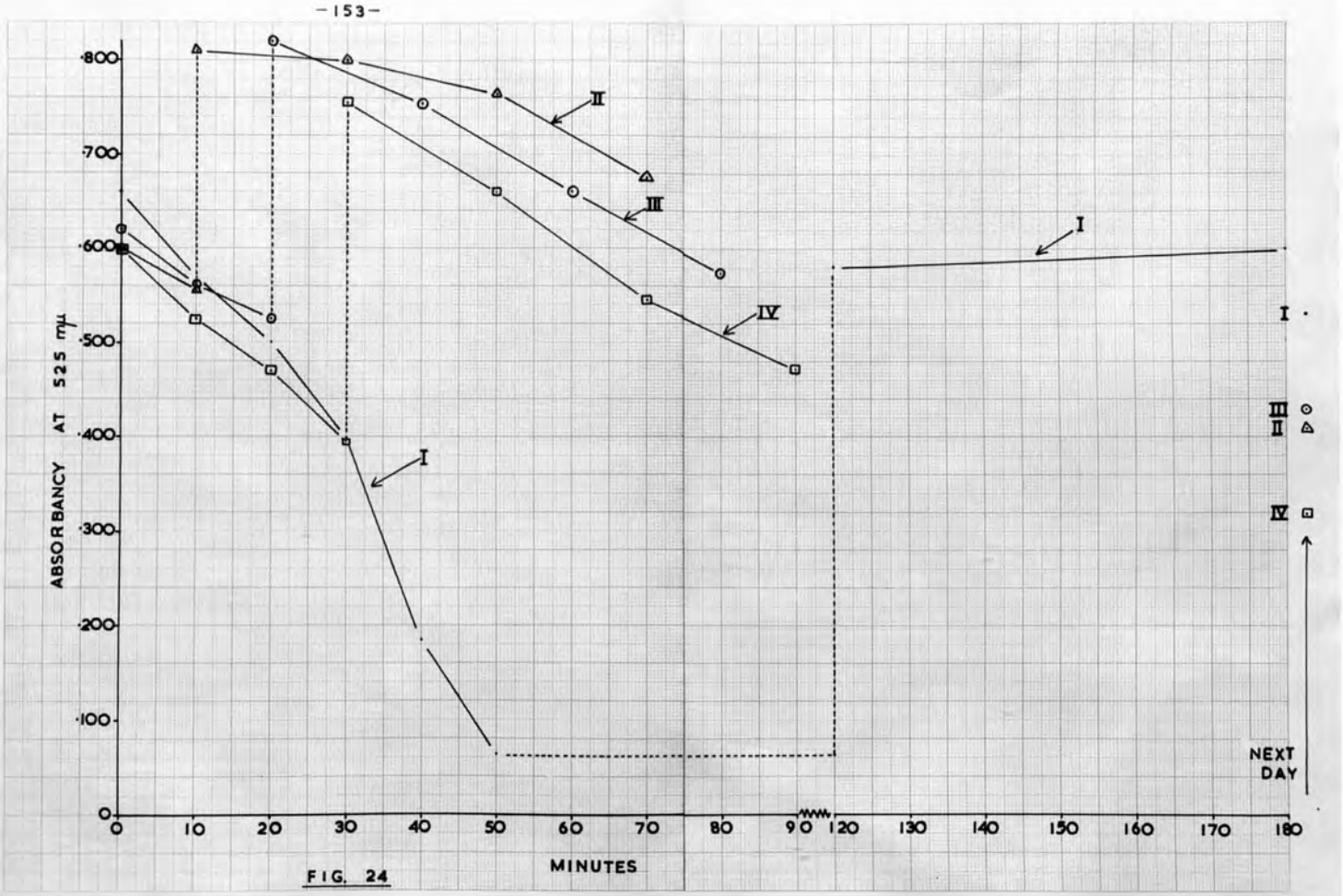

ABSORBANCY AT 525 mμ
·800
·700
·600
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
70
80
90
120
130
140
150
160
170
180
MINUTES
NEXT DAY
I
II
III
IV

FIG. 24

III 1. THE EFFECT ON THE "IAA-OXIDASE" ACTIVITIES OF EXTRACTS (A) AND (B) OF HIGH-SPEED CENTRIFUGING.

Extracts (A) and (B) were prepared as described under "Methods", Section II, and their "IAA-oxidase" activities determined in "Bakhsh" reaction mixtures (the composition of which is given under i. on page 82), as described under "Methods" Sections IV-1 and III-1. 10 ml. quantities of the reaction mixtures were made up and these were sampled at "zero time" and at 10 minute intervals thereafter for a period of 50 minutes. Samples of the extracts (A) and (B) were then centrifuged as described under "Methods", Section V-1, and the "IAA-oxidase" activities of the centrifuged extracts determined as before - except that sampling was continued for 80 minutes. The results are given in figure 25 in which the absorbancy values have been plotted directly.

The graphs of figure 25 indicated that the extracts (A) and (B) retained most of their "IAA-oxidase" activity after treatment by high-speed centrifuging. This confirmed the supposition that the enzyme responsible was a soluble one - that is peroxidase, and was not located in the particulate fraction (for example in the mitochondria). A decrease in activity however did occur with both extracts. This was manifested as an increase in the length of the lag period (extract (A) and extract (B)) and as a decreased rate of reaction (extract (A) only). It was probably caused by allowing the extracts to stand for a relatively long period at room temperature before and after the centrifuging (which was carried out at 10°C).

Both before, and after centrifuging the activity of extract (B) was higher than that of extract (A). Thus the maximum rates attained with extract (B) were slightly faster than those attained with extract (A), while the lengths of the lag periods with (B) were considerably shorter than with (A).

Figure 25. Graphs to show the rates of IAA breakdown in reaction mixtures containing samples of extracts (A) or (B), before and after treatment of the extracts by high-speed centrifuging.

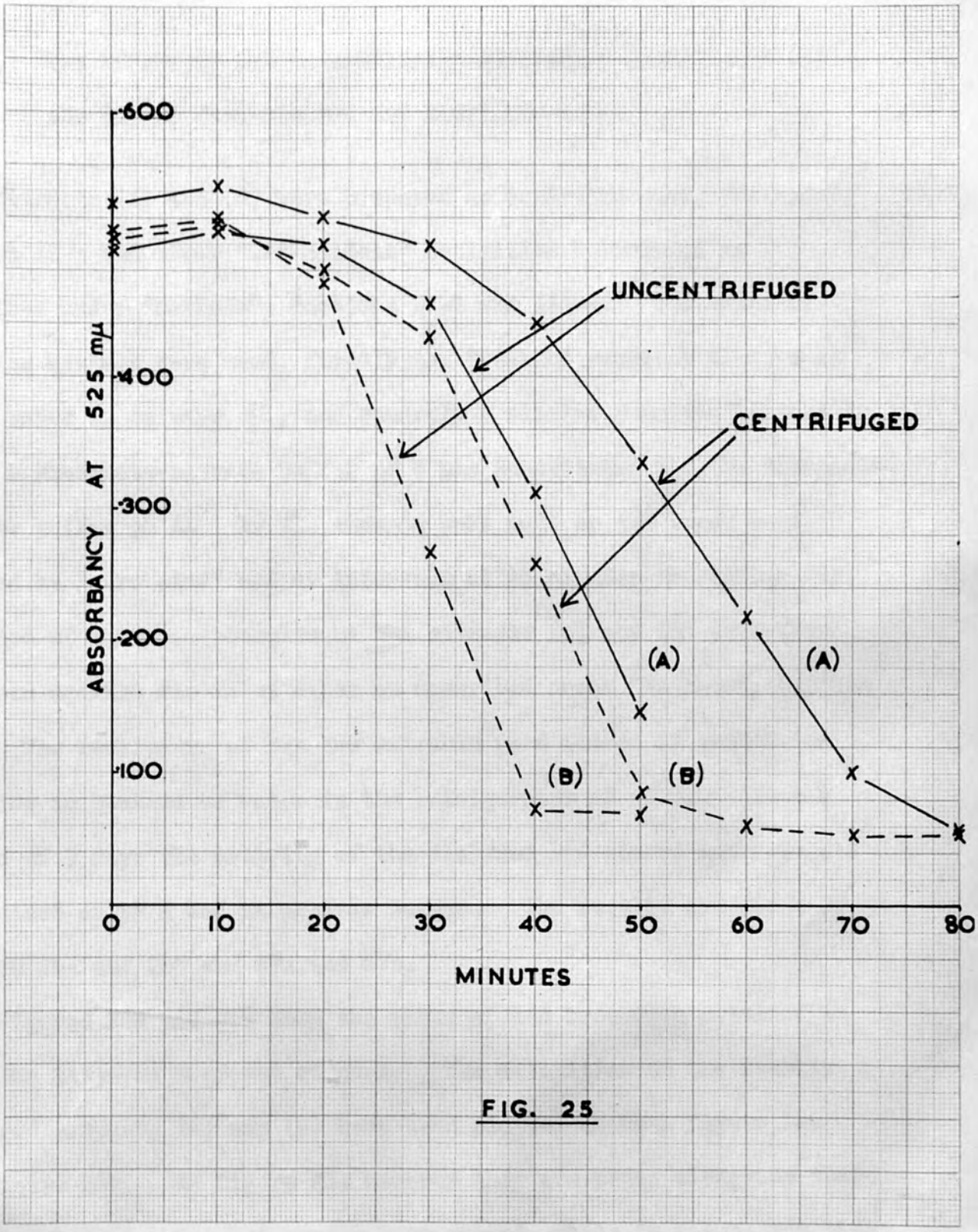

600
400
300
200
100
ABSORBANCY AT 525 mμ
UNCENTRIFUGED
CENTRIFUGED
(A)
(A)
(B)
(B)
0
10
20
30
40
50
60
70
80
MINUTES

FIG. 25

III 2. THE EFFECT ON THE "IAA-OXIDASE" ACTIVITIES OF EXTRACTS (A) AND (B) OF DIALYSIS AGAINST DISTILLED WATER.

Extracts (A) and (B) were prepared as described under "Methods", Section II-1 and their "IAA-oxidase" activities determined as described under "Methods", Sections IV-1 and III-1. Two types of reaction mixture were used, firstly the "Bakhsh" reaction mixture as given under i. on page 82, and secondly a similar reaction mixture in which a phosphate-citrate buffer pH 5 was substituted for the phosphate-citrate buffer pH 4. 10 ml. amounts were made up and these were sampled at "zero time" and at intervals of 15 minutes thereafter for a period of 1 hour. Samples of the extracts (A) and (B) were then dialysed against distilled water as described under "Methods", Section V-2. The remainders of the two extracts were stored in test tubes standing in beakers of water in the refrigerator at +2°C. After a period of 3 days the activity of the dialysed and stored extracts was determined at pH 4 and at pH 5 as before. The results are shown in figures 26a and 26b and 27a and 27b.

Figures 26a and 26b show the rates of IAA breakdown obtained in reaction mixtures buffered at pH 4. The activities of the freshly prepared extracts (A) and (B) have been plotted on both figure 26a and figure 26b. In figure 26a however they are shown alongside those of the extracts which had merely been stored, while in figure 26b they are shown alongside those of the extracts which had been dialysed.

Figure/

Figures 27a and 27b show the corresponding rates of IAA breakdown in reaction mixtures buffered at pH 5.

An examination of figures 26a and 26b indicated that the activity of both extract (A) and extract (B) remained unchanged at the end of a 3 day period of dialysis or storage. Figure 27b on the other hand showed that after dialysis for 3 days, the activity of both extract (A) and (B) had diminished slightly. However, 27a showed that a similar decline had taken place in the stored extracts. A further graph was therefore drawn in which the activities of the stored and dialysed extracts were plotted side by side (figure 28). This showed that the activity of extract (A) which had been stored for 3 days was practically the same as that of extract (A) which had been dialysed for 3 days. In addition the activity of the "stored" extract (B) was almost identical with that of the "dialysed" extract (B). These observations indicated that it was unlikely that the decrease in activity of the dialysed extracts was in fact caused by dialysis. Thus the inactivation which took place in both the dialysed and stored extracts (A) and (B) was probably due to some factor which had acted on all four extracts equally. The most likely explanation seemed to be that all the extracts had been allowed to stand at laboratory temperature for some time - possibly several hours, during the period of dialysis and storage (possibly when the refrigerator was defrosted).

This apparent lack of a change in enzyme activity following dialysis was not the expected result. It had been thought, that in view of the presence/

presence of an endogenous inhibitor in the pea root preparations, the process of dialysis would have led to the removal of the inhibitor and thence to an increase in "IAA-oxidase" activity. Thus Galston (1957) reported that the inhibitor could be collected from breis of pea tissue (that is from the leaf blades and stipules of light-grown plants) by overnight dialysis. An increase in the activity of dialysed preparations was observed by Mudd, Johnson, Burris and Buchholtz (1959) using quack-grass rhizomes and by Waygood, Oaks and Maclachlan (1956a) using wheat leaves. Another phenomenon observed by Mudd et al. and also by Reinert, Schraudolf and Reinert (1957), which was not encountered here, was a shift in the pH optimum of the enzyme preparation from 4 to 5 after dialysis. However, whereas Mudd et al. had found a definite increase in activity of the enzyme (the lag period was found to almost completely abolished) with quack-grass rhizomes, Reinert et al. had found that dialysis of the crude juice obtained from etiolated pea epicotyls (at 4°C for 18 hours with 3 changes of distilled water) caused a 40 - 60% decrease in enzyme activity. Similarly Wagenknecht and Burris (1950), using a preparation very like that of Reinert et al. recorded a ⅓ decrease in activity after overnight dialysis at 7°C against distilled water. It seemed likely that in the present work, if a more efficient method of dialysis had been used, for example one involving a continuous flow of distilled water, and if the process had been carried on for longer, a removal of the inhibitor might have been effected and an increase in enzyme activity obtained. However it was obvious that great care would/

would have to be taken to avoid any increases in temperature, even though only temporary, since these were likely to accelerate inactivation of the enzyme. It was theoretically possible also, that prolonged dialysis might remove the natural activator of the enzyme reported to have been found in peas (Sharpensteen and Galston, 1959) and thus lead to a decrease in enzyme activity, even after the removal of the inhibitor. The removal of manganese might also have this effect. Nevertheless - in the absence of more convincing evidence for the removal of an activator from pea extracts by dialysis - it seemed more probable that the decreases in activity obtained on dialysis by Reinert et al. and by Wagenknecht and Burris - as well as those in the present investigation - were occasioned by some other type of "inactivation".

The differences in the "IAA-oxidase" activities of extracts (A) and (B) were as reported in "Results", Section I. Similarly the differences in the activities of the extracts in the reaction mixtures buffered at pH 4 and pH 5 were as reported in "Results", Section II-1.

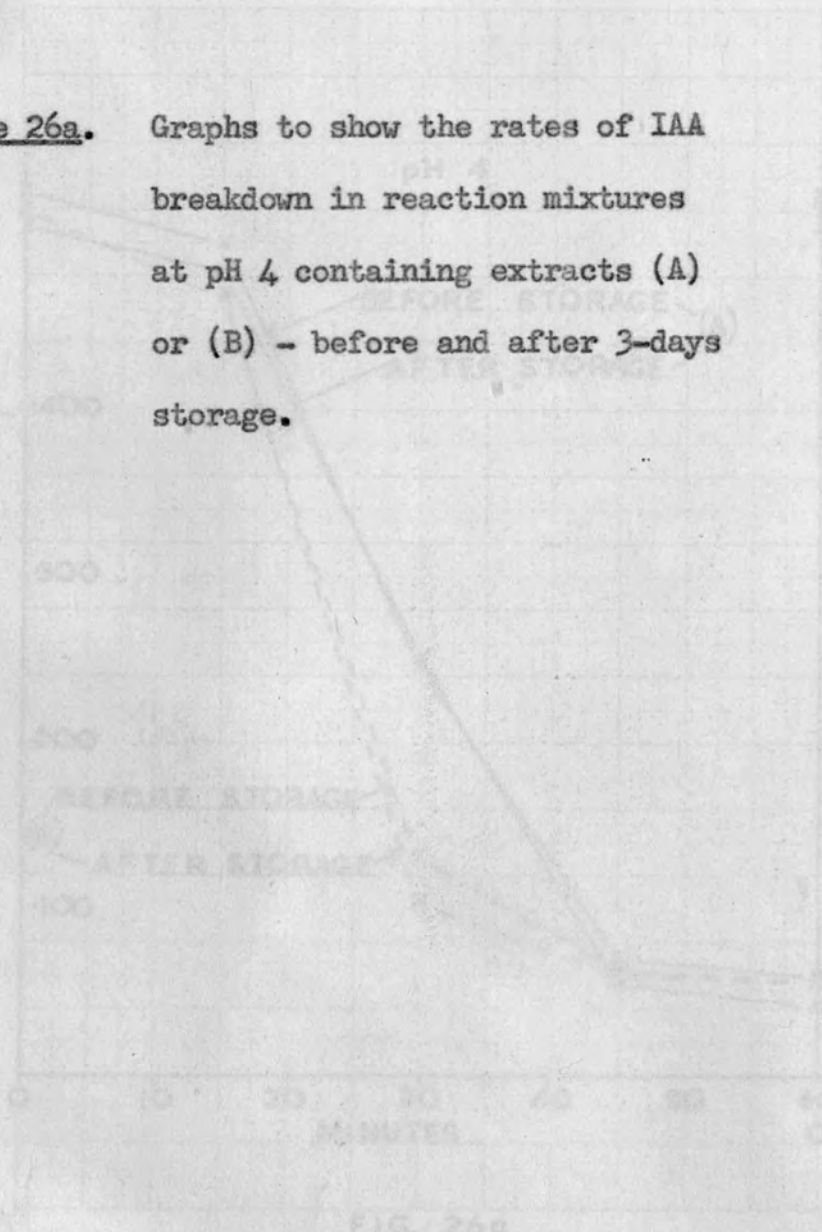


Figure 26a. Graphs to show the rates of IAA breakdown in reaction mixtures at pH 4 containing extracts (A) or (B) - before and after 3-days storage.

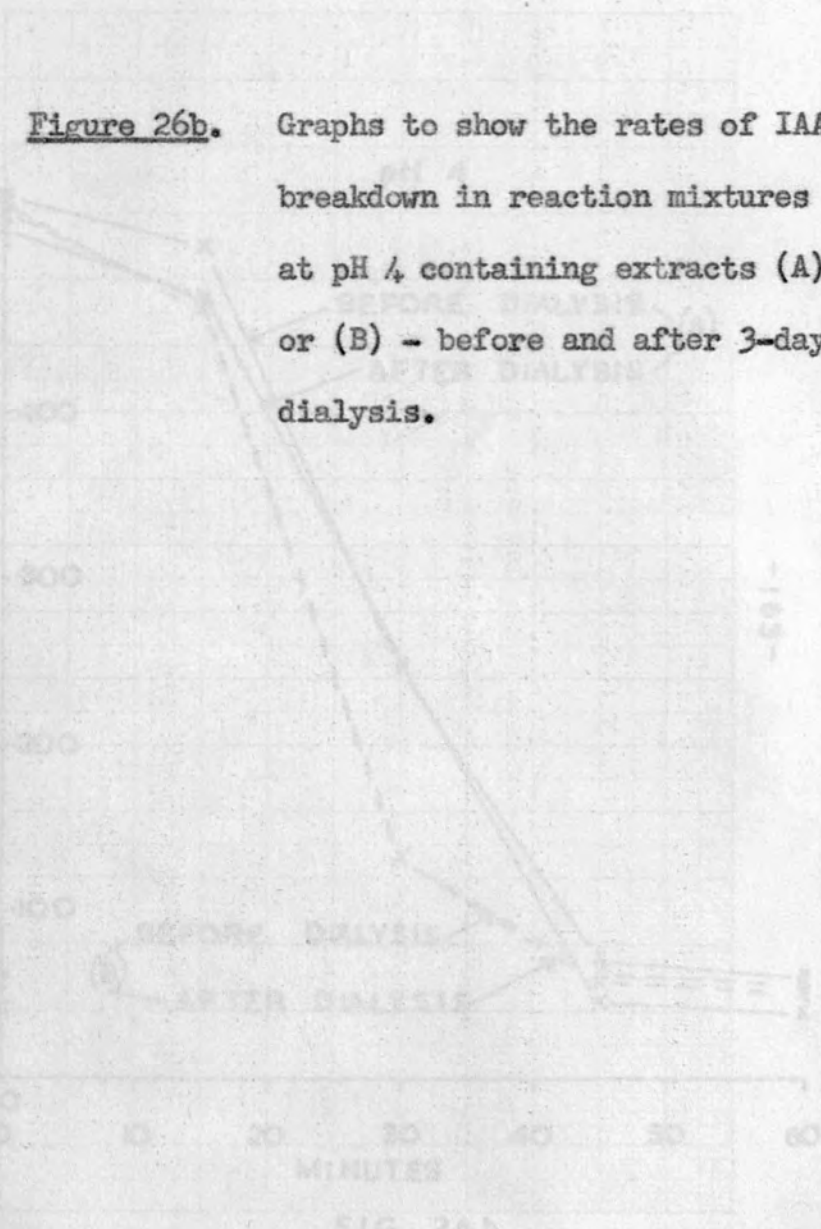


Figure 26b. Graphs to show the rates of IAA breakdown in reaction mixtures at pH 4 containing extracts (A) or (B) - before and after 3-days dialysis.

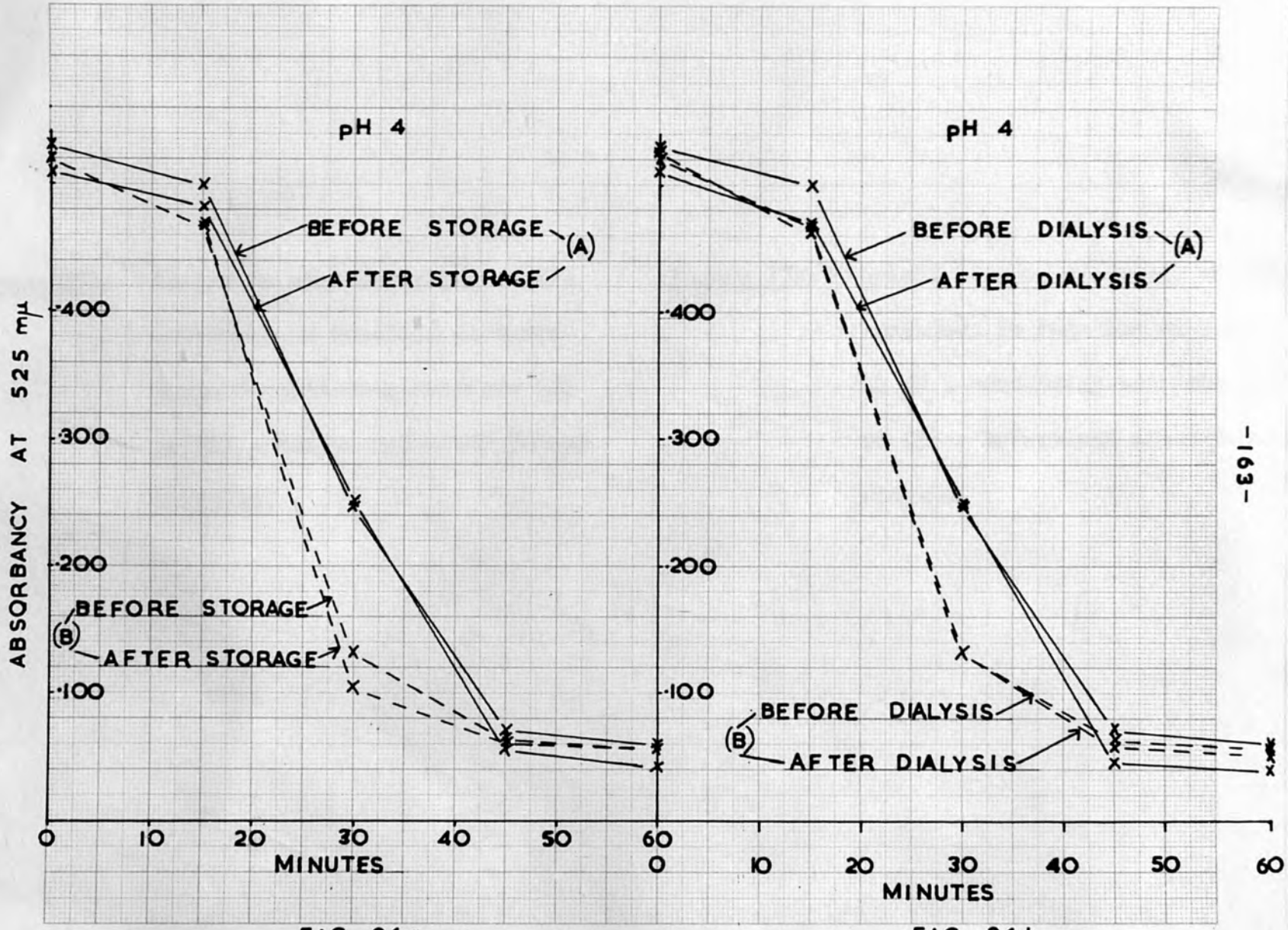


FIG. 26a

FIG. 26b

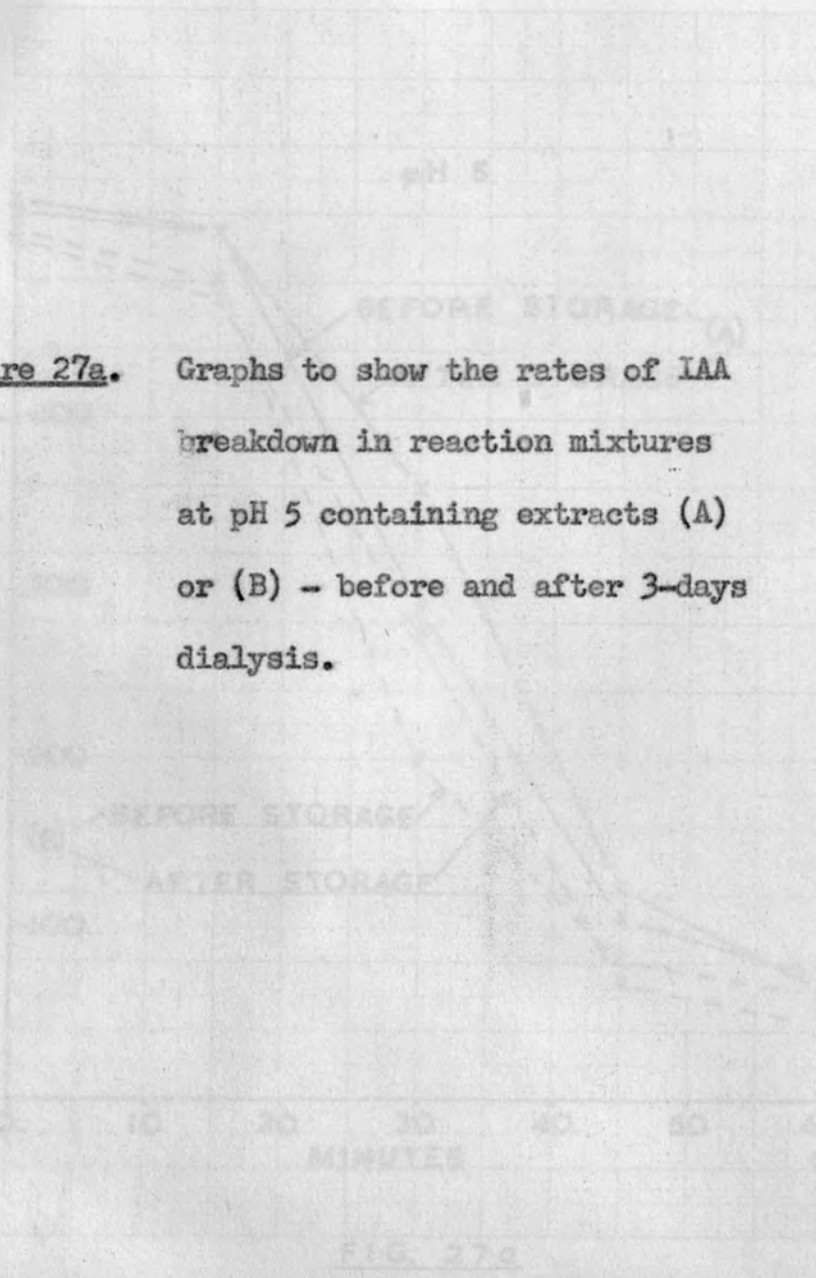


FIG. 27a

Figure 27a. Graphs to show the rates of IAA breakdown in reaction mixtures at pH 5 containing extracts (A) or (B) - before and after 3-days dialysis.

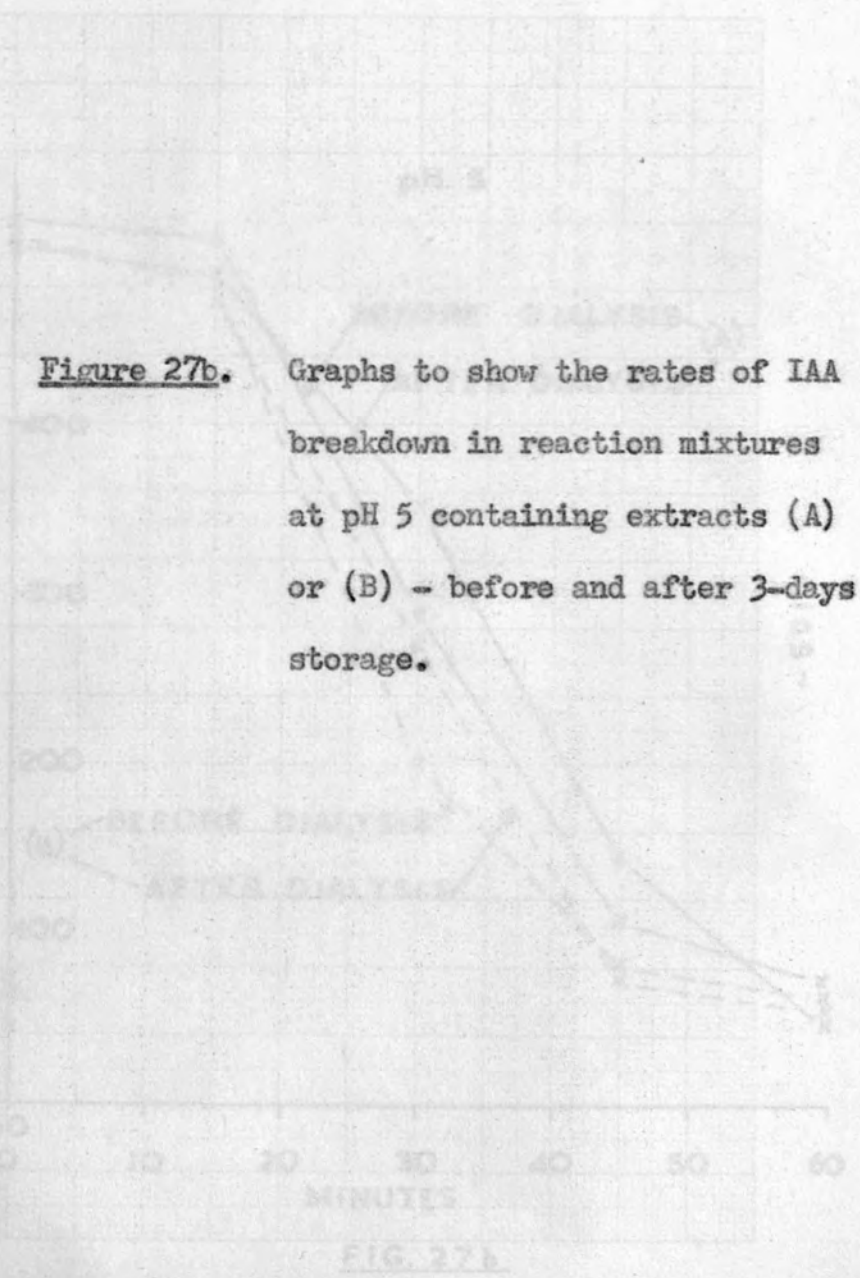


FIG. 27b

Figure 27b. Graphs to show the rates of IAA breakdown in reaction mixtures at pH 5 containing extracts (A) or (B) - before and after 3-days storage.

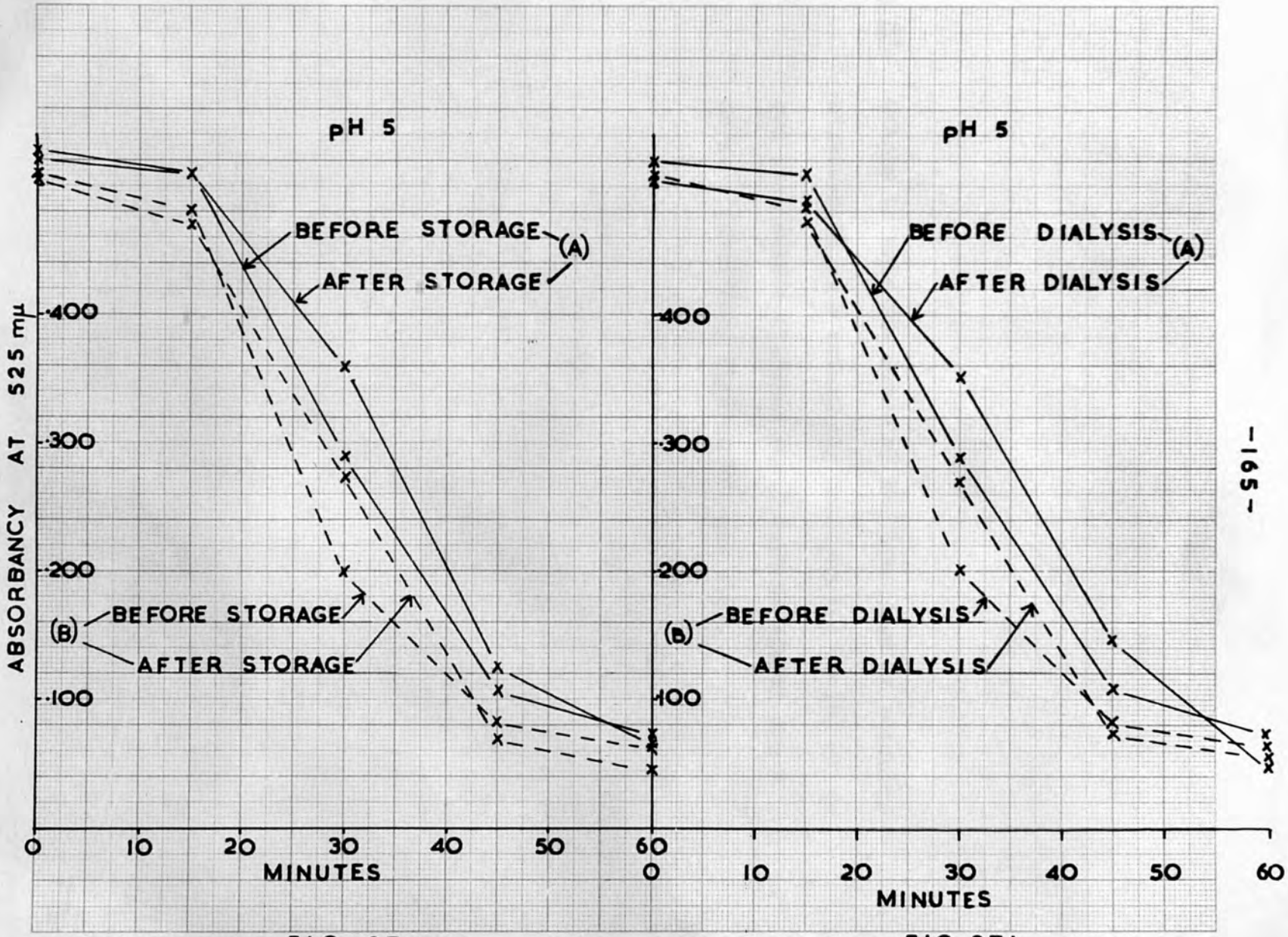


FIG. 27a

FIG. 27b

Figure 28. Graphs to show the rates of IAA breakdown in reaction mixtures at pH 5 containing extracts (A) or (B) which had been either dialysed for 3 days or stored for 3 days. (Data from figures 27a and 27b).

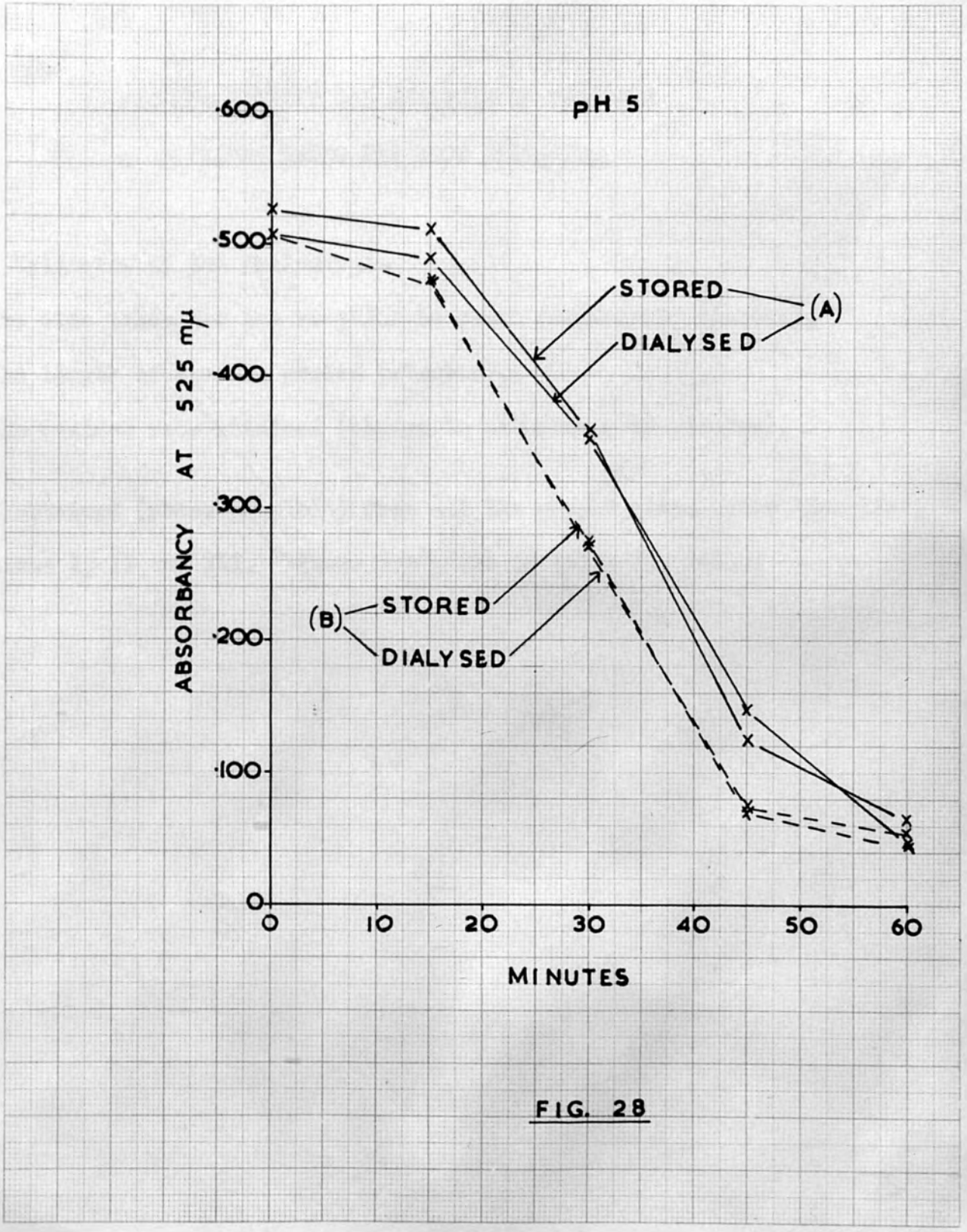

pH 5
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
STORED
DIALYSED
(A)
(B)
STORED
DIALYSED
0
10
20
30
40
50
60
MINUTES

FIG. 28

FURTHER STATISTICAL ANALYSIS OF THE DATA OBTAINED USING PEA ROOT EXTRACTS.

Estimates of the following:-

1. the time taken for the reaction to reach completion (minutes),
2. the length of the lag period (minutes),
3. the maximum rate attained (change in absorbancy/10 minutes),

were obtained (where possible) from all the graphs given under "Results", Sections I, II and III. These are given in table 13, below.

Table 13. (NC = reaction did not go to completion)

Figure.	Time for reaction to reach completion		Length of Lag Period		Maximum Rate Attained.	
	(A)	(B)	(A)	(B)	(A)	(B)
9a	35.25	30.50	15.5	10.0	.240	.241
9b	34.75	28.50	15.0	8.5	.240	.250
9c	34.75	31.00	14.5	12.0	.252	.265
10a	57.00	47.50	24.0	20.0	.148	.172
10b	63.00	45.75	23.5	18.0	.124	.183
10c	59.75	52.00	21.5	19.5	.128	.160
11	NC	102.00	33.0	22.5	.017	.052
12	35.75	31.25	16.5	9.0	.254	.267
19(pH 3)	NC	NC	51.0	38.5	.022	.026
19(pH 4)	30.50	25.75	8.5	8.5	.208	.268
19(pH 5)	35.00	32.00	13.0	12.0	.201	.212
19(pH 6)	NC	NC	31.0	30.5	.076	.078
21(pH 4)	32.50	-	13.5	-	.262	-
21(pH 6)	NC	-	44.5	-	.033	-
22a	46.50	30.50	10.5	6.0	.124	.196
22b	41.00	30.50	15.5	9.0	.177	.228
22c	34.50	30.50	15.0	10.0	.240	.241
22d	30.50	23.50	9.5	5.0	.204	.264
22e	35.00	25.50	14.0	7.5	.228	.265
22f	38.75	28.75	15.5	9.5	.199	.245
24(I)	45.50	-	18.0	-	.216	-
24(II)	NC	-	30.5	-	.045	-
24(III)	NC	-	7.0	-	.048	-
24(IV)	NC	-	4.0	-	.058	-
25 (UC)	NC	40.00	29.0	18.0	.164	.204
25 (C)	73.75	52.00	33.5	26.0	.118	.174
26a(Before)	41.00	33.25	13.5	12.5	.169	.224
26a(After)	44.00	32.25	13.0	13.0	.153	.244
26b(Before)	As 26a	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
26b(After)	44.50	33.25	12.5	12.0	.147	.220
27a(Before)	44.75	37.50	14.5	13.5	.147	.187
27a(After)	48.50	47.00	20.0	12.5	.157	.136
27b(Before)	As 27a	- - - - -	- - - - -	- - - - -	- - - - -	- - - - -
27b(After)	52.25	46.25	19.0	12.5	.137	.133

This data was then examined from the point of view of

a) Differences between the values of the "A" series and the "B" series, and

b) The relationship between the length of the lag period and the maximum rate attained.

For the purposes of a) above, the results of table 13 were grouped arbitrarily into 3 groups according to the maximum rate attained in the "A" series:

Group 1 : Maximum rate .200 to .300

Group 2 : Maximum rate .100 to .200

Group 3 : Maximum rate $<$ .100,

as shown in table 14. (The data from figures 24 (II), 24 (III) and 24 (IV) was omitted since the reaction mixtures contained supplementary amounts of IAA). "t" tests were carried out on the data of each group. The results are shown in table 15.

Table 14. (NC = reaction did not go to completion)

	Figure.	Time for reaction to reach completion		Length of Lag Period		Maximum Rate Attained.	
		(A)	(B)	(A)	(B)	(A)	(B)
Group 1	9a	35.25	30.50	15.5	10.0	.240	.241
	9b	34.75	28.50	15.0	8.5	.240	.250
	9c	34.75	31.00	14.5	12.0	.252	.265
	12	35.75	31.25	16.5	9.0	.254	.267
	19(pH 4)	30.50	25.75	8.5	9.5	.208	.268
	19(pH 5)	35.00	32.00	13.0	12.0	.201	.212
	22c	34.50	30.50	15.0	10.0	.240	.241
	22d	30.50	23.50	9.5	5.0	.204	.264
	22e	35.00	25.50	14.0	7.5	.228	.265
Group 2	10a	57.00	47.50	24.0	20.0	.148	.172
	10b	63.00	45.75	23.5	18.0	.124	.183
	10c	59.75	52.00	21.5	19.5	.128	.160
	22a	46.50	30.50	10.5	6.0	.124	.196
	22b	41.00	30.50	15.5	9.0	.177	.228
	22f	38.75	28.75	15.5	9.5	.199	.245
	25 (UC)	NC	40.00	29.0	18.0	.164	.204
	25 (C)	73.75	52.00	33.5	26.0	.118	.174
	26a (Before)	41.00	33.25	13.5	12.5	.169	.224
	26a (After)	44.00	32.25	13.0	13.0	.153	.244
	26b (After)	44.50	33.25	12.5	12.0	.147	.220
	27a (Before)	44.75	37.50	14.5	13.5	.147	.187
	27a (After)	48.50	47.00	20.0	12.5	.157	.136
	27b (After)	52.25	46.25	19.0	12.5	.137	.133
Group 3	11	NC	102.00	33.0	22.5	.017	.052
	19(pH 3)	NC	NC	51.0	38.5	.022	.026
	19(pH 6)	NC	NC	31.0	30.5	.076	.078

Table 15. Means and "t" values obtained in a comparison of the data for the "A" and "B" series. (Table 14). The number of degrees of freedom is given in brackets.

*** = Significant at 0.1%.

** = Significant at 1%.

* = Significant at 5%.

DATA	MEANS :	A	B	"t" VALUES
TIME FOR REACTION TO REACH COMPLETION	Group 1.	34.00	28.72	4.220 (16) ***
	Group 2.	50.33	39.73	2.816 (24) **
	Group 3.	-	-	-
LENGTH OF LAG PERIOD	Group 1.	13.5	9.2	(d=2.608 (12) *)
	Group 2.	19.0	14.4	1.963 (26)
	Group 3.	38.3	30.5	0.798 (4)
MAXIMUM RATE ATTAINED	Group 1.	.230	.253	2.485 (16) *
	Group 2.	.149	.193	3.828 (26) ***
	Group 3.	.038	.052	0.463 (4)

Note. The variances of the values representing the length of the lag period in group 1 were shown by an "F" test to be significantly different. "d" was therefore determined instead of "t", using the formula/

formula, $d = \dfrac{\bar{x}_1 - \bar{x}_2}{\sqrt{\dfrac{s_1^2}{n_1} + \dfrac{s_2^2}{n_2}}}$,

and also the corresponding number of degrees of freedom (f), from the formula,

$$f = \frac{1}{\dfrac{u^2}{n_1 - 1} + \dfrac{(1-u)^2}{n_2 - 1}} \quad \text{where } u = \frac{s_1^2/n_1}{s_1^2/n_1 + s_2^2/n_2}$$

These are the values (d and f) given in the square brackets in table 15 above and in table 18.

An examination of table 15 showed that as found in "Results", SectionI, the time taken for the reaction to reach completion was significantly lower in the "B" series than in the "A" series (Groups 1 and 2). This appeared to have been due to differences both in the length of the lag period and also in the maximum rate attained. (The "t" value for the length of the lag period in Group2 also approached significance : t = 1.936 with 26 degrees of freedom. Of 5% value of t = 2.056). Failure of one or more of the "t" values to reach significance in the remaining groups was probably attributable to the very heterogeneous nature of the experiments.

The above result was in accord with the finding of Section I - as far as the lower enzyme concentration was concerned. In Section I however it was found that at the higher enzyme concentration - only a difference between the length of the lag period was observed. Nevertheless/

theless it seemed advisable to treat the data of Section I with particular reserve - on account of the small number of samples involved.

The data for the lengths of the lag periods, and the maximum rates attained given in table 14 was also subjected to regression analysis - in order to determine the relationship between these two factors. The values for the "A" and the "B" series were analysed separately and statistically significant values were obtained for the estimated regression coefficient (b) in each case (where x = length of lag period, and y = maximum rate attained). These were b = -0.005142 ("A" series) and b = -0.007053 ("B" series). The value for b ("B" series) was higher than that for b ("A" series), but the difference was not significant.

$$\left(t = \frac{b_1 - b_2}{s\sqrt{\frac{1}{\Sigma_1(x - \bar{x}_1)^2} + \frac{1}{\Sigma_2(x - \bar{x}_2)^2}}} \right.$$ with $n_1 + n_2 - 4$ degrees of freedom.

$$= 1.458 \text{ with } 51 \text{ degrees of freedom.}$$

$$\left. \text{Cf. 5\% value of t (60 d. of f.)} = 2.000 \right).$$

Itwas concluded therefore that there was an inverse linear relationship between the length of the lag period and the subsequent maximum rate attained in any reaction mixture - a result which was expected from a subjective assessment of the graphs obtained.

Further discussion of the results outlined here will be reserved until the analysis of the additional data to be given below has been described.

Additional data.

A further set of data similar to thatalready obtained from "Results", Sections I, II and II, is given below (table 16). It was obtained from experiments which are not described elsewhere. However the experimental conditions were similar to those used in Section I-1 i, and only brief details are given to indicate where they differed (table 17). An additional group (Group 0) has been included which contains the values from experiments in which the maximum rate obtained in the "A" series - expressed as change in absorbancy / 10 minutes - was between .300 and .400.

Table 16. (NC = reaction did not go to completion).

	Experiment	Time for reaction to reach completion		Length of Lag Period		Maximum Rate Attained.	
		(A)	(B)	(A)	(B)	(A)	(B)
Group 0	1	16.50	14.75	7.5	4.0	.478	.448
	2	17.00	13.50	7.0	4.3	.476	.518
	3	20.00	17.75	7.3	6.5	.390	.440
	4	21.75	19.50	8.5	8.0	.356	.424
	5	19.25	15.00	6.3	4.0	.392	.450
Group 1	6	32.00	20.00	12.5	6.5	.214	.326
	7	22.50	33.50	12.0	8.5	.217	.343
	8	25.50	20.25	9.0	7.5	.290	.414
	9	28.25	16.75	10.8	5.3	.256	.410
Group 2	10	41.25	33.50	13.5	12.5	.169	.224
	11	N C	N C	22.0	17.0	.182	.197
	12	43.00	39.00	12.5	11.0	.151	.167
	13	44.00	34.00	12.0	12.5	.151	.220
	14	49.50	48.00	19.8	16.5	.137	.133
	15	44.75	38.00	14.5	13.5	.147	.187
	16	50.00	47.50	19.8	13.5	.157	.136
	17	51.00	42.75	19.3	16.5	.146	.178
	18	74.25	45.50	25.3	20.5	.109	.212
	19	51.50	29.75	19.3	8.5	.164	.248
	20	46.00	26.25	18.5	6.8	.194	.268
Group 3	21	N C	N C	38.5	43.0	.036	.048
	22	N C	N C	45.5	43.5	.041	.045
	23	N C	N C	37.5	38.5	.048	.067
	24	106.00	45.75	33.0	15.0	.069	.164
	25	94.00	57.50	38.0	16.3	.090	.129
	26	103.00	61.75	36.0	26.0	.084	.136
	27	83.50	43.00	32.0	15.3	.095	.178
	28	N C	69.50	51.5	29.8	.073	.130
	29	N C	57.50	54.8	21.5	.070	.136

Table 17. Experimental conditions used in experiments 1 - 29 of table 16 (where they differed from those described under "Results", Section I-1 i on page 82 .)

Experiment.	Experimental Details (1) = Brei made up to 100 ml. instead of 150 ml. (2) = Incubation at room temperature instead of 25°C.
1, 2.	(1)
3, 4.	Culture solutions renewed after 24 hours. (1).
5.	Seeds germinated in sand for 3 days.
6.	(1) (2)
7, 8.	Enzyme diluted (2 parts enzyme : 1 part distilled water) (1)
9.	Plants given 1 hour light treatment before harvest. Enzyme diluted (2 parts enzyme : 1 part distilled water) (1).
10.	
11.	(1) (2)
12, 13.	Enzyme dialysed for 3 days.
14.	Enzyme dialysed for 3 days. pH of reaction mixture 5.
15, 16.	pH of reaction mixture 5.
17.	Culture solutions not renewed (2).
18.	Enzyme diluted (1 part enzyme : 1 part distilled water) (1).
19.	1 day - old roots (1)
20.	1 day - old roots. Plants given 1 hour light treatment before harvest.(1)
21, 22.	pH of reaction mixture 6.

Table 17. (Continued).

Experiment	Experimental Details.
23.	Enzyme dialysed for 6 days. pH of reaction mixture 5.
24, 25.	1 day - old roots. Enzyme diluted (1 part enzyme : 1 part distilled water) (1).
26.	1 day - old roots. Plants given ½ hour light treatment before harvest (and IAA renewed). Enzyme diluted (1 part enzyme : 1 part distilled water). (1).
27.	1 day - old roots. Plants given 1 hour light treatment before harvest. Enzyme diluted (1 part enzyme : 1 part distilled water). (1).
28.	1 day - old roots. Plants given 1 hour light treatment before harvest (and IAA renewed). Enzyme diluted (1 part enzyme : 1 part distilled water). (1).
29.	1 day - old roots. Plants given 5 hours light treatment before harvest (and IAA renewed). Enzyme diluted (1 part enzyme : 1 part distilled water). (1).

This data was analysed exactly as described for the data of table 14. The results of the "t" tests are given in table 18. Again significant differences were obtained in all three categories between the "A" series and the "B" series. Significant values were again obtained for the estimated regression coefficients. These were b =-0.006933 ("A" series) and b =-0.009743 ("B" series). Again the value for b ("B" series) was higher than that for b ("A" series), but once more the difference was not significant. ($t = 1.631$ with 54 degrees of freedom. Cf. 5% value of t (60 d. of f.) = 2.000).

Table 18. Means and "t" values obtained in a comparison of the data for the "A" and "B" series. (Table 16). The number of degrees of freedom is given in brackets.

*** = Significant at 0.1%.

** = Significant at 1%.

* = Significant at 5%.

DATA	MEANS :	A	B	"t" VALUES
TIME FOR REACTION TO REACH COMPLETION	Group 0	18.90	16.10	1.912 (8)
	Group 1	27.06	22.63	1.049 (6)
	Group 2	48.53	38.38	2.319 (18) *
	Group 3	96.63	52.00	6.281 (6) ***
LENGTH OF LAG PERIOD	Group 0	7.32	5.36	2.215 (8)
	Group 1	11.08	6.95	3.979 (6) **
	Group 2	17.86	13.53	2.484 (20) *
	Group 3	40.79	27.66	2.781 (16) *
MAXIMUM RATE ATTAINED	Group 0	.418	.456	1.275 (8)
	Group 1	.244	.373	4.458 (6) **
	Group 2	.155	.196	(d = 2.877 (15) *)
	Group 3	.067	.115	(d = 1.540 (9))

Regression analysis of the combined data from tables 14 and 16.

Since the regression coefficients obtained for the "B" series were higher than those obtained for the "A" series (data of table 14 and data of table 16), and it was observed that the differences, although not significant, were yet approaching significance, the data from tables 14 and 16 was pooled and regression coefficients worked out from the combined data for the "A" series and the "B" series. The values were, b = - 0.006233 ("A" series) and b = - 0.008768 ("B" series) and the difference was in fact found to be significant at the 5% level (t = 2.118 with 109 degrees of freedom. Cf. 5% value of t = 1.980 (with 120 d. of f.)). The regression lines based on these values are given in figures 29a and 29b together with the 95% confidence limits. The individual values from tables 14 and 16 are also plotted in these figures.

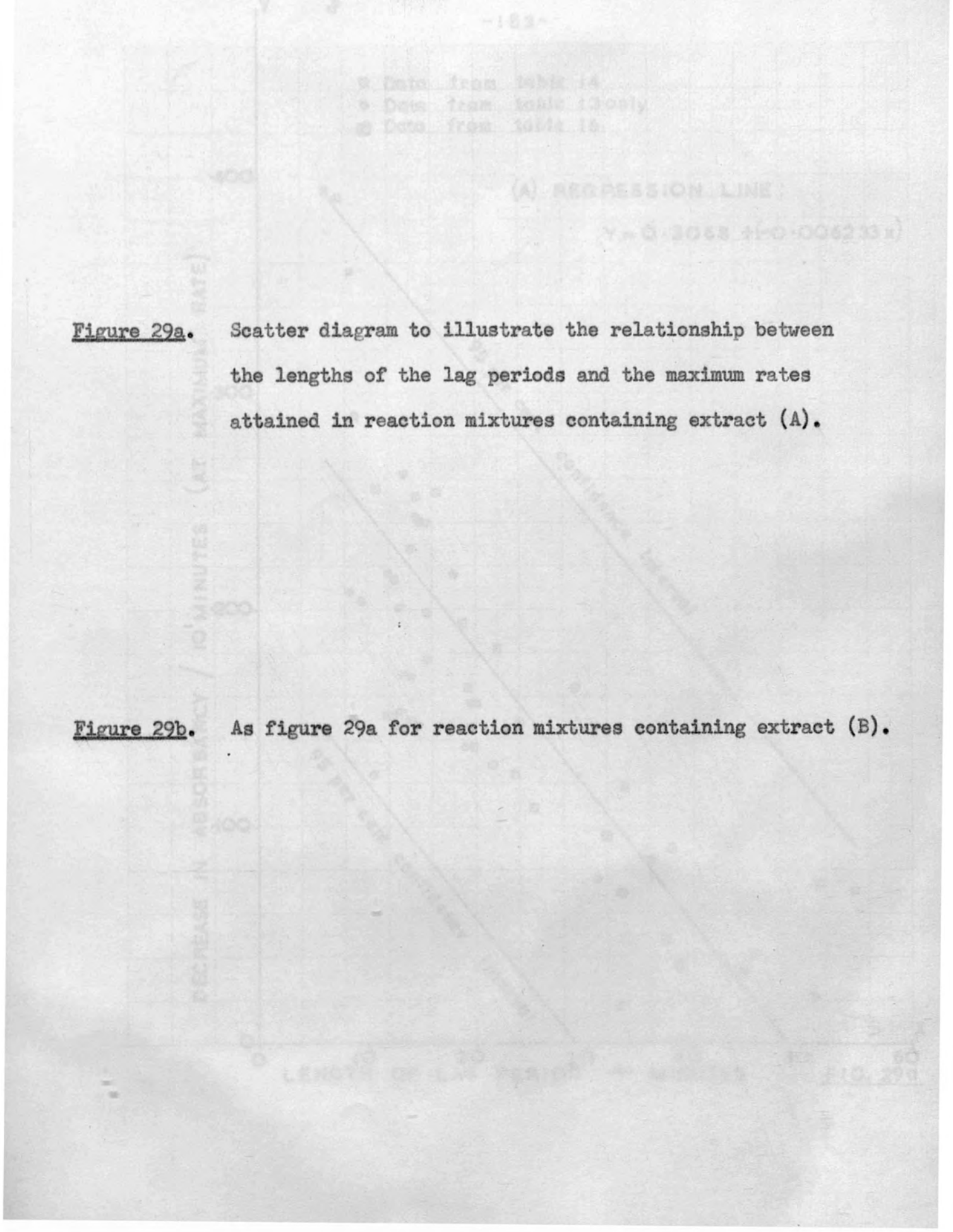


Figure 29a. Scatter diagram to illustrate the relationship between the lengths of the lag periods and the maximum rates attained in reaction mixtures containing extract (A).

Figure 29b. As figure 29a for reaction mixtures containing extract (B).

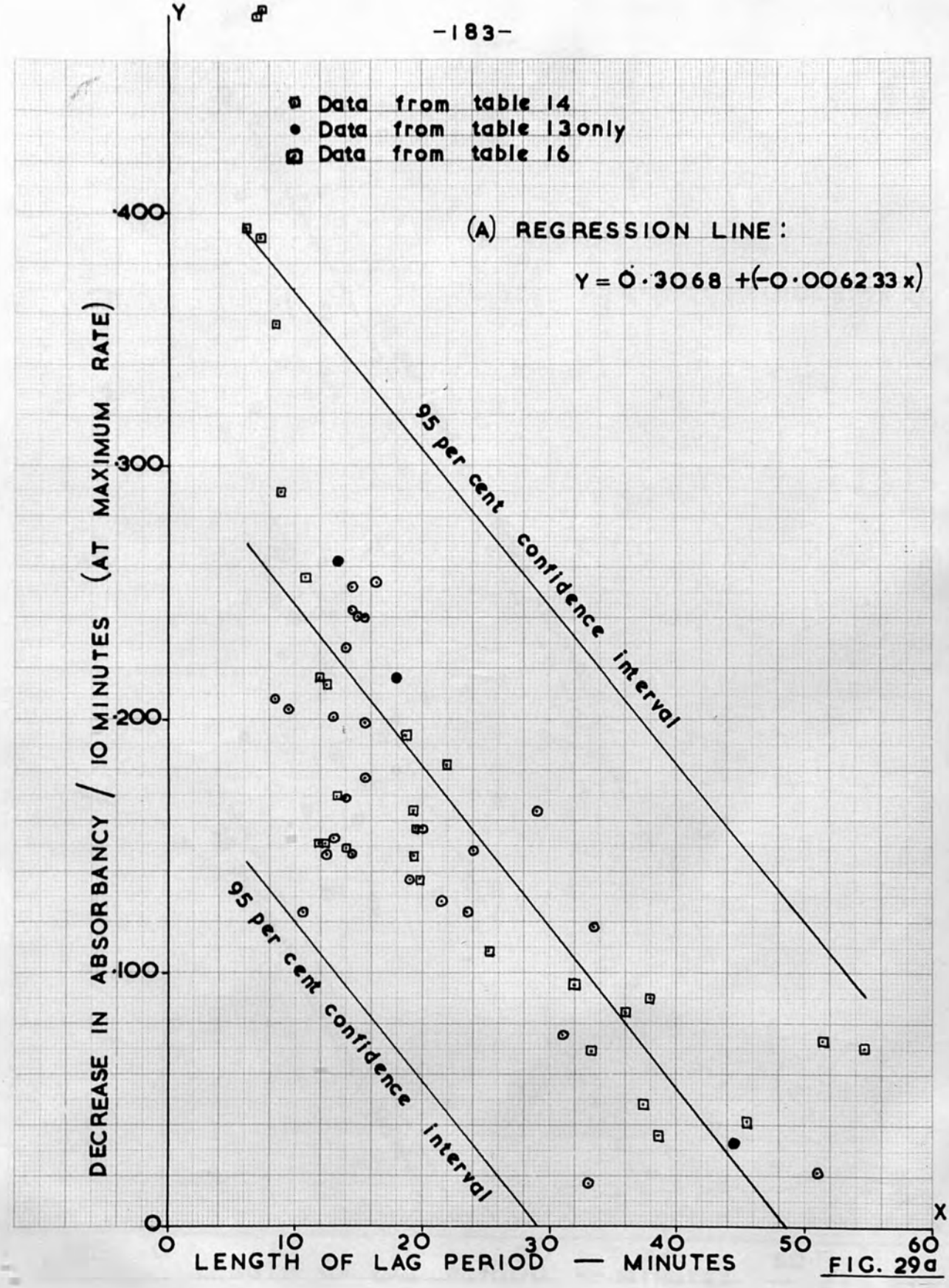

Y
Data from table 14
Data from table 13 only
Data from table 16
(A) REGRESSION LINE:
Y = 0·3068 +(-0·006233 x)
95 per cent confidence interval
95 per cent confidence interval
DECREASE IN ABSORBANCY / 10 MINUTES (AT MAXIMUM RATE)
400
300
200
100
0
X
0
10
20
30
40
50
60
LENGTH OF LAG PERIOD — MINUTES

FIG. 29a

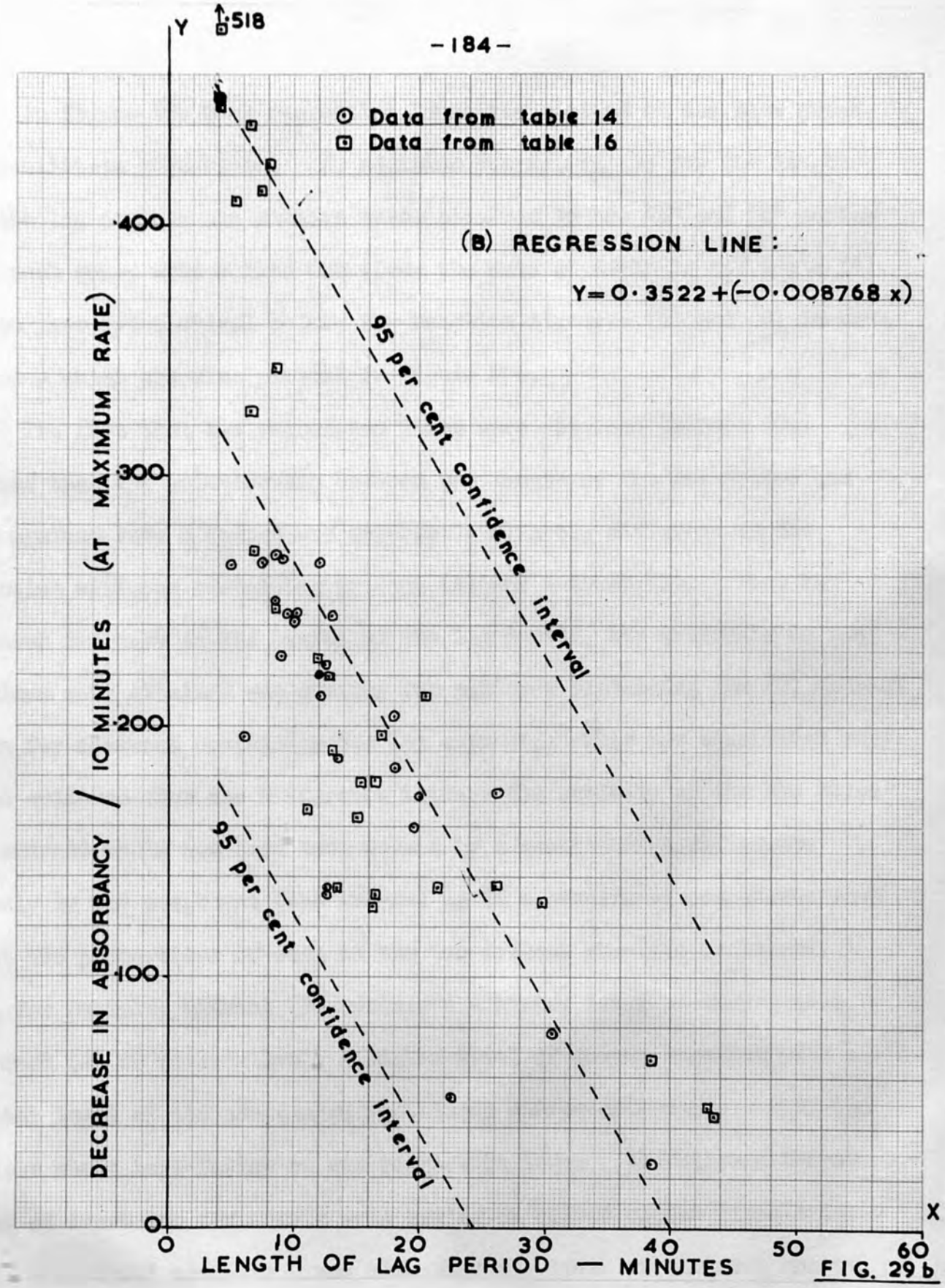

Y
518
Data from table 14
Data from table 16
(B) REGRESSION LINE:
Y = 0·3522 + (−0·008768 x)
95 per cent confidence interval
95 per cent confidence interval
DECREASE IN ABSORBANCY / 10 MINUTES (AT MAXIMUM RATE)
400
300
200
100
0
X
0
10
20
30
40
50
60
LENGTH OF LAG PERIOD — MINUTES

FIG. 29b

In figure 29c both regression lines are plotted on the same graph to facilitate comparison. In addition average values for the lengths of the lag periods and maximum rates attained in the "A" and "B" series for each group were worked out (from the data of table 14 and table 16). These gave hypothetical points for reaction mixtures (A) and (B) in each group - which are also plotted in figure 29c.

The fact that two regression lines were obtained (figure 29c) showed that the relationship between the length of the lag period and the maximum rate attained was apparently slightly different in the reaction mixtures containing extract (A) and extract (B). Thus for a given increase in the length of the lag period, the drecrease in the maximum rate attained was greater for mixtures containing the (B) extract than for mixtures containing the (A) extract. This was considered very good evidence that the difference between the activity of the two types of extract could not have been cuased by a quantitative difference merely in one component (the enzyme) or in a complex of components such that the proportions of each in the two systems remained unaltered. If, for example, extract (B) contained a higher level of enzyme than extract (A) or alternatively a higher level of enzyme together with a higher level of its associated inhibitors and/or activators - then the points would have fallen on one regression line. It followed therefore that at least two components were having an effect on the "indoleacetic acid - oxidase" activity - and also that the level of at least one of these was not the same in the two types of extract. (If there were differences/

Figure 29c. Diagram to show the relationship between the average values obtained in Groups 0 - 3 for the length of the lag period and the maximum rate attained - in reaction mixtures of the "A" and "B" series - (data from tables 14 and 16), and the regression lines obtained from the individual values (see figures 29 and 29b).

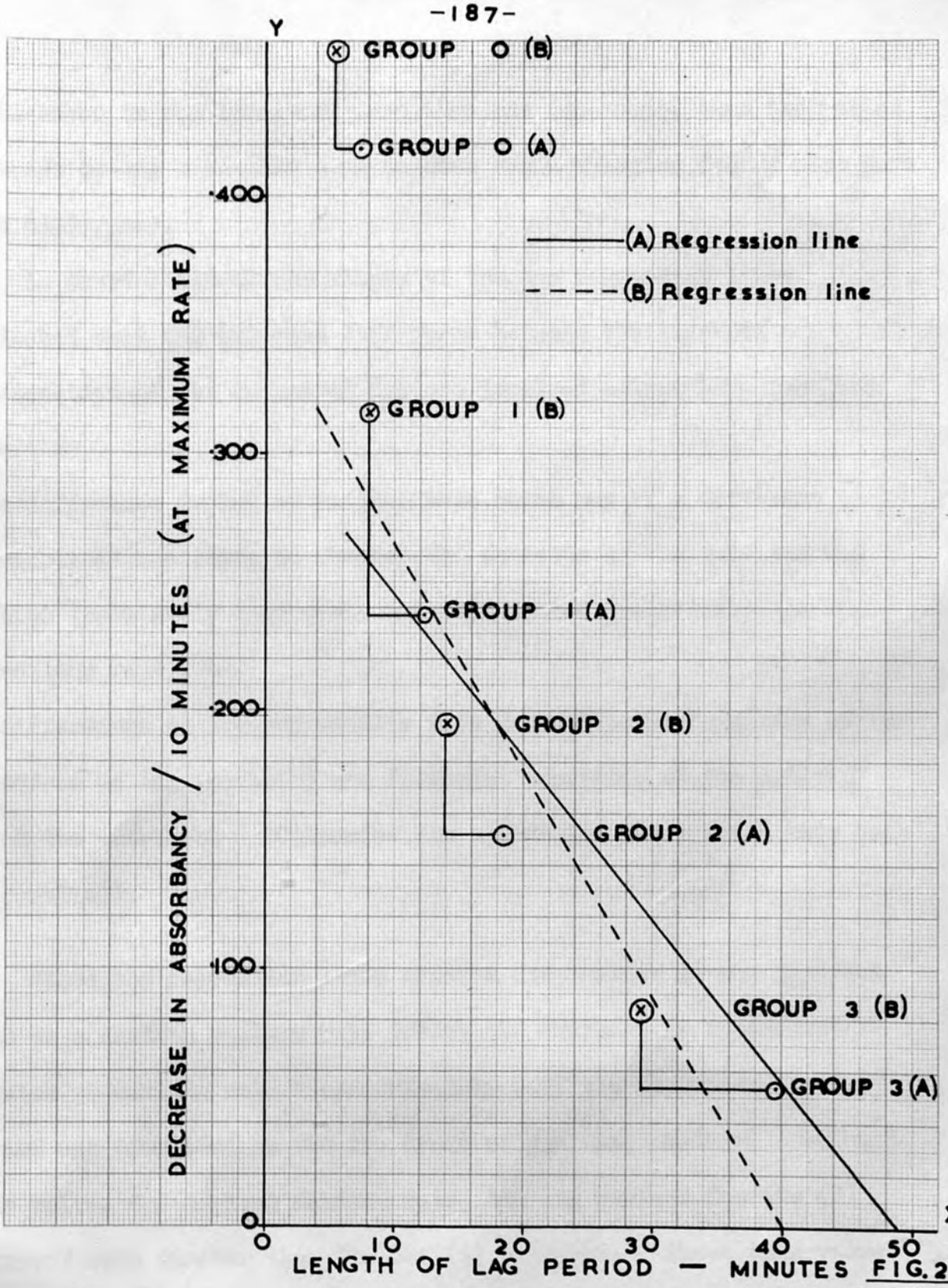

Y
GROUP 0 (B)
GROUP 0 (A)
400
(A) Regression line
(B) Regression line
GROUP 1 (B)
300
GROUP 1 (A)
200
GROUP 2 (B)
GROUP 2 (A)
100
GROUP 3 (B)
GROUP 3 (A)
0
X
0
10
20
30
40
50
DECREASE IN ABSORBANCY / 10 MINUTES (AT MAXIMUM RATE)
LENGTH OF LAG PERIOD — MINUTES

FIG. 29c

differences in the levels of more than one component, then the ratios of these to one - another - or to some non - changing factor must have been different).

An examination of the slopes of the two regression lines indicated that the inherent difference between the extracts - irrespective of the number of factors involved - must have resulted in either,

1) a difference in lag or maximum rate which was of a different magnitude according to whether the activity of the extracts was high (relatively high maximum rate), or low (relatively low maximum rate), or

2) differences in lag and maximum rate such that the magnitude of the effect of one (or both) was different according to the activity of the extracts. (Otherwise the regression lines would have been parallel).

Since the regression lines crossed one another it was apparent that at a certain maximum rate (where the decrease in absorbancy/10 minutes at maximum rate = approximately 0.2) the corresponding lag period was identical in the two types of reaction mixture. Below this value, for a given maximum rate, the lag periods for the (B) extracts were shorter than for the (A) extracts. Above this value, however, for a given maximum rate, the lag periods for the (B) extracts were longer than for the (A) extracts. Nevertheless with the pairs of/

of reaction mixtures used - containing the same amount of either extract (A) or extract (B) - the maximum rates were invariably higher and the lag periods shorter with the (B) extracts. (As shown by the average values plotted in figure 29c).

The above facts may be explained by assuming that the relationship of maximum rate and length of lag period to enzyme concentration (= concentration of the extract) are as shown in the diagram below.

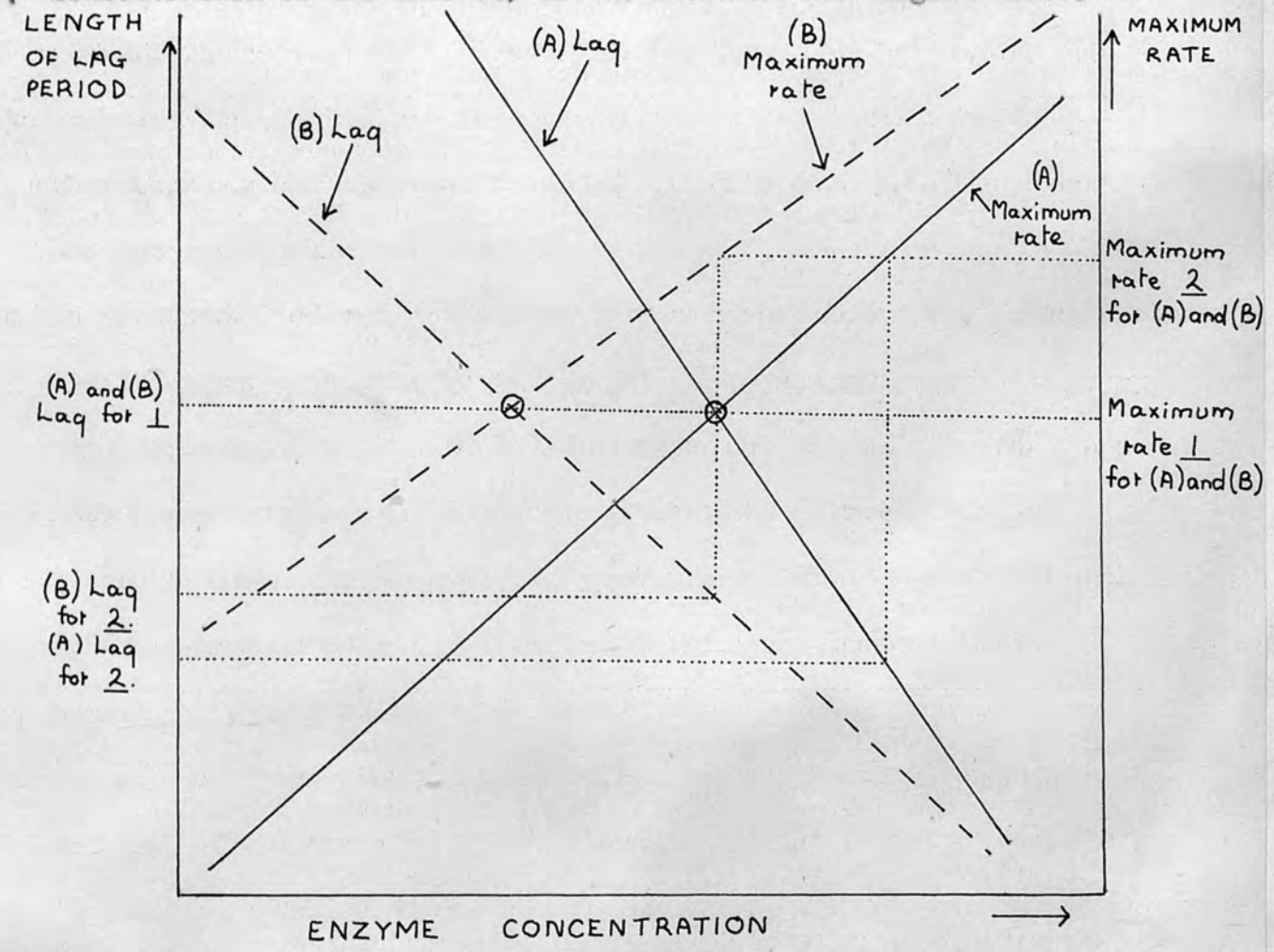


Diagram to show the suggested relationship between enzyme concentration and i. length of the lag period, and ii. the maximum rate attained, for the extracts (A) and (B).

The maximum rates are shown to vary directly and the lag periods inversely, with enzyme concentration for both types of extract. However,

1. The rate of variation in the (A) extracts is greater than in the (B) extracts in each case. Thus for a given increase in enzyme concentration, the increase in maximum rate and decrease in the lag period are both greater with an (A) extract than with a (B) extract.
2. When the (A) extract and the (B) extract are considered individually, the change in lag period with enzyme concentration is greater than the corresponding change in maximum rate.
3. At relatively low concentrations of enzyme the difference between the lag periods in the (A) and (B) extracts is large compared to the difference between the maximum rates whereas at relatively high concentrations of enzyme the position is reversed and the differences in the lag periods is small compared with the difference in the maximum rates. (This is illustrated quite well by the horizontal and vertical lines of figure 29c - connecting the "hypothetical points" for 4 different concentrations of the (A) and (B) extract.)
4. At concentrations of enzyme slightly higher than the ones used in the experiments described, it is expected that the lag periods would be negligible, while the difference in maximum rates were still quite considerable. (When the regression lines of figure 29c are/

are extrapolated to zero lag, the maximum rates (expressed as change in absorbancy/10 minutes) for the (A) and (B) extracts are 0.305 and 0.350 respectively.)

An important point is the fact that the two lines for each extract (maximum rate and lag) intersect at the same maximum rate and lag (these points of intersection are marked by circles on the diagram) - so that at a certain enzyme concentration which is lower for the (B) extract than the (A) extract, the lags and maximum rates are the same (see also the point of intersection of the regression lines in figure 29c). From this point a given increase in the maximum rate (from (1) to (2) in the diagram) is accompanied by a greater decrease in the lag period for (A) than (B) and the lag period for (B) is therefore longer. A decrease in the maximum rate on the other hand is accompanied by a greater decrease in the lag period for (B) than (A) so that that for (B) is shorter.

The effect of enzyme concentration on the maximum rates and lags was not specifically investigated, but when, for example, the values obtained for maximum rate and lag at two different enzyme concentrations were plotted in this way (the data of experiments 2 and 18, in table 17), the graph shown in figure 29d was obtained. It may be seen that there was a good correspondence with the hypothetical one - and indeed if the slope of the line for the (A) lag period were slightly greater, then the two would be identical.

If the deductions made above are correct, then it would be expected/

Figure 29d. Graph to show the relationship between the concentration of the extract and i. the length of the lag period, and ii. the maximum rate attained for extracts ('A') and ('B') - data of experiments listed as 2 and 18 in table 17.

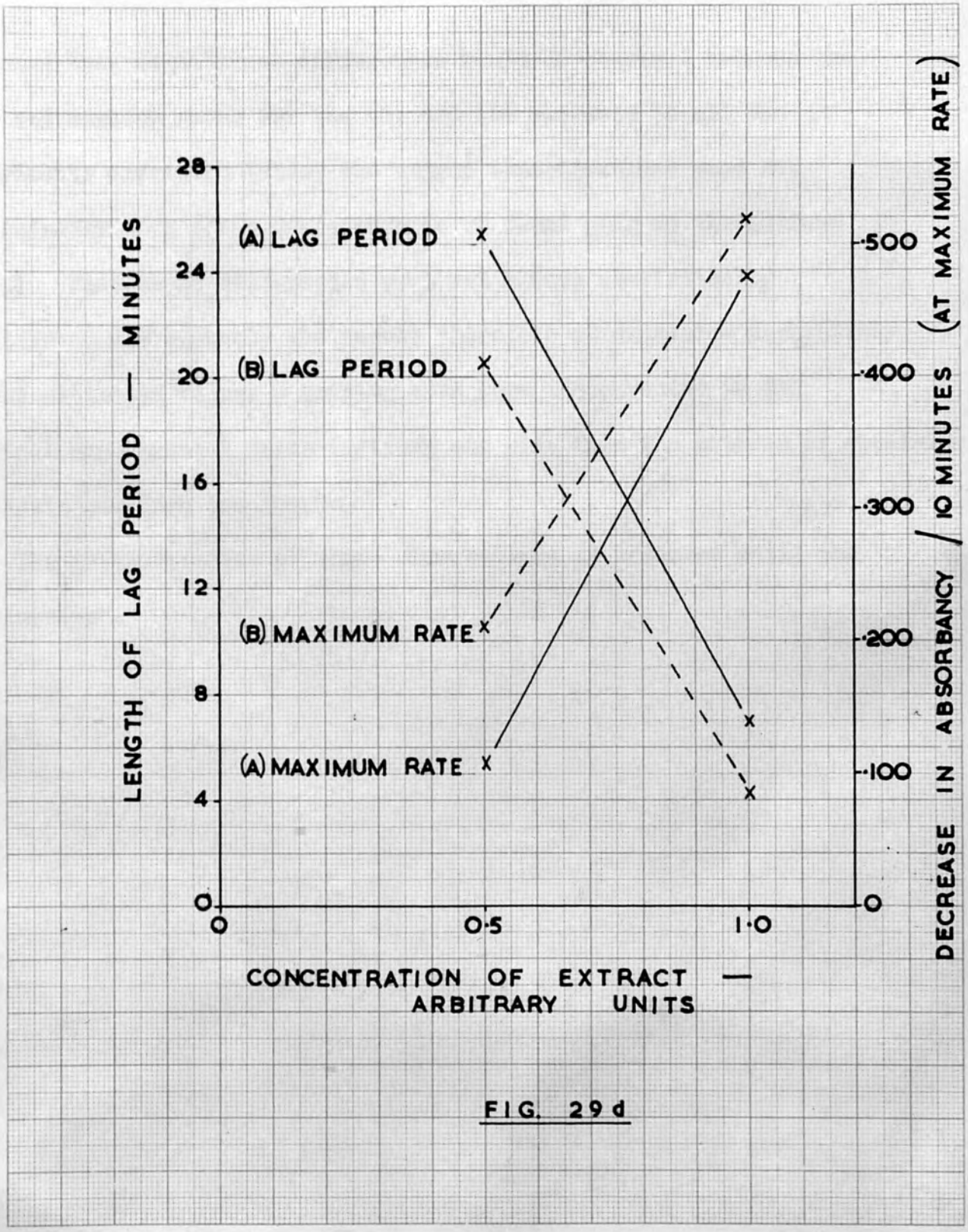

LENGTH OF LAG PERIOD — MINUTES
28
24
20
16
12
8
4
0
(A) LAG PERIOD
(B) LAG PERIOD
(B) MAXIMUM RATE
(A) MAXIMUM RATE
0
0·5
1·0
CONCENTRATION OF EXTRACT — ARBITRARY UNITS
·500
·400
·300
·200
·100
0
DECREASE IN ABSORBANCY / 10 MINUTES (AT MAXIMUM RATE)

FIG. 29 d

expected that significant differences would be observed between the lags and maximum rates for the (A) and (B) extracts in all the experiments described (since the enzyme concentrations were not sufficiently high for a difference to be found only in the maximum rate.) That these differences were not always statistically significant was probably the result therefore of excessive variability in the estimated values - arising both from inaccuracies in the original experimental determinations and also from the methods of obtaining the estimates from the graphs.

The interpretation of these observations is reserved until the "Discussion".

IV. A COMPARISON OF THE "IN VIVO IAA-OXIDASE ACTIVITIES" OF ROOTS OF ETIOLATED PEA SEEDLINGS GROWN IN WATER AND ROOTS OF ETIOLATED PEA SEEDLINGS GROWN IN IAA SOLUTION 10^{-7}G./ML.

Occasion 1. Pea seedlings were grown in water and in IAA solution 10^{-7}g./ml. as described under "Methods", Section I. Whole roots were harvested after 1 day and after 2 days and their "IAA-oxidase" activities determined as described under "Methods", Sections IV-2 and III-1. The reaction mixtures were sampled at "zero time" and after 45 minutes and 90 minutes (roots of plants harvested after 1 day) or at "zero time" and at 15 minute intervals for a period of 45 minutes (roots of plants harvested after 2 days). The results are given in figures 30a and 30b.

Occasion 2. Pea seedlings were grown in water and in IAA solution 10^{-7}g./ml. as described under "Methods", Section I. Whole roots were harvested after 2 days, and their "IAA-oxidase" activities determined as described under "Methods", Sections IV-2 and III-1. A number of plants were also allowed to grow for a further 24 hours - without any further renewal of the water and IAA solution. Whole roots were harvested from these and their "IAA-oxidase" activities also determined. All the reaction mixtureswere sampled at "zero time" and after 20, 40, 60 and 70 minutes. The results are given in figure 31a and 31b.

The results showed that the "in vivo IAA-oxidase activities" of the roots harvested after 2 days and 3 days growth in IAA solution were/

were higher than those of roots of the same ages grown in water - (figures 30b, 31a and 31b).

The "in vivo IAA-oxidase activities" of the roots harvested after 1 day were lower than thoseof the roots harvested after 2 and 3 days. The activities of the roots harvested after 2 days were variable - those of both the water - and IAA - grown roots being considerably higher on occasion 1 than on occasion 2. The activities of the roots harvested after 3 days were of the same order as those of the roots harvested after 2 days.

The term "in vivo IAA-oxidase activity" has been used for the "IAA-oxidase" activity observed under the conditions described - as was done by Galston and Dalberg (1954). However it seemed extremely unlikely that the bulk of the IAA being broken down was actually entering the root, and being broken down in situ there - although it was probable that some of the IAA was in fact destroyed in this way. Thus, judging from the results obtained in the next section ("Results", Section V) it seemed likely that the greater part of the IAA-breakdown was taking place in the external solution - where the very effective activator of IAA oxidation -DCP - together with any enzyme excaping into the mixture - or located on the outer surface of the roots - would form a powerful IAA-oxidase system. (The components of the reaction mixture may have had a damaging effect on the cells of the root - thus causing the enzyme to be set free).

The difference between the activities of the roots grown in water and those grown in IAA solution was somewhat difficult to explain. It/

it seemed rather surprising in the first place that it should be shown up so consistently in these very crude experiments. If, as suggested, the bulk of the IAA oxidation was taking place in the external solution, the difference might have been due to the ease with which a component of the oxidase system (or an inhibitor) was being released from the cells, rather than to actual concentration differences of such substances in the tissues. Alternatively the DCP might have penetrated into the tissues of the IAA-grown roots more readily than into those of the water-grown roots - thus causing either an enhanced rate of IAA breakdown there or a greater release of some component of the oxidase system (as mentioned above) into the external solution. In fact, the most likely state of affairs was thought to be that a rapidly diffusing substance - such as an inhibitor - was present at a higher concentration in the tissues of the roots grown in water, than in the tissues of the roots grown in IAA solution, and that it diffused out into the external solutions in quantities which were proportional to these concentrations - leading thereby to the observed marked differences in the "IAA-oxidase" activities. Although merely speculation, it was this idea which finally prompted the decision to examine the inhibitor levels of the extracts (A) and (B) - as was done in Section VI. (The inhibitor levels of the "ambient solutions" was unfortunately not examined).

Figure 30a. Graphs to show the "invivo IAA-oxidase activities" of the roots of plants grown for 1 day in water or in IAA solution 10^{-7}g./ml. (Occasion 1).

Figure 30b. Graphs to show the "in vivo IAA-oxidase activities" of the roots of plants grown for 2 days in water or in IAA solution 10^{-7}g./ml. (Occasion 1).

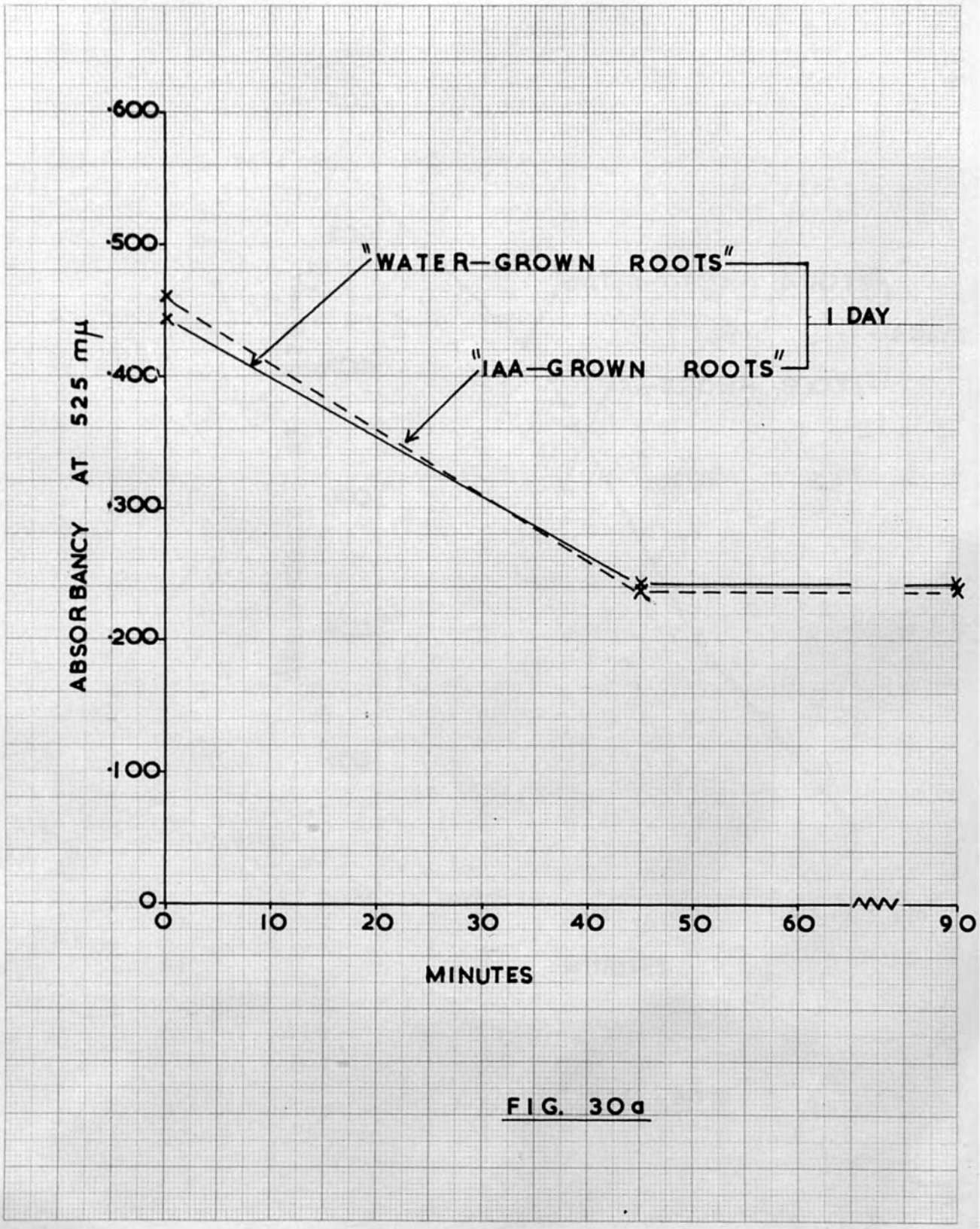

·600
·500
·400
·300
·200
·100
0
ABSORBANCY AT 525 mμ
"WATER-GROWN ROOTS"
"IAA-GROWN ROOTS"
1 DAY
0
10
20
30
40
50
60
90
MINUTES

FIG. 30a

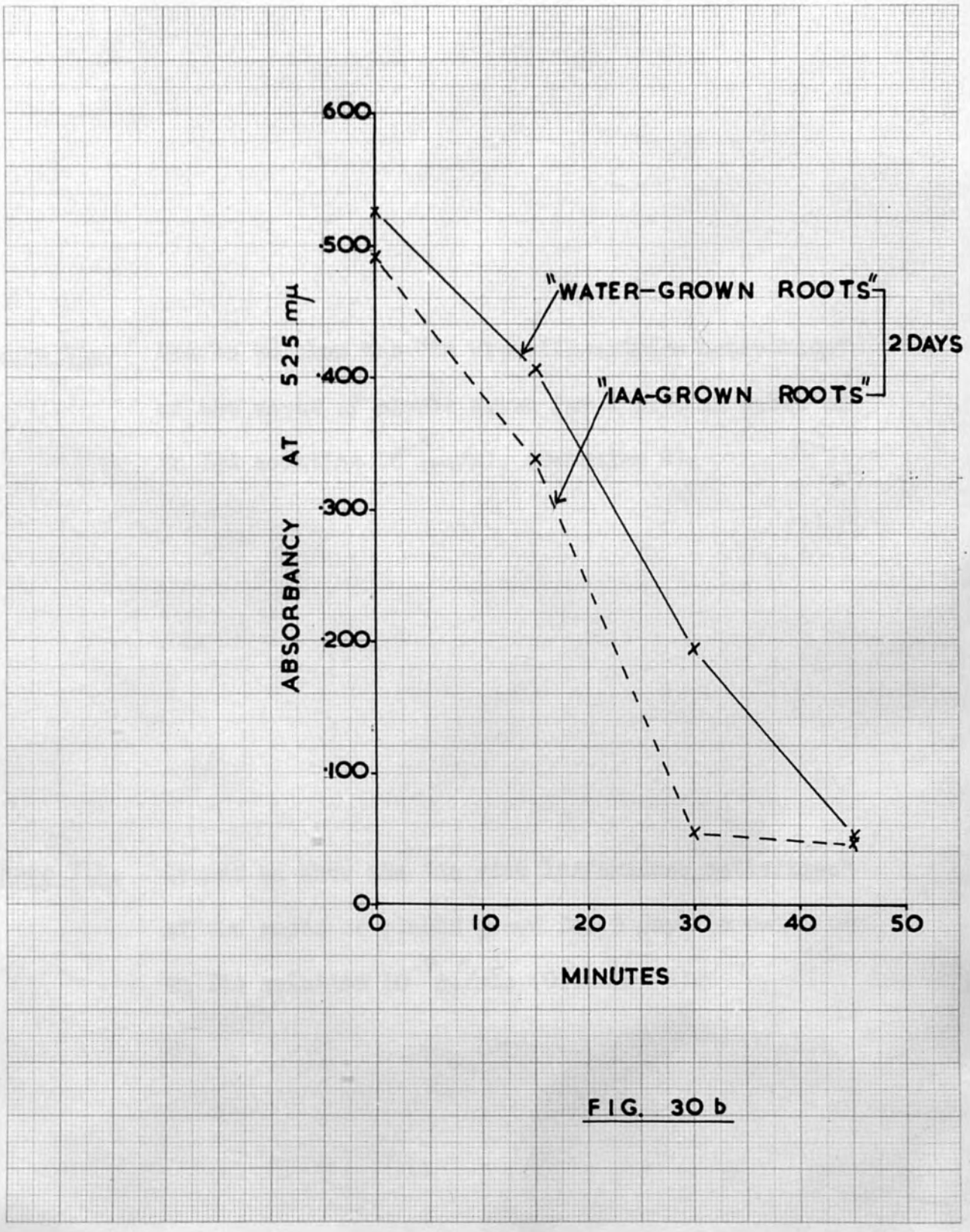

600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
"WATER-GROWN ROOTS"
"IAA-GROWN ROOTS"
2 DAYS
0
10
20
30
40
50
MINUTES

FIG. 30 b

Figure 31a. Graphs to show the "in vivo IAA-oxidase activities" of the roots of plants grown for 2 days in water or in IAA solution 10^{-7} g./ml. (Occasion 2).

Figure 31b. Graphs to show the "in vivo IAA-oxidase activities" of the roots of plants grown for 3 days in water or in IAA solution 10^{-7} g./ml. (Occasion 2).

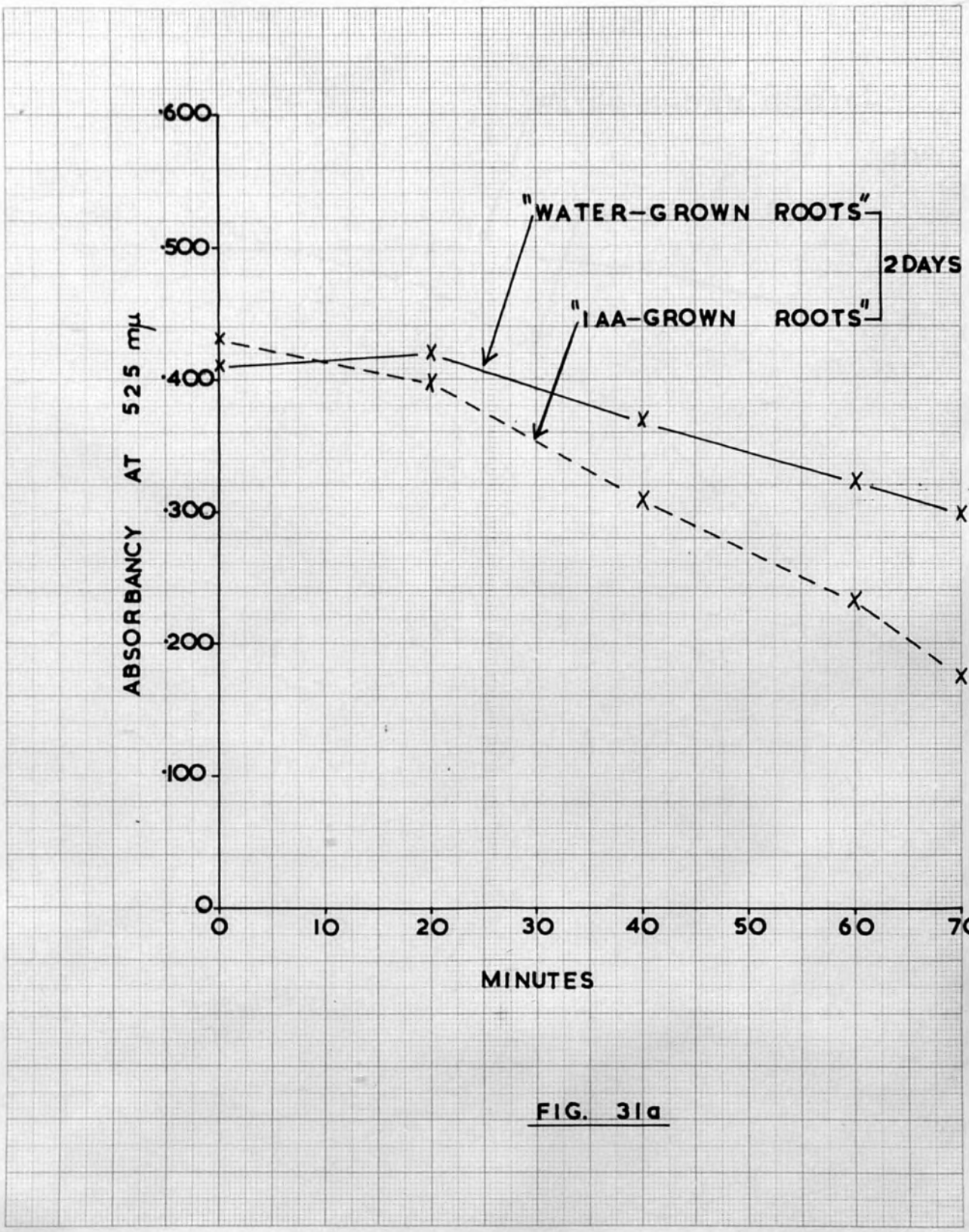

"WATER-GROWN ROOTS"
2 DAYS
"IAA-GROWN ROOTS"
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
70
MINUTES

FIG. 31a

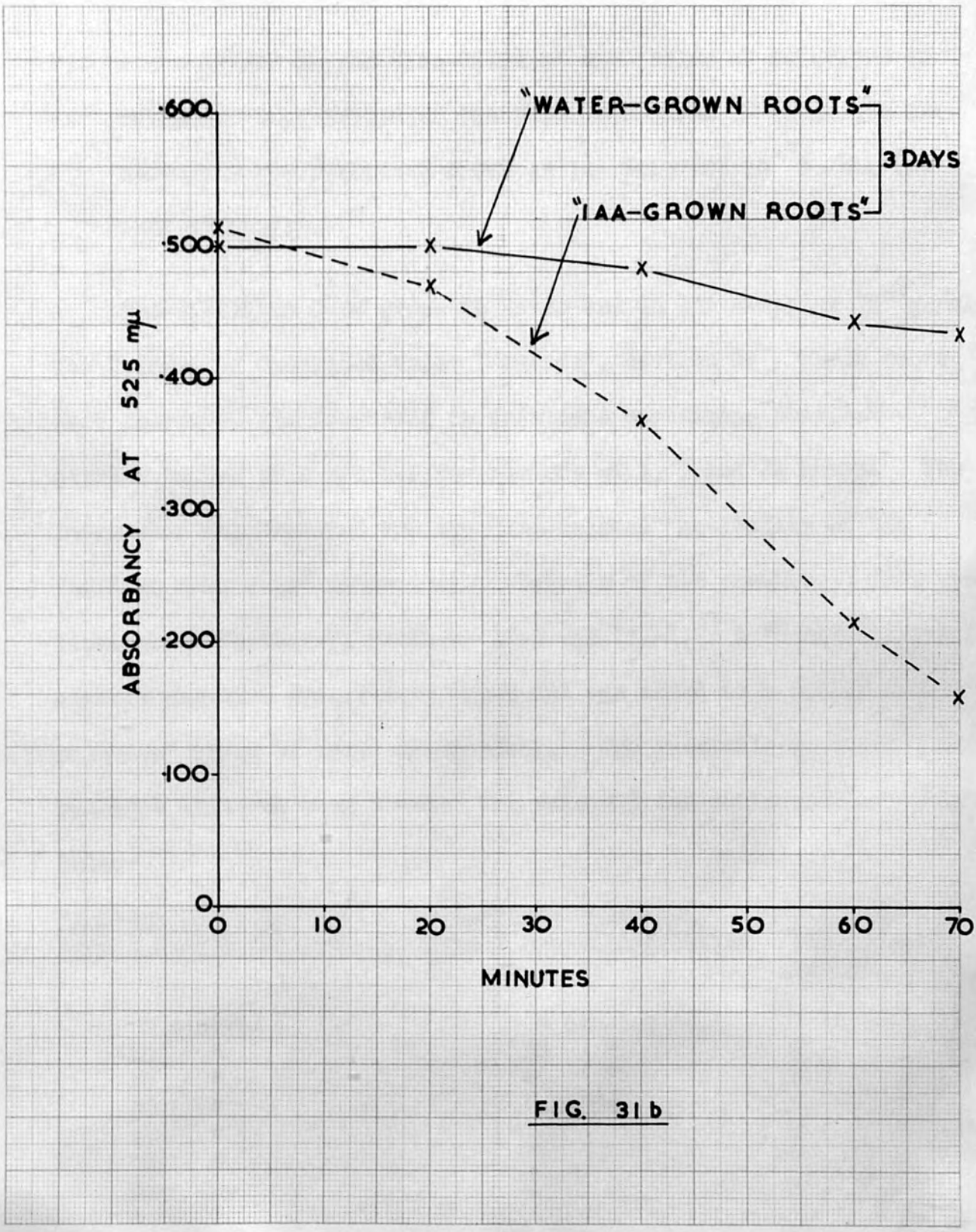

"WATER-GROWN ROOTS"
3 DAYS
"IAA-GROWN ROOTS"
600
500
400
300
200
100
0
ABSORBANCY AT 525 mμ
0
10
20
30
40
50
60
70
MINUTES

FIG. 31 b

V. A COMPARISON OF THE "IAA-OXIDASE" ACTIVITIES OF SOLUTIONS IN WHICH ROOTS OF ETIOLATED PEA SEEDLINGS GROWN IN WATER AND OF ETIOLATED PEA SEEDLINGS GROWN IN IAA SOLUTION 10^{-7}G./ML. HAD BEEN INCUBATED.

Pea seedlings were grown in water and in IAA solution 10^{-7}g./ml. for 2 days, as described under "Methods", Section I. Whole roots were harvested and incubated in solutions containing phosphate-citrate buffer pH 4 and DCP for periods of 1, 3 and 24 hours. The roots were then removed and the "IAA-oxidase" activities of the "ambient solutions" determined. Details of the procedures used are given under "Methods", Sections IV-3 and III-1. A "control" reaction mixture was also prepared in which IAA was added to a boiled "ambient solution" (from 24 hours incubation). The intervals at which the reaction mixtures were sampled for reisudal IAA estimation are shown below:-

"Ambient solution" from 1 hour's incubation.	"Ambient solution" from 3 hours' incubation	"Ambient solution" from 24 hours' incubation	
		Unboiled	Boiled
0 minutes	0 minutes	0 minutes	0 minutes
10 "	30 "	10 "	60 "
20 "	60 "	20 "	
30 "	Next day	30 "	
40 "		40 "	
70 "		50 "	
Next day		60 "	

The results are given in figures 32a, 32b and 32c.

Determination of the effect of 1. decreasing the number of roots and, 2. increasing the concentration of the DCP, on the "IAA-oxidase" activities of the "ambient solutions".

Roots of plants which had been grown in water for 2 days were used for this experiment. Two groups of 12 roots were incubated for 24 hours as described under "Methods", Section IV-3, in solutions to which a DCP solution either of 4×10^{-4} g./ml. or of 10^{-3} g./ml. was added. The "IAA-oxidase" activities of the resulting "ambient solutions" were then determined as described under "Methods", Sections IV-3 and III-1. A further two groups, each of 6 roots, were incubated for 24 hours in the solutions to which a DCP solution either of 4×10^{-4} g./ml. or of 10^{-3} ./ml. was added*. The "IAA-oxidase" activities of the resulting "ambient solutions" was then determined as before. The intervals at which the reaction mixtures were sampled for residual IAA estimation are shown below.

"Ambient solutions" in which 12 roots had been incubated.	"Ambient solutions" in which 6 roots had been incubated.
0 minutes	0 minutes
10 "	60 "
20 "	90 "
30 "	
40 "	
50 "	
60 "	
90 "	

The results are shown in figure 33.

* (The concentrations of DCP in the reaction mixtures were therefore 5×10^{-5} g./ml. and 1.25×10^{-4} g./ml.).

The results showed that the "ambient solutions" prepared by incubating the roots for 1 hour contained very little IAA-oxidising activity (figure 32a). A certain amount of oxidation did take place in the first hour after the addition of the IAA, and after a much longer period had elapsed (about 24 hours) it was found that this amount had increased slightly. There appeared to be no marked difference between the activity of the "ambient solution" prepared by incubating the roots grown in water and of that prepared from those grown in IAA solution. Thus the activity of the "ambient solution" prepared from the IAA-grown roots was the higher of the two during the first hour, but the total amount of IAA broken down in it was found to be smaller.

The results obtained using "ambient solutions" prepared by incubating the roots for 3 hours (figure 32b) were similar, except that firstly the activity of the "ambient solution" prepared from the roots grown in IAA solution was slightly lower than that of the corresponding solution prepared from the roots grown in water, and secondly that when the two reaction mixtures were examined next day - it was found that the IAA had been completely destroyed in the one containing "ambient solution" prepared from IAA-grown roots.

The results obtained using "ambient solutions" prepared by incubating the roots for 24 hours (figure 32c) showed that these solutions contained a very active IAA-oxidase system. Thus the IAA added was completely destroyed after 1 hour. The activity of the "ambient/

"ambient solution" prepared using the roots of plants grown in IAA solution was greater than that prepared using the roots of plants grown in water. No IAA breakdown was observed in the "control" reaction mixtures which contained samples of the "ambient solutions" which had been boiled.

When the graphs of figures 32a and 32b were compared with those of figures 30b and 31a in the previous section ("Results", Section IV) showing the "in vivo IAA-oxidase activities" of roots harvested after 2 days, it became obvious that the breakdown of IAA was faster in the presence of the roots than in the "ambient solutions" - which suggested that breakdown of IAA was occurring at the surface of the roots or actually in the root tissues - to a considerable extent (unless it was assumed that the IAA itself was promoting the release of a component of the oxidase system into the external solution). On the other hand, the complete breakdown of IAA, in the "ambient solution" prepared by incubating IAA-grown roots for only 3 hours (figure 32b) showed how rapidly the external solution could acquire enzyme activity. (The thermolabile nature of the oxidising system was demonstrated by boiling).

The results of the final experiment in this section (figure 33) showed that the activity of the "ambient solutions" prepared by incubating the roots of the water-grown plants for 24 hours was dependent both on the number of roots incubated and on the concentration of the DCP in the mixture. Thus the activities of the "ambient solutions" prepared by incubating 12 roots were greater than those prepared by incubating 6 roots. Also the activities of the "ambient solutions"

to/

to which DCP solution at a concentration of 10^{-3}g./ml. had been added were greater than those of the "ambient solutions" to which DCP at a concentration of 4×10^{-4}g./ml. had been added. This effect of the higher DCP concentration in causing a higher "IAA-oxidase" activity must have been caused by its action in promoting the release of some component of the "IAA-oxidase" system - presumably the enzyme itself. It could not have been merely an effect on IAA breakdown, after the removal of the roots, since this concentration of DCP was shown to be supra-oprimal - at least when enzyme extracts were used (see "Results", Section II-2).

Figure 32a. Graphs to show the "IAA-oxidase" activities of "ambient solutions" in which whole roots had been incubated for 1 hour.

Figure 32b. Graphs to show the "IAA-oxidase" activities of "ambient solutions" in which whole roots had been incubated for 3 hours.

Figure 32c. Graphs to show the "IAA-oxidase" activities of "ambient solutions" in which whole roots had been incubated for 24 hours.

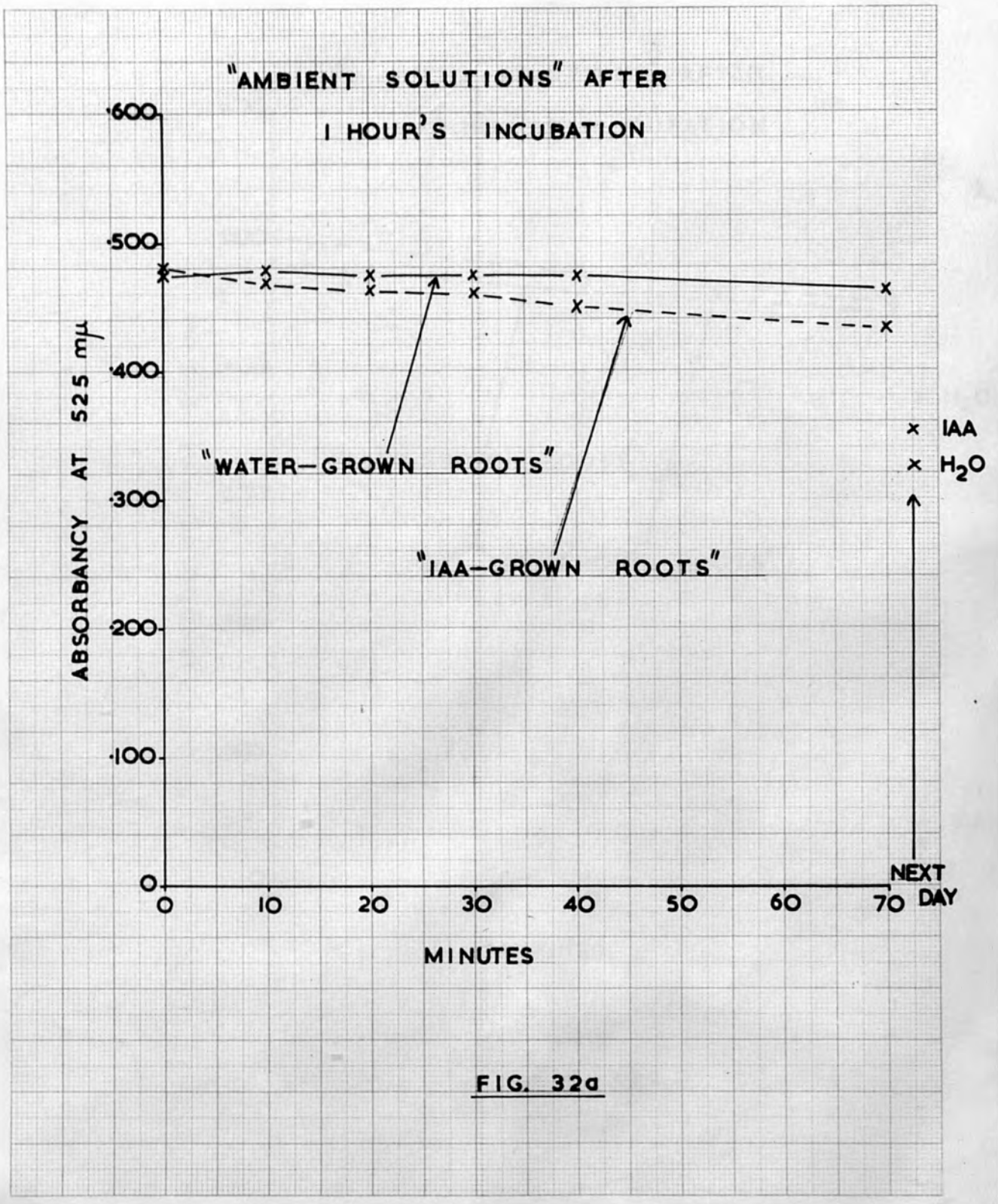

"AMBIENT SOLUTIONS" AFTER
1 HOUR'S INCUBATION
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
"WATER-GROWN ROOTS"
"IAA-GROWN ROOTS"
× IAA
× H2O
NEXT DAY
0
10
20
30
40
50
60
70
MINUTES

FIG. 32a

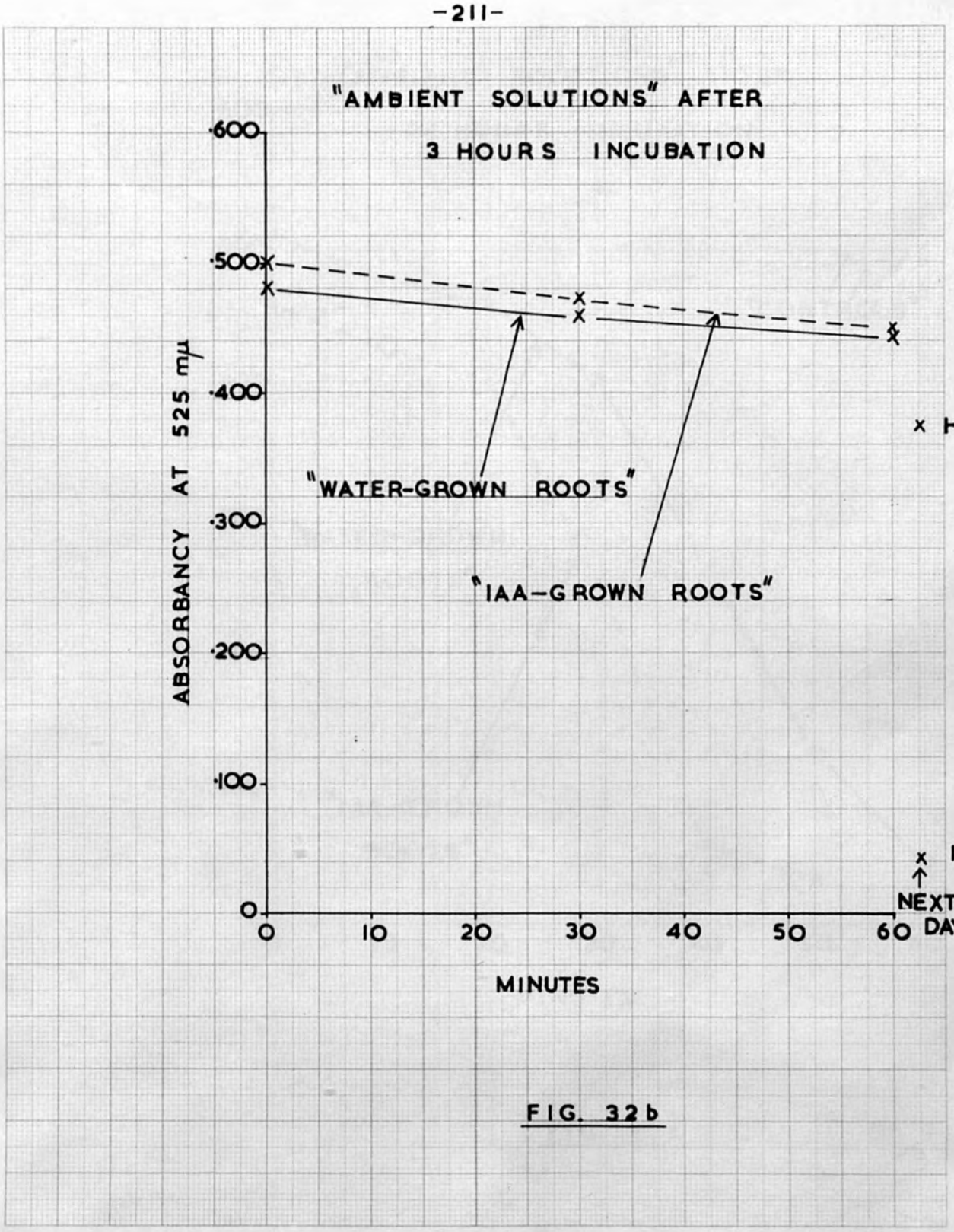

"AMBIENT SOLUTIONS" AFTER
3 HOURS INCUBATION
ABSORBANCY AT 525 mμ
600
500
400
300
200
100
0
0
10
20
30
40
50
60
MINUTES
"WATER-GROWN ROOTS"
"IAA-GROWN ROOTS"
x H2O
x IAA
NEXT DAY

FIG. 32b

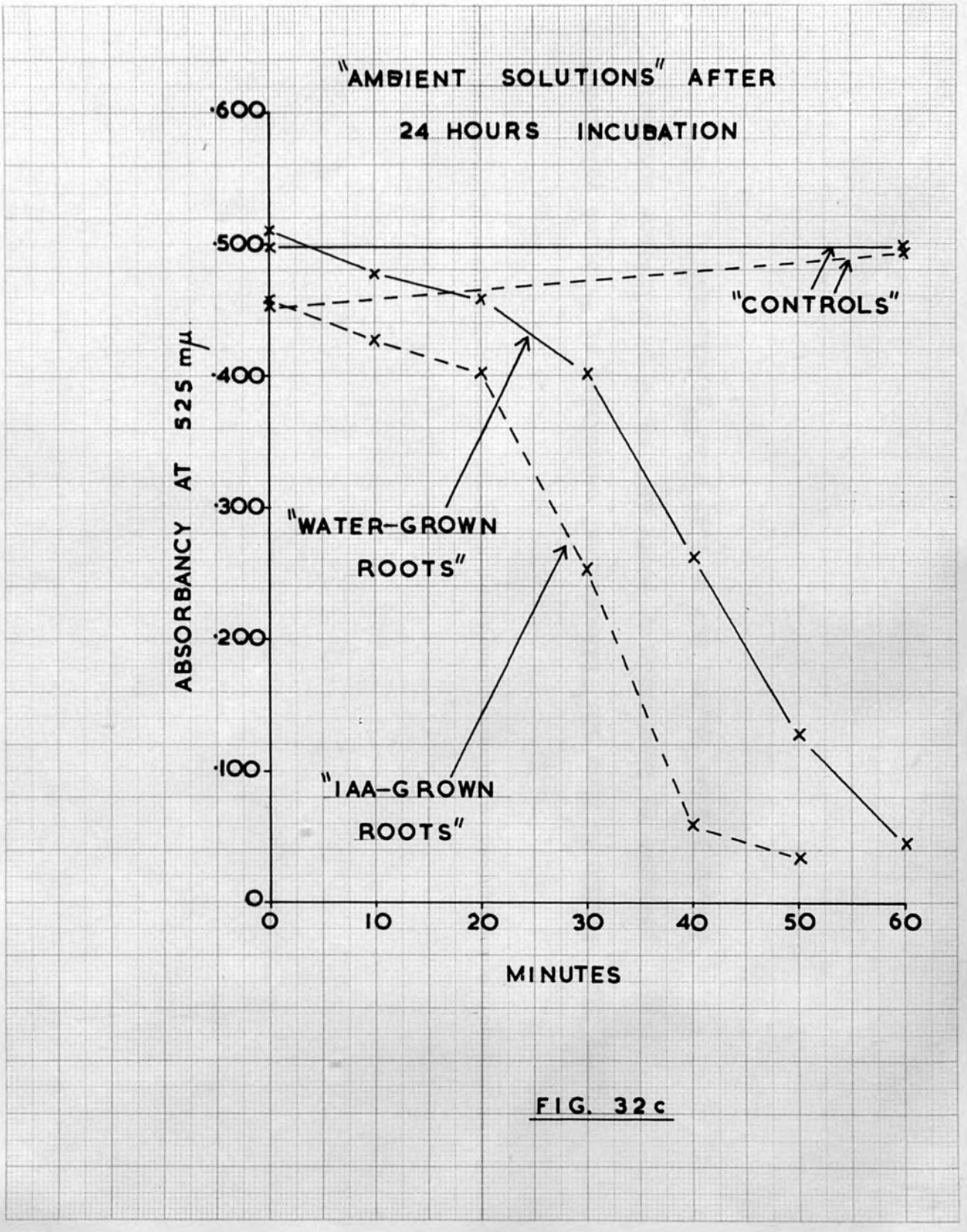

"AMBIENT SOLUTIONS" AFTER
24 HOURS INCUBATION
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
"CONTROLS"
"WATER-GROWN ROOTS"
"IAA-GROWN ROOTS"
0
10
20
30
40
50
60
MINUTES

FIG. 32c

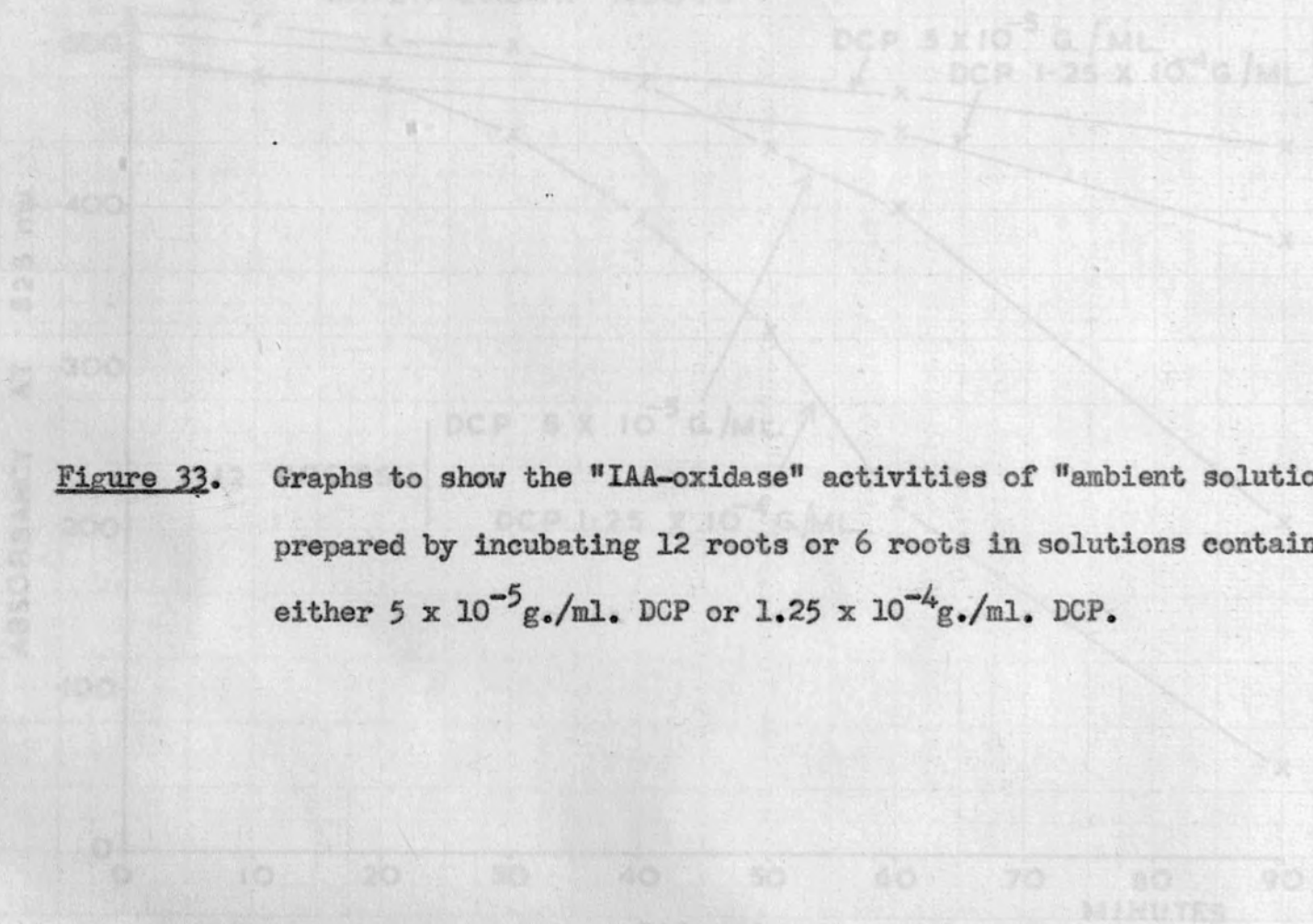


Figure 33. Graphs to show the "IAA-oxidase" activities of "ambient solutions" prepared by incubating 12 roots or 6 roots in solutions containing either 5 x 10^{-5}g./ml. DCP or 1.25 x 10^{-4}g./ml. DCP.

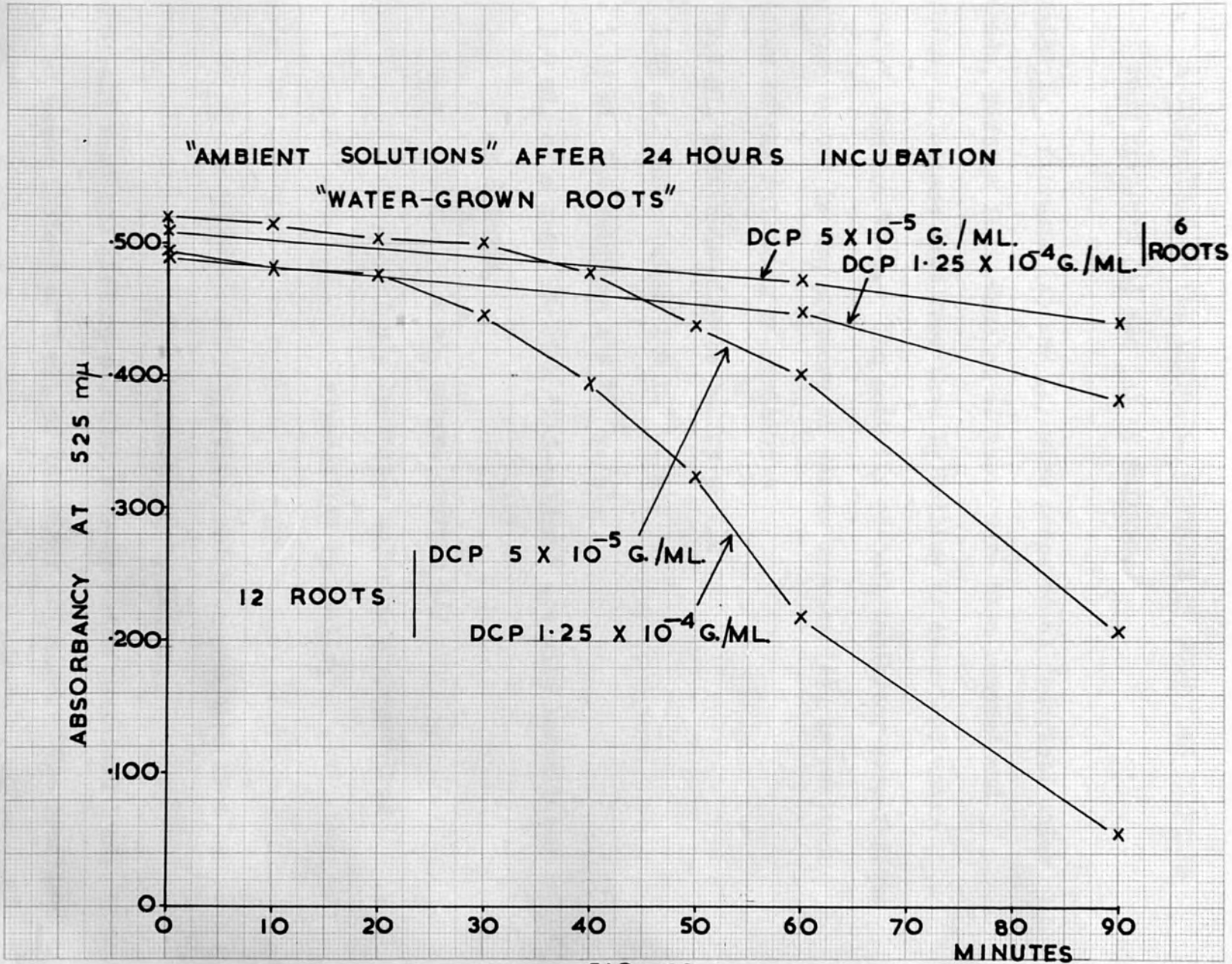

"AMBIENT SOLUTIONS" AFTER 24 HOURS INCUBATION
"WATER-GROWN ROOTS"
DCP 5 X 10⁻⁵ G./ML.
DCP 1·25 X 10⁻⁴ G./ML.
6 ROOTS
DCP 5 X 10⁻⁵ G./ML.
DCP 1·25 X 10⁻⁴ G./ML.
12 ROOTS
ABSORBANCY AT 525 mμ
·500
·400
·300
·200
·100
0
0
10
20
30
40
50
60
70
80
90
MINUTES

FIG. 33

VI 1. A COMPARISON OF THE INHIBITOR CONTENTS OF SOLUTIONS PREPARED FROM EXTRACTS (A) AND (B) BY BOILING.

Occasion 1. Extracts (A) and (B) were prepared as described under "Methods, Section II. Samples of these were then removed and inhibitor solutions made from them by boiling as described under "Methods", Section VI-1. The activity of both inhibitor preparations was then tested in the presence of enzyme extract (A), in reaction mixtures of the following composition:-

2.5 ml. phosphate-citrate buffer pH 4.0

1.25 ml. DCP solution 4 x 10^{-4}g./ml.

1.25 ml. IAA solution 4 x 10^{-4}g./ml.

5.0 ml. of a solution with "IAA-oxidase" activity constituted as follows,

4 ml. enzyme extract (A)

1 ml. inhibitor solution prepared from extract (A) by boiling

or

inhibitor solution prepared from extract (B) by boiling.

A "control" reaction mixture was also made up which contained 1 ml. distilled water instead of 1 ml. of inhibitor solution. The reaction mixtures were incubated as described under "Methods", Section IV-1, and the rate of IAA breakdown determined as described under "Methods", Section III-1. The reaction mixtures were sampled at "zero time" and at/

at 15 minute intervals thereafter for a period of 1½ hours. The results are given in figure 34. (Note change of scale on the abscissa.)

Occasion 2. As on "occasion 1", except that the reaction mixtures contained 5.0 ml. of a solution with "IAA-oxidase" activity constituted as follows:-

3 ml. enzyme extract (A)

2 ml. inhibitor solution prepared from extract (A) by boiling

or

inhibitor solution prepared from extract (B) by boiling,

and also the reaction mixtures were sampled at "zero time" and at 30 minute intervals thereafter for a period of 2½ hours. The results are shown in figure 35.

Occasion 3. Duplicate extracts ('A') and ('B'), referred to as extracts ('A_1'), ('A_2'), ('B_1') and ('B_2'), were prepared as described under "Methods", Section II, except that the centrifuged extracts were made up to a volume of 100 ml. instead of 150 ml. Inhibitor solutions were then prepared from them by boiling as described under "Methods", Section VI,1 and the activity of these tested in reaction mixtures containing either enzyme extract ('A_1') (inhibitor solutions prepared from ('A_1') and ('B_1'), or enzyme extract ('A_2') (inhibitor solutions prepared from ('A_2') and ('B_2')). "Control" reaction mixtures were also prepared containing the enzyme extract ('A_1') or ('A_2') and distilled water. In each case 2 ml. enzyme extract was combined with/

with 3 ml. of inhibitor preparation or 3 ml. of distilled water. The reaction mixtures were sampled at "zero time" and at intervals of 30 minutes thereafter for a period of 2½ hours. The results are given in figures 36a and 36b.

The results* showed that in every case the inhibitory activity of the solution prepared from extract (B) or ('B') by boiling was somewhat lower than that ot is counterpart prepared from extract (A) or ('A'). The difference was more marked when the rate of IAA breakdown was relatively slow (figures 35, 36a, 36b) than when the rate was relatively fast (figure 34).

* See also table 19, page 239.

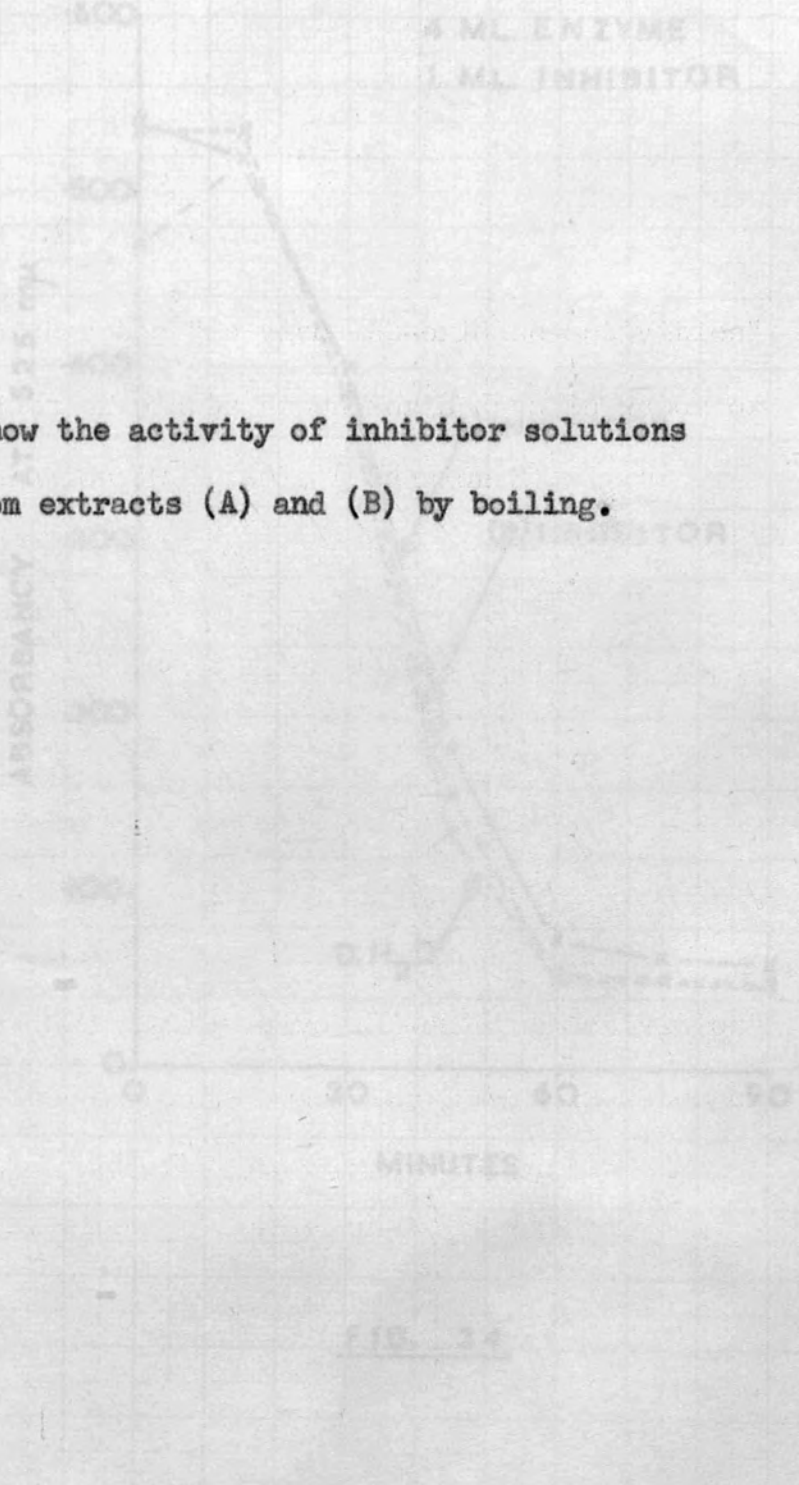


Figure 34. Graphs to show the activity of inhibitor solutions prepared from extracts (A) and (B) by boiling.

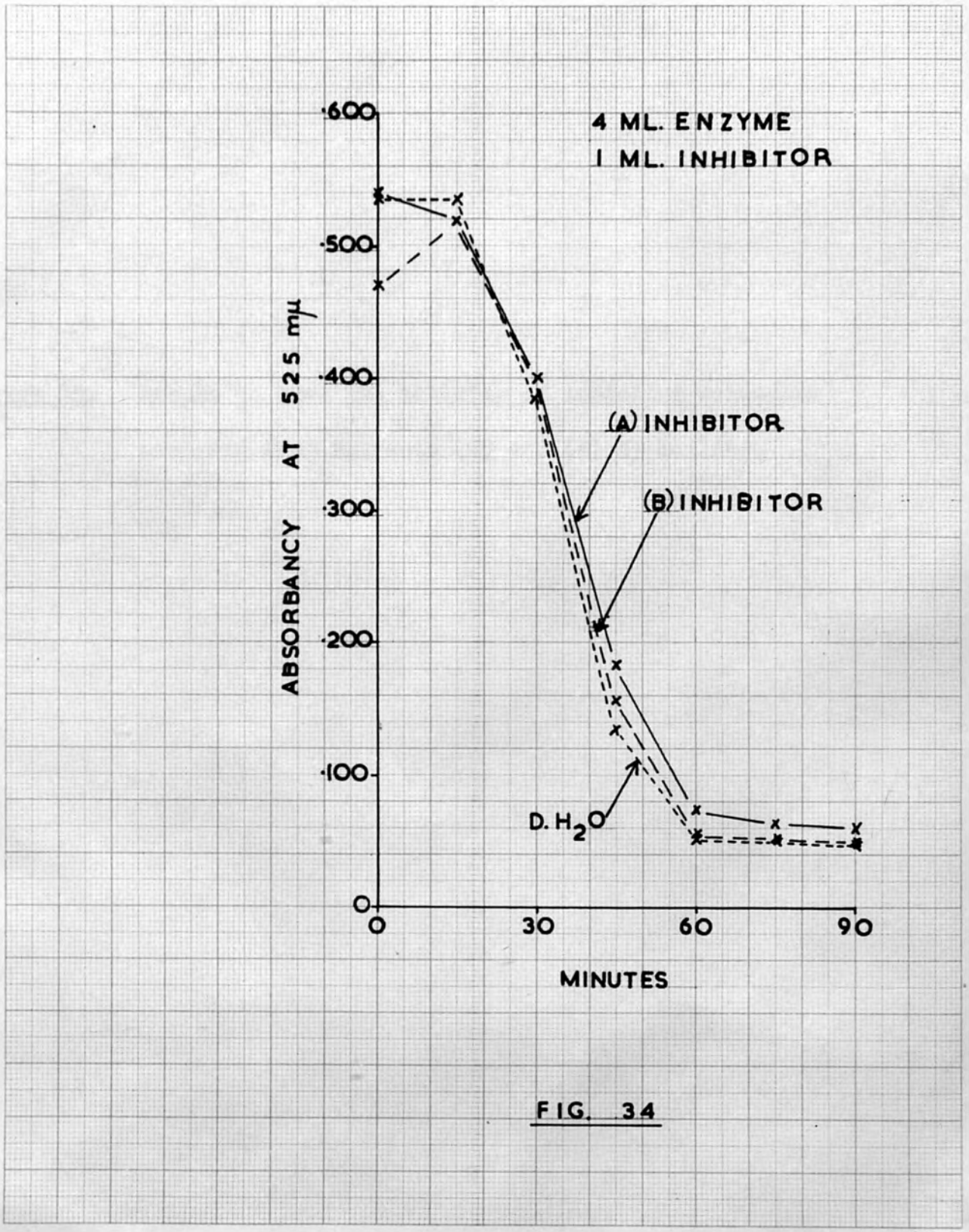

4 ML. ENZYME
1 ML. INHIBITOR
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
(A) INHIBITOR
(B) INHIBITOR
D. H_2O
0
30
60
90
MINUTES

FIG. 34

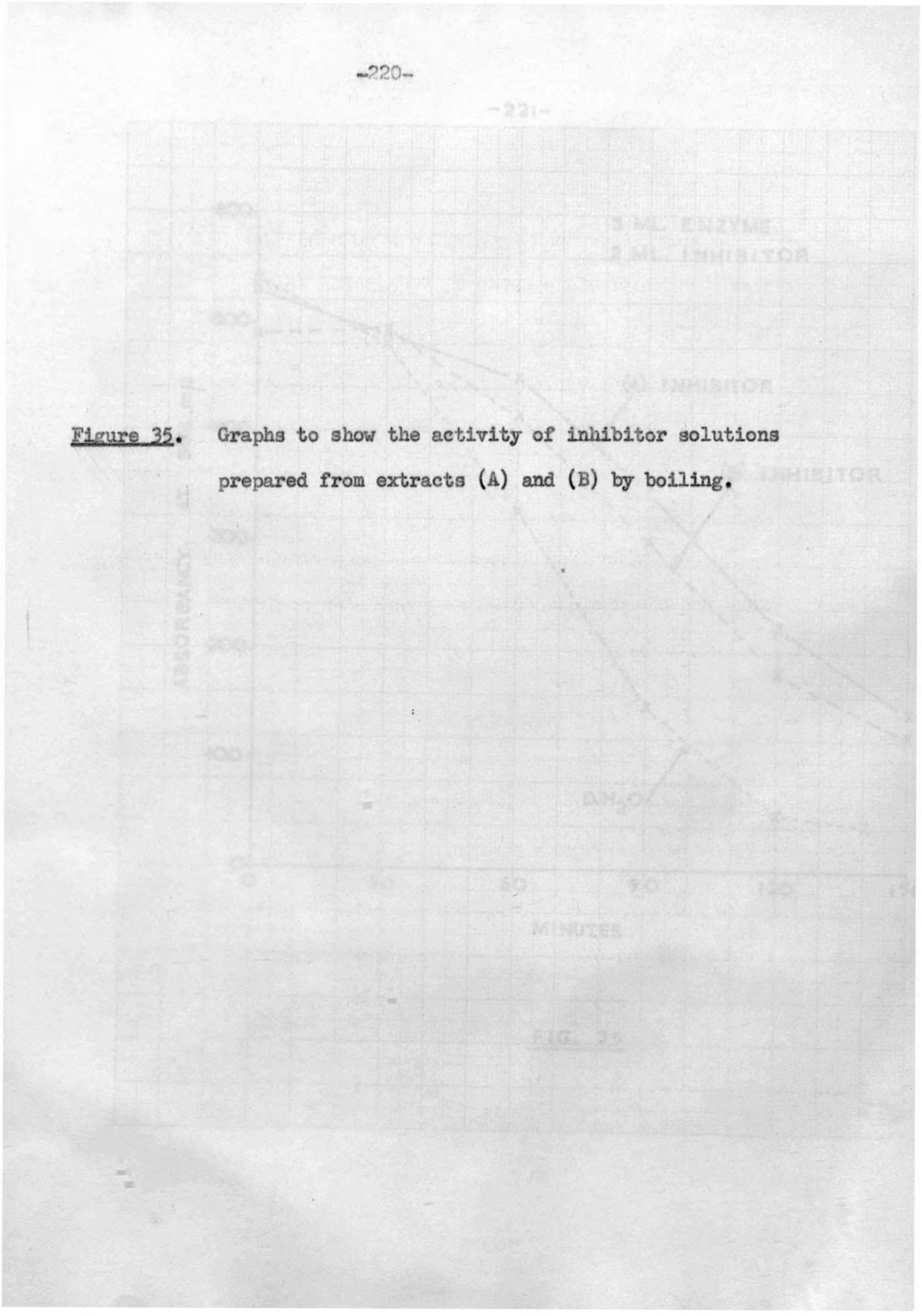


Figure 35. Graphs to show the activity of inhibitor solutions prepared from extracts (A) and (B) by boiling.

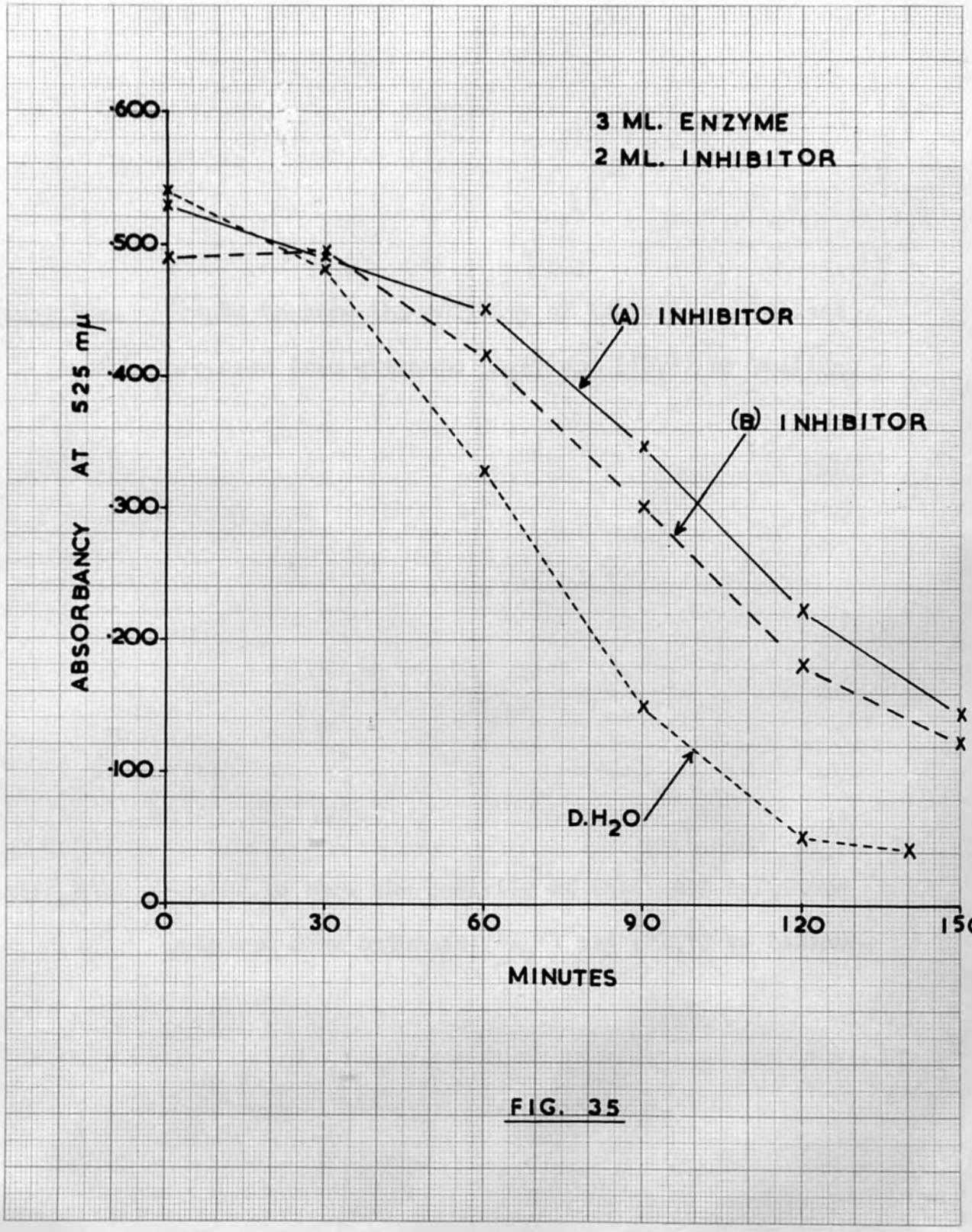

3 ML. ENZYME
2 ML. INHIBITOR
(A) INHIBITOR
(B) INHIBITOR
D.H2O
ABSORBANCY AT 525 mμ
600
500
400
300
200
100
0
0
30
60
90
120
150
MINUTES

FIG. 35

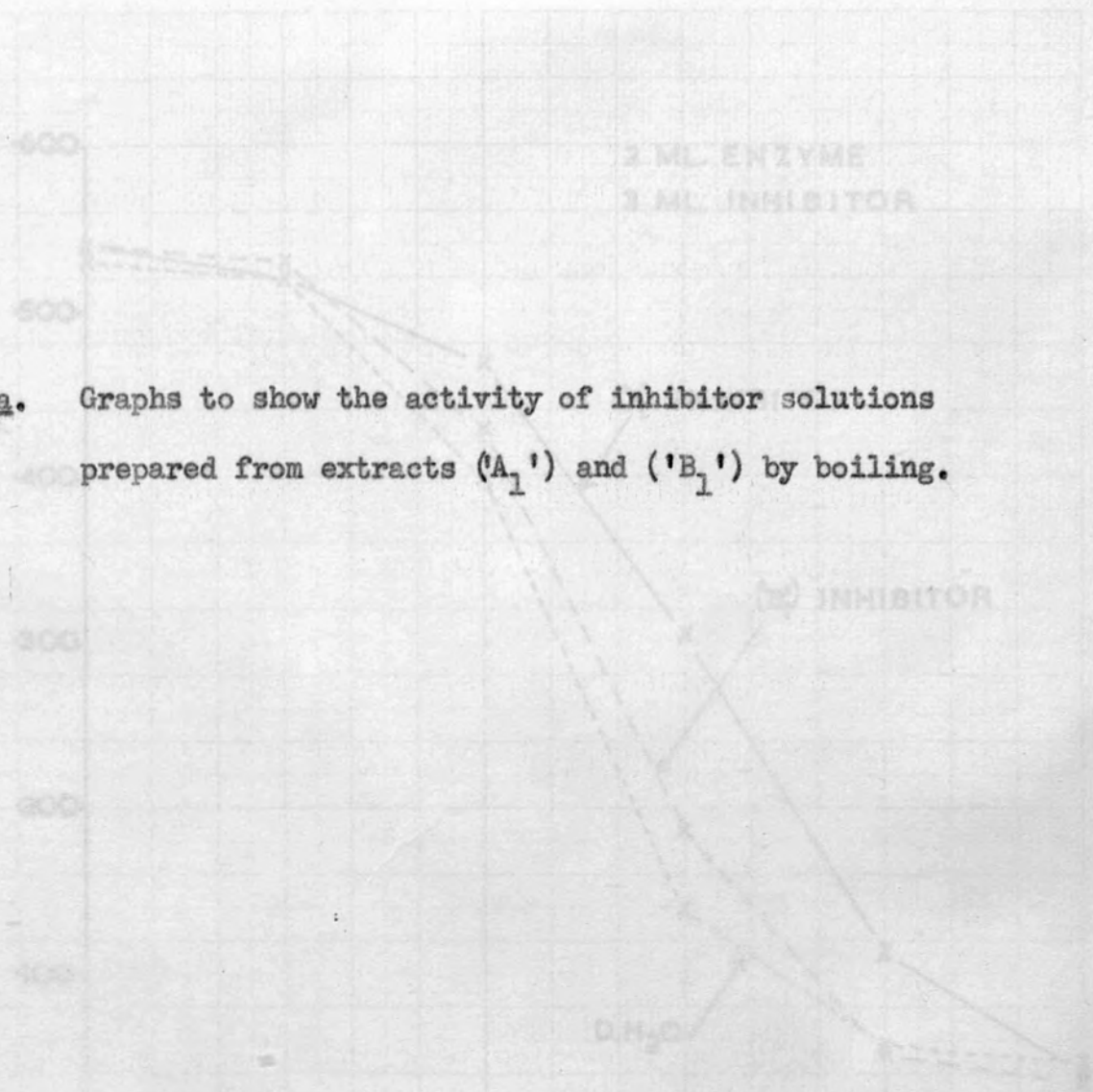


Figure 36a. Graphs to show the activity of inhibitor solutions prepared from extracts ('A_1') and ('B_1') by boiling.

Figure 36b. Graphs to show the activity of inhibitor solutions prepared from extracts ('A_2') and ('B_2') by boiling.

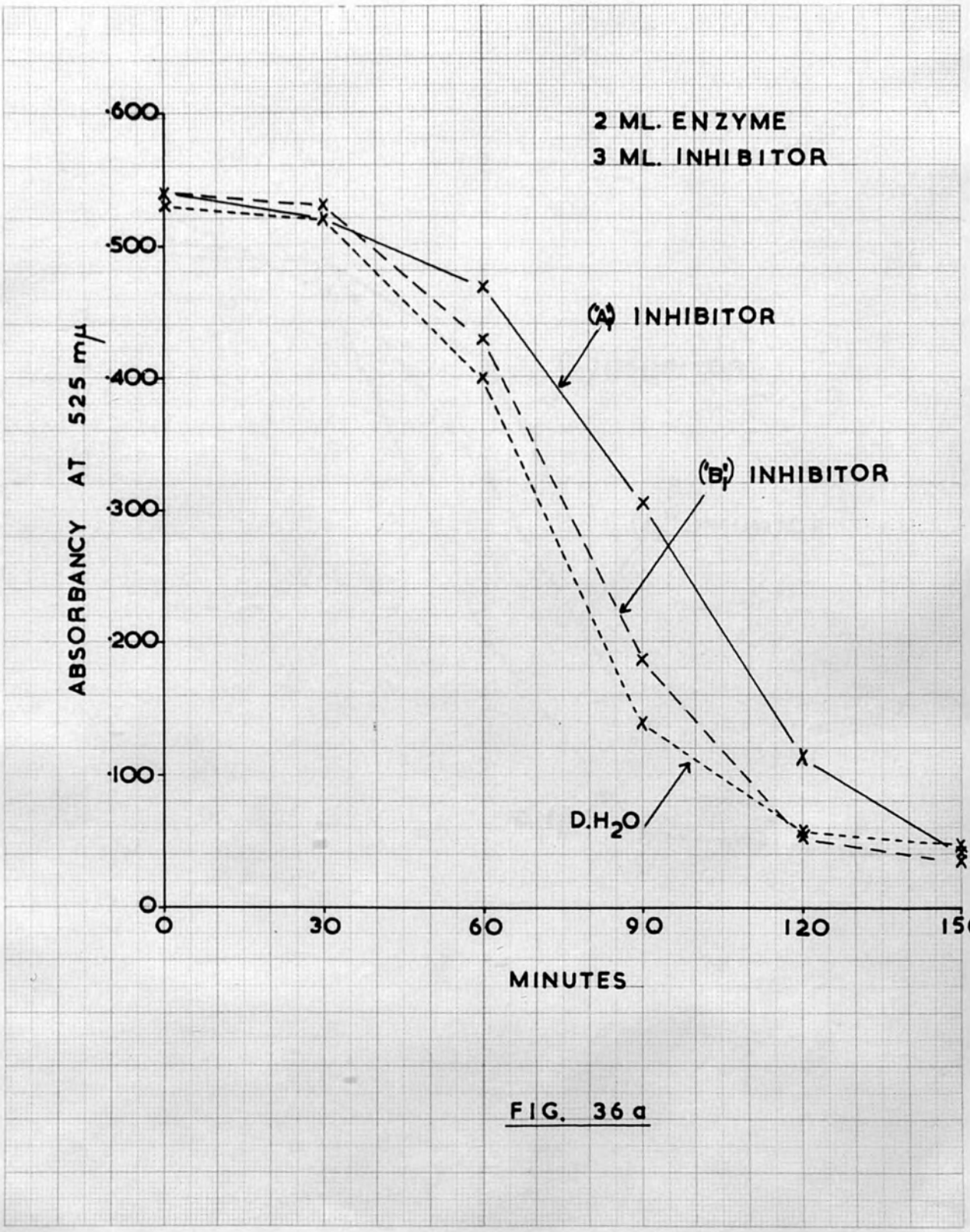

2 ML. ENZYME
3 ML. INHIBITOR
(A) INHIBITOR
(B) INHIBITOR
D.H2O
ABSORBANCY AT 525 mμ
MINUTES
0
100
200
300
400
500
600
0
30
60
90
120
150

FIG. 36 a

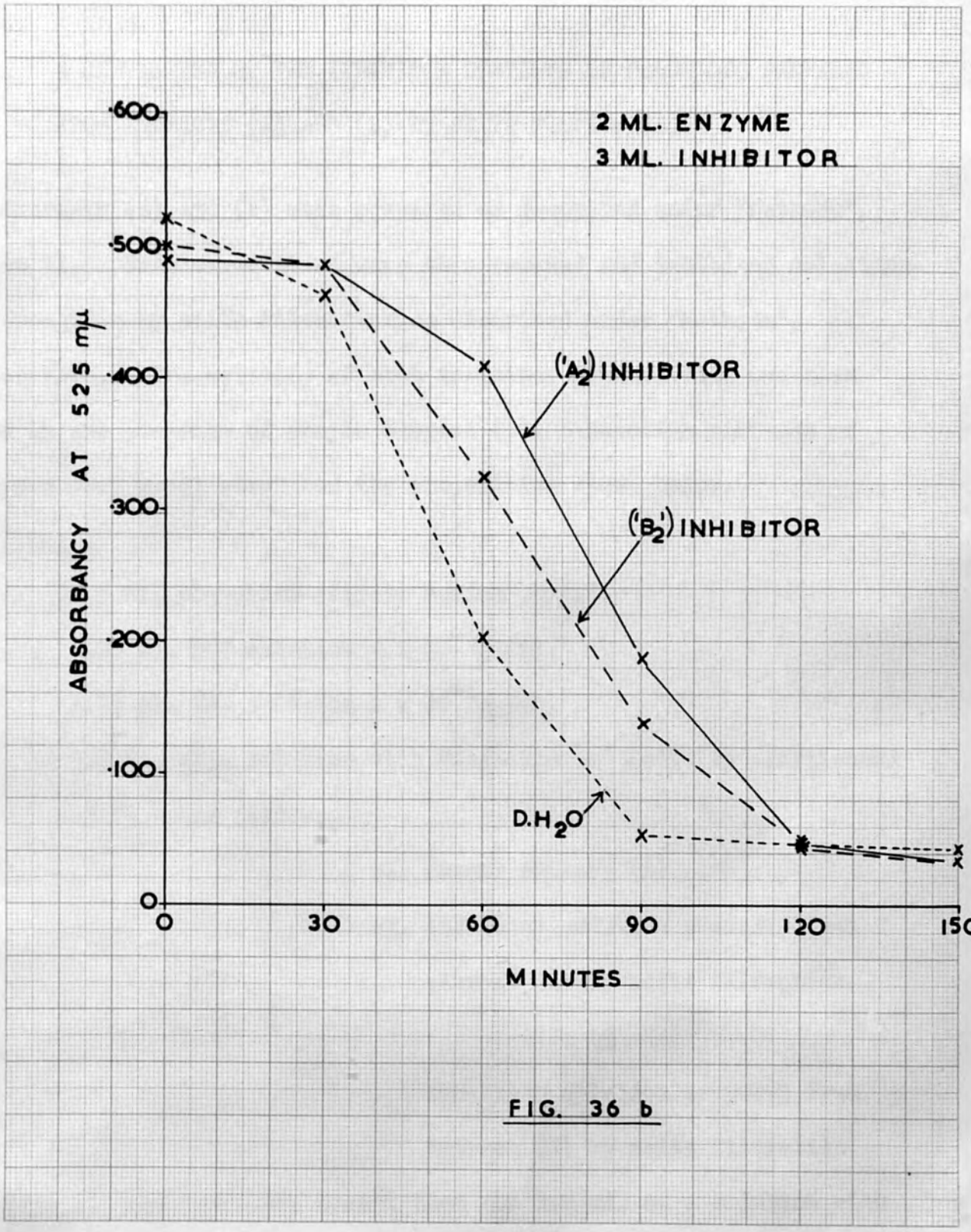

2 ML. ENZYME
3 ML. INHIBITOR
('A2') INHIBITOR
('B2') INHIBITOR
D.H2O
ABSORBANCY AT 525 mμ
600
500
400
300
200
100
0
0
30
60
90
120
150
MINUTES

FIG. 36 b

VI. 2. A COMPARISON OF THE INHIBITOR CONTENTS OF SOLUTIONS PREPARED FROM EXTRACTS (A) AND (B) BY SEITZ FILTRATION.

Extracts (A) and (B) were prepared as described under "Methods", Section II. Samples of these were then removed and inhibitor solutions made from them by seitz filtration as described under "Methods", Section VI-2. The activity of both inhibitor preparations was then tested in the presence of enzyme extract (A). Reaction mixtures of two types were used, which had the composition shown below.

Type 1.

2.5 ml. phosphate-citrate buffer pH 4.0.

1.25 ml. DCP solution 4 x 10^{-4} g./ml.

1.25 ml. IAA solution 4 x 10^{-4} g./ml.

5.0 ml. of a solution with "IAA-oxidase" activity constituted as follows,

4 ml. enzyme extract (A)

1 ml. inhibitor solution prepared from extract (A) by seitz filtration

or

inhibitor solution prepared from extract (B) by seitz filtration.

Type 2. As "type 1", except that the 5.0 ml. of a solution with "IAA-oxidase" activity was constituted as follows,

3 ml. enzyme extract (A)

2 ml. inhibitor solution prepared from extract(A) by seitz filtration.

or

inhibitor solution prepared from extract (B) by seitz filtration.

"Control" reaction mixtures were also made up which contained 1 ml. (type 1) or 2 ml. (type 2) distilled water instead of the corresponding quantities of inhibitor solution. The reaction mixtures were incubated as described under "Methods", Section IV-1 and the rate of IAA breakdown determined as described under "Methods", Section III-1. The "type 1" reaction mixtures were sampled at "zero time" and at 20 minute intervals thereafter for a period of 1 hour 40 minutes. The "type 2" reaction mixtures were sampled at "zero time" and at 30 minute intervals thereafter for a period of $2\frac{1}{2}$ hours. The results are shown in figures 37a and 37b.

The results* showed that the inhibitory activity of the solution prepared from extract (B) by seitz filtration was also lower than that of the solution prepared from extract (A). The difference was found to be very small when the rate of IAA breakdown was relatively fast (figure 37a). However when the inhibitory activity of the same two solutions was tested under conditions in which the rate of IAA breakdown was slower, a marked difference between the (A) and (B) solutions was observed (figure 37b).

The proportions of enzyme extract and inhibitor solutions used in this experiment were the same as these used in the first two experiments of the previous section ("Results", Section VI-1). Thus the results/

* See also table 19, page 239.

results shown in figure 37a were comparable with those of figure 34, and those shown in figure 37b and those of figure 35. In fact the graphs of figure 37b and figure 35 were remarkable similar, whereas those of figures 37a and 34 differed markedly. (Not only was the activity of the enzyme extract - as shown in the "control" reaction mixture - greater in the experiment of figure 34 than that of the enzyme extract used in the experiment of figure 37a, but the inhibitory activities of both inhibitor solutions also appeared to be less.)

As pointed out above, the results described under "Results", Sections VI-1 and VI-2 were very variable, and it was therefore not possible to decide whether or not the inhibitor solutions obtained by seitz filtration were identical (quantitatively and qualitatively) with the inhibitor solutions obtained by boiling. Thus, although an examination of figures 37a and 34, and of figures 37b and 35 suggested that the inhibitor solutions prepared by seitz filtration were in general more active than those prepared by boiling, an examination of figures 37b and 35 alone showed that this difference was not great. However, on the basis of these results it was tentatively concluded that the inhibitor solutions obtained by the two methods were comparable - the inhibitory compound(s) being both heat stable and water stable.

Figure 37a. Graphs to show the activity of inhibitor solutions prepared from extracts (A) and (B) by seitz filtration. ("Type 1" reaction mixture).

Figure 37b. Graphs to show the activity of inhibitor solutions prepared from extracts (A) and (B) by seitz filtration. ("Type 2" reaction mixture).

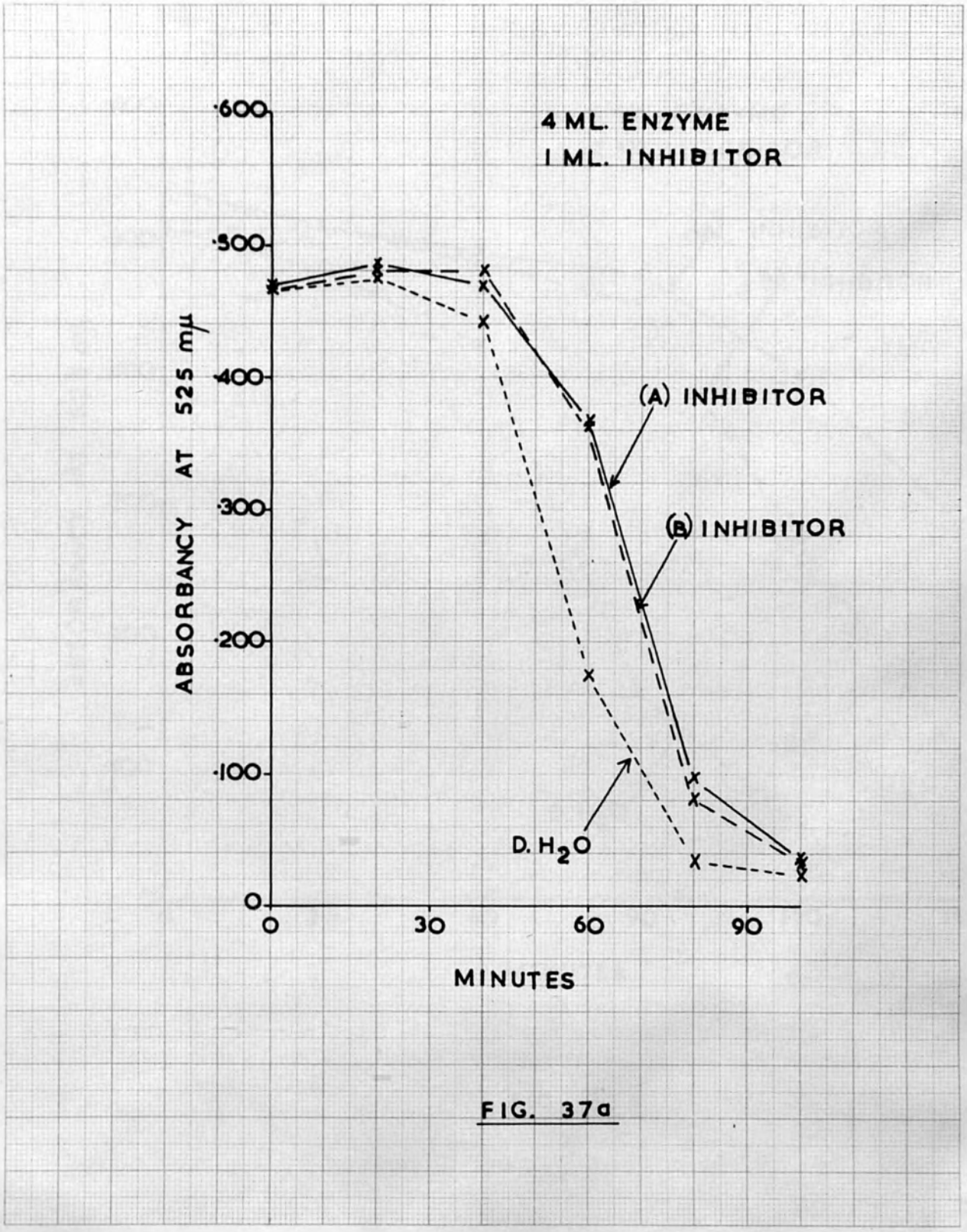

4 ML. ENZYME
1 ML. INHIBITOR
(A) INHIBITOR
(B) INHIBITOR
D. H_2O
ABSORBANCY AT 525 mμ
600
500
400
300
200
100
0
0
30
60
90
MINUTES

FIG. 37a

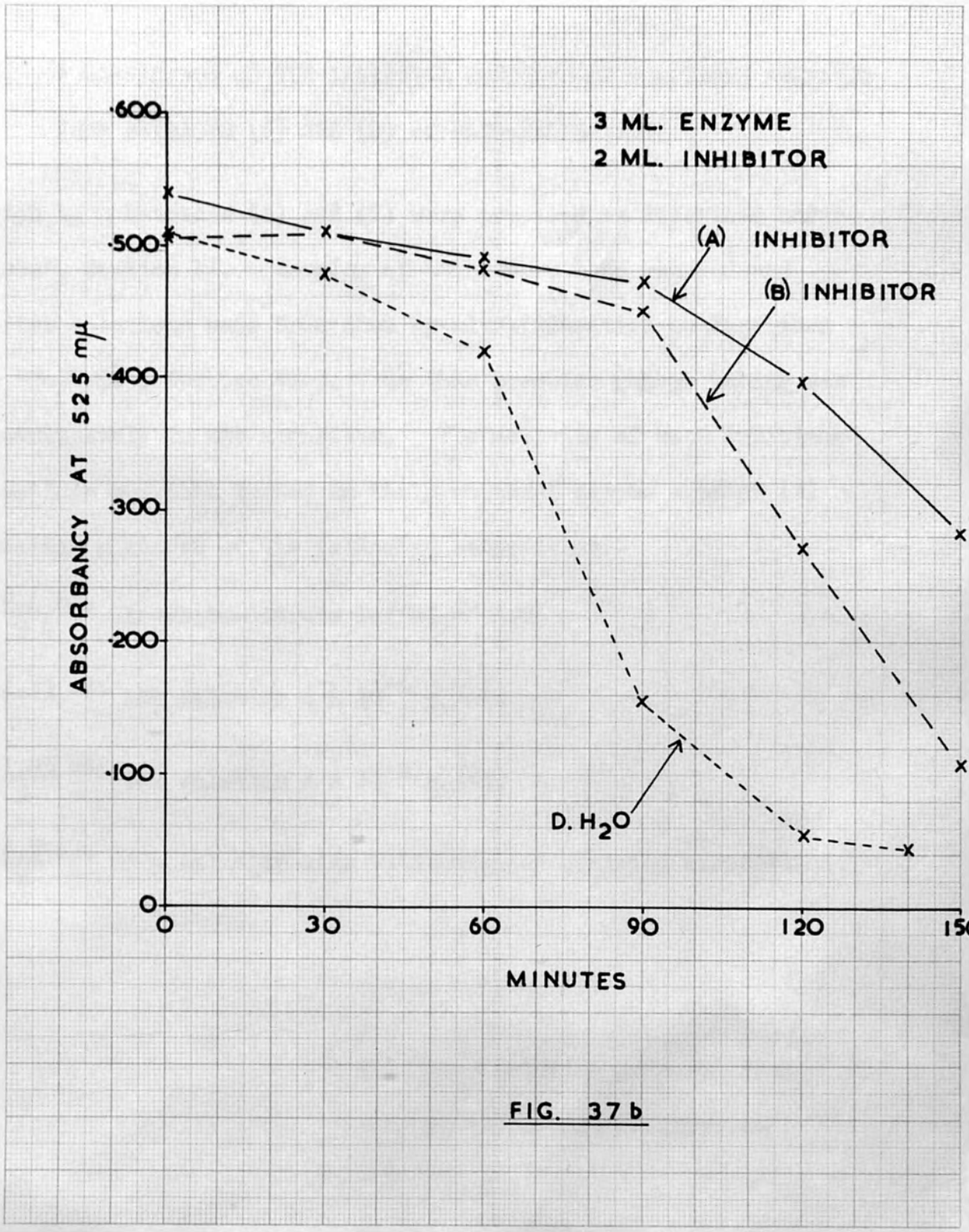

3 ML. ENZYME
2 ML. INHIBITOR
(A) INHIBITOR
(B) INHIBITOR
D. H_2O
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
0
30
60
90
120
150
MINUTES

FIG. 37 b

VI. 3. A COMPARISON OF THE INHIBITOR CONTENTS OF SOLUTIONS PREPARED FROM EXTRACTS (A) AND (B) BY ULTRAFILTRATION.

Occasion 1. Extracts (A) and (B) were prepared as described under "Methods", Section II. Samples of these were then removed and inhibitor solutions made from them by ultrafiltration as described under "Methods", Section VI-3. On this occasion rubber tubing was used exclusively in the apparatus. The activity of both inhibitor preparations was then tested in the presenceof enzyme extract (A) in reaction mixtures of the following composition:-

$\frac{2.5}{2}$ ml. phosphate-citrate buffer pH 4.0

$\frac{1.25}{2}$ ml. DCP solution 4×10^{-4} g./ml.

$\frac{1.25}{2}$ ml. IAA solution 4×10^{-4} g./ml.

$\frac{5.0}{2}$ ml. of a solution with "IAA-oxidase" activity constituted as follows.

$\frac{4 \text{ ml.}}{2}$ enzyme extract (A)

$\frac{1 \text{ ml.}}{2}$ inhibitor solution prepared from extract (A) by ultrafiltration

or

inhibitor solution prepared from extract (B) by ultrafiltration.

A "control" reaction mixture was also made up which contained $\frac{1}{2}$ ml. distilled/

distilled water instead of ½ ml. inhibitor solution. The reaction mixtures were incubated as described under "Methods", Section IV-1 and the rate of IAA breakdown determined as described under "Methods", Section III-1. The reaction mixtures were sampled at "zero time" and at 15 minute intervals thereafter for a period of 1½ hours. The results are given in figure 38. (Since the inhibitor preparation from extract (B) was made and tested two days later than the one from extract (A), a second "control" reaction mixture was made up to check that the enzyme extract (A) had not become inactivated. The results for this are also shown in the figure).

Occasion 2. Extracts ('A') and ('B') were prepared as described under "Methods", Section II-1 except that the centrifuged extracts were made up to a volume of 100 ml. instead of 150 ml. Samples of these were then removed and inhibitor solutions made from them by ultrafiltration as described under "Methods", Section VI-3. However, on this occasion polythene tubing was used instead of rubber tubing. The activity of the inhibitor preparations was then tested in the presence of enzyme extract ('A') in reaction mixtures of the following composition:-

$\frac{2.5}{6}$ ml. phosphate-citrate buffer pH 4.0

$\frac{1.25}{6}$ ml. DCP solution 4×10^{-4} g./ml.

$\frac{1.25}{6}$ ml. IAA solution 4×10^{-4} g./ml.

$\frac{5.0}{6}$ ml. mf a solution with "IAA-oxidase" activity constituted as follows,

$\frac{2}{6}$ ml. enzyme extract ('A')

$\frac{3}{6}$ ml. inhibitor solution prepared from extract ('A') by ultrafiltration

or

inhibitor solution prepared from extract ('B') by ultrafiltration.

A "control" reaction mixture was also made up which contained $\frac{3}{6}$ ml. distilled water instead of $\frac{3}{6}$ ml. inhibitor solution. The reaction mixtures were incubated as described under "Methods", Section IV-1, and the rate of IAA breakdown determined as described under "Methods", Section III-1. The reaction mixtures were sampled at "zero time" and 60 minutes and 90 minutes later. The results are given in figure 39.

The results* showed (figures 38 and 39) that the inhibitory activities of the solutions prepared from extracts (B) or ('B') by ultrafiltration were also lower than those of solutions prepared from extracts (A) or ('A').

The proportions of enzyme extract and inhibitor solution used in the experiment shown in figure 38 were the same as those used in an experiment in each of the previous two Sections - those shown in figure 34 ("Results", Section VI-1) and figure 37a ("Results", Section VI-2). However it was obvious that the inhibitory activities of the solutions/

* See also table 19, page 239.

solutions prepared from extracts (A) and (B) by ultrafiltration were greater than those of the comparable solutions prepared by boiling and by seitz filtration. This suggested therefore that some extraneous inhibitor was present in the ultrafiltrates. Moreover, it was thought that the contaminant might have been the whitish substance present on the inner surface of the rubber tuing of the ultrafiltration apparatus. Thus, when the apparatus was dismantled, the release of the vacuum in the tube containing the ultrafiltrate, caused an amount of this substance to be sucked in through the side arm, and although most of it was seen to be deposited on the walls of the tube in the region of the side-arm, some of it very probably fell down into the ultrafiltrate. In the experiment shown in figure 39 therefore, the rubber tubing was replaced by polythene tubing. The reaction mixture used here was comparable with those used in the experiments shown in figures 36a and 36b ("Results", Section VI-1). An examination of the three figures (36a, 36b and 39) showed that these were all rather similar. In addition the graphs of figure 39, under discussion, resembled those of figure 35 ("Results", Section VI-1.) even more closely. (The rates of IAA breakdown in the experiment of figure 35 were similar to those in the experiment of figure 39, although the composition of the reaction mixtures was slightly different). It was concluded that the use of polythene tubing was probably effective in eliminating the extraneous inhibitor, and also that the inhibitor solutions prepared by ultrafiltration were comparable with those prepared by boiling and by seitz filtration.

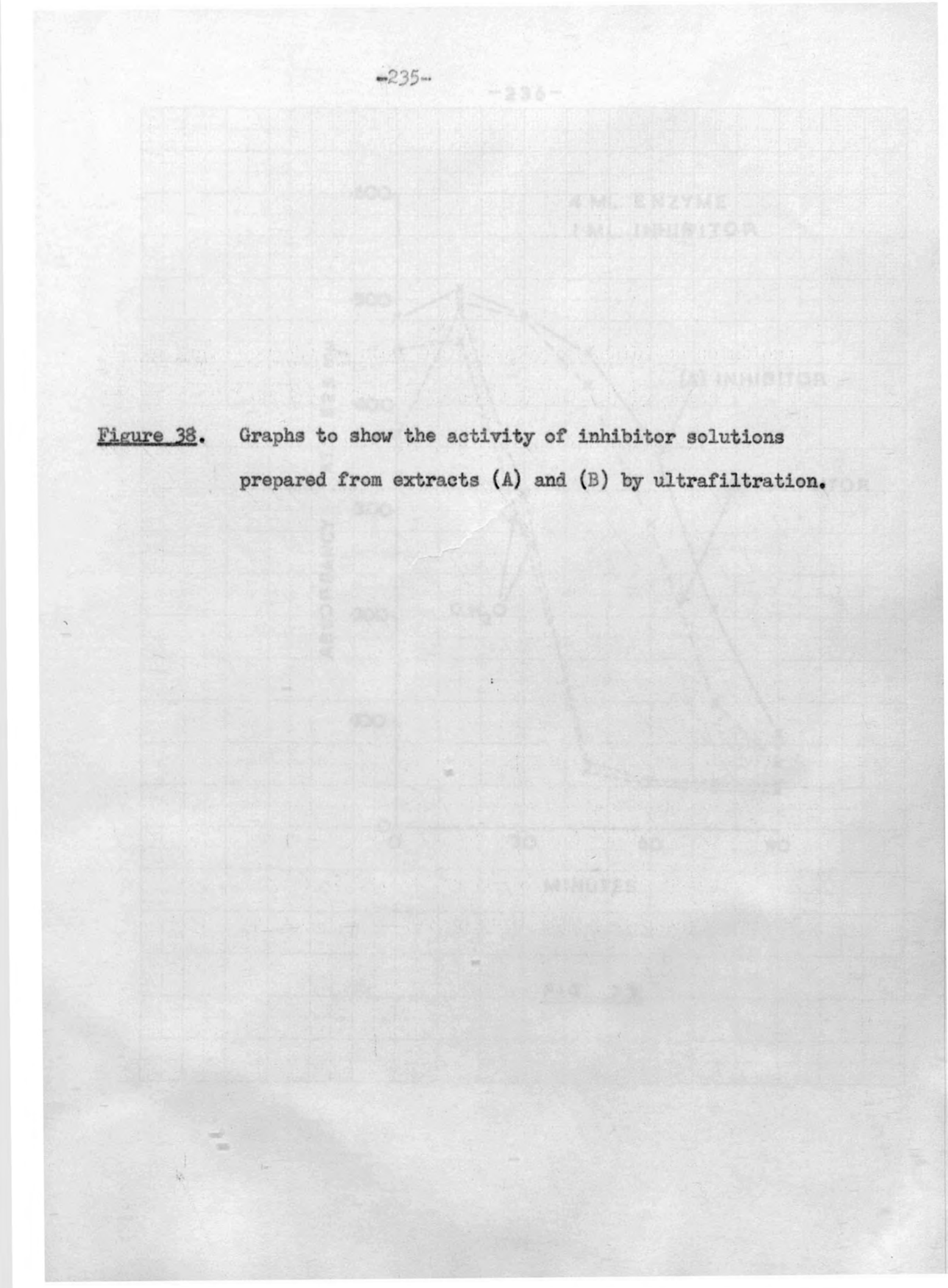


Figure 38. Graphs to show the activity of inhibitor solutions prepared from extracts (A) and (B) by ultrafiltration.

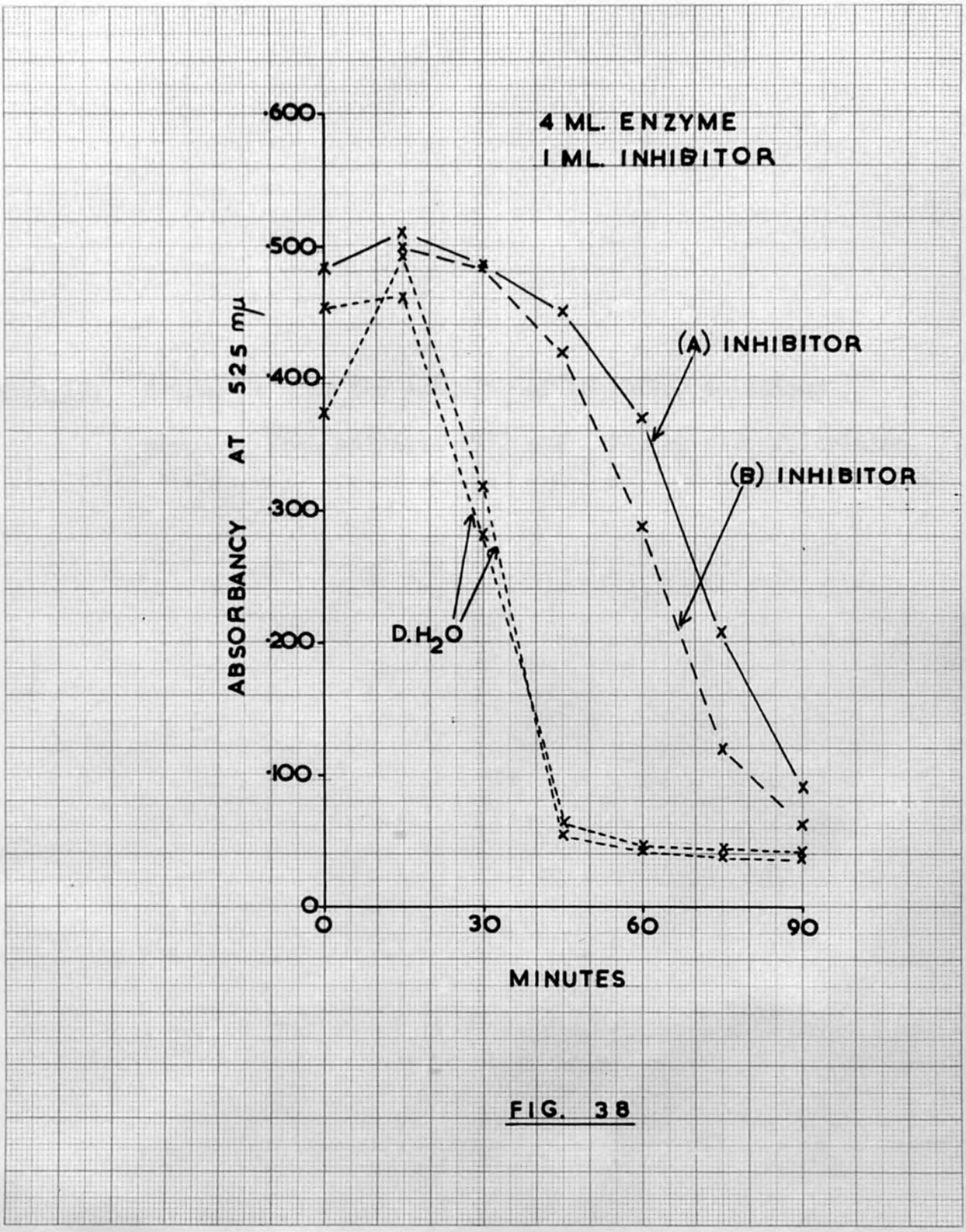

4 ML. ENZYME
1 ML. INHIBITOR
(A) INHIBITOR
(B) INHIBITOR
D. H_2O
ABSORBANCY AT 525 mμ
600
500
400
300
200
100
0
0
30
60
90
MINUTES

FIG. 38

Figure 39. Graphs to show the activity of inhibitor solutions prepared from extracts ('A') and ('B') by ultrafiltration.

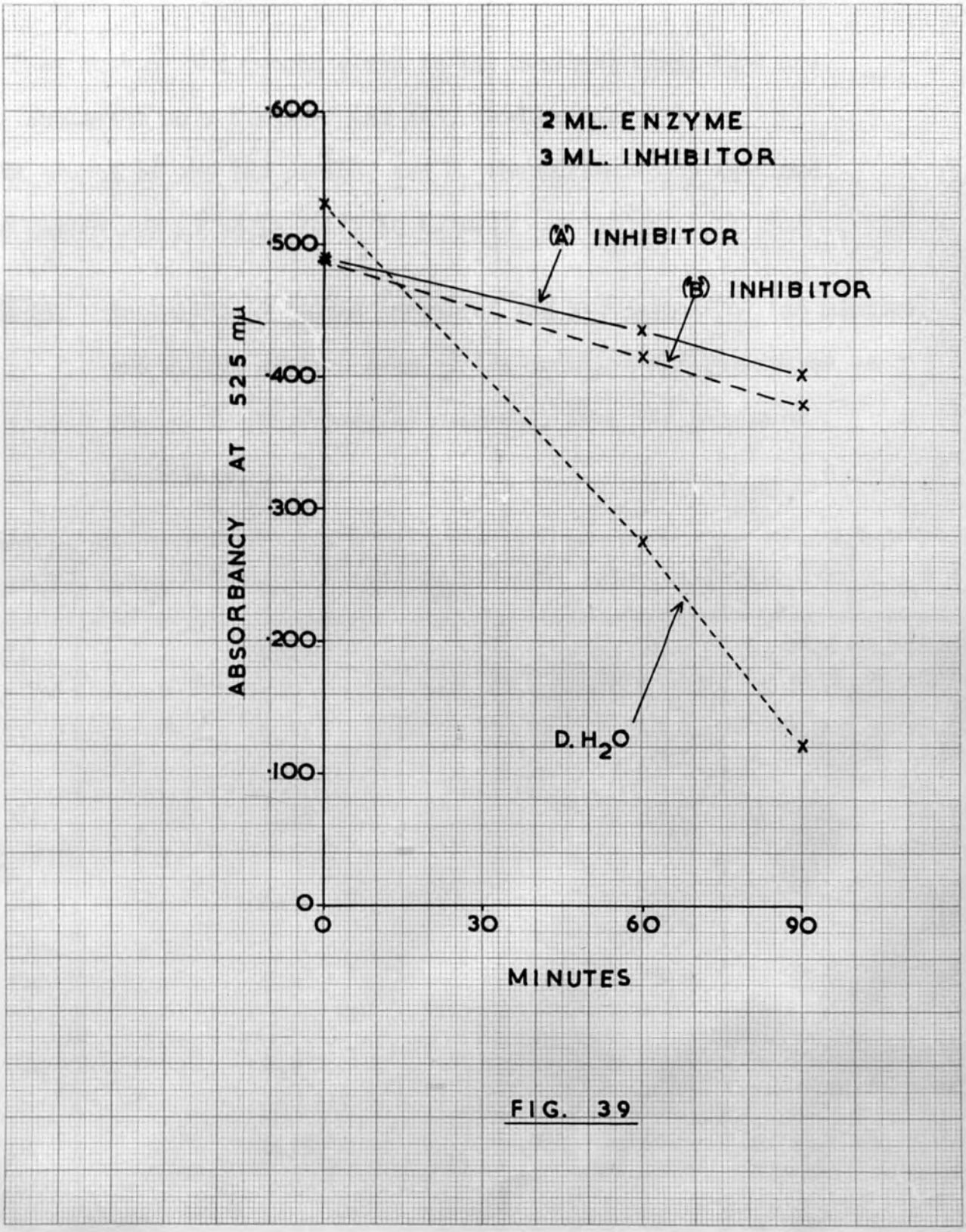

2 ML. ENZYME
3 ML. INHIBITOR
(A) INHIBITOR
(B) INHIBITOR
D. H_2O
ABSORBANCY AT 525 mμ
·600
·500
·400
·300
·200
·100
0
0
30
60
90
MINUTES

FIG. 39

From each of the graphs given in the Sections VI-1, VI-2 and VI-3, estimates were obtained (as described on page 85) for 1. the length of the lag period, and 2. the maximum rate attained. These values are given in table 19 below.

TABLE 19. Estimates of 1. the length of the lag period "L" (in minutes) and 2. the maximum rate of the reaction "R" (expressed as change in absorbancy/10 minutes), from the graphs of figures 34 to 39.

FIGURE	RATIO $\frac{\text{VOLUME OF INHIBITOR SOLUTION}}{\text{VOLUME OF ENZYME}}$	(A)or('A')	(B)or('B)	D.H_2O
34	0.25	L. 21.0 R. .145	L. 22.0 R. .163	L. 22.0 R. .168
35	0.67	L. 46.0 R. .041	L.44.0 R. .041	L. 25.0 R. .059
36a	1.49	L. 53.0 R. .063	L. 47.0 R. .080	L. 45.0 R. .087
36b	1.49	L. 49.5 R. .074	L. 33.0 R. .063	L. 23.0 R. .086
37a	0.25	L. 53.0 R. .135	L. 52.0 R. .140	L. 37.5 R. .134
37b	0.67	L. 83.5 R. .038	L. 80.5 R. .059	L. 50.0 R. .088
38	0.25	L. 50.0 R. .108	L. 44.0 R. .112	L. 1.80 R. .144
39	1.49	L. 12.5 R. .015	L. 6.0 R. .012	L. 13.0 R. .052

From the values given in table 19 average values were obtained for the length of the lag period (in minutes) and the maximum rate attained (expressed as change in absorbancy/10 minutes) for reaction mixtures with an inhibitor/enzyme ratio of 0.25 (figures 34, 37a and 38) and for reaction mixtures with an inhibitor/enzyme ratio of 0.67 (figures 35 and 37b). The values obtained from figures 36a and 36b, and 39 were not averaged. (The reaction mixtures of figures 36a and 36b contained enzyme extracts ('A') and ('B') instead of (A) and (B), while there were insufficient points on the graphs of figure 39 to give comparable estimates). The average values were then plotted against inhibitor/enzyme ratio as shown in figure 40.

The graphs of figure 40 showed that with an increase in the ratio volume of inhibitor solution/volume of enzyme, the lag period increased and the maximum rate attained decreased. A comparison of the graphs for the reaction mixtures containing inhibitor solutions with those for the reaction mixtures containing the corresponding volumes of distilled water showed that these effects could be attributed largely, but by no means entirely to the lower concentration of enzyme present in the reaction mixtures with the higher inhibitor/enzyme ratios. It was clear however that the presence of the higher concentration of inhibitor caused both an increase in the lag period and a decrease in the maximum rate attained. In addition the effect of the inhibitor on the lag period appeared to be greater than its effect on the maximum rate while the latter effect appeared to decrease with an increase in the/

the enzyme concentration. There was therefore a slight resemblance between the graphs of figure 40 and the diagram on page 189. Again it was evident that the activity of the inhibitor solutions prepared from extract (B) was lower than that of those prepared from extract (A). This lower activity manifested itself in the form of less marked effects both on the lag period and on the maximum rate.

The results described in Sections VI-1, VI-2 and VI-3 confirmed the report of Tang and Bonner (1948) and of Galston (1957) that pea tissues contain a powerful inhibitor of the "IAA-oxidase" enzyme. The finding that the ultrafiltrates caused inhibitions of the enzyme activity was considered to constitute evidence for the existence of a striking difference between the activator/inhibitor relationships of pea extracts and of waxpod bean root extracts. (Kenten (1955) found that ultrafiltrates obtained from the latter contained an activator). The fact that the inhibitor solutions obtained from the (B) extracts by all three methods - boiling, seitz filtration and ultrafiltration, had lower activities than those obtained from the (A) extracts was thought to indicate that the higher activity of the untreated (B) extract was a direct result of this lower inhibitor content.

Figure 40. Graphs to show the relationship between the ratio

$$\frac{\text{volume of inhibitor solution}}{\text{volume of enzyme}}$$

and

1. the length of the lag period and
2. the maximum rate attained in reaction mixtures containing inhibitor solutions prepared from extracts (A) or (B).

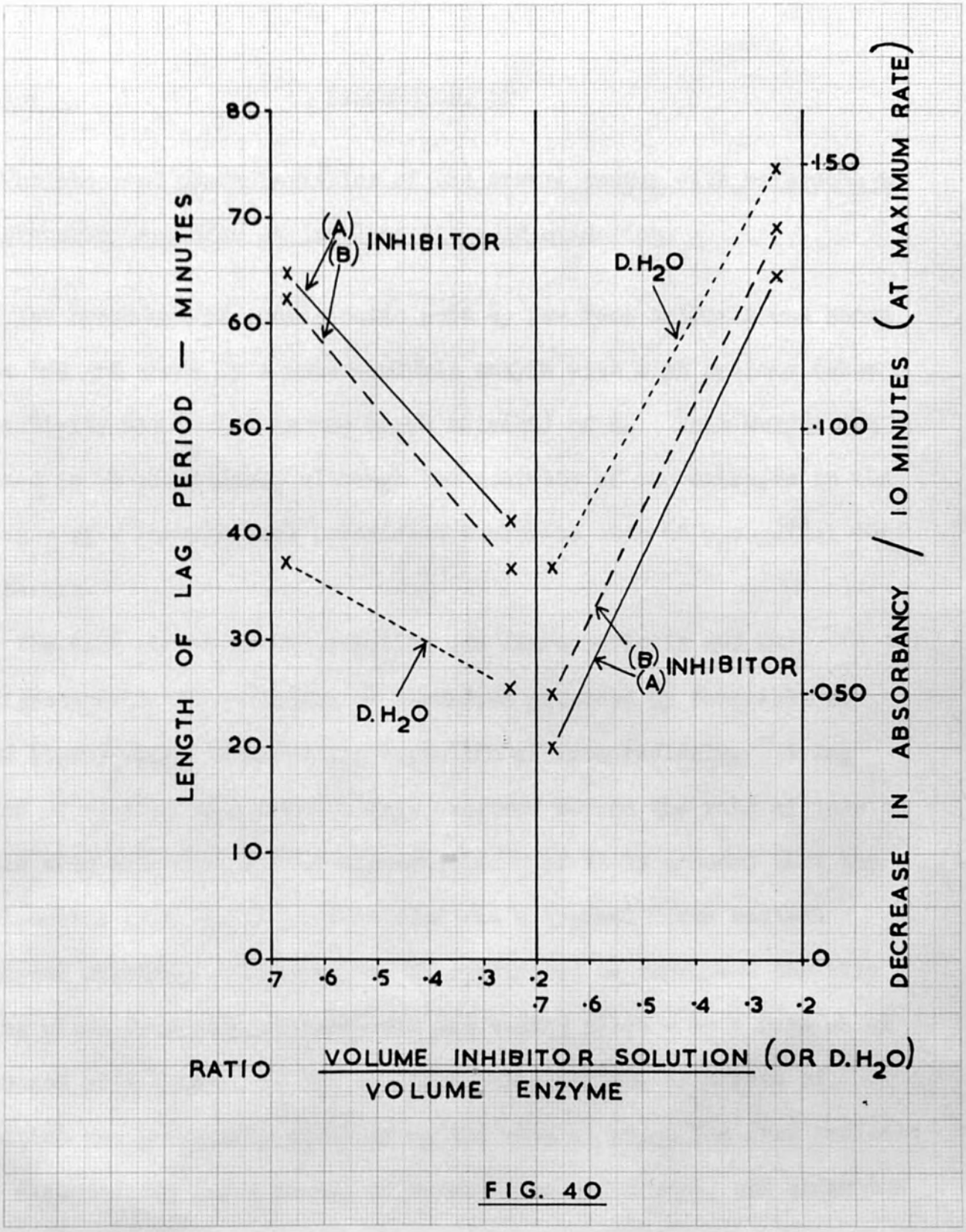

LENGTH OF LAG PERIOD — MINUTES
80
70
60
50
40
30
20
10
0
(A)
(B) INHIBITOR
D. H_2O
D. H_2O
(B)
(A) INHIBITOR
·150
·100
·050
0
DECREASE IN ABSORBANCY / 10 MINUTES (AT MAXIMUM RATE)
·7 ·6 ·5 ·4 ·3 ·2
·7 ·6 ·5 ·4 ·3 ·2
RATIO VOLUME INHIBITOR SOLUTION (OR D. H_2O) / VOLUME ENZYME

FIG. 40

Chapter 4. DISCUSSION.

The observed characteristics of the enzyme system with reference to the probable mechanism of indoleacetic acid oxidation.

The breakdown of indoleacetic acid by pea root extracts was shown to be brought about by a water-soluble enzyme with a pH optimum (when its activity was tested in the crude extract) of 4. This enzyme was assumed to be peroxidase, although the activity of the extracts in the presence of a conventional peroxidase substrate such as pyrogallol was not tested.

The time course of the reaction was characteristic and was consistent with the mechanism of breakdown proposed by Yamazaki and Souzu (1960) which involved the formation of free radicals. A lag period of varying length was always observed before the rate of indoleacetic acid oxidation became appreciable. If it is assumed that the indoleacetic acid oxidation proceeded via a typical "free radical catalysed reaction", this lag may be considered to represent the time during which free radical formation was taking place - at a rate which increased with time. At the end of the lag period, this rate of formation was presumably equalled by the rate at which the free radicals were disappearing (as a result of termination reactions), and under the steady state conditions thus achieved, the reaction velocity for the indoleacetic acid breakdown became constant. The "induction phase" may/

may also have corresponded with the time during which the "Peroxidase Complex II" of Yamazaki and Souzu was being formed. Further it was observed that the enzyme became inactivated - apparently towards the end of the reaction (see "Results", Section II, page 147). This probably resulted from a reaction between the enzyme and IAA^1 radicals (as suggested by Yamazaki and Souzu for an anaerobic system).

The activity of the system was greatly enhanced by the addition of 2,4 - dichlorophenol. This,according to the interpretation of Yamazaki and Souzu was because phenol radicals (produced by peroxidation of the phenol) reacted with indoleacetic acid to give IAA^1 radicals and this reaction was faster than the peroxidase - catalysed one between indoleacetic acid and H_2O_2 to give the IAA^1 radicals. A heat-stable inhibitor of the indoleacetic acid oxidising system was shown to be present in the extracts ("Results", Section VI). The nature of this substance was unknown, but it was considered likely that it consisted of a mixture of polyphenols - compounds which are recognised as powerful inhibitors of free-radical catalysed reactions - on account of their tendency to give rise to <u>inactive</u>, resonance-stabilised radicals.

The reaction proceeded in the absence of added H_2O_2. Hence it was assumed that H_2O_2 was being formed from the substrate, as described by Yamazaki and Souzu. (It was not known whether the endogenous manganese in the extracts was having an activating effect on this process - by the suggested promotion of a reaction between IAA^1 radicals and molecular oxygen). In the present system therefore, in which indoleacetic/

indoleacetic acid was oxidised, the enzyme peroxidase was acting as a so-called "direct oxidase".

Information from the chromatographic data concerning the breakdown products of the enzymatic oxidation of indoleacetic acid, and possible intermediates in the reaction.

Of the compounds located by their colour reactions on the chromatograms of figures 14a, 14b, 16 and 17 ("Results", Section I-2), three were considered to be products of the enzymatic oxidation of indoleacetic acid. These were the spots listed as No. 8, No. 9 and No. 10 in the table on page 127. No. 8 and No. 10 (Rf values .77 and .86) gave colours with Salper and Ehrlich reagents, while No. 9 (Rf .80) gave a colour only with Van Eck reagent. Whereas the identity of Nos. 8 and 10 was unknown, No. 9 was thought to be indolealdehyde.

The origin of the remaining compounds (Spots Nos. 1, 4, 5, 6 and 7) on the above chromatograms was uncertain. Three alternatives suggested themselves:

1. They were formed from indoleacetic acid by the action of the "indoleacetic acid-oxidase" system - in which case they could be regarded as "intermediates" in the reaction.
2. They were present as impurities in the commercial preparation of indoleacetic acid which was used for the experiments. If this were so, then there was evidence that certain of them could be substrates for the "indoleacetic acid-oxidase" system - namely those/

those which were not present in the reaction mixtures when the indoleacetic acid oxidation had proceeded to completion.

3. They were compounds which had been formed from indoleacetic acid non-enzymatically either during the process of ether extraction or during chromatography.

As far as spots No. 1 and 4 were concerned, it was the third alternative which appeared most likely. The evidence for this was as follows:-

1. Both these spots were found on the chromatograms of the "control" reaction mixtures - in which indoleacetic acid breakdown had not taken place (figure 14c) - and also on the chromatograms of the "marker" solutions of indoleacetic acid in methanol (figure 15).
2. They were present on the chromatograms only when the solution applied at the starting point contained indoleacetic acid itself (figures 14a, 14b and 16).
3. No evidence was obtained that they accumulated with time and then disappeared - as might be expected if alternative 1. above were the correct one - though it might be argued that the rate of the reaction under the conditions used was too fast for this to be observed.

Since the compounds in question, spots Nos. 1 and 4, werefound both on the "control" chromatograms and on the "marker" chromatograms, it was concluded that they must have arisen from indoleacetic acid during chromatography rather than during extraction. It must be noted, that this finding was not/

not in agreement with that of Melchior (1958) who presented evidence which suggested that these two compounds were in fact intermediates (see page 34 of the "Introduction").

It was thought on the other hand, that the origin of the compound of spot No. 6 could be explained in terms of alternative 2. above. Thus this spot was present on the "control" and "marker" chromatograms, and was always found on chromatograms sprayed with Salper or Ehrlich reagents, regardless of whether or not the starting spot had contained indoleacetic acid (figures 14a, 14b, 14c, 16 and 17). The status of the compound of spot No. 7 was much less certain. It was only found on one chromatogram, that sprayed with Ehrlich reagent (figure 16). As it was present in reaction mixtures which did not contain indoleacetic acid it was probably an impurity in the original indoleacetic acid - in which case its absence from the chromatograms representing the samples of reaction mixtures extracted at 50 and 60 minutes was merely caused by the fact that its concentration had fallen below the minimum value giving a colour reaction (this was weak in any case). (The compound of spot No. 0 - found only on the "marker" chromatograms (figure 15) - was also probably an impurity in the indoleacetic acid. However it was presumably present in so small a concentration that it only gave rise to a detectable spot when very large amounts of indoleacetic acid were chromatographed).

The origin of the spot No. 3 which gave a yellow colour with Van Eck reagent and was found to have approximately the same Rf as indoleacetic/

indoleacetic acid has already been discussed ("Results", Section I-2, page 130). It was thought to be attributable to indolealdehyde - formed on the paper from indoleacetic acid - presumably during the process of drying the chromatograms.

The existence of a difference between the "indoleacetic acid-oxidase" activities of extracts (A) and (B).

It has been shown ("Results", Sections I, II and III) that the "indoleacetic acid-oxidase" activities of aqueous extracts made from the roots of etiolated pea seedling grown for two days in indoleacetic acid solution 10^{-7}g./ml. ((B) extracts) were greater than the activities of similar extracts made from the roots of etiolated pea seedlings grown for two days in water ((A) extracts). Thus the "time taken for the reaction to reach completion" was found to be shorter in reaction mixtures containing the (B) extracts than it was in similar reaction mixtures containing the (A) extracts. Also, when the progress curves for the breakdown of indoleacetic acid in the presence of the two types of enzyme extract were compared, certain differences were observed. In reaction mixtures containing (B) extracts, it was found that the lag period was always shorter, and the maximum rate attained in the reaction higher, than in reaction mixtures containing (A) extracts.

Further differences between the activities of the (A) and (B) extracts were revealed when the relationship between the length of the lag period and the maximum rate attained for each type of extract was examined/

examined. (Statistical analyses on page 174 et. seq.). Thus although an inverse linear relationship was found in each case, the regression lines for y (maximum rate attained) on x (length of the lag period) were slightly different (see figure 29c on page 187). The slope of the regression line given by the data for the (B) extracts was greater than the slope of that given by the data for the (A) extracts. This meant that for a given increase in the length of the lag period, the decrease in the maximum rate was greater with the (B) extract than with the (A) extracts.

From a study of the difference in slope of the two regression lines, and their position in relation to one another on the graph, a number of deductions were made concerning the relationship of the lags and maximum rates to enzyme concentration (see the diagram on page 189). These were that with increasing enzyme concentration:

1. The rate of <u>increase</u> of the maximum rate was greater for the (A) extracts than for the (B) extracts.
2. The rate of <u>decrease</u> of the lag period was greater for the (A) extracts than for the (B) extracts.
3. When the (A) extract and the (B) extract were considered individually, the change in lag period with enzyme concentration was greater than the corresponding change in maximum rate.
4. At relatively low concentrations of enzyme the difference between the lag periods in the (A) and (B) extracts was large compared with the difference between the maximum rates, whereas at relatively high/

high concentrations of enzyme the position was reversed and the difference in the lag periods was small compared with the difference in the maximum rates.

On this basis it was possible to explain the fact that at a given concentration (which was different for the two extracts) the lags and maximum rates for (A) and (B) would apparently be identical, whereas for a given maximum rate above that found at the point of intersection of the regression lines (figure 29c) the lag for (B) would be longer than that for (A), and for a given maximum rate below this value, the lag for (B) would be shorter.

Explanation of the difference.

A difference in inhibitor levels was demonstrated experimentally ("Results", Section VI) - that of the (B) extract being lower than that of the (A) extract. It seemed probable that this at least to some extent accounted for the differing activities. If it were assumed that the inhibitory activity could be ascribed to one chemical compound, then it would be necessary to assume that this affected both the lag period and the maximum rate. This may have been so. On the other hand, since Sharpensteen and Galston (1959) and Galston (1959) presented evidence which suggested that the inhibitor from peas was a complex mixture of substances, it seemed more likely that the inhibitor preparation obtained in the present work also contained more than one component. Although/

Although the chemical nature of this preparation was not investigated - it was considered likely that phenolic substances were involved as suggested by Galston (1959) for the pea inhibitor, and also by Waygood, Oaks and Maclachlan (1956b) for a similar inhibitor from wheat leaves, and by Gortner and Kent (1953) for one from pineapple. It seemed possible moreover that some components of the mixture affected chiefly the lag phase, while others affected chiefly the maximum rate. (Waygood, Oaks and Maclachlan, (1956b), made a distinction between inhibitors of the "catechol type" which merely extended the lag period, and those such as hydroquinone and scopoletin - which acted as "chain transferrers" and thus affected the "steady state reaction rate".) In any case it was considered that the inhibition could almost certainly be interpreted in terms of the competition of the inhibitor with indoleacetic acid for the enzyme and/or hydrogen peroxide.

In order to account for the suggested relationship between the "activity of the inhibitor" and the enzyme concentration (see the diagram on page 189), it was assumed that the activity of one or more of the components of the inhibitor mixture was dependent on the inhibitor and/or enzyme : substrate ratio. (The ratio enzyme to inhibitor would be constant for a given (A) or (B) extract). It is of interest to note that some evidence was actually obtained for precisely this kind of situation by Gortner and Kent (1953) - although they used a pineapple extract which was in several respects very different from the peroxidase - containing extracts derived from other sources. Thus they found that the/

the activity of the pineapple inhibitor was greatest at high inhibitor : substrate ratios and lowest at low inhibitor : substrate ratios (when the substrate concentration was varied in the presence of a given amount of inhibitor). Presumably, in the present work, since the effect was in fact the reverse of the above, it was the ratio of "inhibitor + enzyme" : substrate which was important. This may have been because, under the conditions used, the enzyme was particularly susceptible to inactivation at low concentrations.

The difference in slope of the curves for the (A) and (B) extracts in the diagram on page 189 was thought to be attributable either to a difference in the make up of the inhibitor in the two types of extract, or merely to a difference in the ratio of inhibitor : enzyme + indole-acetic acid (which would be different for the reaction mixtures containing the (A) and (B) extracts).

When the regression lines of figure 29c are extrapolated back to zero lag period, the maximum rate of the reaction with the (B) extract is greater than that for the (A) extract. This would at first sight appear to suggest that the (B) extract contained a higher level of enzyme or activator (possibly even a cofactor) than the (A) extract. Convincing evidence that the peroxidase levels of extracts made from indoleacetic acid-treated tissues are in fact higher, has however not so far been reported. The same is true for activator levels - although the existence of a natural activator is well known. Thus an activator - which was also thought to be phenolic - was detected by/

by Kenten (1955) in yellow waxpod bean extracts, and by Waygood, Oaks and Maclachlan (1956a) in wheat leaf extracts. Also the inhibitor from pineapple was later fractionated (Gortner, Kent and Sutherland, 1958) to give an ester of ferulic acid - which was a powerful inhibitor of the "indoleacetic acid-oxidase", and an ester of p-coumaric acid which had activating properties. Using pea extracts Sharpensteen and Galston (1959) and Galston (1959) claimed to have isolated a heat stable "co-factor" which could be separated (Galston, 1959) into two inactive fractions which yielded the original activity on recombination. Sondheimer and Griffin (1960) showed that a boiled extract of etiolated pea epicotyls could counteract the inhibition brought about by adding chlorogenic acid. However in the present work it was thought likely that the inhibitory properties of the boiled extracts ("Results", Section VI) were the most important. Thus it was considered that the observed extrapolation phenomenon could be explained in terms of an inhibitor which affected only the rate - at least at high concentrations of enzyme. Indirect evidence was available which indicated that the difference between the activities of the two extracts was not caused to any extent by a different level of activator. Thus the optimum concentration of 2,4-dichlorophenol ("Results", Section II-2) was the same for both extracts - so that it appeared that the activation produced by 2,4-dichlorophenol was proportional to the enzyme + inhibitor component of the two extracts. (The activity of the (B) extract was higher than that of the (A) extract at the optimum 2,4-dichlorophenol concentration).

The difference between the "in vivo" "indoleacetic acid-oxidase" activities of the "indoleacetic acid-grown" roots and the "water-grown" roots, and between those of the "ambient solutions".

Differences similar to those described above between the extracts (A) and (B) were again found when the activities of whole roots ("Results", Section IV) and of "ambient solutions" ("Results", Section V) were examined. Thus the so-called "in vivo indoleacetic acid-oxidase activities" of "indoleacetic-grown" roots were found to be higher than those of "water-grown" roots (when these were harvested after two and three days), while in one experiment the activity of a solution in which "indoleacetic acid-grown" roots had been incubated for twenty-four hours was higher than that of the corresponding solution in which "water-grown" roots had been incubated. A tentative suggestion was made (see the comments on page 197) that these differences were again attributable to the fact that the two types of tissue contained different levels of a rapidly diffusing water soluble inhibitor of indoleacetic acid breakdown.

The significance of the enhanced "indoleacetic acid-oxidase" activity of the extracts of the "indoleacetic acid-grown" roots in terms of

1. the idea of adaptive enzyme formation.
2. a physiological role in combating high indoleacetic acid concentration.

It has been shown during the course of this investigation, that, as reported by Galston and Dalberg (1954) and by Bakhsh (1956), the "indoleacetic acid-oxidase" activity of extracts made from tissues which had been "pre-treated" with indoleacetic acid, was higher than that of those made from the non-treated controls. However, the finding that there appeared to be a difference in the inhibitor levels of extracts (A) and (B) suggested that the observed difference in "indoleacetic acid-oxidase" activity could be attributed to these and not to a difference in the amounts of enzyme (peroxidase) present. If this were so, then there would be no necessity to postulate that the enzyme "indoleacetic acid-oxidase" was being formed adaptively - in the sense originally intimated by Galston and Dalberg. Furthermore, even if an increase in the peroxidase content of the (B) extracts had been found (such an increase was in fact found by Jensen (1955) using intact pea root sections), in order to establish whether or not adaptive enzyme formation - in the classical sense - had taken place, it would still have been necessary to show that the addition of the substrate was having a direct effect on the formation of the enzyme, causing it to be formed either de novo, or from a previously existing protein precursor/

precursor (possibly another enzyme). In the absence of such information, an alternative explanation would be that the indoleacetic acid was affecting growth or differentiation in some way - and hence promoting changes in the enzyme complement of the tissues indirectly. Since the evidence for the occurrence of adaptive enzymes in the higher plants is at the present time so meagre - coming only from a study of two enzymes - "indoleacetic acid-oxidase" itself and glycolic acid oxidase (Tolbert and Cohan, 1953) it seemed that some alternative explanation - such as the one mentioned above - was more probable. Nevertheless it must be mentioned that Fåhraeus and Tullander (1956) using the fungus Polyporus versicolor were apparently able to demonstrate the "induction" of laccase by the addition of indoleacetic acid. This enzyme was also able to destroy indoleacetic acid - and could therefore be considered as an "indoleacetic acid-oxidase" - though of a completely different type from the one described for higher plants.

Information was also obtained during the course of this work on the other phenomenon observed by Galston and Dalberg - that of the lag period which preceded the rapid breakdown of indoleacetic acid by young tissues (see the diagram on page 42 of the "Introduction"). Thus it was shown here that such a lag period was an inherent feature of the enzymatic oxidation of indoleacetic acid - regardless of the source of the enzyme. Moreover it was shown that the variations in its length could be attributed - at a given pH - to the concentrations of the various components of the system-enzyme, activator and inhibitor - present at/

at the start of the experiment. Hence, firstly, there was thought to be no justification for the assertion of Galston and Dalberg that the reaction proceeded without a lag period when the older "non-growing" tissues were used (an examination of the published graphs - page 42 of the "Introduction" - showed that there was in fact quite a considerable lag period). Secondly, the more marked lag period with the young tissue was thought to be quite consistent with the lower "indoleacetic acid-oxidase" activity of the younger tissues (a feature which Galston and Dalberg had demonstrated in a previous experiment involving the "topographical distribution" of "indoleacetic acid-oxidase". It could not have been the result of the adaptive formation of the enzyme during the course of the reaction.

Although the results of the present work do not support the idea of "adaptive enzyme formation", it seemed possible that the enhanced "indoleacetic acid-oxidase" activity of the "indoleacetic acid-grown" roots might on the other hand account in some way for the phenomenon of the "adaptation" of roots to grow in indoleacetic acid solutions observed by Bakhsh (1956) and by Burström (1957). Thus, an enhanced capacity to destroy indoleacetic acid, induced by the presence of this substance in the culture medium, might have enabled the roots to grow more normally after an initial period of retarded and/or "abnormal" growth. The concentration of indoleacetic acid used here (10^{-7} g./ml.) was such that growth inhibition was expected, while in addition to this, a malformation of the roots was also observed (see figures 2b and/

and 3b under "Methods", Section I, pages 52 & 54). Such a finding could be regarded as very good, though rather indirect, evidence that externally applied indoleacetic acid at any rate was being broken down in vivo.

The question as to whether endogenous indoleacetic acid is likewise broken down must remain unanswered. The role of the "indoleacetic acid-oxidase" enzyme in controlling growth processes - by regulating the concentration of indoleacetic acid in the tissues - is therefore still extremely hypothetical. However, in conclusion, it must be admitted that the negative decision reached in this work on the subjectof the adaptive formation of the "indoleacetic acid-oxidase" enzyme, in response to added indoleacetic acid, in no way excludes the possibility that the enzyme in question is in fact formed adaptively in the tissues in response to endogenous indoleacetic acid. This state of affairs would be very difficult to prove or disprove experimentally. Indeed it seems that a very close relationship may well exist between indoleacetic acid concentration and enzyme activity in vivo. Thus Jensen (1955) was of the opinion that pretreatment with indoleacetic acid caused not only an increase in the peroxidase activity of pea root tissues, but also the premature onset of lignification - a process in which the enzyme peroxidase is thought to be involved. This being so, it is at least conceivable that endogenously supplied indoleacetic acid might perform a similar function in the normal course of differentiation in all lignified tissues. Also, in view of the known effects of indoleacetic acid, not only on differentiation, but also on extension growth/

growth and cambial activity, it seems likely that other examples of this kind of effect - possibly involving other enzymes, might subsequently be discovered. However, once again the objection might be raised that the observed effects on enzyme activity might be indirect ones - brought about by the stimulation of growth or differentiation by some quite independent mechanism.

On the basis of the experimental evidence which has been presented so far, it appears that the hypothesis of adaptive enzyme formation cannot be entertained seriously as an explanation of the response of plant tissues either to applied indoleacetic acid or to endogenous indoleacetic acid. Finally, it must be remarked that this is so in spite of the general attractiveness of the idea as an explanation of the observed diversity of enzyme activity among cells with the same genetically controlled potentialities.

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