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THE BIOCHEMICAL SYNTHESIS OF D-XYLULOSE AND L-RIBULOSE; DETAILS OF THE ACTION OF ACETOBACTER SUBOXYDANS ON D-ARABITOL, RIBITOL AND OTHER POLYHYDROXY COMPOUNDS

V. Moses and R. J. Ferrier

August 1961

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The Biochemical Synthesis of D-Xylulose and L-Ribulose; Details of the Action of *Acetobacter suboxydans* on D-Arabitol, Ribitol and Other Polyhydroxy Compounds.

(Running Title: Action of *Acetobacter suboxydans* on Polyols)

BY V. MOSES AND R.J. FERRIER*

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Bacteria of the genus *Acetobacter* have long been known to oxidise polyols with particular steric configurations to ketoses (Bertrand, 1898 *a*, *b*.). *Acetobacter suboxydans* (Kluyver & de Leeuw, 1924), in particular, is characterised by many very specific quantitative oxidations of this type (Butlin, 1936; Fulmer & Underkofler, 1947; Hann, Tilden & Hudson, 1938), and has been used on a preparative scale to obtain previously unknown or rare substances; *e.g.* in the oxidation of D-gluco-D-ido-heptitol to D-idoheptulose (Pratt, Richtmeyer & Hudson, 1952), D-arabitol to D-xylulose (Prince & Reichstein, 1937), ribitol to L-ribulose (Reichstein, 1934), β-sedoheptitol to L-guloheptulose (Stewart, Richtmeyer & Hudson, 1952), and erythritol to L-erythrulose (Whistler & Underkofler, 1938). Other acyclic sugar derivatives have recently been used successfully as substrates (Jones, Perry & Turner, 1961).

The steric requirements for oxidation of polyols have been defined (Hann *et al.*, 1938; Fulmer & Underkofler, 1947): two contiguous D-secondary hydroxyl groups must be adjacent to the primary hydroxyl group at the bottom of the Fischer projection formulae. Cyclitols have also been found to be

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susceptible to oxidations of this type and rules governing the stereospecificity of the reaction have been put forward (Magasanik, Franzl & Chargaff, 1952; Anderson, Tomita, Kussi & Kirkwood, 1953). Posternak & Ravenna, (1947) showed that A. suboxydans attacked cyclohexane-1:2:3-triols which possessed two cis-adjacent hydroxyls to dihydroxycyclohexanones and this has suggested to us the use of bacterial oxidation for the preparation of oxoglycosides which have previously been obtained only in low yield by non-specific oxidations of the secondary hydroxyl groups in glycosides (Brimacombe et al., 1961) and which are of interest as potential starting substances in the synthesis of rare branched-chain sugars (Burton, Overend & Williams, 1961).

An investigation initiated by the requirements for multi-gram quantities of ketopentoses has been made of the kinetics of polyol oxidations in the organism using Warburg respirometry gas exchange. Paper chromatography was used for product analysis.

EXPERIMENTAL

Growth. Cultures of the organism were maintained and grown on the following medium (g./l.): Difco yeast extract, 5; K_2HPO_4, 1; $MgSO_4 . 7 H_2O$, 0.5; the solution was adjusted to pH 7 and glucose, $CaCO_3$ and agar (each 20) were added. The medium was then autoclaved and after shaking to distribute the chalk evenly was rapidly cooled until solid. The cells were grown in Roux bottles at 28° for 4 days. After growth, they were removed from the agar with distilled water, the suspension was filtered through muslin to remove contaminating pieces of agar, and the bacteria were centrifuged for 30 min. at 2,000 x g. The sediment consisted of two layers: an upper pink layer containing the cells, and a lower brown one of agar debris. The latter packed very firmly and the cells were readily separated from it. The bacteria were washed twice by centrifugation before being suspended in the appropriate medium at the required concentration. The relationship between dry

weight, packed cell volume and opacity was found so that the cell concentrations in suspensions could be determined before performing an experiment.

Preparation of ketopentoses. The growth from two Roux bottles (about 2 ml. of wet-packed cells) was washed and suspended in 150 ml. of aqueous solutions containing 5 g. of substrate, and the reaction mixture divided equally between four 250 ml. flasks, which were flushed with O_2, stoppered, and shaken machanically at 28°. The courses of the oxidations were followed polarimetrically on samples of the reaction mixtures after removal of the bacteria by centrifugation. When oxidation was complete, as indicated by constant optical activity of the supernatants, the organism was removed and the products were obtained simply by evaporation under reduced pressure of the aqueous solution to dryness. The purity of the products was determined chromatographically.

Respirometry experiments. The bacteria were suspended in 0.067 M-KH_2PO_4, adjusted with KOH to pH 6.67; the concentration of cells in the suspension was 25 µl of wet-packed cells (3.4 mg. dry wt./ml). Measurements of O_2 consumption and CO_2 production were performed with the Warburg apparatus using the "direct method"; a correction was made for the CO_2 retained by the buffer, assuming a value of 6.382 for the first association constant for carbonic acid (Umbreit, Burris & Stauffer, 1949). The main compartment of each flask contained 1.6 ml. of cell suspension in phosphate buffer (40 µl. of wet-packed cells) together with 0.1 ml. of the same buffer which contained chloramphenicol (1.0 mg./ml.) in appropriate cases. The substrate (15 µmoles dissolved in 0.3 ml. of buffer) was added from the side-arm as required. The centre-wells of the flasks contained, when necessary, 0.1 ml. of 15% (w/v) KOH to absorb CO_2. The flasks were shaken in a water bath at 30°. A second set of flasks, containing similar quantities of the reactants, was also shaken at 30° and from these samples were removed periodically for chromatographic analysis. Portions (25 µl.) of the media after

centrifugation were spotted on each chromatogram.

Chromatography. One-dimensional paper chromatography was employed for following the reaction products corresponding to various stages of gas exchange, using Whatman No. 4 paper, developed with either phenol:water or n-butanol:propionic acid:water (Benson et al., 1950). For the investigations of the purity of products obtained on a preparative scale, samples were run on Whatman No. 1 paper developed with n-butanol:ethanol:water (4:1:5 v/v; upper phase). Reducing carbohydrate products were located and classified by a phloroglucinol spray (Borenfreund & Dische, 1957). Other compounds were detected with $AgNO_3$.

Chemicals. Ribitol, D-glucitol, D-arabitol and chloramphenicol (chloromycetin) were obtained commercially. L-Ribulose was prepared from ribitol by oxidation with A. suboxydans (see Results); D-ribulose was prepared by treatment of D-arabinose boiling pyridine (Levene & Tipson, 1936) and was purified by gas chromatographic separation of its di-O-isopropylidine derivative. Methyl D-riboside was prepared by heating a solution of D-ribose in methanolic HCl (1% w/v) under reflux to constant optical rotation (3 hr.). The non-reducing syrupy product obtained after neutralisation of the acid with Ag_2CO_3 has $[\alpha]_D$ -47° (concn. 2.0% [w/v] in water) and was shown to contain 75% pyranoside as determined by its consumption of periodate (Aspinall & Ferrier, 1957) and the liberation of formic acid during the oxidation (Anderson & Greenwood, 1955). Methyl β-L-arabopyranoside (m.p. 169-170°) was similarly prepared by the glycosidation of L-arabinose.

RESULTS

Kinetic studies of polyol oxidation

D-Glucitol(sorbitol) and D-arabitol. Both D-glucitol and D-arabitol were rapidly oxidised by washed bacterial cells (Fig. 1); within 30 min. 1 atom of oxygen was consumed per molecule of substrate supplied. Thereafter the rate of oxygen uptake

INSERT FIG. 1 NEAR HERE

was somewhat reduced but continued without a pause until 60-70% of the oxygen required for complete oxidation of the polyol to carbon dioxide and water had been consumed; this took about 7 hr. Carbon dioxide evolution commenced within 30 min. after addition of the substrate to the cells, though the rate of release was very slow during the period in which the first atom of oxygen per molecule of substrate was being consumed. The rate of its production gradually increased for about 30 min. until the R.Q. was near unity during the second stage of oxidation. Paper chromatographic analysis of the supernatant medium from the time one atom of oxygen per molecule of substrate had been consumed until the end of the experiment showed only sorbose or xylulose (from D-glucitol and D-arabitol, respectively) as products of the reactions. The amounts of these sugars gradually decreased with time, and they had disappeared entirely when respiratory activity ceased. No other products were observed chromatographically. Polarimetry showed that the D-isomer of xylulose was formed; Visser't Hofft (1925) found that the sorbose produced is the L-isomer.

Ribitol. This substance was also oxidised rapidly, one atom of oxygen being consumed per molecule of added substrate within one hr. (Fig. 2). There was
INSERT FIG. 2 NEAR HERE
no evolution of carbon dioxide at this stage, and chromatographic analysis of the supernatant medium revealed the presence of ribulose as the sole product. This was shown polarimetrically to be the L-isomer.

Respiratory activity then ceased except for a low rate of endogenous respiration, and was eventually resumed after a lag period of about 6 hr. The resumed respiration had a R.Q. of one, and proceeded for at least 5 days, by which time about 58% of the oxygen required for total oxidation had been absorbed; the rate of oxygen consumption showed no sign of decreasing at the end of 5 days. Chromatographic analysis of the supernatant medium during the second stage of oxidation showed the presence only of decreasing quantities of ribulose. These observations on the kinetics of ribitol oxidation suggested that the delay between the first

and second stages of the oxidation might be due to the formation of an adaptive enzyme system required to continue the metabolism of L-ribulose produced in the first stage of ribitol oxidation. This possibility was examined in a number of ways.

The oxidation of ribitol by the organism was studied in the presence of chloramphenicol at a concentration of 50 µg./ml. This antibiotic is well known to be a specific inhibitor of protein synthesis, and while not affecting the activity of pre-formed enzymes, prevents processes such as enzyme adaptation which involve the manufacture of new enzyme molecules (Brock, 1961). Experiment showed that chloramphenicol had no effect on any stage of the oxidation of D-glucitol or D-arabitol, nor did it affect the first stage of ribitol oxidation during which one atom of oxygen was utilised per molecule of substrate supplied, with the quantitative formation of L-ribulose. Even pre-incubation of cells with chloramphenicol for 24 hr. prior to the addition of ribitol had no action on the first stage of oxidation of the polyol. However, during the second stage of ribitol metabolism, chloramphenicol inhibited both the oxygen uptake and the carbon dioxide liberation, and increased the lag period between the end of the first stage and the beginning of the second stage from 6 hr. to about 54 hr. (Fig. 2). Eventually, after about 100 hr., the rates of gas exchange with and without chloramphenicol became equal.

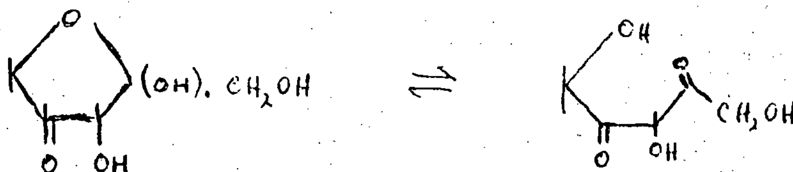
The oxidation of L-ribulose itself by the bacteria closely paralleled the second stage of ribitol oxidation, both as regards oxygen uptake and carbon dioxide release; i.e., there was a lag period of several hr. before oxidation commenced (Fig. 2). Chromatography at time intervals during the oxidation of L-ribulose showed only decreasing amounts of this substance in the medium.

A further batch of cells was incubated with ribitol for 5 days to promote enzyme adaptation, then washed several times and incubated separately with ribitol and L-ribulose in the Warburg apparatus. These cells demonstrated no lag

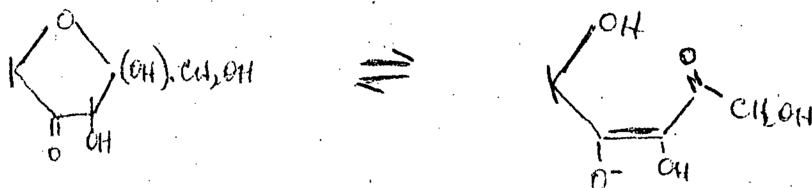
period between the first and second stages of ribitol oxidation; in the second stage the rate of oxygen uptake remained lower than during the first stage, the situation resembling those with D-glucitol and D-arabitol. There was no lag period before the oxidation of L-ribulose, which again closely paralleled the second stage of ribitol oxidation, indicating that adaptation had already taken place during the pre-incubation period (Fig. 3). Further, adapted cells,

INSERT FIG. 3 NEAR HERE

in contrast to unadapted bacteria, began to release carbon dioxide as soon as they were supplied with either ribitol or L-ribulose, demonstrating again their capability for metabolising L-ribulose with no lag period. The capability of the cells to oxidise D-ribulose was investigated. Only the D-isomer has the necessary chemical configuration for the characteristic oxidation of a secondary alcoholic group. D-Ribulose showed a kinetic pattern of gas exchange similar to those of D-glucitol and D-arabitol (Fig. 1): a rapid first stage oxidation corresponding to about one atom of oxygen per molecule of sugar, followed by a slower second stage (which proceeded without a lag period) during which carbon dioxide was produced and oxygen absorbed with an R.Q. in the range 1.1-1.2. Carbon dioxide was formed as soon as the cells were supplied with the sugar. Chromatography of the supernatant medium showed, in contrast to L-ribulose, the formation of a reaction product which would be expected to be 1:3:5-trihydroxy-pentane-2:4-dione (assuming the ketose to oxidise in the acyclic form). The structure of the product was not proved unequivocally, but some evidence was obtained for it being the β-diketone. A sample of the substrate (15 mg.) was oxidised under conditions known from respirometry to be optimal for production of an intermediate and after removal of the cells the supernatant showed carbonyl absorption in the ultraviolet (λ_{max} 285 mμ, log ε 1.71) suggesting the presence of the equilibrium I:



Addition of alkali to the solution caused reversible enhanced absorption and a hypsochromic shift (λ_{max} 270 mμ, log ϵ ca. 1.65). This is attributed to absorption from the acyclic species in the equilibrium II:



Methyl D-riboside. Very slow oxidation of this substance took place. In the course of some three days one atom of oxygen was absorbed per molecule of glycoside, with no production of carbon dioxide. This was followed by a period in which some carbon dioxide was produced together with oxygen absorption, until after about 9 days, when the experiment was terminated, about 14% of the oxygen required for complete oxidation had been absorbed (Fig. 4). As the rate of oxygen

INSERT FIG. 4 NEAR HERE

uptake was so low, it was difficult to determine whether any increase in the rate took place as a result of enzyme adaptation. However, in the presence of chloramphenicol, the total oxygen absorption in 5 days was about 20% less than the corresponding value in the absence of the antibiotic, suggesting that adaptation was less pronounced than in the case of ribitol. Further, there was no lag period, either before oxidation, or after one atom of oxygen per

molecule of substrate had been absorbed. Chromatographic analysis of the supernatant medium showed the presence of a product formed in the course of the first four days; this gradually decreased in quantity during the subsequent period of incubation, and simultaneously a second product made its appearance. The second product appeared from chromatographic data to be acidic, and was not examined further. In order to investigate the first product in greater detail the glycoside, $[\alpha]_D$ -47° was oxidised on a larger scale (125 mg. in 10 ml.), the reaction being followed polarimetrically to constant rotation ($[\alpha]_D$ -24°, 5 days). The supernatant after removal of the cells was shown to contain primarily the initial product which ran on chromatograms as an elongated streak, all of which gave a yellow colour with 2:4-dinitrophenylhydrazine. The solution had λ_{max} 287 mμ, log ϵ 2.24, changing in 0.1 N-sodium hydroxide to λ_{max} 310 mμ, log ϵ 2.61. These figures are consistent with the molecule having a 2-hydroxycyclohexanone structure as the positions of maximum absorption in neutral and alkaline solutions are similar to those quoted for adipoin (Auterhoff & Zeisner, 1953). Methyl-3-oxoglucoside is reported to have an absorption maximum at 285 mμ, shifting initially to 320 mμ in alkaline solution (Theander, 1958). The syrup obtained on evaporation of the supernatant after removal from the cells, gave, on reduction with sodium amalgam and hydrolysis with sulphuric acid, mainly ribose, with a small amount of arabinose. This indicates that bacterial oxidation occurred at $C_{(2)}$.

Methyl-β-L-arabopyranoside. This glycoside was oxidised even more slowly than methyl riboside. After 9 days, no carbon dioxide had been produced, while the oxygen uptake corresponded to about one atom per molecule of substrate supplied. During the first four days of incubation the total oxygen uptake was some 25% lower in the presence of chloramphenicol than in its absence, a finding similar to that with methyl riboside. Two products were formed from methyl arabinoside which were chromatographically similar to those from methyl riboside, but no further investigations were made of these substances.

Preparation of multi-gram quantities of ketoses.

When oxidation was complete (Table 1) evaporation of the solvent under reduced
INSERT TABLE 1 NEAR HERE
pressure after removal of the bacteria gave the ketoses as clear, pale yellow syrups in yields greater than 96%. The sugars had identical chromatographic properties to those of authentic ketopentoses and were further characterised by interconversion in good yields to their corresponding phenylosazones. The final specific rotations were $[\alpha]_D$ -29.0° and $+16.2^{\circ}$ for L-ribulose and D-xylulose; values quoted in the literature are respectively -33° and $+16.6^{\circ}$ (Levene & Tipson, 1936). The ribulose was chromatographically pure but the xylulose was shown to contain, from early stages in the oxidation, small amounts of an aldopentose (probably xylose).

DISCUSSION

Constitutive and adaptive enzymes involved in polyol oxidation.

The kinetic data reported above of the gas exchanges and product formation suggest that cells grown on a medium containing yeast extract and glucose as carbon sources, possessed a constitutive enzyme system capable of oxidising ribitol to L-ribulose. Continued incubation of the cells in the presence of L-ribulose stimulated the production of adaptive enzymes which permitted the further metabolism of the pentulose to carbon dioxide. Support for this contention was provided by the inhibition of L-ribulose oxidation by chloramphenicol, and the ready oxidation of ribitol and L-ribulose, with no lag period, by cells which had been pre-adapted in the presence of L-ribulose produced from ribitol. The oxidation of ribitol and L-ribulose by these pre-adapted cells was unaffected by chloramphenicol, demonstrating that the adaptive enzymes, once formed, were not inhibited by the antibiotic. The metabolism of D-glucitol and D-arabitol by unadapted cells showed that the organism possessed constitutive enzymes capable not only of oxidising these alcohols to L-sorbose and D-xylulose, respectively, but also of subsequently oxidising these sugars to carbon dioxide. However, the formation of L-sorbose and D-xylulose was much more rapid than their utilisation, and this accounted for large amounts of these substances being released from the cells into the medium.

Oxidative metabolism of the methyl glycosides was slow, and appeared to be largely constitutive, since chloramphenicol reduced the rates of gas exchange with these substrates by only 20-25%. There was in addition no sign of any increase in the rate of oxidation of the glycosides after a long incubation in the absence of chloramphenicol (Fig. 4), in contrast to the pattern obtained with ribitol and L-ribulose (Fig. 2). The effect of chloramphenicol on the oxidation of D-ribulose was not studied, but from kinetic curves of the gas exchange the oxidation appeared to be entirely constitutive.

The use of *Acetobacter suboxydans* in polyol oxidation

As a preparative tool for the oxidation of an appropriate secondary alcohol to a ketone, the present study has shown that four types of situation might be encountered with compounds which are oxidised by *A. suboxydans*. The first, and experimentally most satisfactory of these, is exemplified by ribitol. With this substance there is a quantitative oxidation of the susceptible secondary alcohol grouping to the corresponding ketonic group, followed by a long lag period prior to the complete oxidation of the initial product. Thus there is little danger for a considerable period of products being lost as a result of their further metabolism.

The second situation is illustrated by D-glucitol, D-arabitol, and D-ribulose. Here, although the first stage of oxidation is quantitative, the second stage starts before the first is finished and there is no lag period between the two. It therefore becomes desirable to stop the reaction as soon as the first stage has been completed, because continued incubation will result in a loss of the desired product, although this will remain pure.

The methyl glycosides are examples of the third possibility, in which oxidation proceeds very slowly and may possibly not be complete before the cells deteriorate unless a relatively high cell:substrate ratio is used.

The gas exchanges and analyses indicate that carbonyl products are formed but the metabolism of these glycosides to specific compounds is complicated by the eventual appearance of secondary products. The difficulty with which the oxidation proceeds and the known lability of the initial carbonyl products (Theander, 1958) would seem to preclude this as a method for the synthesis of oxoglycosides.

Fourthly, there are substances which undergo oxidation, but from which the bacterium produces no chromatographically detectable product. Such substances are D-xylulose, L-ribulose and L-sorbose. It appears in this case that once these substances enter the metabolic apparatus of the organism, no product is released from the cells until the substrate has been completely oxidised to carbon dioxide.

It is of interest and convenience that these oxidations proceed satisfactorily in a medium of distilled water, so facilitating isolation of the pure products.

SUMMARY

1) An aqueous suspension of Acetobacter suboxydans readily oxidises D-arabitol and ribitol to D-xylulose and L-ribulose, respectively, in the absence of added buffer or growth factors. While the ribulose is pure, the xylulose shows slight contamination.

2) The mechanisms of the primary oxidation of polyols to ketoses and of these ketoses have been investigated and have been shown to vary greatly with substrate structure.

3) The organism was shown to oxidise two methyl glycosides to oxo-derivatives but under conditions which are most unfavourable for preparative work.

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Table 1. Preparation of L-ribulose and D-xylulose

Ribitol or D-arabitol (5 g.) was incubated at 28° with about 300 mg. dry wt. of cells in 150 ml. of distilled water under an atmosphere of O_2 gas.

Ribitol		D-Arabitol	
Hr.	$[\alpha]_D$	Hr.	$[\alpha]_D$
24	-0.76	15	+0.28
48	-0.92	30	+0.50
72	-0.96	54	+0.54
96	-0.96	72	+0.54

ABSTRACT

The biochemical synthesis of D-xylulose and L-ribulose; details of the action of *Acetobacter suboxydans* on D-arabitol, ribitol and other polyhydroxy compounds. V. Moses and R.J. Ferrier: Biochem. J., (Lawrence Radiation Laboratory, Univ. of California, Berkeley, Calif., U.S.A.)

An aqueous suspension of *Acetobacter suboxydans* readily oxidises D-arabitol and ribitol to D-xylulose and L-ribulose, respectively, in the absence of added buffer or growth factors. While the ribulose is pure, the xylulose shows slight contamination. The mechanisms of the primary oxidation of polyols to ketoses and of these ketoses have been investigated and have been shown to vary greatly with substrate structure. The organism was shown to oxidise two methyl glycosides to oxo-derivatives but under conditions which are most unfavourable for preparative work.

Captions for Figures.

Fig. 1. Oxidation of D-arabitol after deduction of endogenous respiration. Each flask contained 5.4 mg. dry wt. of cells and 15 μmoles of substrate in 2.0 ml. of 0.067 M-K phosphate buffer, pH 6.67. Substrate added at time 0. A, O_2 uptake; B, CO_2 evolution. D-Glucitol and D-ribulose showed similar curves.

Fig. 2. Oxidation of ribitol and L-ribulose by unadapted cells in the presence and absence of chloramphenicol. Endogenous respiration deducted. Each flask contained 5.4 mg. dry wt. of cells and 15 μmoles of substrate in 2.0 ml. of 0.067 M-K phosphate buffer, pH 6.67. Chloramphenicol concn., 50 μg./ml. Substrate added at time 0. A, O_2 uptake with ribitol in absence of chloramphenicol; B, O_2 uptake with ribitol in presence of chloramphenicol; C, CO_2 evolution with ribitol, and O_2 uptake and CO_2 evolution with L-ribulose, all in the absence of chloramphenicol; D, CO_2 evolution with ribitol in the presence of chloramphenicol.

Fig. 3. Oxidation of ribitol and L-ribulose by pre-adapted cells. Endogenous respiration deducted. Each flask contained 5.4 mg. dry wt. of cells and 15 μmoles of substrate in 2.0 ml. of 0.067 M-K phosphate buffer, pH 6.67. Substrate added at time 0. A, O_2 uptake with ribitol; B, CO_2 evolution with ribitol, and O_2 uptake and CO_2 evolution with L-ribulose.

Fig. 4. Oxidation of methyl-D-riblpyranoside. Endogenous respiration deducted. Each flask contained 5.4 mg. dry wt. of cells and 15 μmoles of substrate in 2.0 ml. of 0.067 M-K phosphate buffer, pH 6.67. Substrate added at time 0. A, O_2 uptake; B, CO_2 evolution.

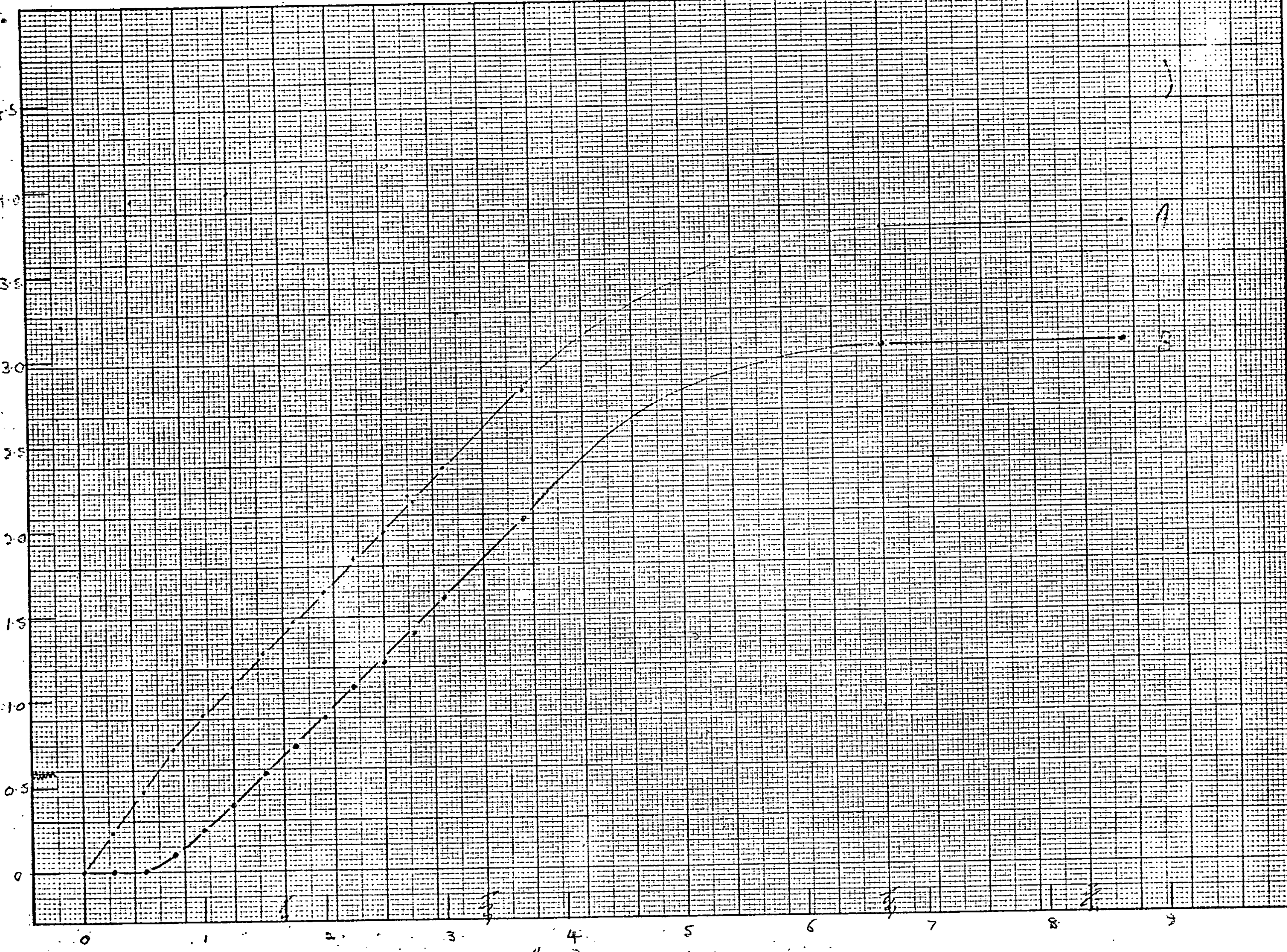

Fig 1

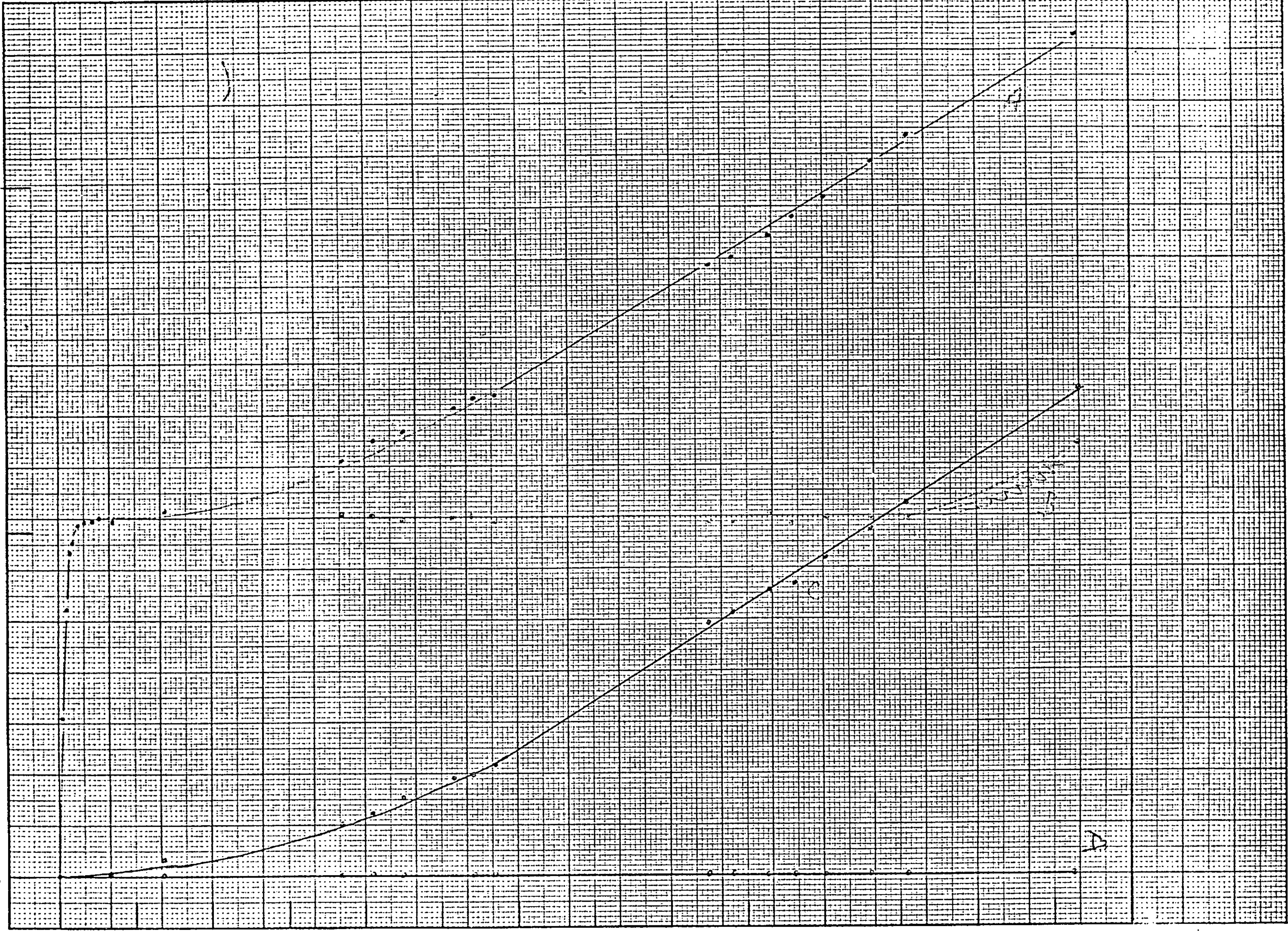

Fig 2

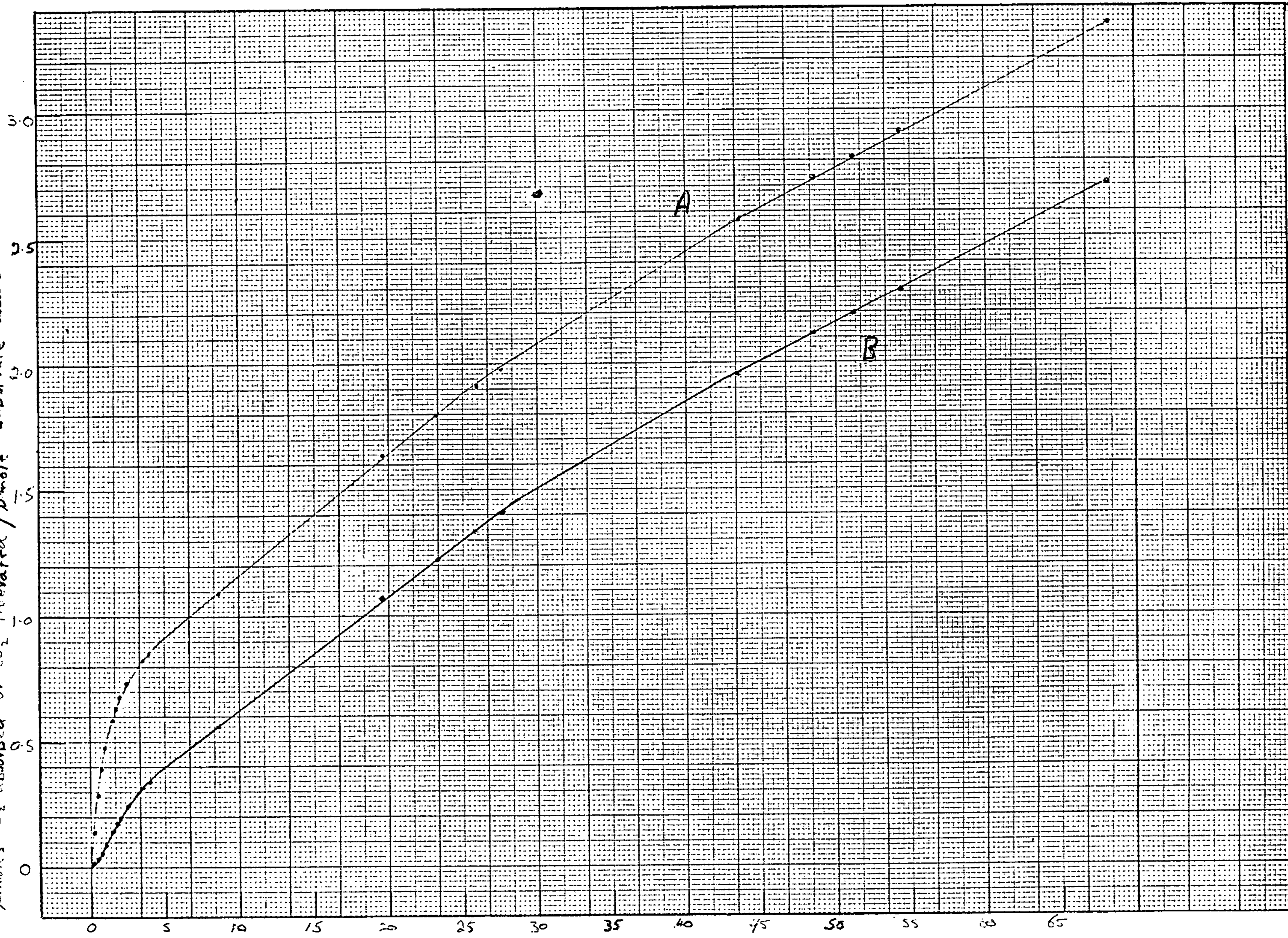

Fig 3

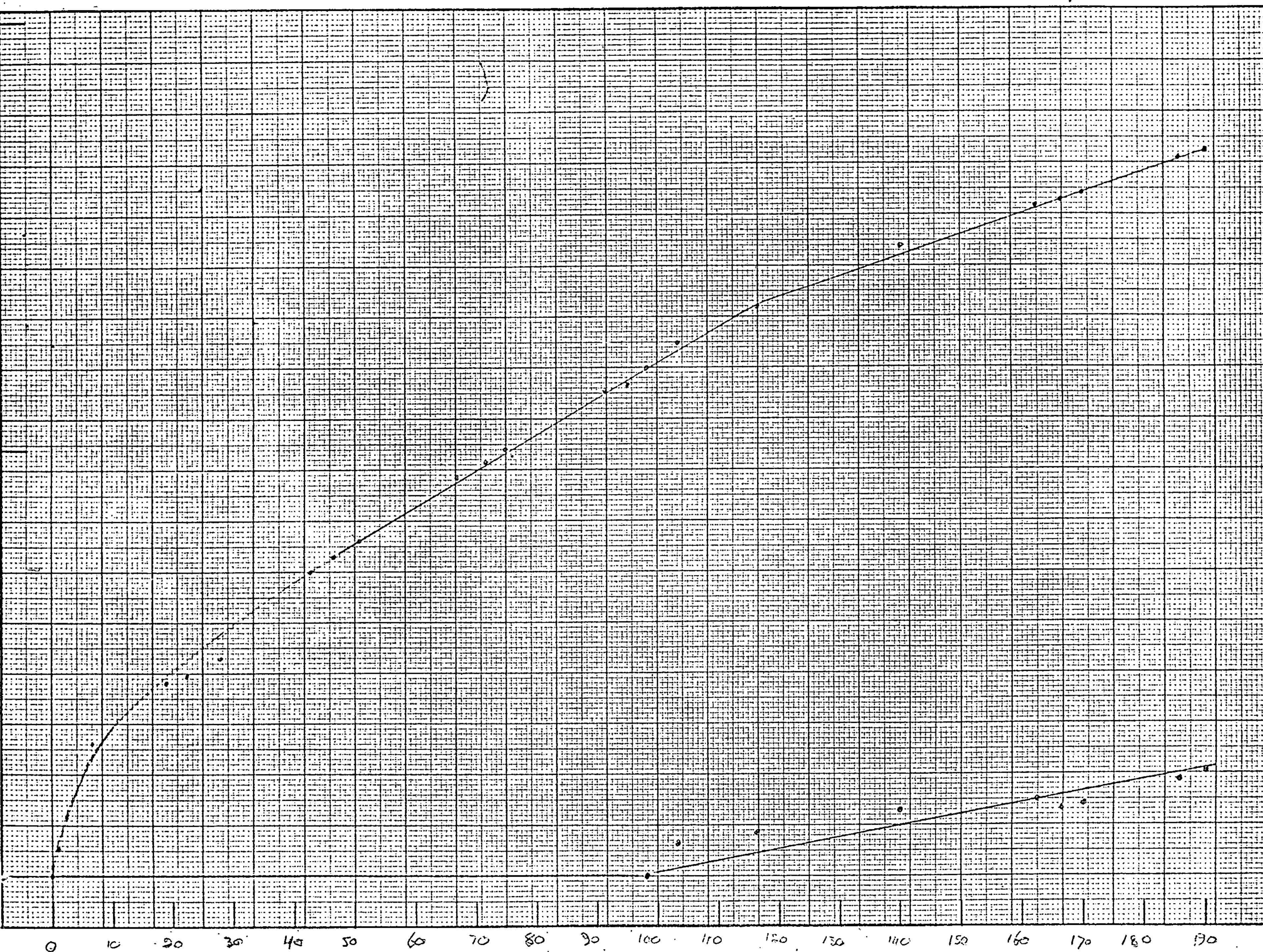

Fig 4

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