10

SOME OBSERVATIONS ON THE OXYGEN ABSORPTION OF PORK MUSCLE

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SUMMARY

The oxygen absorption of fresh pork has been studied. The curve may be represented by two components, one a straight continuous line, the other a discontinuous exponential curve.

The significance of these two components are considered together with the effect of various substrates, inhibitors, co-enzymes, pH, and carbon dioxide evolution.

INTRODUCTION

The development of lactic acid from glycogen in rigor mortis, has been widely studied and is now familiar. Very little however, is known about the post-rigor chemistry that then goes on, albeit at a slower rate. That changes do take place however, is quite apparent, viz the "ageing of beef". We in our laboratories have frequently noted the slow disappearance of lactic acid from stored, post-rigor meat. This disappearance of lactate does not appear to be due to bacterial action since it occurs even at $-20^{\circ}F$.

Another and more notable property of post-rigor muscle is that it is still continuously absorbing O_2. The following Warburg studies on Pork muscle were therefore undertaken in the hope of at least paving the way for a better understanding of what changes, if any, take place in stored, post-rigor muscle.

Andrews et al[1] & Grant[2] have investigated the surviving enzyme activities of post-rigor muscle, showing the succinic dehydrogenase system to be by far the greatest residual activity. This however, only implies that there is a potential activity there. Whether there is any

actual activity or not depends on the presence of its substrate. Although these workers have stressed the abundance of this enzyme in post-rigor muscle, it should be apparent that it need not necessarily be of any importance at all, unless its substrate also is present. The much smaller activity of say lactic dehydrogenase, may well be of much more importance, because it alone has a large reservoir of its substrate naturally present. All other enzymes present, however great their potential activity might be, will only play such a part as is determined by the amount of their substrates present, or the rate of formation in any respiration cycle. Knowing the economies and due provisions which nature usually fashions, it is tempting to suggest that the enzymes in meat are present in the proportions best suited to their functioning in the live animal, and not to any comparatively slow residual post-rigor and aerobic activity.

The presence of a large potential activity of the succinic dehydrogenase may therefore be a lead to what is required in the living tissue, but is not in our opinion any indication of its importance in the respiration of carcase tissue particularly post-rigor.

Urbin and Wilson[3] have recently studied the post-mortem oxygen requirements of beef tissue at 1°C, and interpret their findings as a steady rate of enzymatic oxygen absorption, together with an evolution of carbon dioxide both as an initial 'surge' and as a steady rate. The apparent initial surge of oxygen absorption is explained partly as an artefact caused by pre-formed gaseous carbon dioxide in the tissue escaping, thus causing a diminution of sample volume, which would be measured as an oxygen absorption.

EXPERIMENTAL

All experiments were conducted in a conventional Warburg apparatus at 37°C. Flask contents were always 1gm of freshly minced tissue + 2mls of buffer etc. in air, with 0.4mls of potassium hydroxide solution on a roll of filter paper in the centre well, unless otherwise stated.

RESULTS

1. Typical Curves

Over the short periods of time usually used in work with tissue slices etc. an exponential type of curve is obtained. When these periods were extended to 2-3 hrs. a quite definite straightening of the line occured, the oxygen then being absorbed at a steady constant rate. A typical curve is shown in Fig.1. That this continued steady absorption, superimposed on the exponential curve, was not due to bacterial growth, is shown by:-

a) That if the time was further prolonged, then eventually a definite sharp increase was found (Fig.2) due to the influence of bacterial growth.

b) If rather contaminated or much handled meat was used, a curve was found, where the increase due to bacterial growth occured before the exponential phase was complete.

c) In the presence of toluene, the same type of normal curve was obtained. The normal oxygen absorption curve is therefore presumed to be not materially affected by bacterial growth up to approx. 3 hrs., if normal fresh clean muscle is used.

2. Equation of typical curve

The data did not appear to fit any exponential or other form of curve; the best general fit always being obtained with the use of two components, i.e. a straight line continuous component, assumed to have been straight from the start, leaving a residual curve which appeared

to be truly exponential. These components are shown in Fig.3.

On this basis then, the general equation of the normal curve can be calculated

a) The continuous component will be given by the equation of a straight line going through the axes,

$$\text{i.e. } y_1 = at$$

where y is the oxygen absorbed in this component in t mins., and 'a' is a constant.

b) The discontinuous component will be given by the exponential equation,

$$y_2 = b\text{-}be^{-kt}$$

where y_2 is the oxygen absorbed in this component in 't' mins., 'b' 'k' being constants.

c) The equation of the full curve therefore becomes:-

$$y = y_1 + y_2 = at + b\text{-}be^{-kt}$$

This curve has been found to be generally applicable to all samples (more than a hundred) examined. The only slight departure from this that is sometimes found is a slight curving of the straight line component after some time. This is undoubtably because the straight line is really the first part of a very large exponential curve, which for all normal purposes may be considered as a straight line. Only when the total activity is very weak can this effect be noticed within 3 hrs.

THE CONSTANTS 'a' 'b' & 'k' OF THE EQUATION.

From the proceeding it may be seen that the value of 'a' represents the slope of the continuous straight line component, and represents directly the rate of this component. This should not be affected by any reasonable delay period i.e. during mincing, weighing out, and equilibriating in the Warburg flask prior to taking measurements.

The value of 'b' however, representing the asymptotic

maximum amount of oxygen absorbed in the discontinuous component, will be effected largely by variations in the time before measurement is commenced. The values of 'b' must therefore be interpreted with caution, and standardisation of timing and exposure conditions of samples before the Warburg taps are closed, is essential if any comparison of 'b' values is to be made.

The values of 'k' representing the rate of change of slope of the exponential component should be independent of the starting point.

3. EFFECT OF THE FORM OF THE MUSCLE SAMPLE

A comparison of chopped muscle, sliced muscle and minced muscle was made with the results shown in Fig.4.

The fastest oxygen absorption occured with the minced meat. This effect is also demonstrated in Fig.5 together with the influence of the amount of meat in the flask. Per gram of muscle, the highest rate was obtained using 1gm. of minced muscle in the Warburg flask.

It was sometimes found, particularly with rather acid meat (pH 5.4) that duplicates did not invariably agree. With this type of meat there is usually some exudation of fluid present, and it was thought that this could lead to bad sampling in the weighing out of the 1gm portion, causing the activity to vary slightly. This would be so if the activity was concentrated more in the meat juices. Further experiments were therefore conducted to elucidate this point.

Minced muscle (pH 5.3) was compared with the juice expressed direct by centrifuging lightly, and with the insoluble residue after extracting and washing twice with excess saline, allowing one hour to elapse with each extraction. The results are shown in Fig.6 and indicate that most of the meat activity was present in the expressed juice.

This indicated that more accurate results might be obtained by always using the expressed juice. However, this juice can only be expressed by centrifuging if the pH of the muscle is low. An experiment was therefore conducted to see if a 50% juice could be used, by extracting (and diluting) with phosphate buffer. The results are shown in Fig. 7 and demonstrate that the increased handling and exposure of the juice has led to a very significant loss of activity. The lability of the juice appears to be too great for this method to be used with success.

EFFECT OF INHIBITORS

a. Malonate.

Malonate which classically inhibits succinic dehydrogenase, showed no inhibition of the oxygen absorption, as shown in Fig.8.

b. Fluoride & Azide (Fig.9)

Fluoride appeared to reduce the continuous rate 'a', but increased the curved component 'b'. This same effect was also demonstrated to a greater extent by azide. The increase however, was restricted to the b value, the value of k remaining unchanged.

c. Cyanide

The addition of (3×10^{-2}M) cyanide had no detectable affect.

EFFECT OF ADDED SUBSTRATES

a. Acids of the Krebs Cycle.

Oxygen absorption curves were obtained in the presence of Lactate, Pyruvate, Succinate, Fumarate, Oxalo-Acetic and α-keto-glutaric acid. Of the above acids only succinate and fumarate had any effect (Fig.10). The rate of the 'a' value was unchanged, but the 'b' values showed slight increases.

b. Other Substrates:

Glucose, cystein, glycollate, tryptophane, and creatine all were found to have no effect on the oxygen absorption curve, apart from a very slight lowering.

EFFECT OF OXYGEN CARRYING PIGMENTS

a. Myoglobin. Fig.11.

Myoglobin had a slight depressing effect on both components, proportional to the myoglobin concentration.

b. Cytochrome.

The addition of 0.05% Cytochrome c had no effect on the oxygen absorption.

EFFECT OF pH

Three muscles from different carcases (chosen for their pH values) were examined with the results shown in Fig.12. The oxygen absorption progressively increased with the increase of pH of the meat. Both components showed this increase.

One sample of meat was then examined in a range of phosphate buffers with the results shown in Fig.13. Increase of pH again increased both components of the oxygen absorption. The buffering power of the meat however, was so great that the eventual pH values in the flask were all much nearer to the original meat pH value of 5.5 than was expected.

EFFECT OF ADDED CO-ENZYMES ETC.

The effects of some of these compounds on the oxygen absorption curve are shown in Fig.14.

A.T.P. & A.D.P. both had no effect.

Nicotinamide also showed no effect. Co-enzyme A gave a slightly increased value of 'a' but had no effect on the discontinuous rate, i.e. the 'b' value.

TPN had no effect on the 'a' value but slightly increased the b value.

DPN gave a well marked acceleration of the 'a' value (doubled), with no effect on 'b'.

CARBON DIOXIDE EVOLUTION

In general, the carbon dioxide evolution was always almost equal to the oxygen absorption, i.e. the respiratory quotient was normally 1.0 or just below.

DISCUSSION

Significance of the Two Components.

The fact that the oxygen absorption can be represented by two separate components, one continuous, and the other discontinuous, suggests many possible explanations, and some alternatives are considered below:-

a) AN ARTEFACT INTRODUCED DURING MEASUREMENT

a1) Urbin & Wilson (2) using cubes of tissue at 1°C explained the initial surge of oxygen uptake that they observed similar to our discontinuous component, as being a diminution of sample volume, brought about by the gradual release of preformed gaseous carbon dioxide. This volume change would be interpreted as an uptake of oxygen.

It is apparent however from the results under our conditions of minced tissue, suspension in fluid, and 10-15 mins. equilibriating in the Warburg before closing the tops, that the presence of preformed carbon dioxide is not very likely. This faster rate continues under our conditions for 1-1½ hrs., and as may be seen from Fig.4 using coarsely chopped tissue instead of a fine mince does not delay or increase this discontinuous component at all. This component was also still present when the meat juice itself was used. On this evidence therefore, a change of sample volume due to preformed

carbon dioxide cannot be responsible for the initial exponential component of the oxygen absorption curve, at least under our conditions.

a2) As the meat is measured in the form of an aqueous dispersion, the oxygen has to be transported to the cells via the aqueous media in addition the cells are undergoing gradual dialysis by the aqueous media.

The faster initial rate of oxygen absorption could therefore be due to the difference between the absorption rates of muscle during and after the dialysis with the suspending fluid. If this is so, the rate of oxygen absorption should reach the steady rate quicker with a fine mince than with a coarser mince. Also the expressed juice would not be expected to show this effect, and in addition, delaying the start (before adding fluid) should not alter the curve. In fact none of these effects are obtained, and it appears reasonable to assume that the two components are actually present and represent true rates of oxygen absorption, not artefacts introduced during measurement.

b) COMPLETE UTILISATION OF ONE SPECIFIC SUBSTRATE

If one specific substrate is oxidised at a faster rate than the others, and this substrate is only present in a limiting amount, the initial fast rate would then slow down exponentially as this particular substance was removed, finally attaining the steady rate of the oxidation of the remaining pool of substrates. i.e. a curve similar to the one obtained in practice would be explainable on this basis. Most of the possible substrates that were tried however did not affect the rate. Succinate and Fumarate, the two exceptions were found to increase the discontinuous component, i.e. increased the 'b' values as shown by the following values from Fig.10.

	a	b
Meat alone	1.0	84
" & succinate	1.0	150
" & Fumarate	1.0	110

These values indicate that the fumarate and particularly the succinate are preferentially used in the discontinuous component. The increase of the b values was also shown up in the carbon dioxide values, showing that it was more than just dehydrogenation causing the increase.

These results may therefore be interpreted as a possible explanation of the normal oxygen absorption curve. If it is assumed that an oxidation cycle as for example, the Krebs' cycle is responsible for the oxygen absorption, then this cycle is not operating in the interior of carcase meat where the system is anaerobic. Under these conditions, some small reservoir or pool of substrate for each step in the cycle could conceivably be accumulated according to the anaerobic conditions. On mincing this material and starting the aerobic cycle, a faster rate could be obtained at first while these substrate concentrations gradually come to the equilibrium strengths of the aerobic cycle. Thus a curve of the type obtained normally, may be explained. Further, as in any cycle like this, one reaction may be rate limiting. The addition of substrates to the other steps will not influence the rate, and only the addition of the substrate produced in the rate limiting step and those for subsequent steps, will be expected to produce an overall increase of the whole cycle. Now in the Krebs' cycle, succinate to fumarate and fumarate to oxalo-acetic are the last stages, and these are the only two substrates which were in fact found to increase the rate of the cycle. In addition as Andrews (1) & Grant (2) have shown, succinic dehydrogenase is present in large excess as far as the

aerobic cycle is concerned, i.e. there would be no reservoir of succinate. It would be very probable then that the decarboxylation and oxidation of α-keto glutaric acid to form succinate, would be the rate limiting step in the cycle.

The anaerobic accumulation of small amounts of succinate and/or fumarate may therefore be the reason for the faster initial uptake of oxygen in freshly exposed meat.

c) UTILISATION OF ONE SPECIFIC CO-ENZYME.

If one specific co-enzyme present in excess at the start, was gradually used up, the rate would fall to a steady value depending on the general rate of cyclic resynthesis or regeneration of the active form.

In fact, of the co-enzymes and related substances added, none had any real effect on the 'b' value. TPN did increase this slightly but the effect was not great enough to confirm this possible explanation.

d) TWO DISTINCT ENZYMATIC PATHWAYS.

The presence of two separate and distinct oxidative pathways, with one system being more rapid, but also more labile than the other, could also explain most of the findings.

This concept is also supported by the evidence from the addition of inhibitors. Thus azide and fluoride reduced the continuous 'a', but increased the 'b' value. This increase of 'b' could be explained, for, if two simultaneous systems were competitive for the substrates, then it would be expected that decreasing one could have the effect of increasing the other. The fact that the 'k' values were found to be the same, supports this also, because it infers that the curve was the same but was able to continue for longer, because the other inhibited pathway was not removing its usual quota of the substrate.

CONCLUSIONS.

The oxygen absorption of post-rigor muscle appears to have two distinct components.

These do not seem to be an artefact introduced during measurement, and are thought to be most likely due to either the utilization of one pre-formed substrate, or to the presence of two distinct oxidative pathways. It is hoped to examine these alternatives in further work.

REFERENCES.

(1) Andrews, M. M., Guthneck, B. T., McBride, B. H., and Schweigert, B. S. J. Bio. Chem., 194, 715 (1952)

(2) Grant, N. H. Food Research. 20, 250 (1955)

(3) Urbin, M. C., and Wilson, G. D. J. Food Sc., 26, 314 (1961)

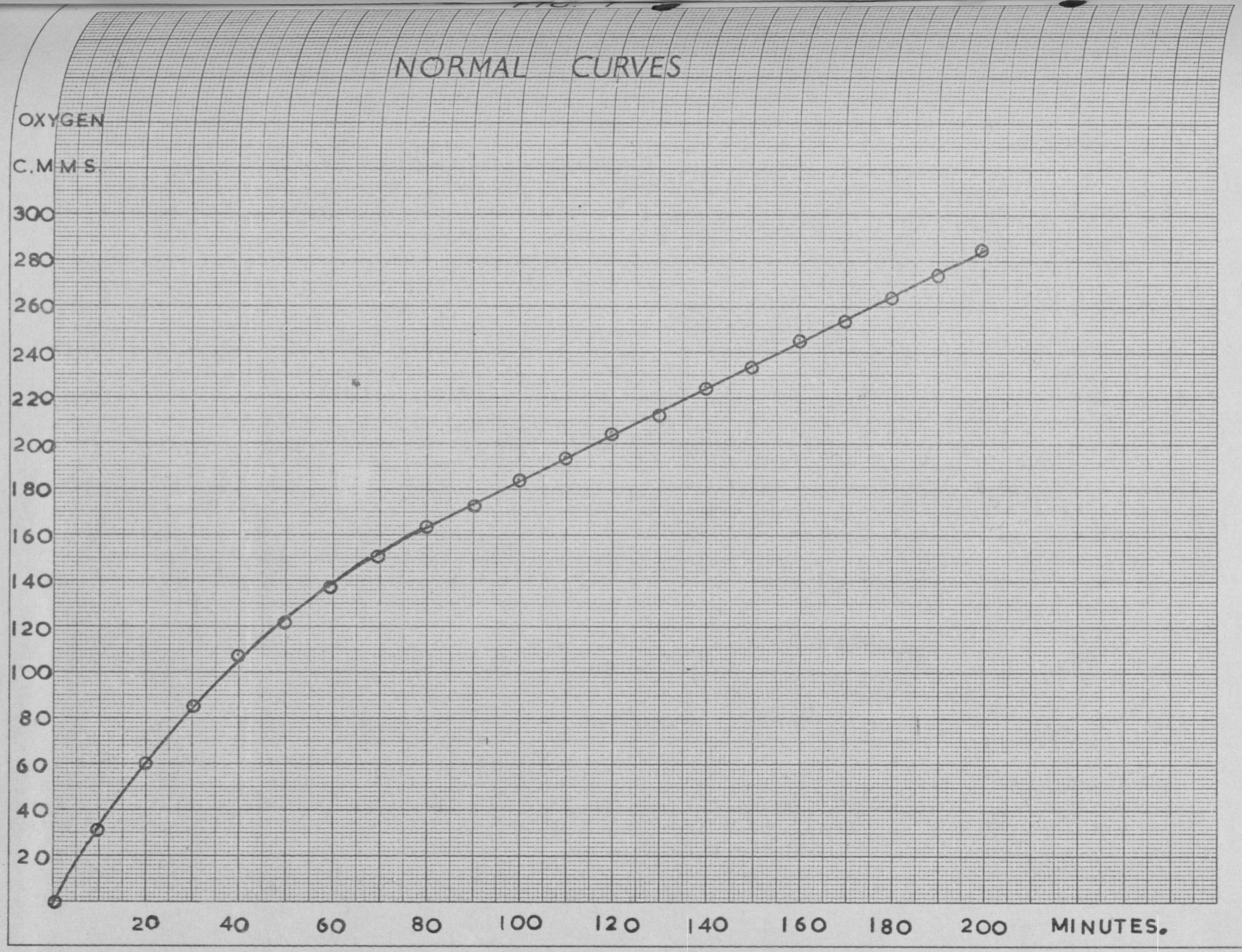
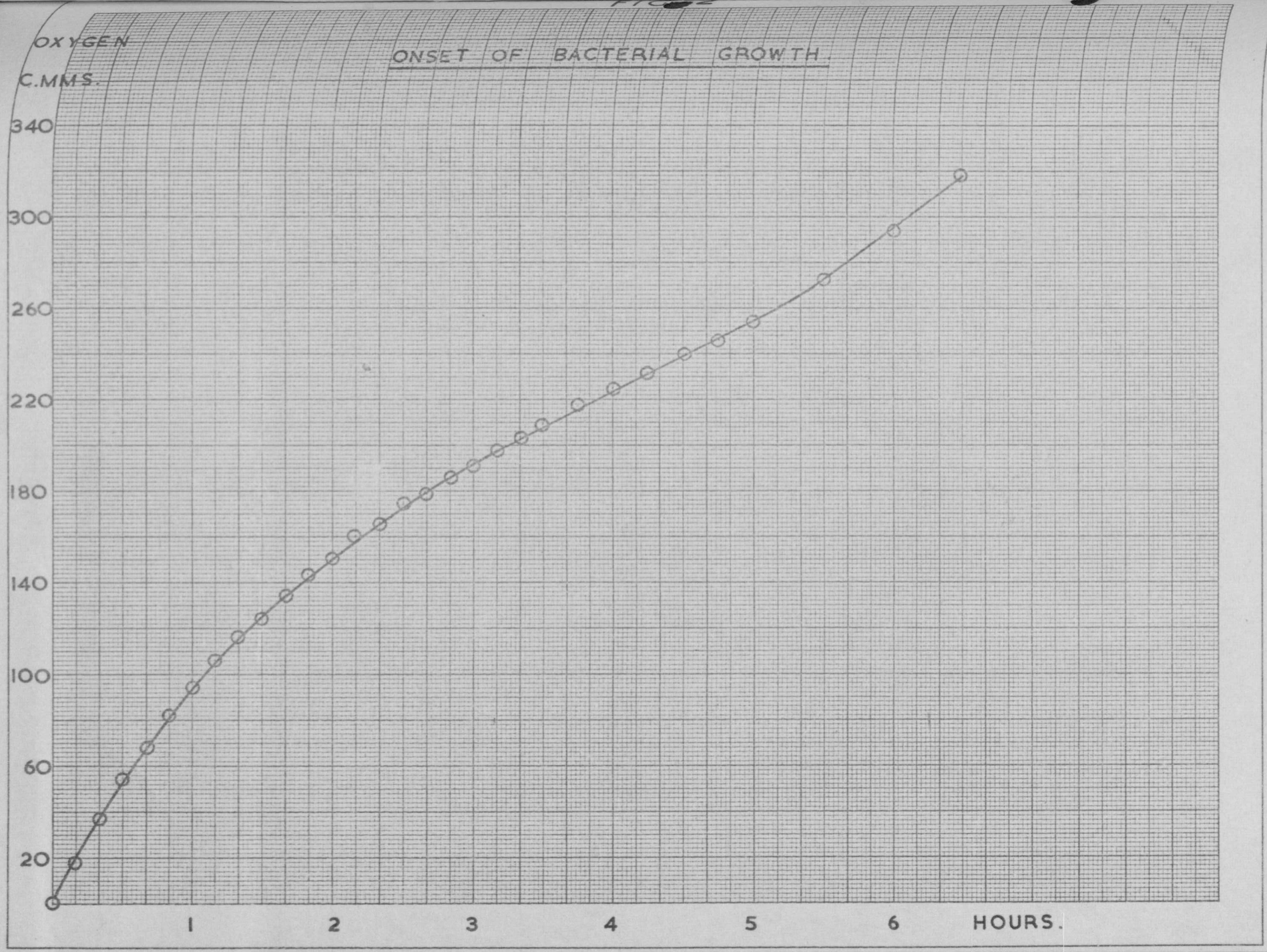

NORMAL CURVES
OXYGEN
C.M.M.S.
300
280
260
240
220
200
180
160
140
120
100
80
60
40
20
20
40
60
80
100
120
140
160
180
200
MINUTES.

FIG 2


ONSET OF BACTERIAL GROWTH.
OXYGEN
C.MMS.
340
300
260
220
180
140
100
60
20
1
2
3
4
5
6
HOURS.

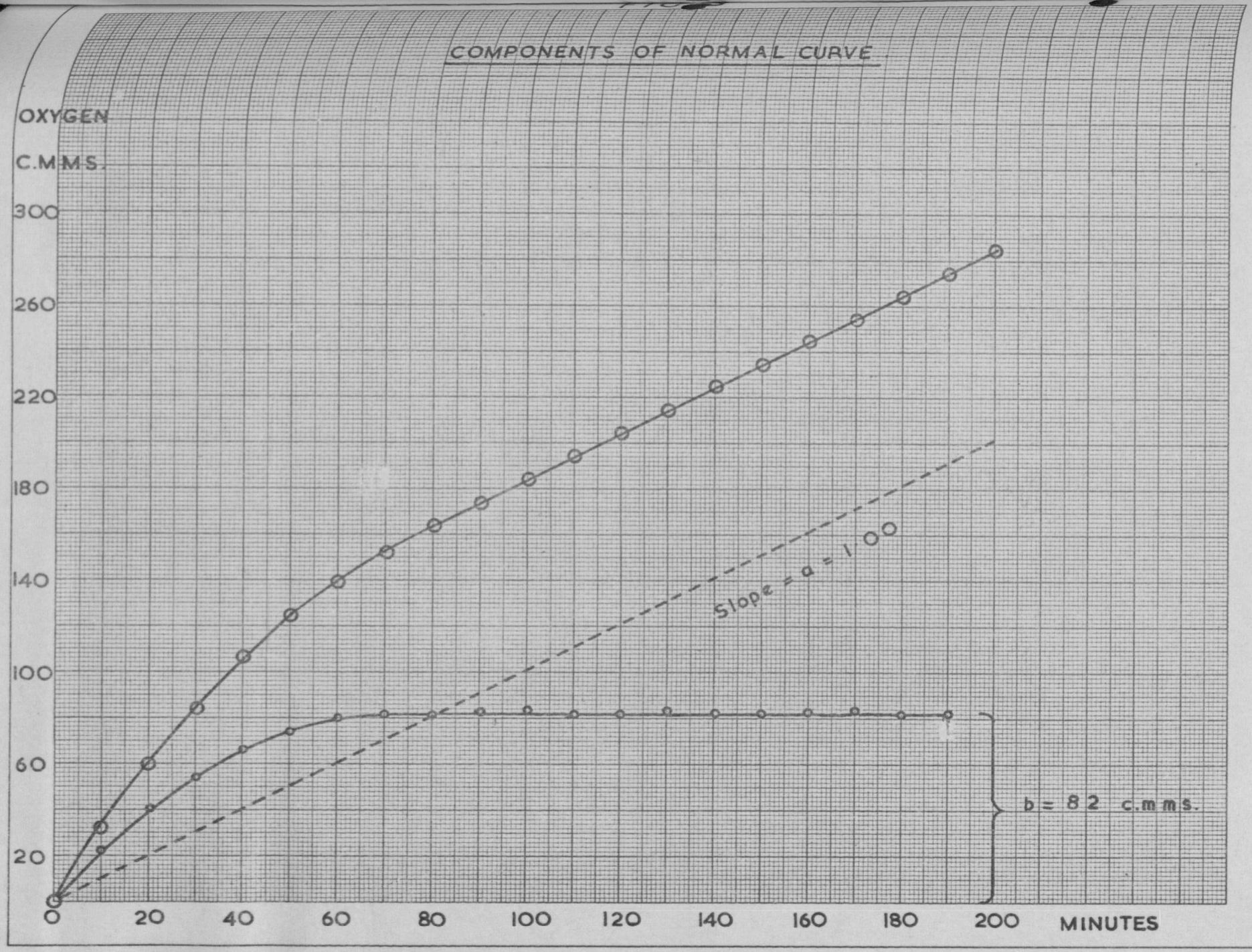
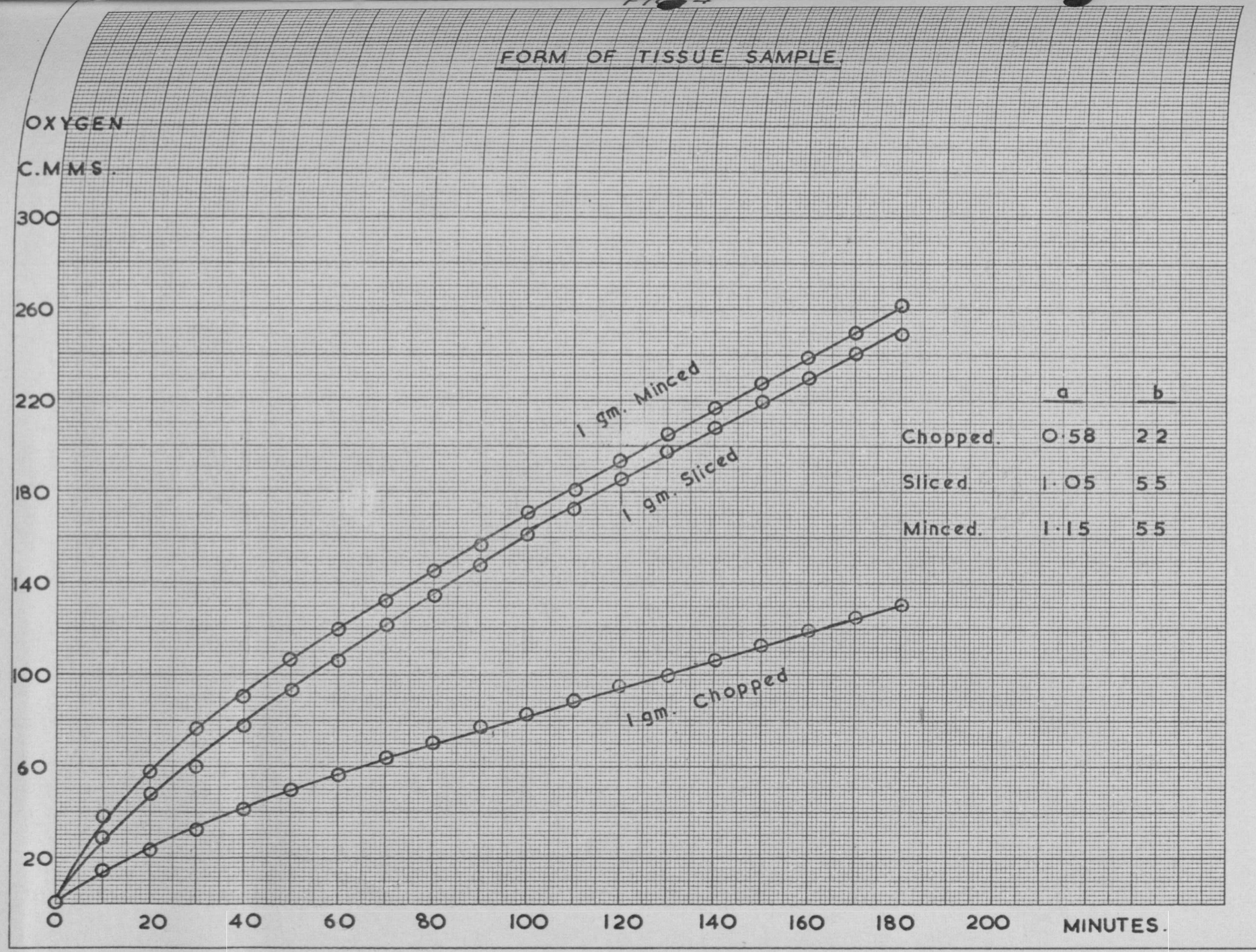
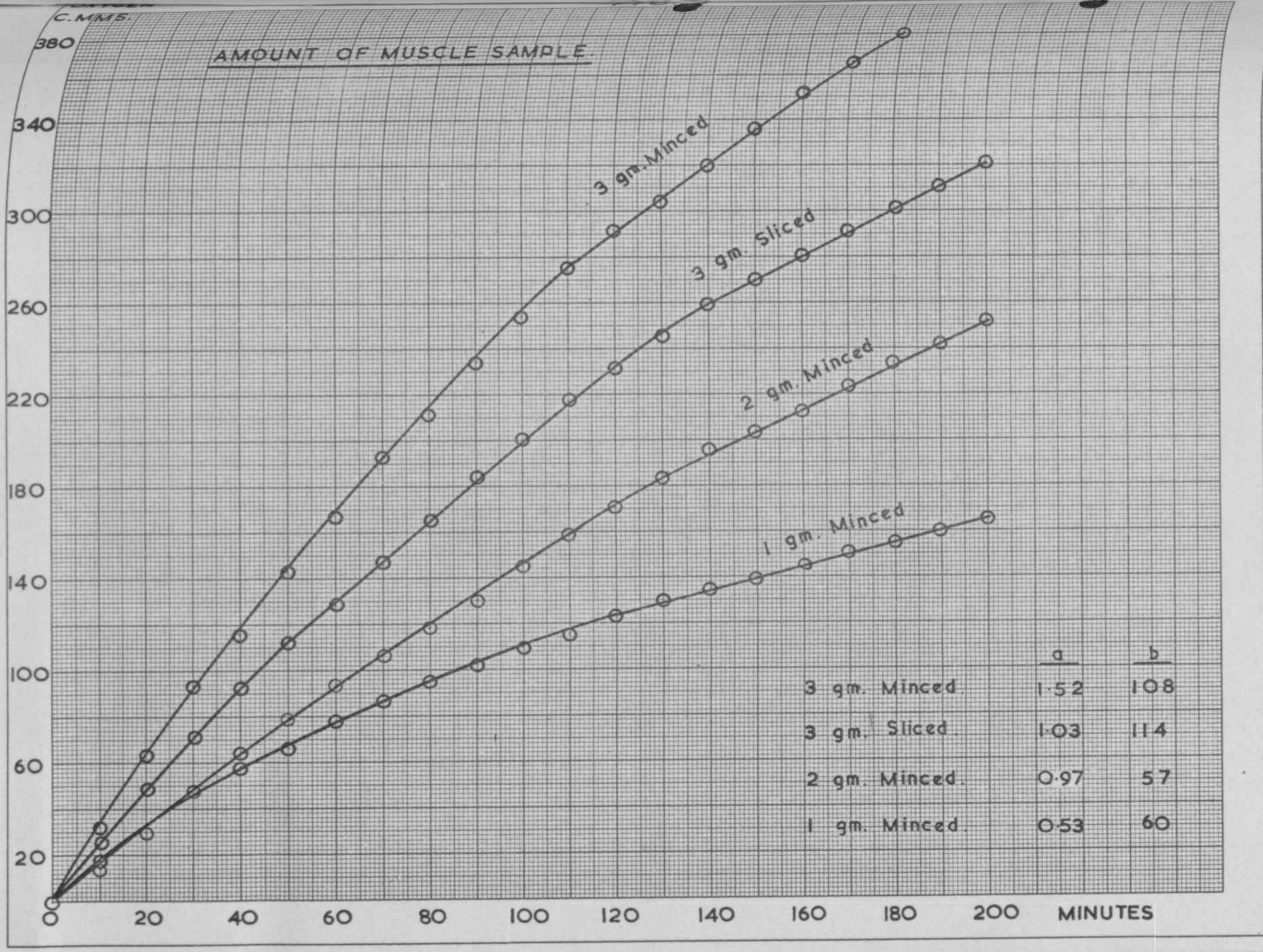

COMPONENTS OF NORMAL CURVE
OXYGEN
C.M.M.S.
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES
Slope = a = 1.00
b = 82 c.m.m.s.

FIG 4


FORM OF TISSUE SAMPLE.
OXYGEN
C.MMS.
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES.
1 gm. Minced
1 gm. Sliced
1 gm. Chopped
a
b
Chopped. 0·58 22
Sliced. 1·05 55
Minced. 1·15 55


C.MMS.
AMOUNT OF MUSCLE SAMPLE.
380
340
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES
3 gm. Minced
3 gm. Sliced
2 gm. Minced
1 gm. Minced
a
b
3 gm. Minced 1·52 108
3 gm. Sliced 1·03 114
2 gm. Minced. 0·97 57
1 gm. Minced. 0·53 60

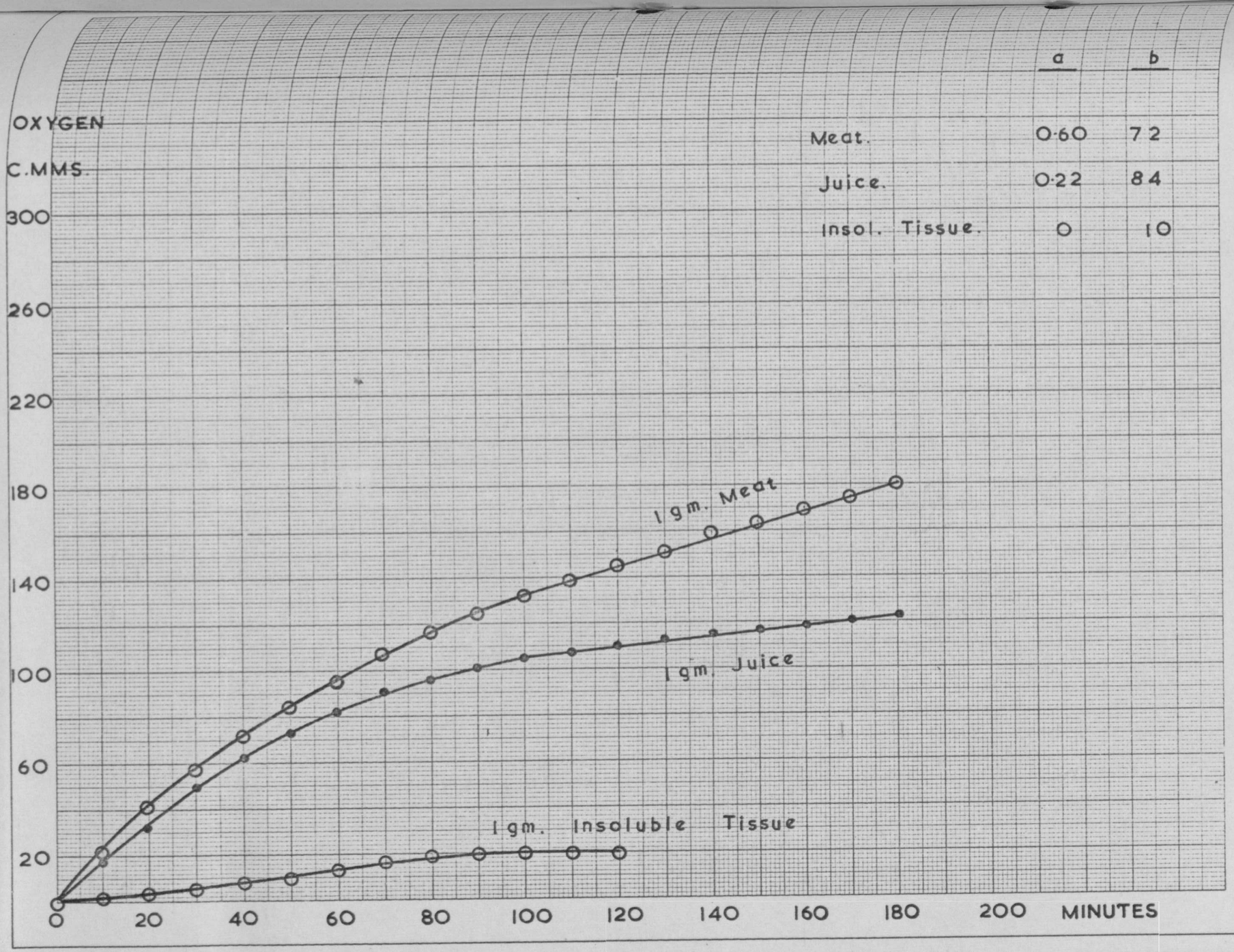

OXYGEN
C.MMS.
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES
1 gm. Meat
1 gm. Juice
1 gm. Insoluble Tissue
a
b
Meat. 0·60 72
Juice. 0·22 84
Insol. Tissue. 0 10

FIG. 7

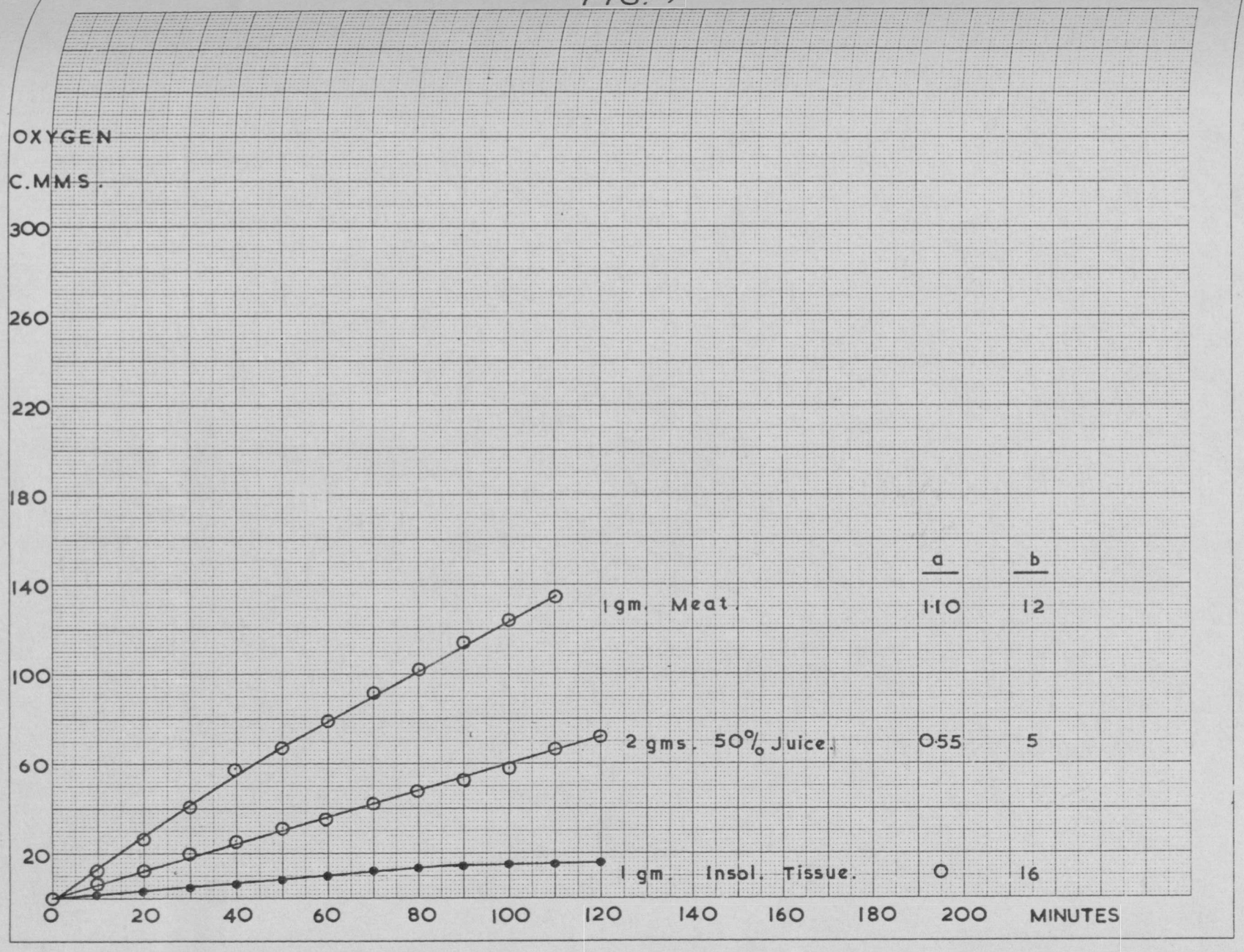
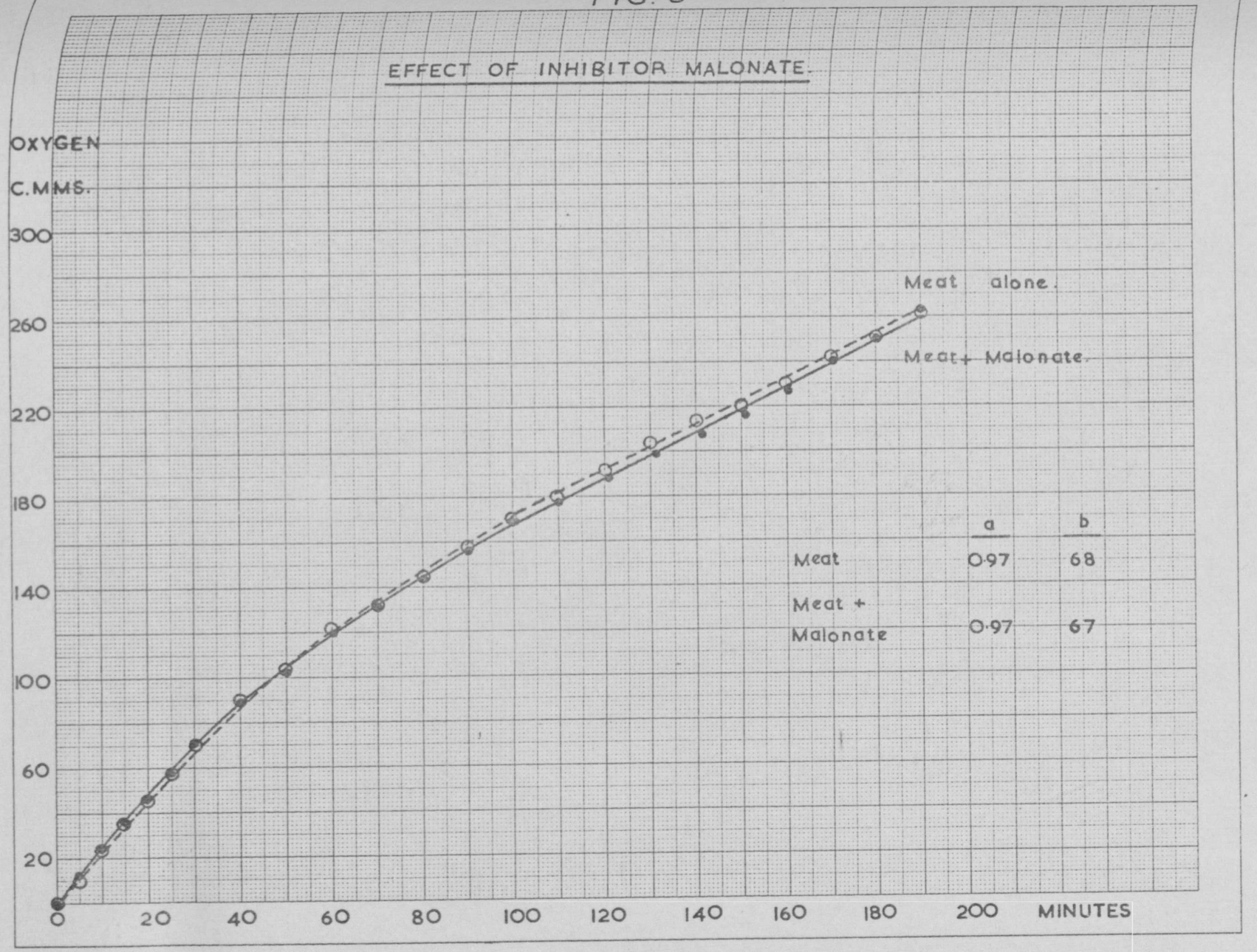
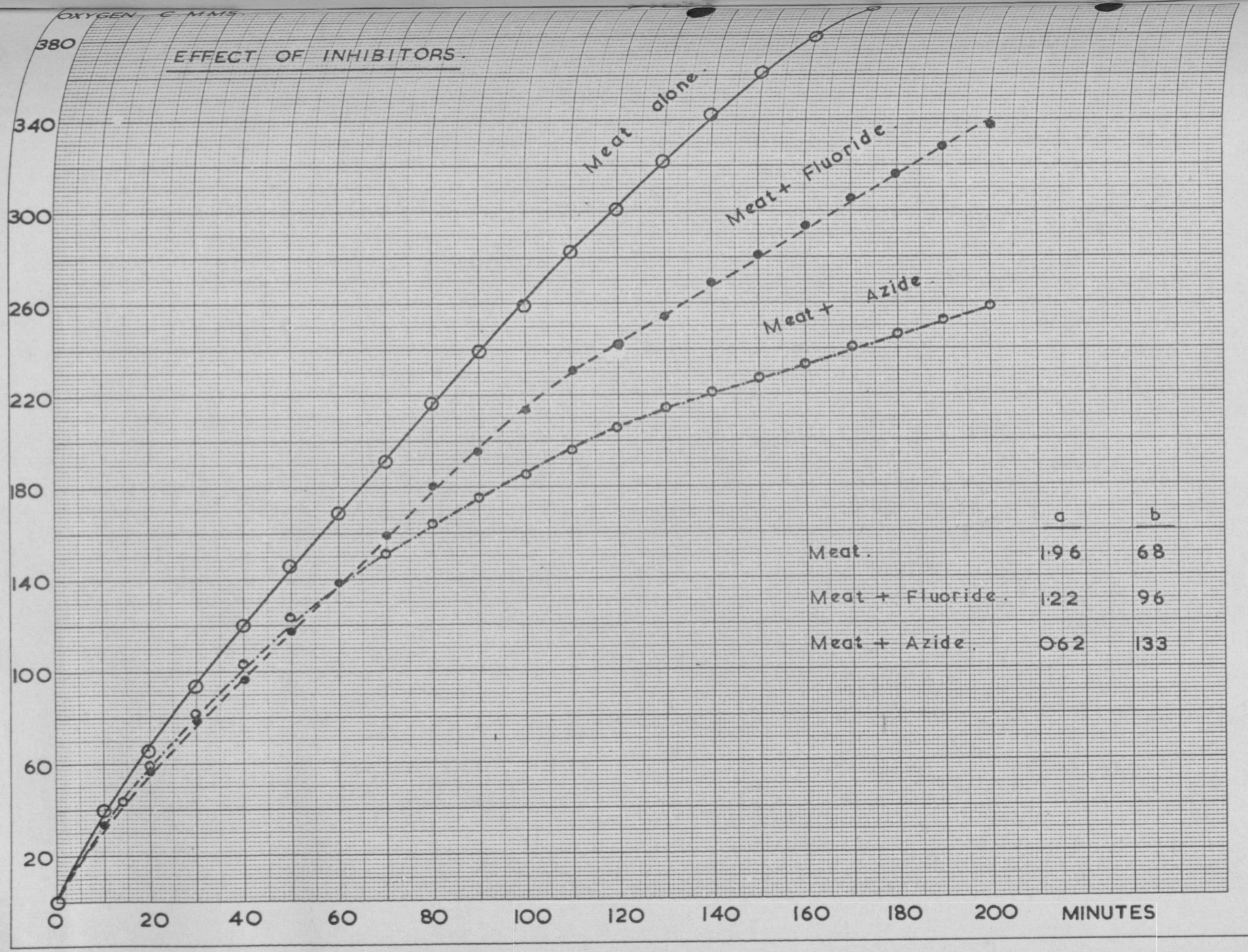

OXYGEN
C.MMS.
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES
a
b
1 gm. Meat.
1·10
12
2 gms. 50% Juice.
0·55
5
1 gm. Insol. Tissue.
0
16

FIG. 8


EFFECT OF INHIBITOR MALONATE.
OXYGEN
C.MMS.
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES
Meat alone.
Meat+ Malonate.
a
b
Meat
0·97
68
Meat +
Malonate
0·97
67


OXYGEN C MMS
EFFECT OF INHIBITORS.
380
340
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES
Meat alone.
Meat + Fluoride.
Meat + Azide.
a
b
Meat. 1·96 68
Meat + Fluoride. 1·22 96
Meat + Azide. 062 133

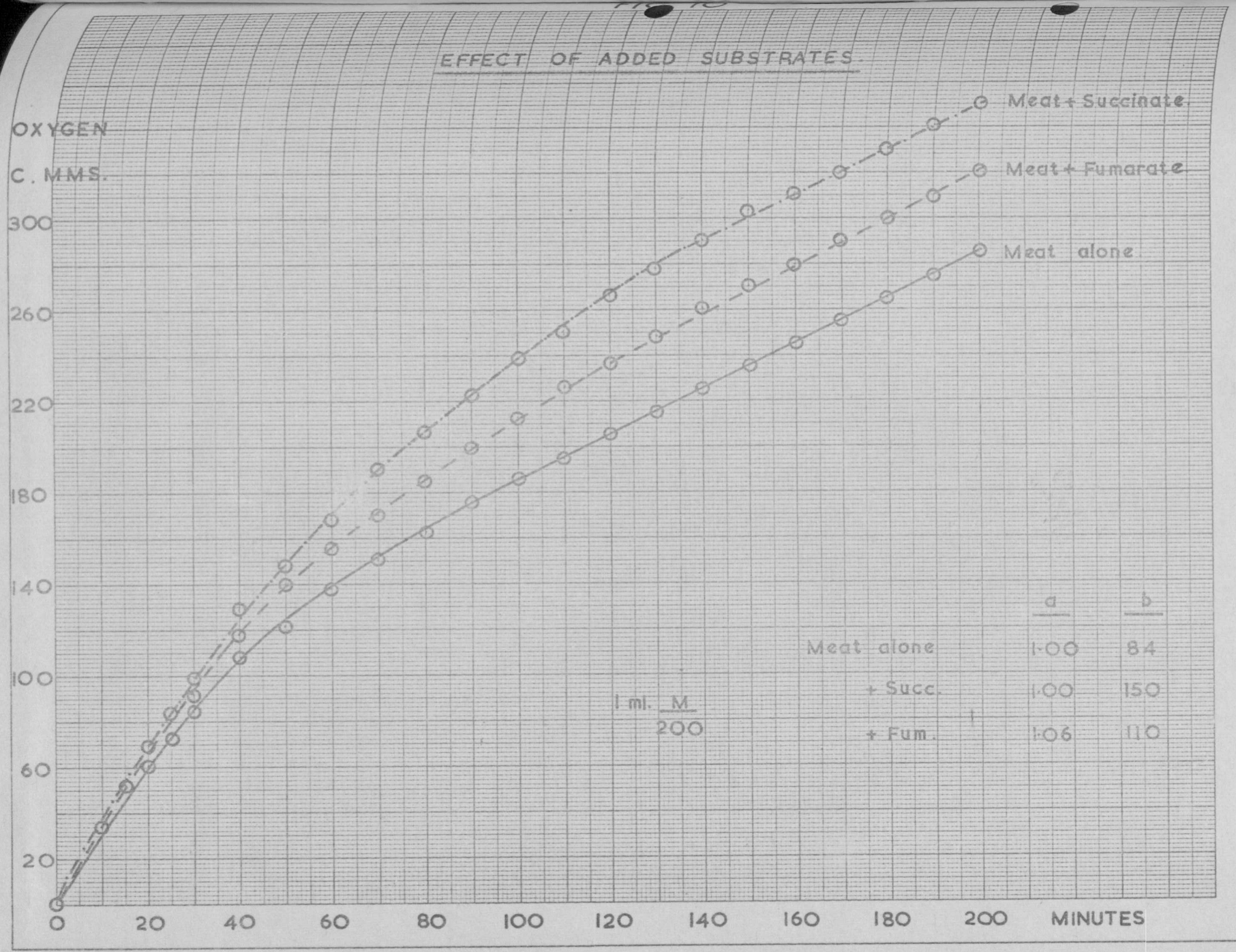
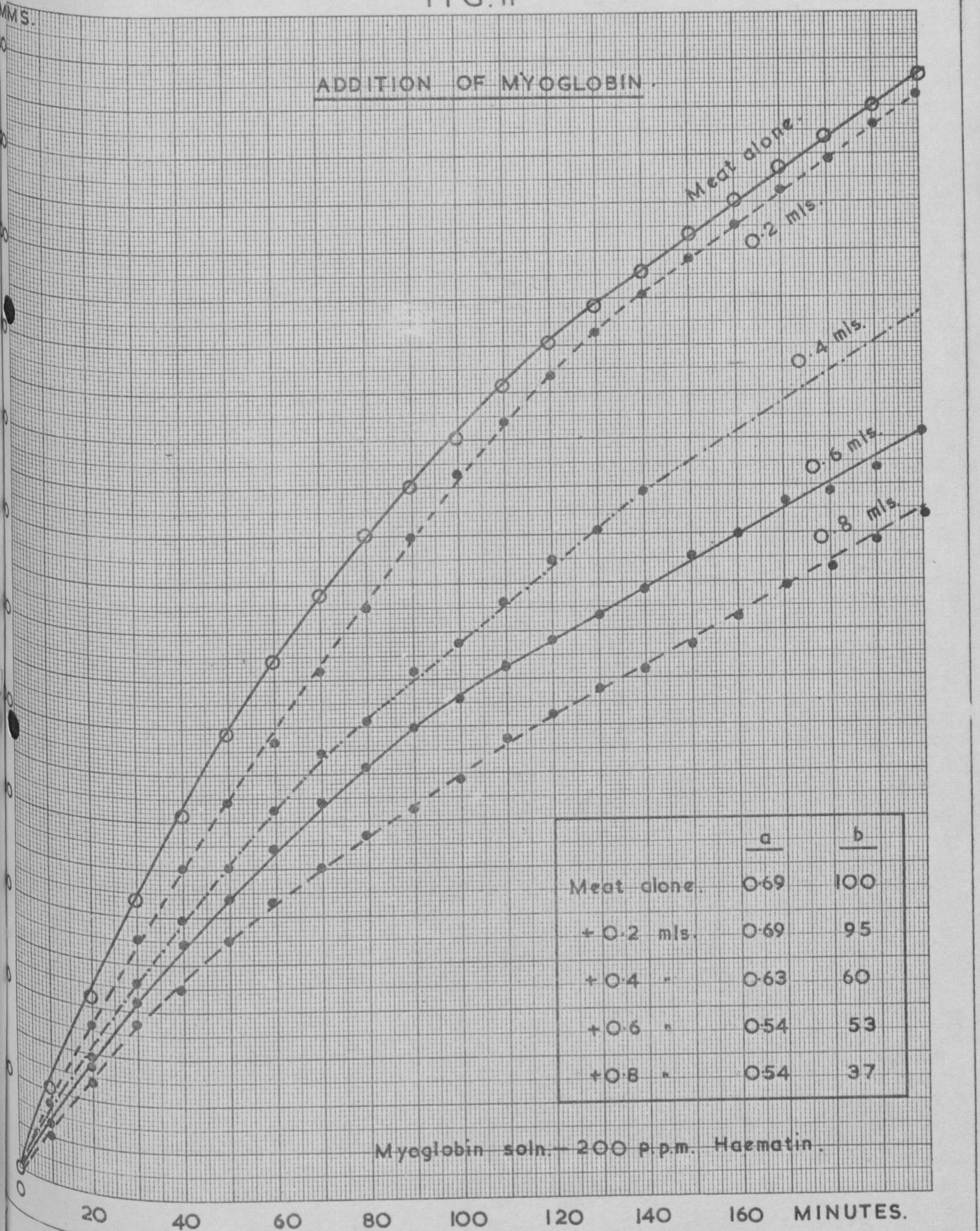

EFFECT OF ADDED SUBSTRATES.
OXYGEN
C. MMS.
300
260
220
180
140
100
60
20
0
20
40
60
80
100
120
140
160
180
200
MINUTES
Meat + Succinate
Meat + Fumarate
Meat alone
1 ml. M/200
a
b
Meat alone 1·00 84
+ Succ. 1·00 150
+ Fum. 1·06 110

FIG. II

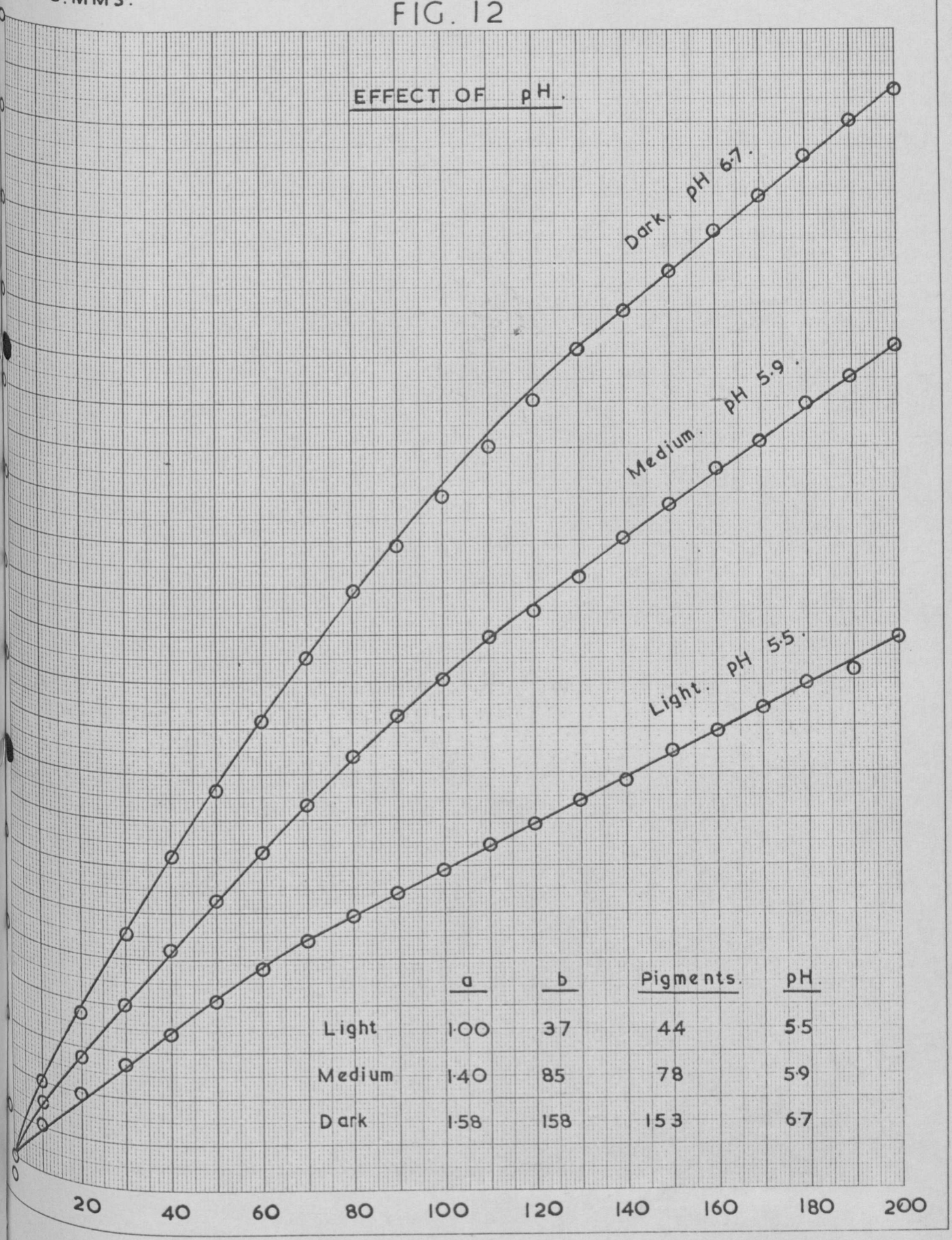


	a	b
Meat alone.	0·69	100
+ 0·2 mls.	0·69	95
+ 0·4 "	0·63	60
+ 0·6 "	0·54	53
+ 0·8 "	0·54	37

Myoglobin soln. — 200 p.p.m. Haematin.

YGEN C.MMS.

FIG. 12



	a	b	Pigments.	pH.
Light	1·00	37	44	5·5
Medium	1·40	85	78	5·9
Dark	1·58	158	153	6·7

EFFECT OF pH. FIG. 13

pH.	a	b
5·0	0·15	1
5·5	0·27	3
6·0	0·27	7
6·5	0·49	18
7·0	0·57	59
7·5	0·65	62
8·0	0·67	90

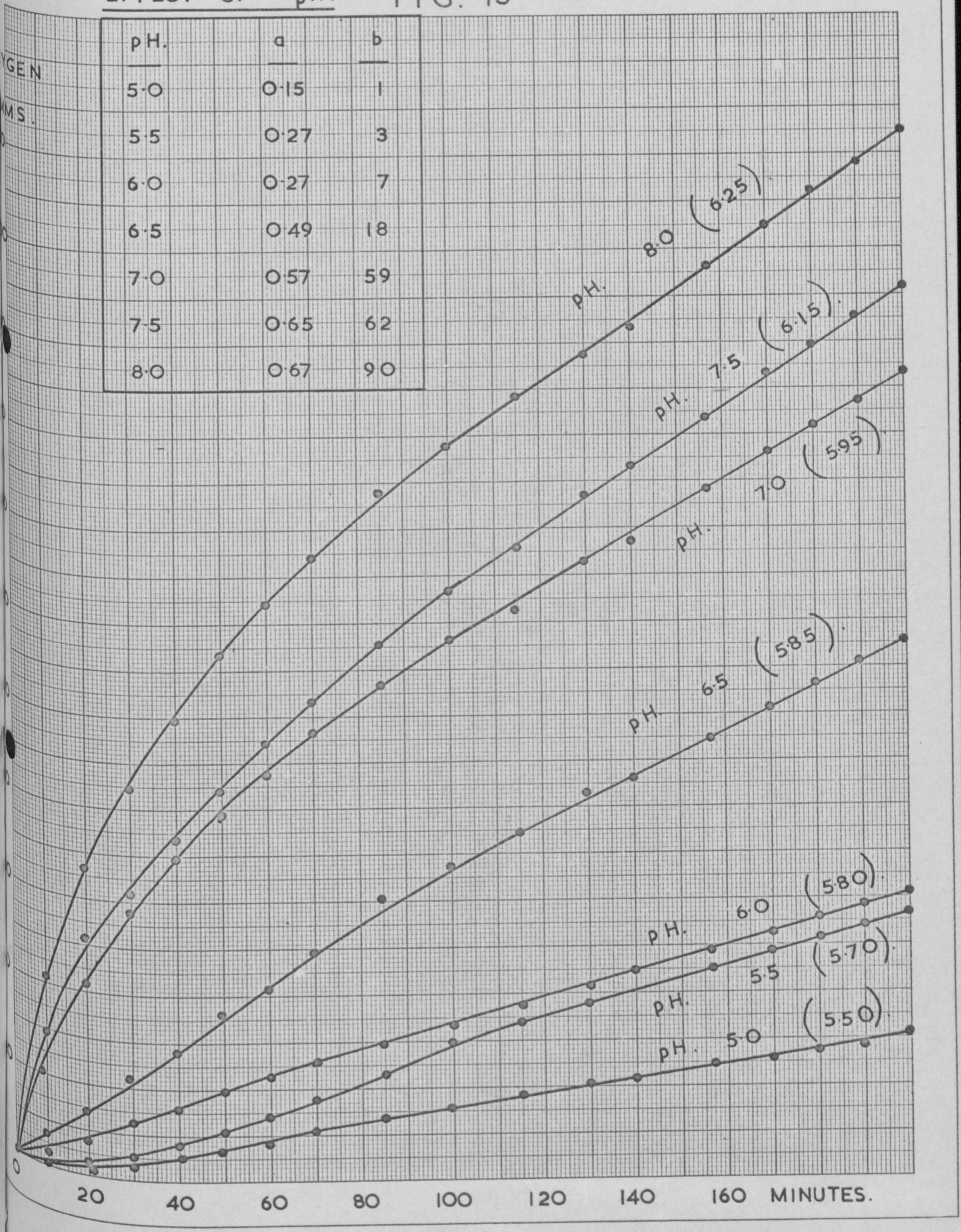
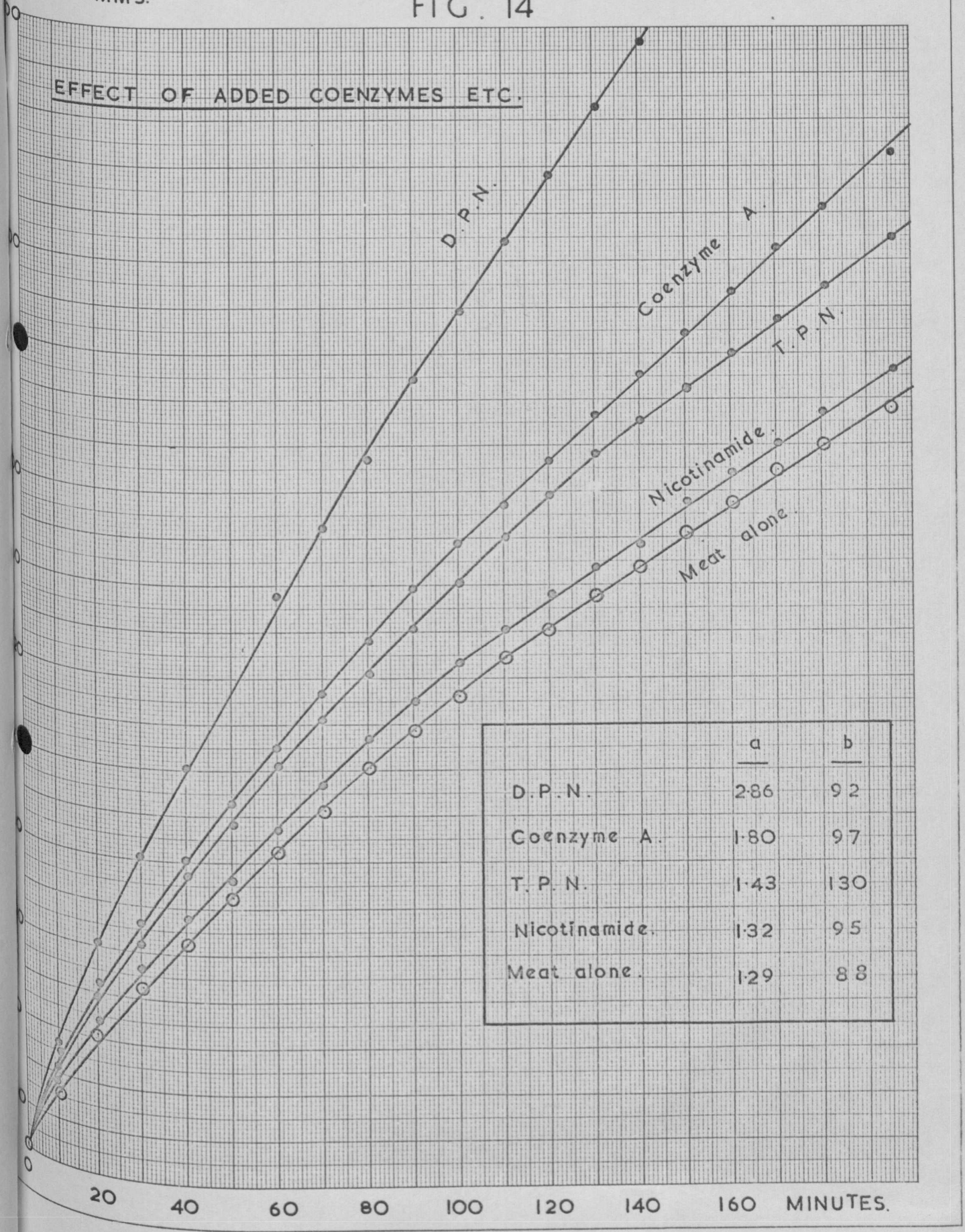


FIG. 14



	a	b
D.P.N.	2·86	92
Coenzyme A.	1·80	97
T.P.N.	1·43	130
Nicotinamide.	1·32	95
Meat alone.	1·29	88