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BIOCHEMICAL PROPERTIES OF PORK MUSCLE IN RELATION TO CURING

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CORRIGENDA

page 1 line 34 delete 'of oxygen'
page 2 line 42 for 'an' read 'any'
page 7 line 22 for 'inclusions' read 'inclusion'
page 15 line 29 for 'an' read 'as'
page 16 line 25 for 'larger' read 'large'
page 18 line 16 for 'aammonium' read 'ammonium'
page 24 line 3 (below table) for 'soline' read 'saline'

The work described in this Report is being carried out for the United States Department of Agriculture with the aid of funds made available under U.S. Public Law 480. The work is not completed and it is hoped to present at future European meetings further interim reports of progress.

Grateful acknowledgement is made of the financial assistance which has made this work possible.

General Introduction

It was shown by Haldane[1] that the characteristic colour developed during the curing of meat is due to the formation of nitrosomyoglobin by the reaction of nitric oxide with the reduced form of the muscle pigment. Satisfactory colour development thus involves the prior formation of both reactants, the reduced pigment by conversion of the oxy- and met- forms and the nitric oxide by the reduction of nitrite, either introduced as such during curing or itself formed by reduction of nitrate introduced as saltpetre. The necessity for reducing conditions has been demonstrated by Brocks[2], who showed that sodium nitrite reacted with hemoglobin to yield exclusively nitrosohemoglobin only in the presence of a reducing agent such as dithionite; in the absence of a reducing agent, equimolecular proportions of nitrosohemoglobin and methemoglobin were produced, the brown colour of the latter predominating in the mixture.

The nature and sources of the reducing systems operative during commercial curing are not clearly established. Bacterial reduction of both nitrate and nitrite is known[3] and chemical reducing agents (ascorbic and erythorbic acids) are used in commercial curing as processing aids. The present investigation is concerned with the significance of the surviving biochemical systems of the meat itself from this point of view. Two main lines of approach can be envisaged. In the first place, the ability of the muscle system to effect reduction will be reflected in the oxidation-reduction potential of the system. Knowledge of the level and degree of variation of this potential is thus of interest, although it must be recognised that in a system as complex as meat muscle, electrode measurements must be largely empirical in character. Efforts are being made to establish conditions under which reproducible and meaningful values of oxidation-reduction potential in meat muscle can be obtained. These efforts are described in the first part of the Report.

A more specific approach is through the study of the surviving enzyme systems. It is known that the interior of large blocks of meat muscle becomes anaerobic through the scavenging of oxygen of oxygen by these enzyme systems and the survival of specific respiratory enzymes has been demonstrated[4,5]. It was originally proposed to use measurements of endogeneous respiration of pork muscle as an index of the activity of these enzymes but it is now felt to be necessary also to search for enzyme systems capable of effecting the specific reductions involved. The progress of this search to date is described in Part II.

PART I - ELECTRODE POTENTIAL STUDIES

Introduction

The oxidation-reduction potential of simple systems can be measured either by means of redox dyes or potentiometrically, the electrical method having the advantage that it does not introduce a further oxidation-reduction system. When an isolated gold or platinum electrode is introduced into a tissue it tends to capture or release elctrons until it is in equilibrium with the oxidation-reduction potential prevailing in the tissue. In practice a definite potential is taken up, which can be measured, provided no current is allowed to flow[6].

Measurements of the potential of a platinum spear electrode inserted into a block of horse muscle under a paraffin wax seal have been made by Barnes and Ingram[7]. These workers found a progressive fall in potential from an initial value of approximately +200 mv to -150mv or below. Preliminary experiments using platinum electrodes of similar pattern inserted at intervals into pork muscle maintained under a layer of paraffin indicated that the potentials acquired by these electrodes were influenced by factors other than the oxidation-reduction potential of the systems.

Subsequent studies have been directed towards establishing the conditions necessary for the observation of significant values of electrode potentials presenting a true indication of the oxidation-reduction potential of the system; for this purpose aqueous meat extracts have been employed in place of muscle blocks to eliminate heterogeneity.

Experimental

Electrode potential determinations:

Electrodes of platinum (of the spear type of Barnes and Ingram), and of gold, silver and aluminium in the form of wire, were inserted into both pork muscle blocks and aqueous pork muscle extracts maintained under a layer of liquid paraffin. Potential measurements were made with a potentiometer of input resistance 10^{10} ohms (Model 23A Direct Reading pH meter, Electronic Instruments Ltd., Richmond, Surrey) using a calomel reference electrode and an agar-potassium chloride bridge. pH determinations were made with the same instrument, using a glass electrode.

In later experiments, anaerobic conditions have been maintained with a positive pressure of argon introduced through copper tubing, and connections from the electrodes have been made in co-axial cable; after defatting with carbon tetrachloride, the glass tubing supporting the electrodes has been treated with several coats of Repelcote Silicone water repellent (Hopkin and Williams, Chadwell Heath, Essex), to reduce an electrical leakage due to moisture. Under these conditions the

introduction of a 100 megohm damping resistor across the potentiometer has been without effect upon the potential observed, indicating that the system is of adequate impedance for such measurements.

Through the courtesy of Dr. D. B. Cater of the Department of Radiotherapeutics, University of Cambridge, an opportunity arose to examine the potentials exhibited by micro electrodes of platinum, gold and stainless steel[8] upon insertion into a pork extract maintained anaerobically under a stream of nitrogen in the apparatus of Dr. Cater and his co-workers[9]. The potentials thus obtained were measured with an apparatus of input resistance of at leat 10^{12} ohms, and recorded by means of a Cambridge automatic recording galvanometer. In the course of these tests additions were made of a 0.01 % solution of potassium - 5:5' - indigo-disulphonate (added in 1.0 ml. aliquots to 20 ml. of meat extract); prior to addition the dye solutions were bubbled with nitrogen for at least 15 minutes to remove dissolved oxygen.

Preparation of aqueous extracts:

Pork muscle from the hock of the animal (obtained by local purchase usually within 24 hours after slaughter) was minced and ground with an equal weight of water in a pestle and mortar; the clear red supernatant obtained by centrifugation at 2500 r.p.m. has been employed after filtration for studies requiring a homogeneous fluid.

Results

Figs 1a and 1b typify the behaviour of platinum spear electrodes and of hard drawn gold wire electrodes after insertion into pork muscle tissue (sow shoulder meat) maintained under a layer of paraffin at room temperature. The pattern of decay was substantially reproduced by electodes inserted at intervals, approximately two hours being required in some instances for equalization of potentials within 25-50mv. After this period electrode potentials remained substantially unaltered up to 24 hours.

The maximum variation between the potentials of three platinum spear electrodes inserted simultaneously into a homogeneous pork extract was $\pm$10 mv. The insertion of such electrodes at intervals produced patterns (Fig. 2) similar to those obtained with muscle tissue in that a considerable variation of potential existed between successively inserted electrodes at any one time; this variation was not due to lack of homogeneity, as it was not affected by agitation of the solution. The rapid initial fall in potential shown by the electrodes in the muscle blocks was, however, not always reproduced in the extracts. No appreciable alteration in pH occurred during the periods of test. Generally similar results were obtained with gold wire electrodes (Fig. 3). Silver wire electrodes appeared to show less tendency for initial drift, but discrepancies between

FIG 1a PLATINUM SPEAR ELECTRODES

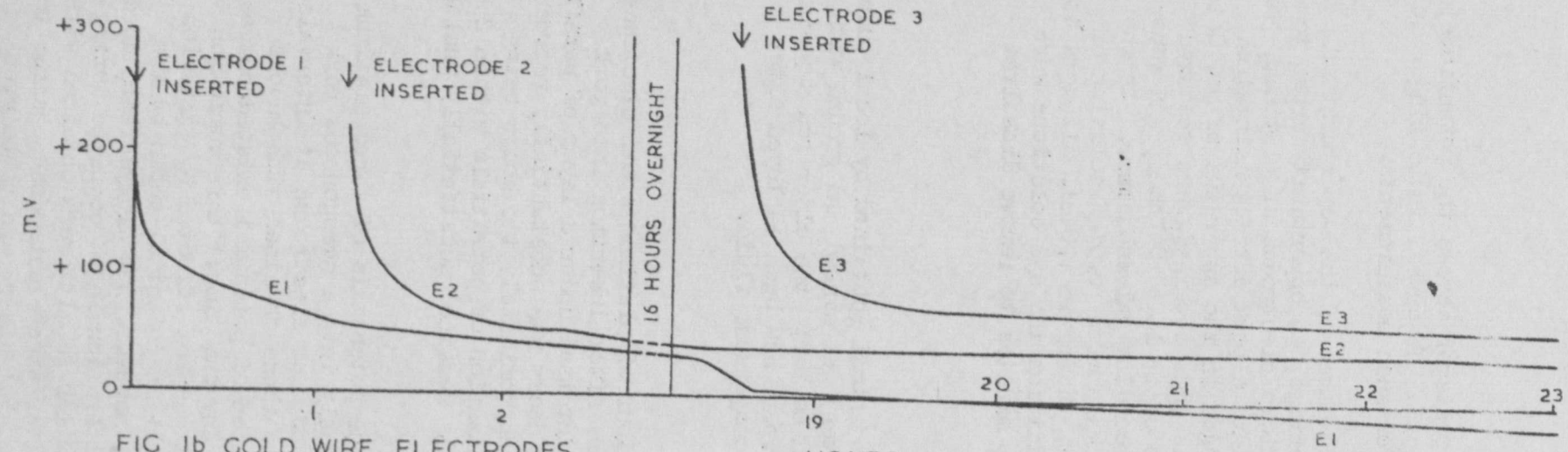


FIG 1b GOLD WIRE ELECTRODES

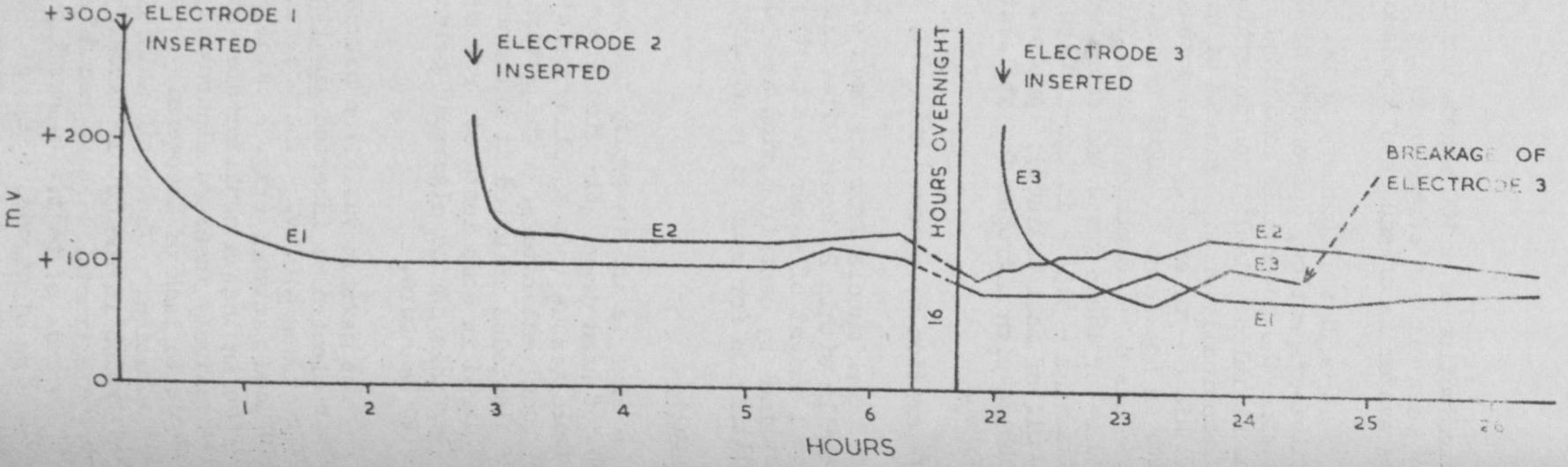


FIG.1. THE POTENTIALS OF a) PLATINUM SPEAR AND b) GOLD WIRE ELECTRODES

FIG. 1. THE POTENTIALS OF a) PLATINUM SPEAR AND b) GOLD WIRE ELECTRODES

FIG. 2

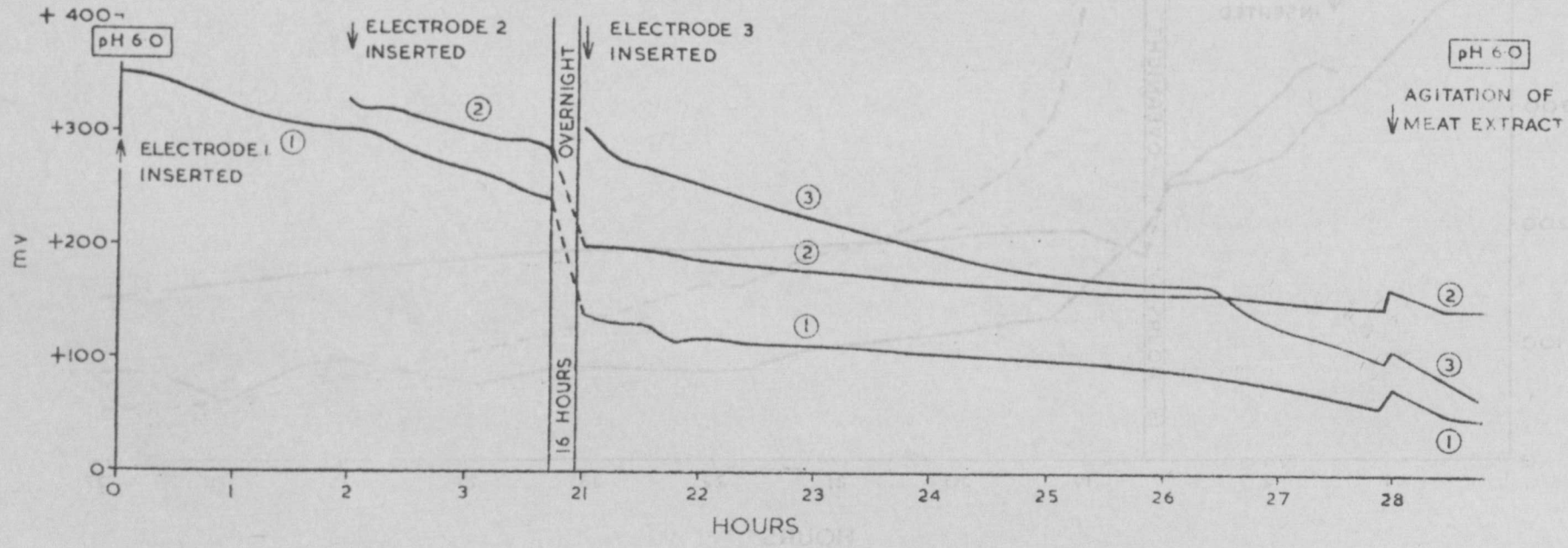


FIG. 2. THE POTENTIALS OF PLATINUM SPEAR ELECTRODES AFTER INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE UNDER PARAFFIN

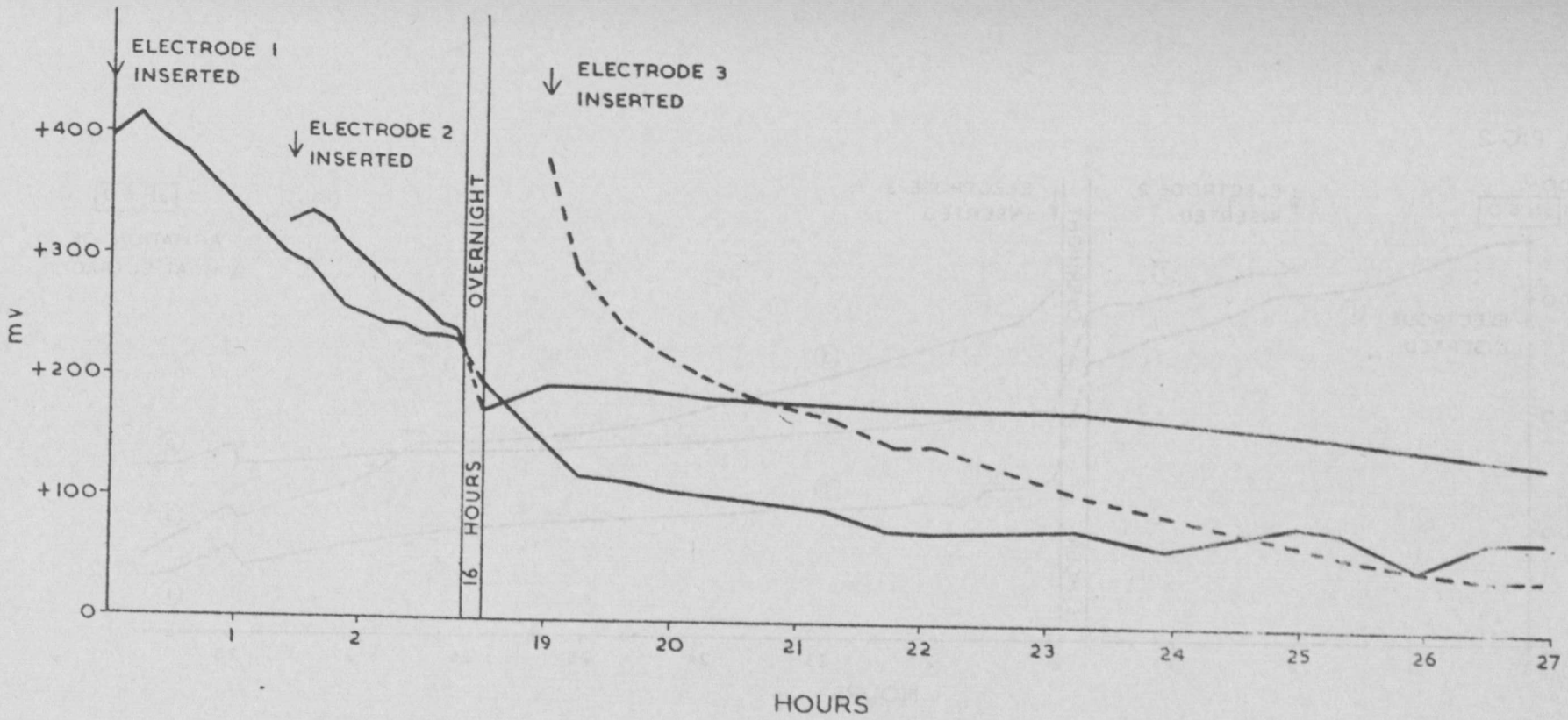


FIG. 3. THE POTENTIALS OF GOLD WIRE ELECTRODES AFTER INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE UNDER PARAFFIN.

successively inserted electrodes were again found (Fig. 4). Electrodes of aluminium wire gave much higher potentials and were clearly influenced by electrochemical factors other than the redox potential (Fig.5). After 20 hours these high potentials exceeded the maximum potentiometer range of +1.1 volt; further readings were later obtained by extending the range with a shunt.

The treatment of platinum spear electrodes with hydrogen prior to insertion into a fresh aqueous pork extract resulted inimmediate low potentials (Fig. 6), an effect which persisted even when the electrodes were subsequently maintained under a slight pressure of nitrogen for 30-60 minutes before use in an attempt to remove adsorbed hydrogen.

In the later work anaerobic conditions have been maintained in an atmosphere of argon introduced through copper tubing; this alteration has improved the response of platinum electrodes in pork muscle extracts with and without the inclusion of small amounts of methylene blue (Figs 7 and 8), reducing the maximum period for effective equalization of their potentials to the order of 30 minutes. The potentials acquired by these electrodes continued to record a steady drop from about +200 mv with respect to the hydrogen electrode when freshly inserted, levelling off around -250 mv within approximately 20 hours, with no appreciable change of pH under the anaerobic conditions. The platinum electrodes were sensitive to the inclusions of oxygen into the system, all alternations in potential being consistent with the changes in methylene blue colour indicated upon the graph.

Fig. 9 illustrates the decay in the potentials registered by micro electrodes of platinum, gold and stainless steel inserted into a pork extract maintained anaerobically under a stream of nitrogen; these observations were made in the apparatus of Dr. Cater and his co-workers[9]. With platinum and stainless steel the potentials observed fell rapidly from an initial value of about +250 mv, requiring only about 7 hours to reach the value of -400 mv with respect to the hydrogen electrode; although it is not shown on the graph of Fig. 9, these potentials had both levelled off at -400 to -450 mv after 19 hours. The potential of the gold electrode was initially lower and tended to fall more slowly in a stepwise fashion to a reasonably constant value around -300 mv within 11-12 hours. At the lower levels of potential the platinum and stainless steel electrodes were very sensitive to cessation of the stirring with nitrogen gas, showing a marked fall in potential, whereas the gold electrode showed only a very slight response to the altered conditions. Resumption of the flow of nitrogen restored the platinum and stainless steel potentials to their former levels, and the effect is thought to be attributable to the accumulation of hydrogen of bacterial origin. Pork extracts exhibiting low electrode potentials rapidly decolorised small additions of potassium - 5:5' - indigo-disulphonate. Each addition of the dye gave an immediate sharp rise in the potentials of all three electrodes, followed by a rapid fall to a level slightly above the original value. Finally, after several additions, the decolorization of the dye became slower due to its

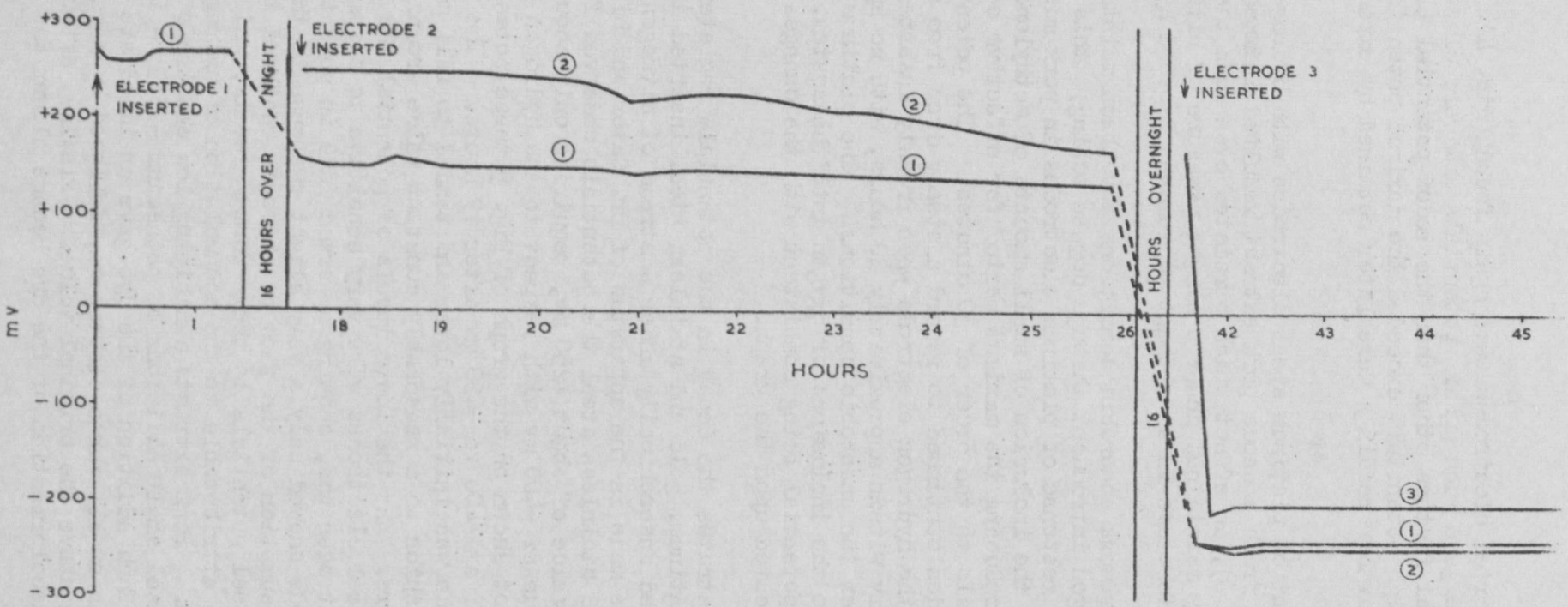


FIG 4 THE POTENTIALS OF SILVER WIRE ELECTRODES AFTER INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE UNDER PARAFFIN

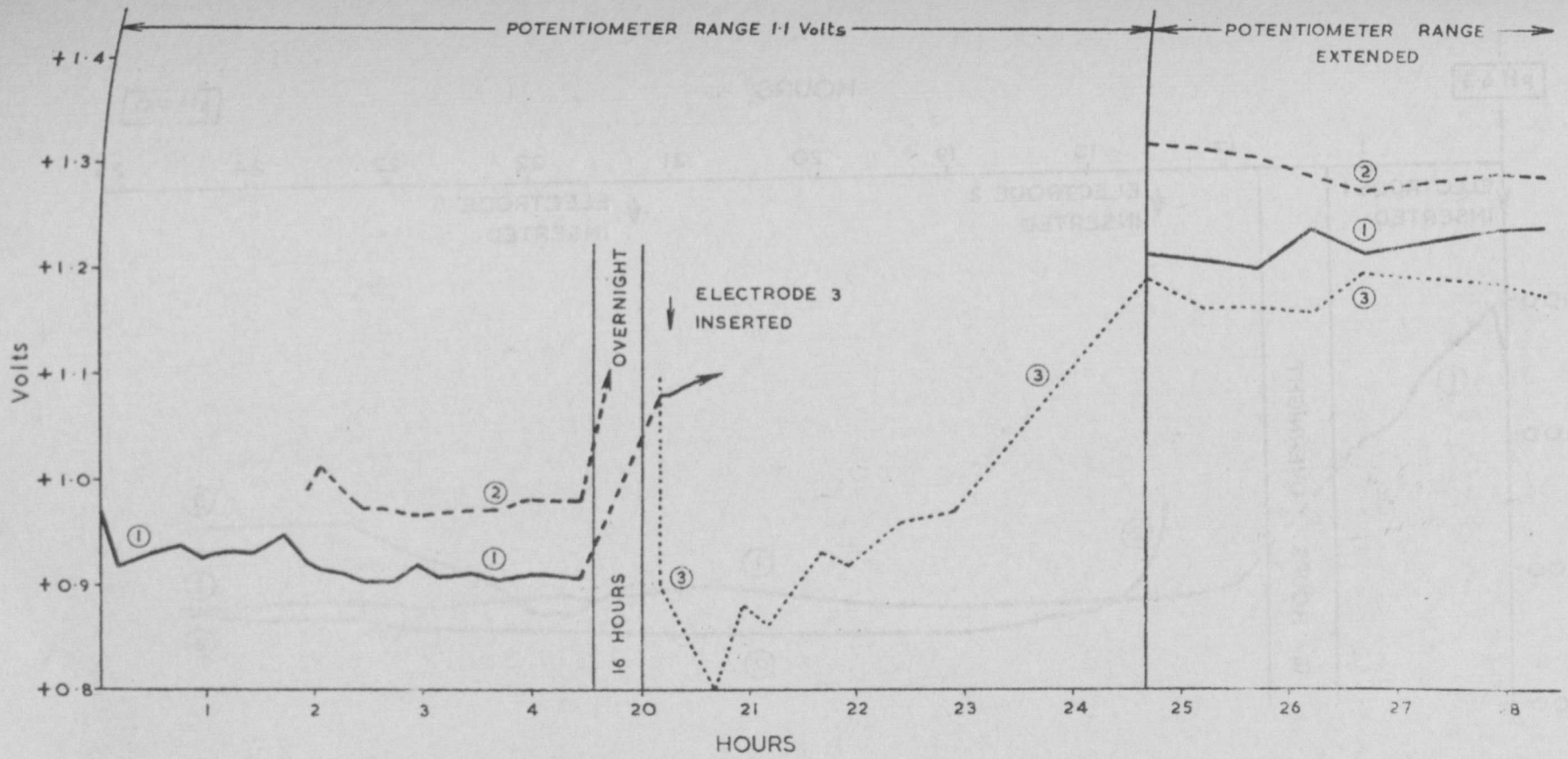


FIG. 5 THE POTENTIALS OF ALUMINIUM WIRE ELECTRODES AFTER INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE UNDER PARAFFIN

MAINTAINED UNDER STRICT ANAEROBIC CONDITIONS

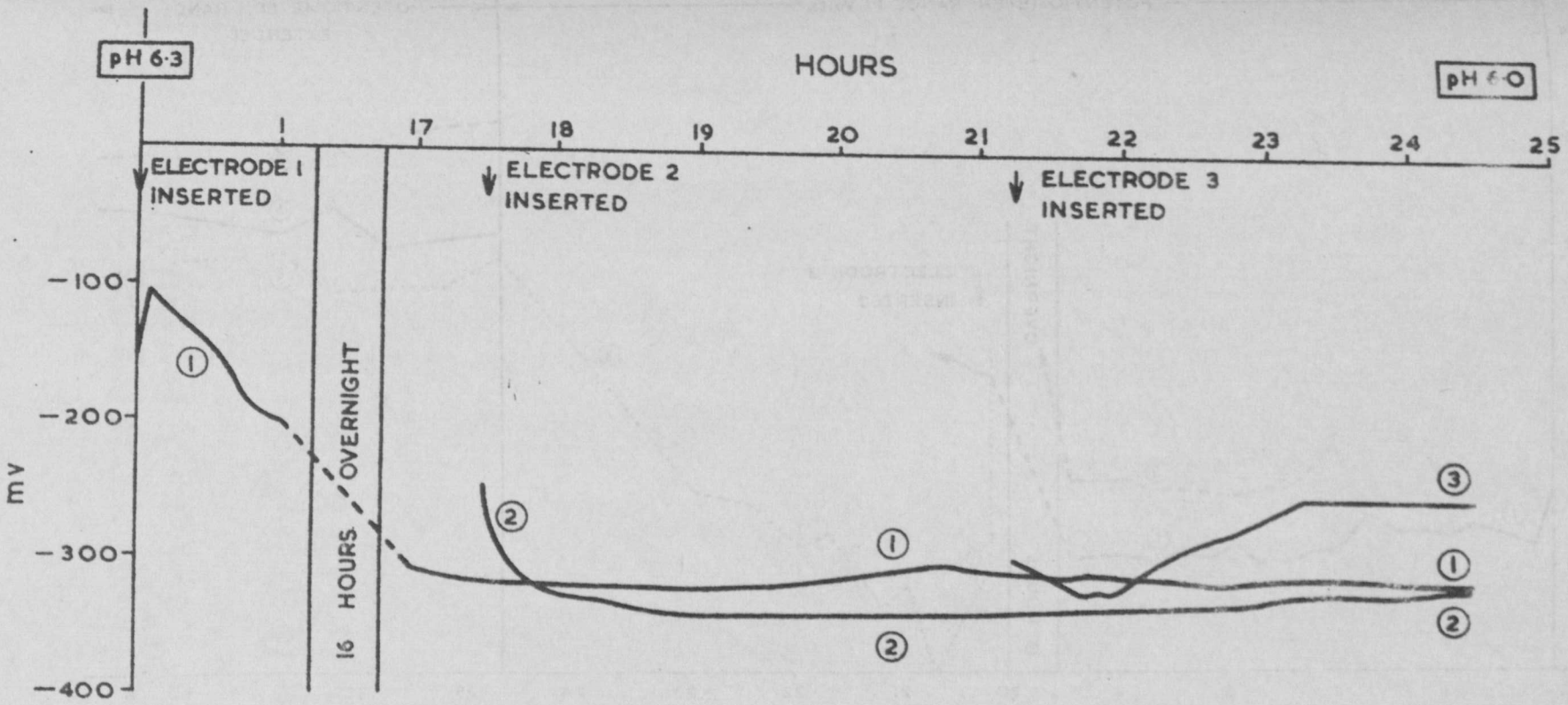


FIG 6. THE POTENTIALS OF PLATINUM SPEAR ELECTRODES TREATED WITH HYDROGEN BEFORE INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE UNDER PARAFFIN

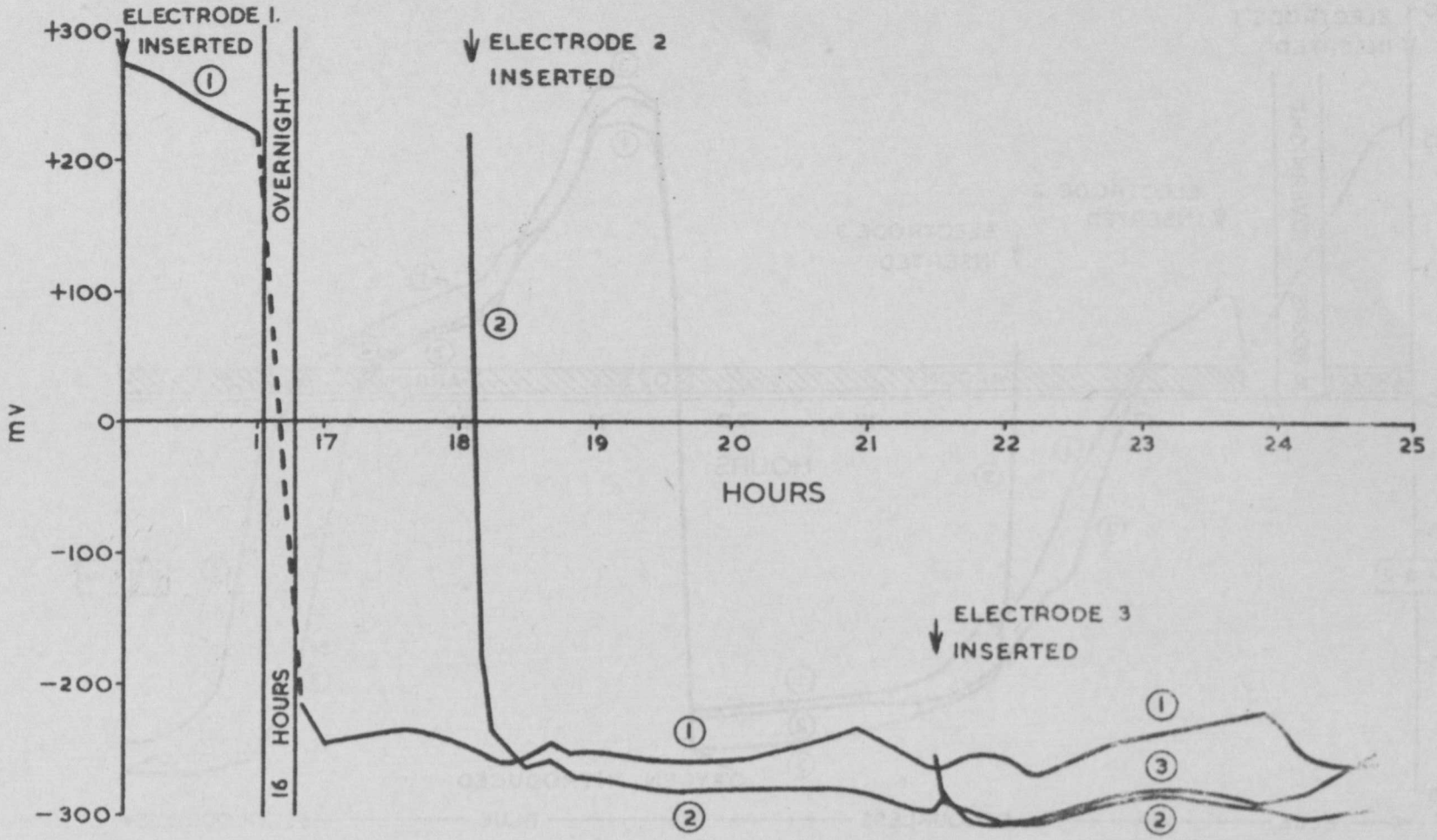


FIG. 7. THE POTENTIALS OF PLATINUM SPEAR ELECTRODES AFTER INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE MAINTAINED UNDER STRICT ANAEROBIC CONDITIONS IN AN ATMOSPHERE OF ARGON.

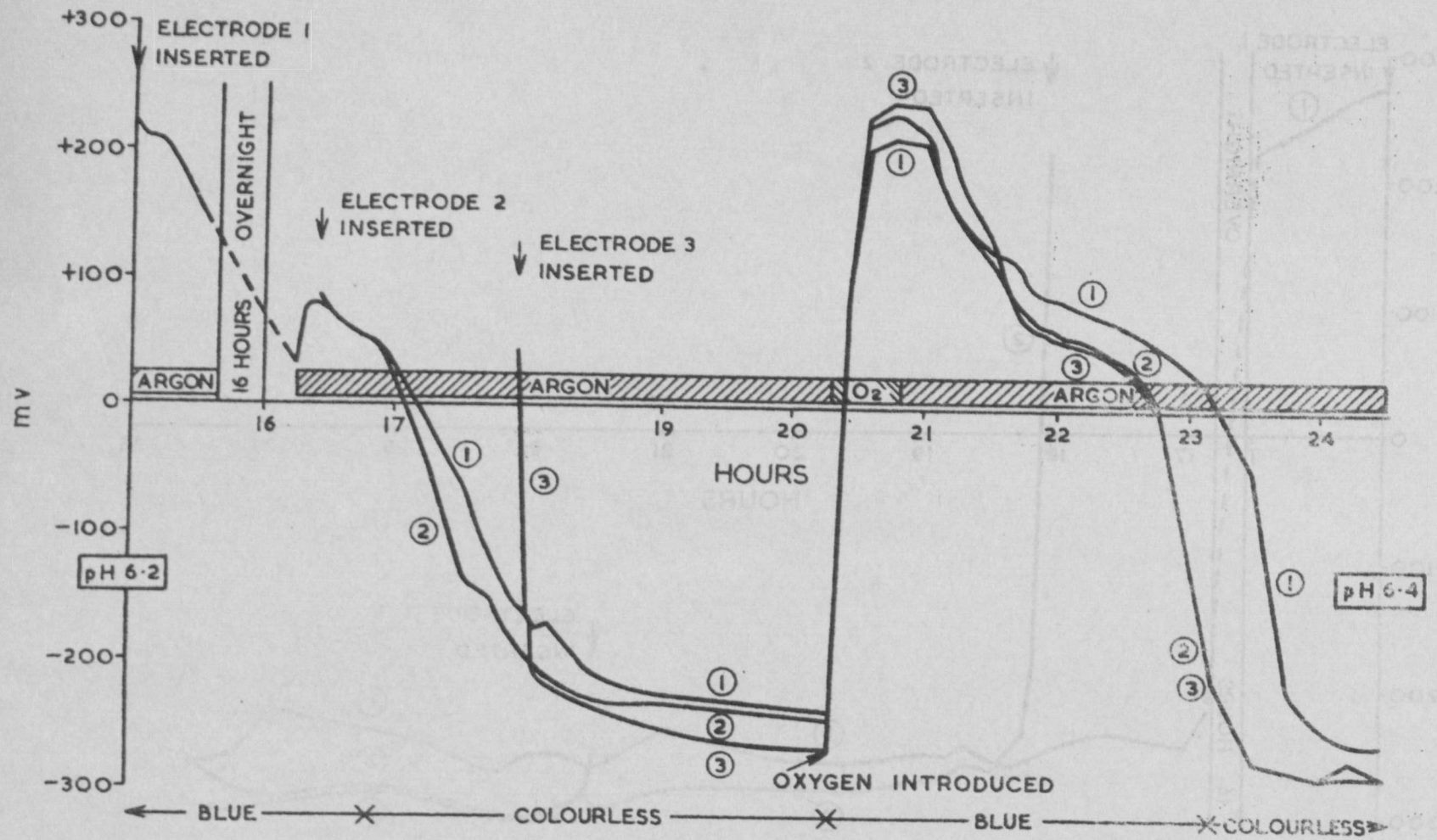


FIG. 8 THE POTENTIALS OF PLATINUM SPEAR ELECTRODES AFTER INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE INCLUDING METHYLENE BLUE AND MAINTAINED UNDER STRICT ANAEROBIC CONDITIONS

FIG. 9

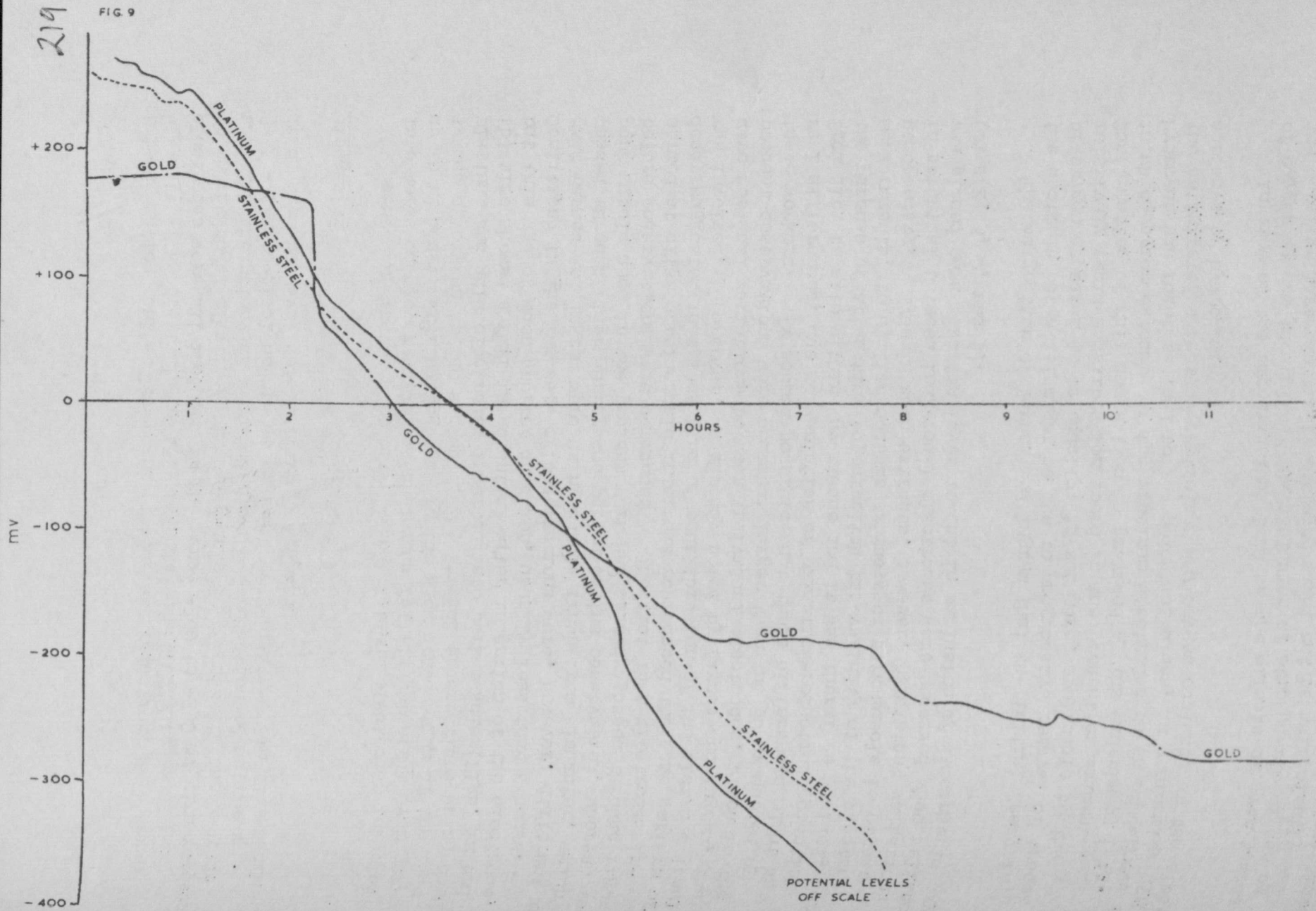


FIG. 9 THE POTENTIALS OF PLATINUM, GOLD & STAINLESS STEEL MICROELECTRODES AFTER INSERTION INTO AN AQUEOUS EXTRACT OF PORK MUSCLE MAINTAINED UNDER STRICT ANAEROBIC CONDITIONS IN AN ATMOSPHERE OF NITROGEN

poising action and at the point of complete reduction, as observed visually, the potentials of the platinum, stainless steel and gold electrodes were all included in the range -200 to -250 mv with respect to the hydrogen electrode, in reasonable agreement with one another and with the potential recorded for 100 % reduction of potassium - 5:5' - indigo-disulphonate at pH 7.0[1°]. Facilities for pH determination were not available in this experiment.

Discussion

Barnes and Ingram (loc cit), using platinum electrodes inserted into horse muscle, recorded a continuous fall in potential over periods up to 12 hours, attributing this to the slow consumption of the last traces of oxygen in the system. The initial observations using both platinum and gold electrodes inserted into pork muscle (Figs, 1a and 1b) also showed a fall in potential after insertion of the electrode, but this was of much shorter duration, usually less than 2 hours. Conditions in the two sets of observations were, however, different in that Barnes and Ingram were using muscle fresh from slaughter, which passed through rigor during the period of the observation, whereas the pork muscle used in the present work had already passed through rigor before observations were commenced. The fact that electrodes inserted at often considerable intervals reproduced the same pattern of decay suggested that some kind of equilibration of the elctrode itself was involved. Moreover, as already noted by Barnes and Ingram, there were residual variations between individual electrodes, which in the present observations were somewhat larger than the 30 mv quoted by these workers. It seemed possible that these differences might be real differences, due to variation of oxidation-reduction potential from site to site within the tissue and it was therefore decided, for the purpose of attempting to establish the validity of the electrode measurements, to utilise aqueous extracts of pork muscle to obtain homogeneity. Even in such extracts, however, persistent variations in potential between individual electrodes were observed when reliance was placed on a paraffin layer to obtain exclusion of atmospheric oxygen (Figs.2, 3, 4, and 5).

The suggestion of Barnes and Ingram that the initial decay in the elctrode potential might be due to progressive removal of traces of oxygen appeared to be equally, if not more, applicable to the relatively rapid equilibration found in the present experiments. Confirmation of this suggestion is provided by the experiment (Fig. 6) in which prior exposure of the platinum electrodes to an atmosphere of hydrogen was found to lead to low potential values, in conformity with the anticipated effect of replacing any oxygen occluded at the electrode surfaces by hydrogen.

In view of the emphasis thus laid on the effects of even traces of oxygen it was thought that the exclusion of atmospheric oxygen by the paraffin layer initially employed might not be sufficiently rigorous. More elaborate steps to ensure anaerobic conditions, involving

the maintenance of a positive pressure of oxygen-free argon, were found to lead to much more rapid equalization of potentials of platinum spear electrodes inserted at intervals into pork extracts (Fig. 7). The improved equalization confirms the importance of exclusion of oxygen and suggests that some of the variations encountered in the earlier tests may in fact have been due to failure of the paraffin covering layer to maintain adequately anaerobic conditions. The sensitivity of the platinum electrodes to oxygen was confirmed by deliberate introduction (Fig. 8).

The comparatively good agreement obtained over the range +150 to -150 mv between the potentials indicated by micro-electrodes of platinum, gold and stainless steel in a homogeneous meat extract under fully anaerobic conditions (Fig. 9) suggests that over this range these electrodes were truly inert. At low potentials, however, it is apparent that platinum and stainless steel, which are both occlusive, have deviated from the non-occlusive gold electrode. As already indicated above, it is thought that this divergence, in which the occlusive electrodes exhibited lower potentials, was due to the presence of hydrogen of bacterial origin. This suggestion is supported by the fact that these electrodes were sensitive to cessation of stirring; the indicated potential fell slowly when stirring ceased and recovered slowly again when stirring was resumed, presumably owing to the accumulation and subsequent dispersal of hydrogen. Furthermore, the fact that the occlusive electrodes were initially higher in potential than the non-occlusive gold electrode is consistent with a larger effect of adsorbed oxygen in the initial stages. It is clear that gaseous occlusion is a potential source of error in electrode measurements of this type; it is therefore imperative to avoid the use of occlusive metals as indicator electrodes and for this reason the use of platinum as an electrode material cannot be regarded as satisfactory.

PART II - RESIDUAL ENZYME STUDIES

Introduction

The persistence of enzyme systems capable of utilizing oxygen in meat muscle tissue after death is shown by the development of anaerobic conditions in large blocks of muscle tissue. The overall activity of these enzyme systems is measured by the endogeneous respiration of the tissues and measurements of endogeneous respiration were used in the earlier stages of this work. The earlier experiments were largely directed towards establishing satisfactory conditions of observation of residual enzyme activity and for this purpose the endogeneous respiration should be a perfectly valid criterion.

The fully intact respiratory chain is, however, probably not a requisite for the activity of enzyme systems capable of reducing nitrite, nitrate or metmyoglobin. The occurrence of nitrite and nitrate reductases in mammalian tissues is not generally recognised, although systems capable of reducing nitrate to nitrite, and nitrite to hyponitrite, hydroxylamine and ammonia have been observed in the liver[11]. Nitrite reductases of bacterial and other origin have been determined manometrically by the evolution of nitric oxide as a result of their action[12].

The enzymic reduction of metmyoglobin has usually been studied spectrophotometriaally by, for instance, the changes in absorption at 630 and 550mμ[13], but Gibson[14] has determined methemoglobin reductase in erythrocytes by a manometric method, using the uptake of carbon monoxide by the reduced pigment formed. As a necessary preliminary to studies of this type, a larger scale extraction of metmyoglobin from pig hearts was carried out. Crystalline preparations of myoglobin have been obtained from horse hearts[15] and from sperm whale[16], a value of 18,000 being reported for the molecular weight of the latter material. Crystallization of pork myoglobin has not yet been reported.

In studies using the purified pig myoglobin evidence was obtained of the formation of a nitric oxide-metmyoglobin complex analagous to the nitric oxide-methaemoglobin complex of Keilin and Hartree[17]. The preparation and properties of this pigment are separately described.

Experimental

Gas exchange studies:

Respiration studies and other experiments involving gas exchange were carried out in a conventional Warburg apparatus employing a shaking rate of at least 120 oscillations per minute. Observations to date have been made at a temperature of 37°C, but it is intended to adapt the apparatus to enable measurements at 5°C to be made. In the early stages of the work considerable difficulty was experienced in obtaining satisfactory replication of the rather small pressure changes involved and this necessitated a careful examination

of the apparatus and technique employed. The main sources of variation prooved to be non-uniformity of temperature and variable 'bedding-in' of the ground glass joints between the manometers and the flasks. The provision of a Uralite baffle above the bath heater elements and the use of vaseline petroleum jelly in place of lanoline for sealing the joints enabled satisfactory replication to be obtained.

Flasks and manometers were calibrated for interchangeable use by the method of Dickens[18]; pH values were determined with a glass electrode coupled to a direct reading meter (Model 23A; Electronic Instruments Ltd., Richmond, Surrey.).

Measurements of endogeneous respiration have generally been made in air, although a few observations were made in oxygen. The results given in the Report were obtained in air, except when specifically stated otherwise. Samples were prepared for testing by mincing (using a small tissue grinder supplied by A. Gallenkamp and Co., Ltd., of London), slicing with a Cathcart hand microtome (all slices being cut transversely to the direction of the muscle fibres) and chopping (with a McIlwain and Buddle tissue chopper[19] made available on loan through the courtesy of Mr. H. Mickle of Gomshall, Surrey). Supporting media employed have included 0.9 % saline solution, two phosphate buffers (Na_2HPO_4(0.1M)/HCl (0.02M) - pH 7.4) and Na_2HPO_4(0.013M)/KH_2PO_4(0.113M) - pH 6.0), a phosphate-citrate buffer (Na_2HPO_4(0.127M)/ citric acid (0.037M) - pH 6.0) and the phosphate-saline buffer of Krebs and Eggleston[20]. The proportion of supporting medium to muscle tissue has varied from 1:1 to 10:1 according to sample size (as necessitated by the particular activity being studied) and the capacity of the Warburg flasks employed.

Attempts to detect reduction of metmyoglobin have been based on the uptake of carbon monoxide by the reduced pigment formed. Detection of nitrite reduction has been based upon differential absorption of the nitric oxide produced, and in this connection three alternative solutions for absorption of nitric oxide have been examined. The alkaline sulphite solution of Divers[21] proved to be much more effective for this purpose than either ferrous sulphate solution or acidified potassium permanganate solution and was accordingly adopted for use. Alkaline sulphite has been reported not to absorb nitrous oxide[22] but has been found to act as a sluggish absorbent for oxygen. The same technique has been applied to the attempted detection of nitrate-reducing activity.

Spectrophotometric measurements:

Spectrophotometric observations on the metmyoglobin-nitric oxide complex and related pigments were made on a Unicam S.P. 500 spectrophotometer, using a 1 cm. glass cell.

Pig heart myoglobin preparation:

10 lb. of washed pig hearts were minced twice mechanically and macerated at room temperature with 2250 ml. distilled water. As much

as possible of the liquid was removed by pressing and the extracts were treated with a slight excess of basic lead acetate and the precipitate separated by centrifuging and filtration. The filtrate was adjusted to pH 6.8 and solid ammonium sulphate was added to give a concentration of 25 % (w/v), equivalent to 34 % of that of saturation at 15°C; a light buff precipitate was centrifuged off and discarded. Increasing the ammonium sulphate concentration to 60 % of saturation gave a dark brown precipitate which was not separated by centrifuging at 3000 r.p.m. but which could be collected by filtration. This precipitate dissolved completely in 3M phosphate buffer of pH 6.8; after exhaustive dialysis against running tap water of an aqueous solution in a Visking cellulose casing the non-diffusible residue was concentrated to dryness by freeze drying (through the courtesy of the Distillers Company Ltd., Burgh Heath, Surrey). Yield : 12 grams.

Attempts to crystallise the product by dialysis of an aqueous solution at pH 6.8 against saturated aammonium sulphate were unsuccessful, resulting in an amorphous powder only.

Results

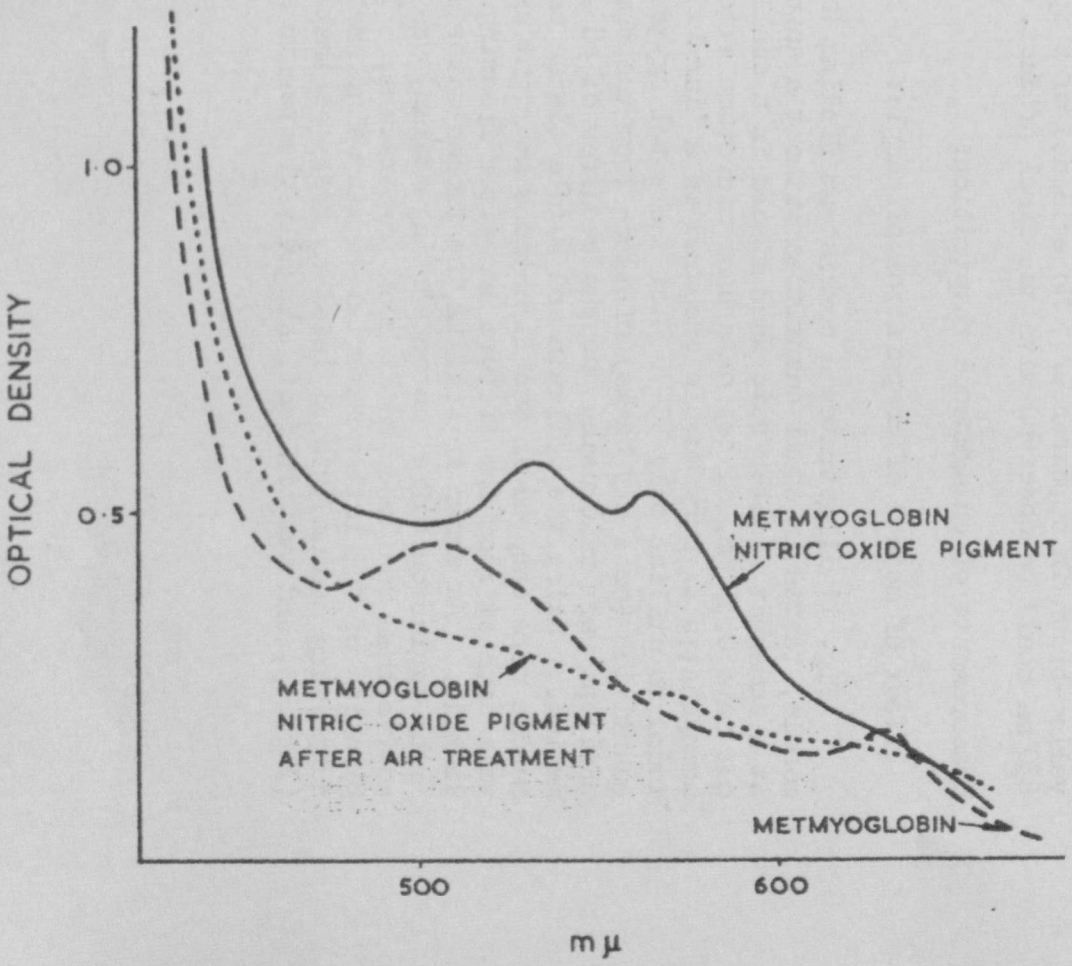
Observations on pig heart myoglobin:

The freeze dried product was dark brown in colour and contained 0.22 % of iron, equivalent to 1 gram atom of metal in 25,000g. 95 % of the material was soluble in distilled water to yield solutions containing 30-40 mg/ml. The absorption spectrum of the aqueous solution (Fig. 10) displayed the characteristic absorption bands (at about 505mμ and 630mμ) of the met pigment, oxidation having taken place during the extraction and purification procedure. Reduction with sodium dithionite yielded a cherry-red pigment showing a diffuse absorption band at about 550mμ (reduced myoglobin) and this reacted with oxygen and carbon monoxide to yield bright red pigments with sharper spectral bands at 544 and 579 mμ and 540 and 576 mμ respectively. It also formed with pyridine a protohemochrome showing bands at 524 and 557mμ in a Hartridge reversion spectroscope.

Measurements of the absorption of the brown met pigment at 630 mμ over a range of concentrations within which Beer's law was obeyed gave a value for the millimolar extinction coefficient (on the basis of 1 atom of iron per molecule) of 1.5 at pH 6.5.

The anaerobic treatment of aqueous solutions of the brown pigment with nitric oxide resulted in the formation of a deep crimson colour, the absorption spectrum of which (Fig. 10) exhibited maxima at 532 and 563 mμ, showing no evidence of the original bands of the metmyoglobin and differing from the nitrosomyoglobin spectrum obtained by treatment with sodium nitrite and sodium dithionite , in which bands at 548 and 580 mμ were observed. This crimson colour was unstable in air,

FIG. 10. ABSORPTION SPECTRUM OF METMYOGLOBIN (– – –),
OF ITS RED COMPLEX WITH NITRIC OXIDE (——),
AND OF THE DECOMPOSITION PRODUCT OF THE COMPLEX IN AIR (·····),
ALL AT A CONCENTRATION OF 3·6 mg/ml.



decomposing rapidly to yield a yellow-brown pigment of absorption spectrum without pronounced peaks (Fig. 10). Light was not necessary for this decomposition and the brown end-product did not react again with nitric oxide to yield any red coloration. Reduction of the red nitric oxide-metmyoglobin complex with sodium dithionite produced a visible change of colour tint and a shift of absorption bands to 546 and 579 mμ corresponding within experimental error to those of nitrosomyoglobin. Similar treatment of the yellow-brown decomposition product of the complex gave no indication visually or spectrophotometrically of reduced myoglobin; no hemochrome formation with pyridine could be detected.

An aqueous solution of nitrosomyoglobin coagulated rapidly at 74°C (165°F) forming a distinctly pink flocculum within 10 minutes. A solution of the red nitric oxide-metmyoglobin complex showed no obvious change after similar heat treatment under anaerobic conditions; heating at 100°C resulted in the formation of a brown precipitate after about ten minutes.

Subjection of the nitric-oxide-metmyoglobin complex to a reduced pressure of 5 mms. of mercury (under argon) produced no visible change of colour within 30 minutes, but after 2 hours the solution had become yellow-brown in appearance. No evidence of a spectral peak at about 630 mμ could be observed with the final product.

Measurements of endogeneous respiration:

Effect of method of preparation of sample:

Fig. 11 illustrates a consistent finding that pork gastrocnemius muscle, tested in equal quantities without a supporting medium, respired at a somewhat greater rate when minced in a small hand mincer than when cut into 0.37 mm. slices on a hand microtome after freezing. 50 % homogenates of pork muscle prepared in a 'Turmix' macerator were also found to utilise only 53-70 % of the total oxygen consumed by comparable quantities (on a dry basis) of mince from the same muscle; these comparisons were made using phosphate buffer of pH 7.4 as supporting medium. Using the McIlwain and Buddle tissue chopper, samples of pork muscle were chopped in two directions at right angles at 0.36 mm. intervals both at room temperature and after freezing solid with solid carbon dioxide. As shown in Fig. 12 the frozen material (in presence of Krebs-Eggleston buffer as supporting medium) was consistently less active than that chopped at room temperature. The endogeneous respiration of the latter material (again using Krebs-Eggleston buffer as supporting medium) was at least as great as that of the minced tissue (Fig. 13) and was, in fact, slightly greater in some experiments.

FIG. 11 THE ENDOGENEOUS RESPIRATION OF PORK MUSCLE MINCE AND SLICES (0·37 m.m.)

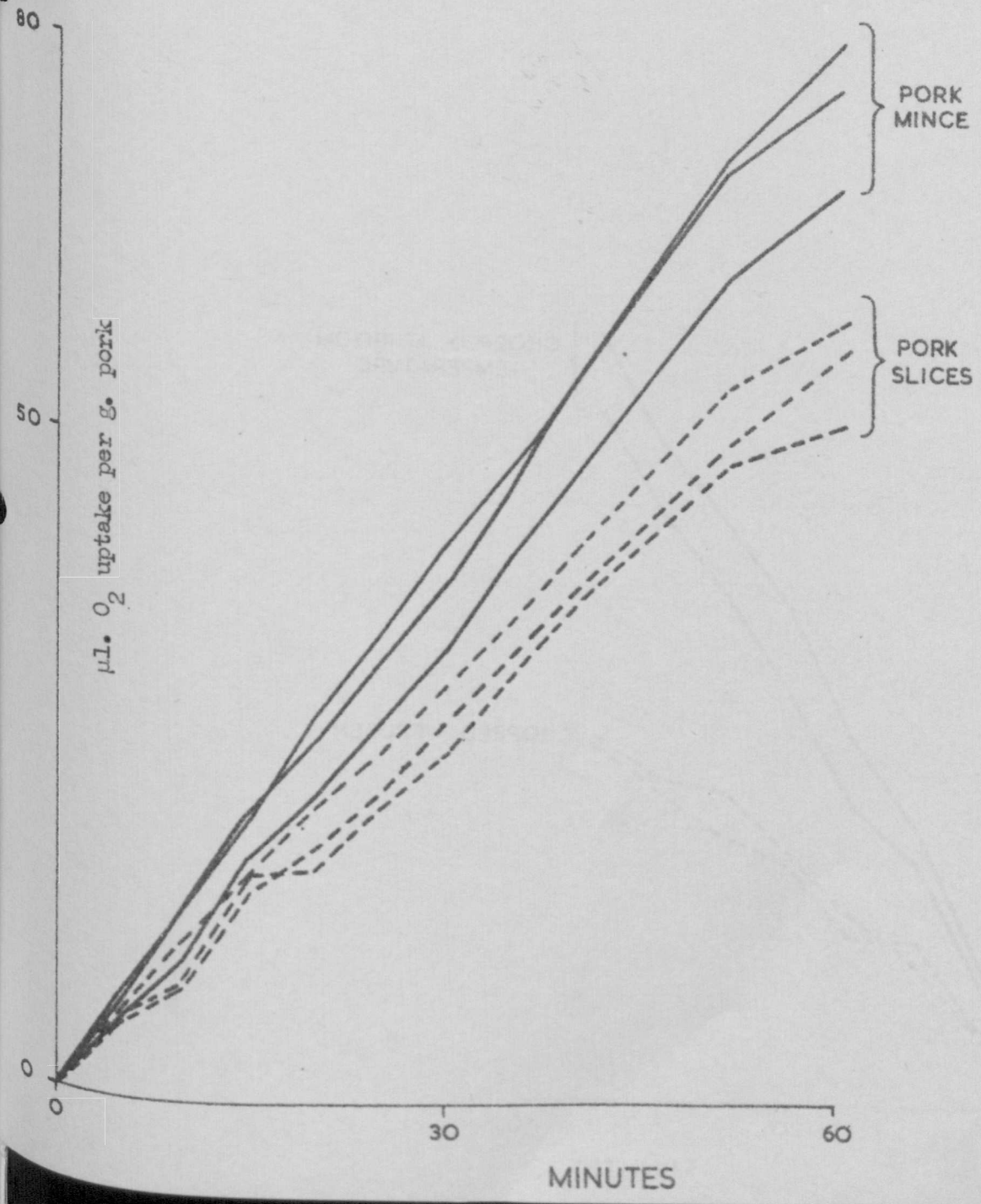


FIG. 12 THE ENDOGENEOUS RESPIRATION OF PORK MUSCLE CHOPPED IN TWO DIRECTIONS AT RIGHT ANGLES AT 0·36 m.m. INTERVALS AT ROOM TEMPERATURE & WHEN FROZEN WITH SOLID CARBON DIOXIDE.

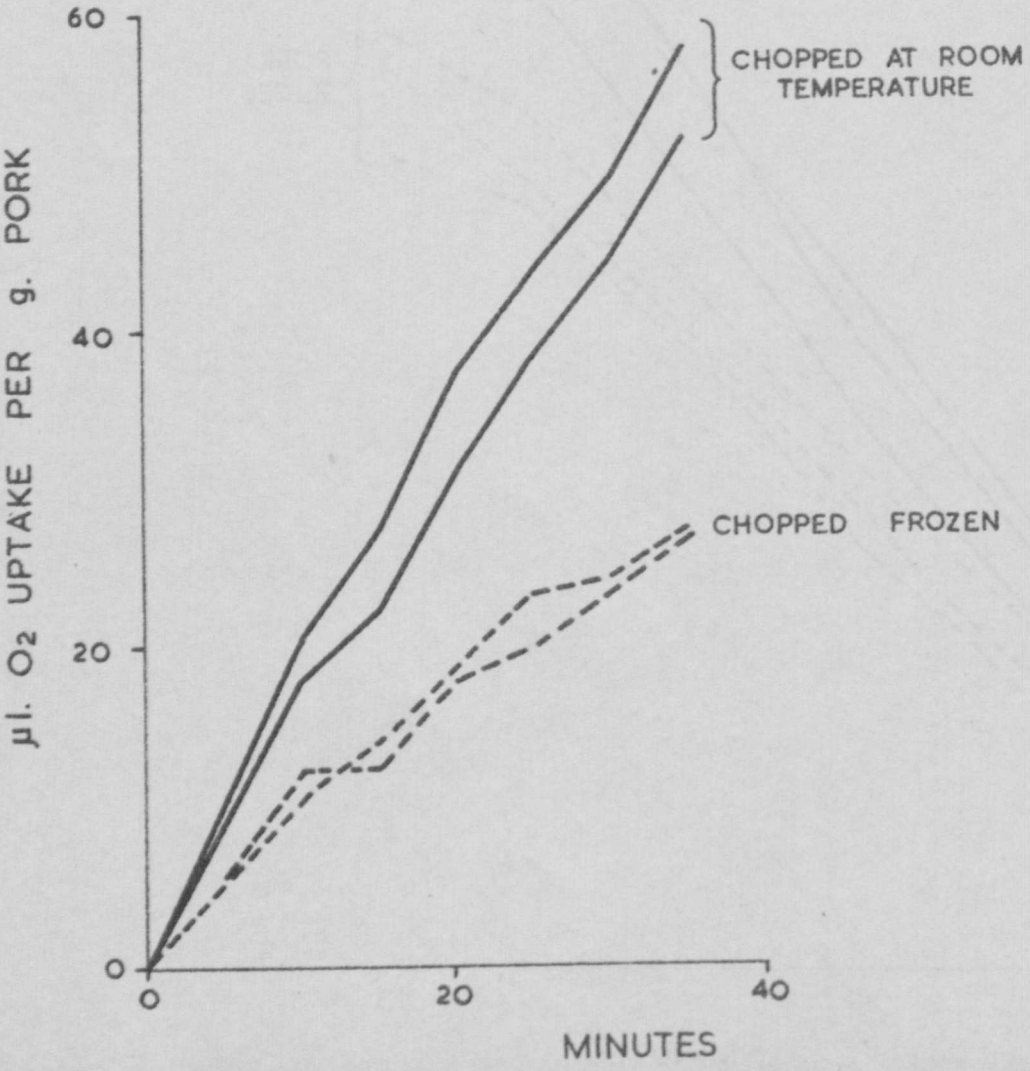
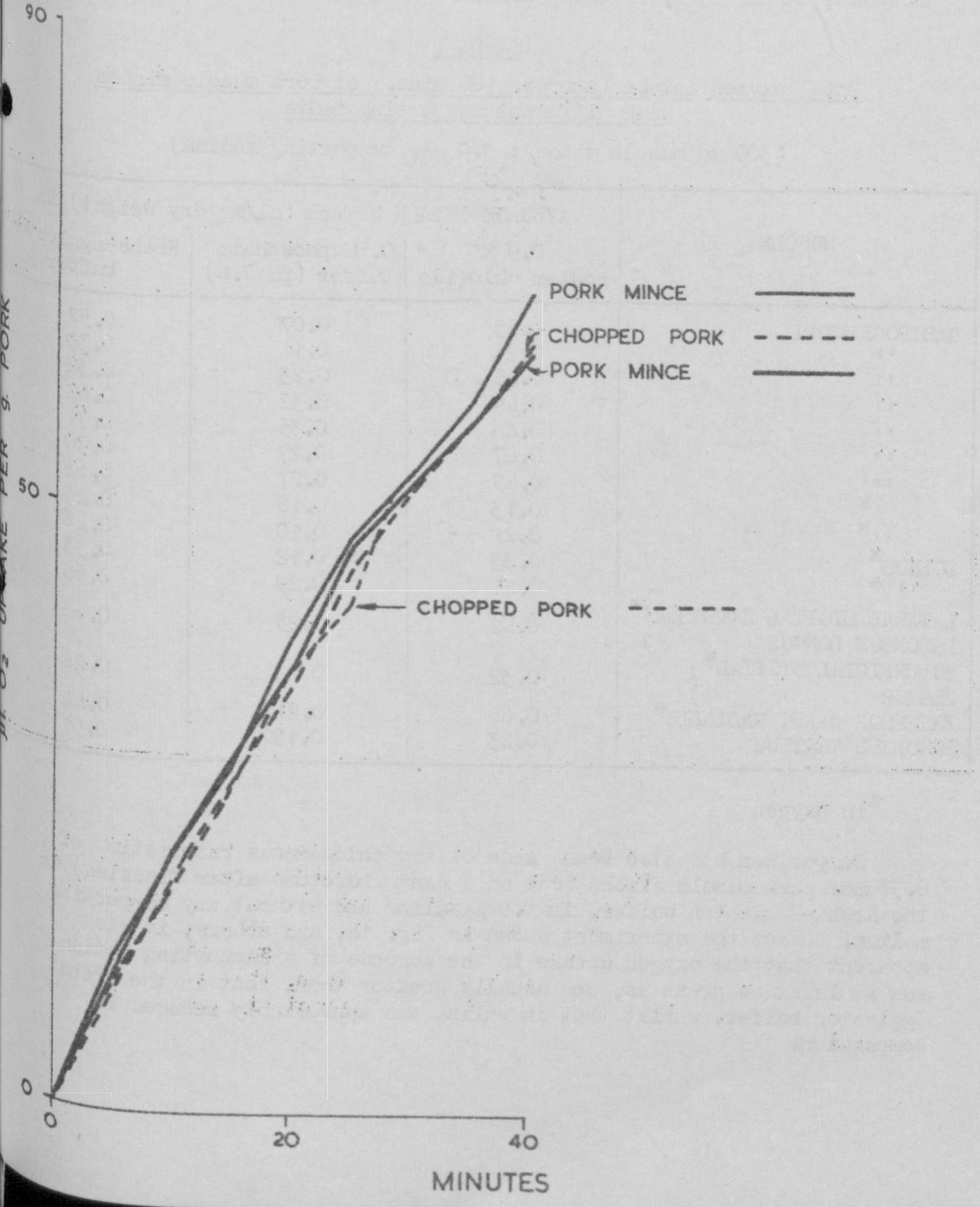


FIG. 13 THE ENDOGENEOUS RESPIRATION OF PORK MUSCLE MINCE AND OF MATERIAL CHOPPED IN TWO DIRECTIONS AT RIGHT ANGLES AT 0·36 m.m. INTERVALS.



Effect of supporting medium:

Table I shows the results of a series of experiments comparing the endogeneous respiration of minced pork muscles in three alternative supporting media. In these experiments the maximum respiration was generally observed in the Krebs-Eggleston buffer. The average uptake of oxygen in 0.9 % sodium chloride was 79 %, and in the 0.1M phosphate buffer 53 %, of this maximum.

TABLE I

Total oxygen uptake (approx. 100 mins.) of pork muscle minces with different supporting media

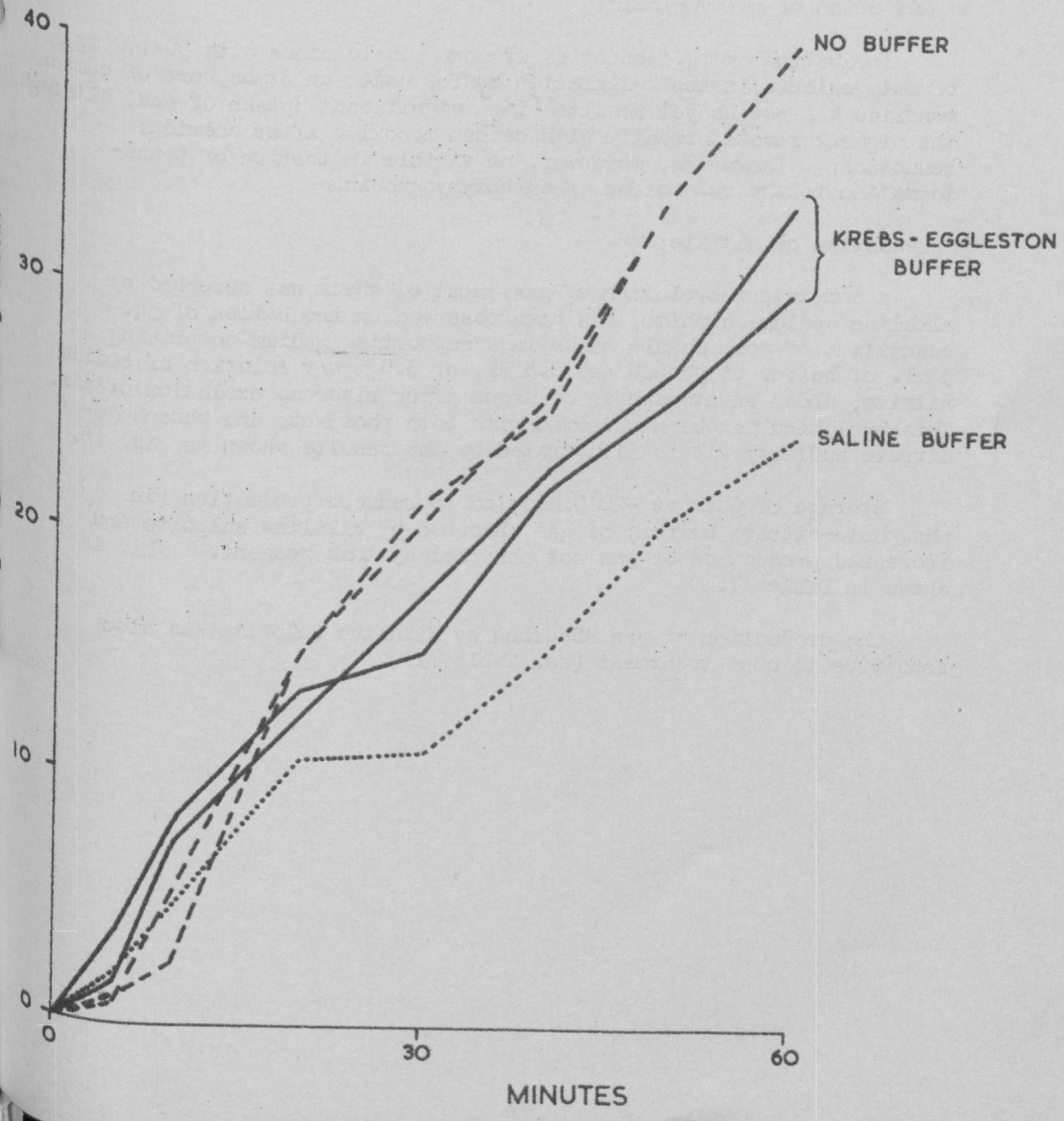
(300 mg muscle mince : 3.0 ml. supporting medium)

MUSCLE	AVERAGE OXYGEN UPTAKE (µl/mg dry weight) in 0.9 % sodium chloride	0.1M phosphate buffer (pH 7.4)	Krebs-Eggleston buffer
GASTROCNEMIUS	0.23	0.07	0.52
"	0.26	0.11	0.30
"	0.28	0.23	0.32
"	0.45	0.32	0.62
"	0.46	0.35	0.71
"	0.47	0.27	0.70
"	0.49	0.27	0.63
*	0.13	0.10	0.26
"*	0.21	0.10	0.26
SOLEUS*	0.33	0.12	0.33
"*	0.41	0.29	0.58
LATERAL DIGITAL EXTENSOR, PERONEUS LONGUS*	0.42	0.25	0.48
SUPERFICIAL DIGITAL FLEXOR*	0.32	0.13	0.26
EXTENSOR CARPI RADIALIS*	0.66	0.21	0.44
PERONEUS TERTIUS	0.23	0.19	0.51

*in oxygen

Comparison has also been made of the endogeneous respiration of 0.37 mm. pork muscle slices (cut on a hand microtome after freezing) in the Krebs-Eggleston buffer, in 0.9 % saline and without any supporting medium. From the experiment shown in Fig. 14, and others, it was apparent that the oxygen uptake in the absence of a supporting medium was at least as great as, and usually greater than, that in the Krebs-Eggleston buffer, whilst that in saline was appreciably reduced in comparsion.

FIG. 14 THE ENDOGENEOUS RESPIRATION OF PORK MUSCLE SLICES (0·37 m.m.) IN 0·9% SALINE, KREBS-EGGLESTON BUFFER AND WITHOUT SUPPORTING MEDIUM.



Separation of mince:

In one experiment, the distribution of endogeneous respiration between water-soluble and water-insoluble portions of a pork mince was followed by macerating with 2 parts of Krebs-Eggleston buffer and centrifuging at 3000 r.p.m. The activity of the supernatant was 15 % and of the solid 21 % of that of the original mince. Recombination in the original proportions gave 50 % of the initial respiration.

Studies on specific reducing systems:

Reduction of metmyoglobin:

Incubation of 1g. quantities of pork muscle mince with 50-100 mg. of metmyoglobin in Krebs-Eggleston buffer under an atmosphere of carbon monoxide has not as yet resulted in a significant uptake of gas, although the pigment reacted readily with carbon monoxide after chemical reduction. There was, moreover, no visible indication of transformation to the red colour of carboxymyoglobin.

Reduction of nitrite:

A consistent evolution of gas, most of which was absorbed by alkaline sodium sulphite, has been observed on incubation of 3g. quantities of pork muscle mince in a supporting medium comprising 3 ml. of buffer of pH 6.0 and 0.5 ml. of 3.0 % w/v solution of sodium nitrite, under an atmosphere of argon after rigorous exclusion of air. This evolution has been observed with both phosphate and phosphate-citrate buffers; it is illustrated by the results shown in Fig. 15.

Storage of pork at -20° C has led to reduced production (in phosphate-citrate buffer) of gas absorbed by alkaline sulphite and increased production of gas not absorbed by this reagent. This is shown in Table II.

The production of gas absorbed by alkaline sulphite was also sensitive to heat treatment (see Table III).

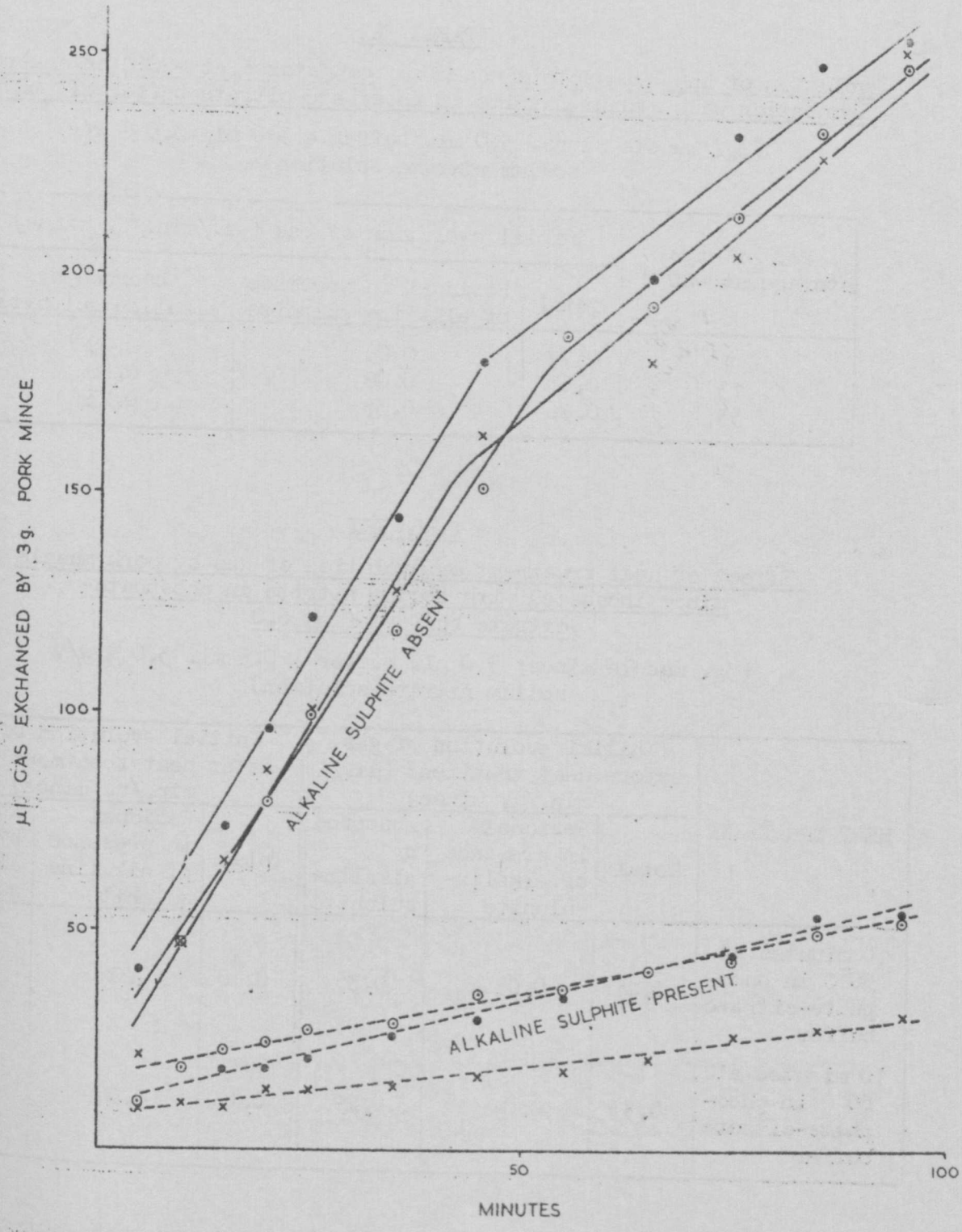


FIG. 15 THE EVOLUTION OF GAS FROM SODIUM NITRITE UPON INCUBATION WITH PORK MINCE IN CITRATE – PHOSPHATE BUFFER pH 6·0, WITH AND WITHOUT THE PRESENCE OF ALKALINE SODIUM SULPHITE

TABLE II

Evolution of gas by muscle mince from pork stored at -20°C on anaerobic incubation with sodium nitrite in phosphate-citrate buffer of pH 6.0

(3g. muscle mince; 3.0 ml. buffer + ~~5.0~~ 0.3 ml. 3.0 % w/v sodium nitrite solution)

No. of days storage at -20°C	Initial evolution of gas (µl./min./g. mince)		
	Total	Residual in presence of alkaline sulphite	Absorbed by alkaline sulphite
1	0.77	0.08	0.69
13	0.48	0.08	0.40
33	0.56	0.33	0.23

TABLE III

Effect of heat treatment on evolution of gas by pork muscle mince incubated with sodium nitrite in phosphate-citrate buffer of pH 6.0

(3g. muscle mince; 3.0 ml. buffer + 0.5 ml. 3.0 % w/v sodium nitrite solution)

HEAT TREATMENT	Initial evolution of gas before heat treatment (µl./min./g. mince)			Initial evolution of gas after heat treatment (µl./min./g. mince)		
	Total	Residual in presence of alkaline sulphite	Absorbed by alkaline sulphite	Total	Residual in presence of alkaline sulphite	Absorbed by alkaline sulphite
10 minutes at 50°C in phosphate-citrate buffer	0.41	0.05	0.36	0.18	0.02	0.16
10 minutes at 80°C in phosphate-citrate buffer	0.33	0.04	0.29	0.13	0.04	0.09

A direct comparison between the phosphate and phosphate-citrate buffers was made on a sample of pork tested after varying periods of storage at -20°C. The results, given in Table IV, indicated that the presence of citrate stimulated the evolution of gas absorbed by alkaline sulphite, although this stimulation was not shown by material stored at -20°C for 33 days.

TABLE IV

Effect of supporting buffer on evolution of gas absorbed by alkaline sulphite by pork muscle stored at -20°C on incubation with sodium nitrite

(3g. muscle mince; 3.0 ml. buffer + 0.5 ml. 3.0 % w/v sodium nitrite solution)

Period of storage at -20°C - days	Initial evolution of gas absorbed by alkaline sulphite µl./min./g. mince		Additional gas evolution in phosphate-citrate µl./min./g. mince	Percentage Stimulation
	In phosphate buffer pH 6.0	In phosphate-citrate buffer pH 6.0		
1	0.28	0.51	0.23	82
4	0.28	0.44	0.16	57
7	0.15	0.24	0.09	60
33	0.22	0.23	0.01	5

Reduction of nitrate:

In experiments to date, anaerobic incubation of 3g. quantities of pork mince (from material active in the presence of sodium nitrite) with 3.0 ml. of phosphate-citrate buffer of pH 6.0 and 0.5 ml. of 3.0 % w/v solution of sodium nitrate has led to a small evolution of gas absorbed by alkaline sulphite. In one experiment, shown in Fig. 16, introduction of sodium nitrite after an incubation period of 90 minutes with sodium nitrate resulted in an increased evolution of gas absorbed by alkaline sulphite together with an evolution of gas not so absorbed.

Discussion

Pig heart myoglobin and derived pigments:

The aim in preparing myoglobin from pig hearts was to obtain an appreciable quantity of a stable product of reasonable purity which had not been denatured. The solubility of the preparation in 3M-phosphate buffer of pH 6.8 indicates that it was essentially free from hemoglobin, since the blood pigment is insoluble in this medium[23]. Upon the basis of a molecular weight of 18,000 reported recently for sperm whale myoglobin, the final freeze dried product contained sufficient iron for a purity of at least 70 %.

As already remarked, spectroscopic studies showed that the pigment had become converted to the met form during the preparation. These studies also showed that the metmyoglobin preparation, after reduction with sodium dithionite, was capable of reacting with oxygen and carbon

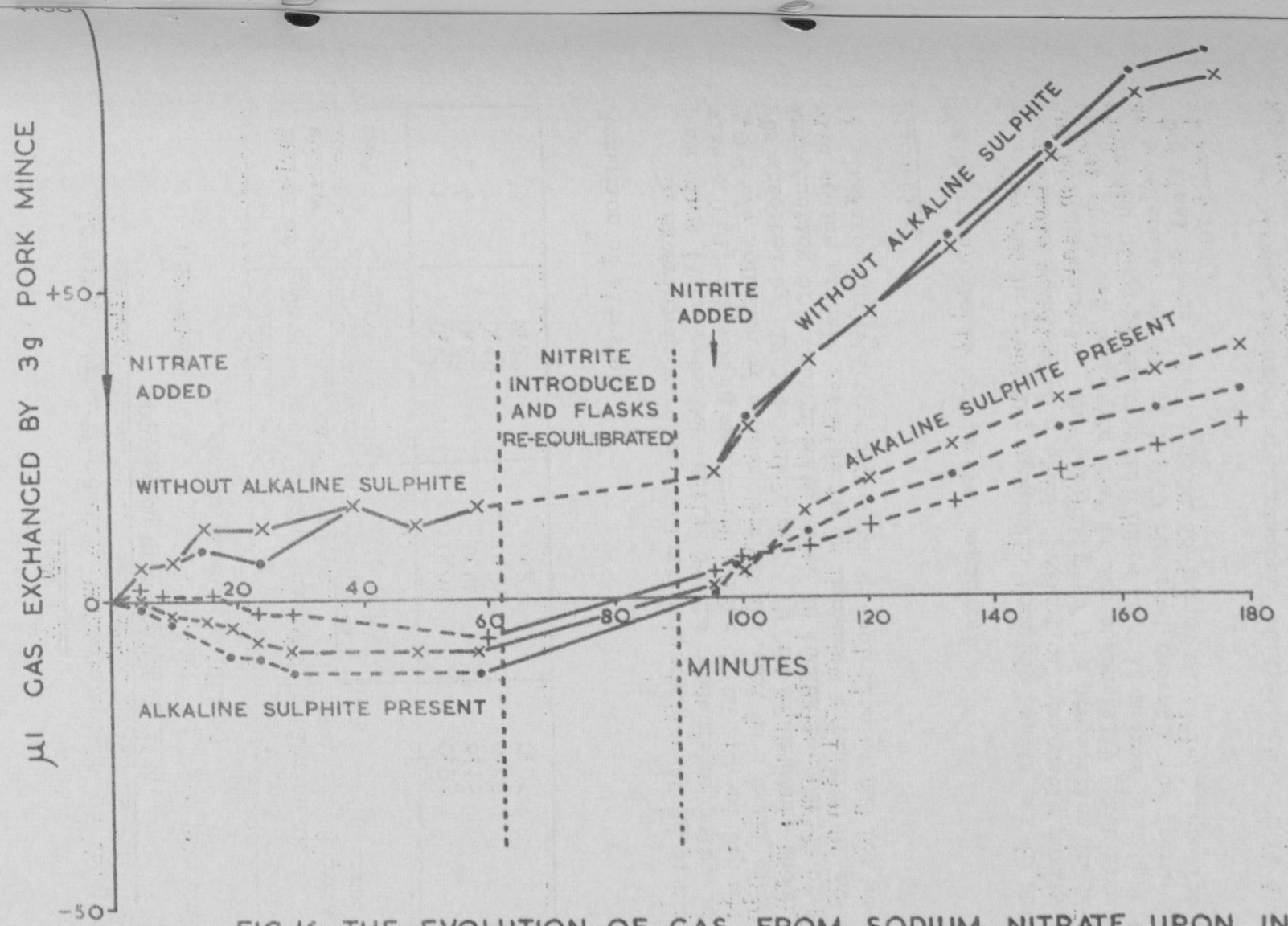


FIG. 16 THE EVOLUTION OF GAS FROM SODIUM NITRATE UPON INCUBATION WITH PORK MINCE IN CITRATE—PHOSPHATE BUFFER pH 6·0 BEFORE AND AFTER THE ADDITION OF SODIUM NITRITE.

monoxide to form the corresponding addition compounds; the peaks of the carboxy compound, for instance, agreed well with the values (542 and 576-579 mμ) recorded for myoglobins from human and other sources[24,25]. The observed millimolar extraction coefficient of 1.5 at pH 6.5 is, however, well below the coefficient of 3.66-3.77 reported at the same wavelength, for horse heart metmyoglobin at pH 6.0-7.1[26].

Keilin and Hartree[17] have reported that nitric oxide reacts with acid methemoglobin to form an unstable red complex whose spectrum exhibits two bands at 531 and 568 mμ which are more distinct than those of the stable nitrosohemoglobin complex. As in the case of the complex of nitric oxide with metmyoglobin now reported, the characteristic absorption bands of the met pigment complex were therefore displaced towards the blue end of the spectrum in comparison with those of the corresponding ferrous pigment. The metmyoglobin compound appears to be much more stable than the complex of Keilin and Hartree; these authors state that "the NO-MHb, soon after its formation, is gradually transformed into a compound which is indistinguishable from NO-Hb and this change is accelerated if the NO in the tube is replaced by nitrogen." By contrast, the metmyoglobin compound was stable under nitrogen (as judged by the persistence of the characteristic absorption bands) for at least 20 hours at room temperature. Furthermore, evacuation of the containing vessel immediately after formation of the nitric oxide-methemoglobin compound is stated to result in almost complete reversion to methemoglobin, whereas with the metmyoglobin compound evacuation (to 5mm of mercury) produced no visible change within 30 minutes. On the other hand, the metmyoglobin compound was rapidly decomposed on exposure to air; on this point no observation is made by Keilin and Hartree. A final point of difference lies in the fact that whereas the methemoglobin compound regenerated methemoglobin on decomposition, the breakdown of the metmyoglobin analogue appeared to be much more extensive; the observed failure to react with pyridine after decomposition suggests that the heme moiety was no longer available.

The nitric oxide-metmyoglobin complex appeared to be less readily denatured on heating than the normal nitrosomyoglobin compound, and survived heating for a short period to a temperature as high as that normally encountered in the cooking of hams. More extensive observations on this point are envisaged.

Endogeneous respiration studies: choice of method of preparation of samples and supporting medium:

Since the present studies are aimed at elucidating the process of curing, in which the reactions take place in intact muscle blocks, it is undesirable on general grounds to introduce more than the necessary minimum of disintegration of the tissue. Early attempts were made to separate intact film bundles (Richardson, et al[27]) but the low activity of pork muscle, and the consequent necessity for using comparatively large amounts of material, made this procedure impossibly time-consuming. Homogenization is highly disruptive of cellular organisation and for

this reason alone is undesirable; in addition, experimental observations showed that homogenization decreased the oxygen uptake of the material. The experiment made on the respiration of the centrifuged mince also serves to emphasise the need to avoid separation of the cell components.

Umbreit[28] has calculated that liver slices no thicker than 0.2mm should be used in air if the diffusion of oxygen into the tissue is not to become the limiting factor; McIlwain, Buchel and Cheshire[29] however have usefully employed brain slices for respiration studies as thick as 0.35mm. Pork muscle slices of 0.2mm thickness are extremely fragile and the number required for any assay of this relatively inactive material would be excessive; in any case, its oxygen uptake is considerably less than that of liver, so that a slice thickness of 0.36mm was not considered to limit the entry of the gas significantly. Umbreit also contends that sketetal muscle does not yield satisfactory tissue slices chiefly because of the large size of the cells and the consequent number damaged per slice, although this criticism is probably less applicable to minces. The mechanical chopper of McIlwain and Buddle[19] was in fact designed originally to disrupt cell structure as little as possible, cutting through the tissue in a regular and reproducible fashion; direct comparison of pork muscle prepared in this chopper against a corresponding mince showed no appreciable reduction of activity in the minced material (Fig. 13). Freezing the material before cutting was found to be clearly detrimental, possibly owing to rupture of cells by ice crystals; apart from the direct comparison of Fig. 12, the lower respiration of the pork slices of Fig. 11 may be attributed to the fact that these slices were cut from frozen material. It was concluded that, for pork muscle, mincing was as effective as slicing in maintaining respiration and, in view of the over-riding advantage in respect of ease of handling, the procedure has been adopted in subsequent work.

The choice of supporting medium must also be influenced ultimately by considerations of practical significance, and on this basis the use of a salt solution is indicated. Physiological saline has been found to give somewhat lower overall respiration than Krebs-Eggleston buffer (Fig. 14 and Table I), but this may be in part a pH effect. The saline solution being practically unbuffered, the pH of the meat-saline mixture will depend mainly on that of the meat itself (normally 5.9 to 6.0) whereas the pH of the mixtures containing buffer will be raised by the contribution of the buffer solution of higher pH (7.4). It does not appear that the respiration was inhibited by the chloride ion itself since the Krebs-Eggleston buffer, which contains chloride, gave considerably higher respirations than did the straight phosphate buffer. On the other hand, the respiration of pork muscle slices was substantially depressed in the presence of 0.9 % saline (Fig. 14).

Studies on specific reducing systems:

Reduction of metmyoglobin:

The absence of detectable reduction of metmyoglobin by pork muscle mince in an atmosphere of carbon monoxide may be due to interference by the carbon monoxide with the enzyme systems. Carbon monoxide could interfere with the cytochromes if the terminal portion of the respiratory chain were required for the reduction of metmyoglobin. In earlier experiments carried out in another connection, reduction of metmyoglobin by pork muscle mince in an atmosphere of argon has been demonstrated spectrophotometrically.

Reduction of nitrite:

The observations reported establish the consistent evolution of a gas capable of absorption by alkaline sulphite solution when pork muscle mince is incubated in the presence of sodium nitrite, but they do not demonstrate that the gas evolved is in fact nitric oxide. It is appreciated that the absorption reagent, being alkaline, would act as an absorbent for carbon dioxide; further work now in hand, which will be reported fully in a later Report has shown by differential absorption that part of the absorbed gas is not carbon dioxide, although attempts to detect nitric oxide specifically have so far been unsuccessful.

The quantities of sodium nitrite used in these experiments are extremely large in relation to curing practice, but it was desired initially to treat the sodium nitrite as a substrate and to provide an overwhelming excess. The reduction in gas-producing activity observed after heat treatment (Table III) does indicate an enzymic mechanism; the experiments so far reported do not resolve the question of whether enzymes of mammalian or bacterial origin are involved, although the appreciable loss in activity after 33 days at -20°C (Table II) is suggestive of a mammalian rather than a bacterial source. The question is being further studied with the aid of antibiotics.

The apparent enhancement of the gas-producing action by citrate is being followed up and developed into a general study of the influence of added substrates.

It is appreciated that the measurement of gas production as an index of nitrite-reducing activity has definite limitations, since there may be simultaneous absorption of nitric oxide by combination within the meat itself. In the first place, some degree of conversion of any pigment present to the nitroso form may be anticipated. According to Scaife[30] the myoglobin content for pork muscle can be up to 0.53 %; on this basis complete conversion of the pigment in the 3g. sample of meat used would require about 22µl. of nitric oxide at 37°C. Evidence has also been obtained of a non-specific absorption of nitric oxide by pork muscle tissue from which the muscle pigments had been removed by extraction with water; total gas uptakes of the order of 20µl./g. wet weight of tissue have

CONCLUSIONS

1. Electrodes of platinum, gold, silver and aluminium inserted into pork muscle and into aqueous extracts of pork muscle excluded from the atmosphere by a layer of liquid paraffin showed a considerable drift of potential over a period of hours, leading to variations between the potentials simultaneously indicated by successively inserted electrodes of the same metal.

2. Maintenance of strict anaeorbic conditions gave more rapid equalisation of potentials between platinum electrodes in aqueous pork muscle extracts, the systems being sensitive to the inclusion of oxygen.

3. Micro-electrodes of platinum, gold and stainless steel acquired comparable potentials over the range +150 to -150 mv with respect to the hydrogen electrode in an unpoised pork extract, and were in agreement with the observed decolorization of potassium-5:5'-indigodisulphonate in a system poised by the dye.

4. Outside this range of potential the occlusive metals (platinum and stainless steel) showed different potentials from the non-occlusive gold. This variation is ascribed to adsorption and the occlusive metals are on this account regarded as unsuitable for use as inert electrodes for the present purpose.

5. It has been found possible to prepare native (undenatured) pig heart metmyoglobin, free from haemoglobin and of about 70 % purity on the basis of a molecular weight of 18,000, by freeze drying an aqueous extract after suitable purification.

6. A new pigment complex has been prepared by direct combination between pig heart metmyoglobin and nitric oxide under anaerobic conditions. This complex is unsta ble in air; its absorption spectrum in aqueous solution has two bands displaced from those of nitrosomyoglobin by 16-17 mμ towards the blue end of the spectrum.

7. The endogeneous respiration of pork muscle comminuted by fine mincing was greater than that after homogenization, and at least as great as that of material prepared by more elaborate methods of slicing and chopping.

8. The endogeneous respiration of pork muscle mince at 37° C was as great in the absence of a supporting medium as in the presence of phosphate-saline buffer of pH 7.4. This buffer was generally more effective than either phosphate buffer or saline alone.

9. The anaerobic incubation at 37° C of pork muscle mince with sodium nitrite at pH 6.0 has resulted in the evolution of gas largely absorbed by alkaline sodium sulphite, believed to contain both carbon dioxide and nitric oxide; this property of pork muscle has been retained up to

been recorded under an atmosphere of pure nitric oxide. The solubility of nitric oxide in water at 37° C and 760mm pressure is reported as 44µl./ml.[23] It cannot be assumed, however, that the observed absorption is due to solubility, since a period of at least 30 minutes was allowed for equilibration before measurements were commenced. In any case it is probable that the absorption would be reduced under the very low partial pressures of nitric oxide obtaining in the gas exchange measurements, particularly in the presence of a specific absorbent for this gas. These limitations are still under review.

It is thought that the evolution of gas not absorbed by alkaline sulphite (see Fig. 15) may be due to de-amination of primary amino groups by the added nitrite, leading to production of nitrogen. Gould[31] has reported that the optimum pH for this deamination is 4.6; at pH 5.5, however, the reaction still persisted in the case of leucine, although it was slow. The increased evolution of non-absorbable gas after storage for 33 days at -20° C (see Table II) may be due to increased accessibility of primary amino groups on the stored material.

Reduction of nitrate:

The action of pork muscle mince on sodium nitrate, as measured by formation of gas absorbed by alkaline sulphite, was markedly less than that on sodium nitrite under the same conditions (Fig. 16). It thus appears that the rate of reaction of any nitrite formed from nitrate was not a limiting factor in the initial gas production.

33 days during storage at - 20°C, was markedly reduced after heat treatment, and was stimulated by the inclusion of citrate into the supporting buffer.

10. A pork muscle mince, active in producing alkaline sulphite soluble gas upon incubation with sodium nitrite at pH 6.0, reacted with sodium nitrate under the same conditions to evolve small quantities of gas showing similar absorption behaviour.

SUMMARY

The formation of the nitroso pigment complex during curing involves reduction both of the meat pigment and of nitrite introduced during the cure. Mammalian muscle contains enzymic reducing systems which in the living animal are involved in the tissue metabolism, and the activity of these systems is known to persist in the tissues after death. The present work attempts to determine the extent to which such systems are involved in the formation of the nitroso pigment during curing.

The ability of the muscle tissues to effect the specific reductions involved is being studied by Warburg manometry and by spectrophotometry. The existence has been demonstrated of a reducing system in pork muscle capable of yielding from sodium nitrite a gas soluble in alkaline sodium sulphite but not in alkali; these properties are characteristic of nitric oxide, but it has not so far been found possible to confirm completely the identity of the gas produced. Little or no activity has been found in the production of a similar gas from sodium nitrate.

Pig metmyoglobin in at least 70 % purity has been isolated from pig hearts. This pigment has been found to combine directly with gaseous nitric oxide under anaerobic conditions, without preliminary reduction. The absorption spectrum of the resulting complex was generally similar to that of nitrosomyoglobin, but the absorption maxima were displaced in the direction of shorter wavelength. Unlike nitroso-myoglobin, the new complex decomposed at once in contact with air.

Preliminary observations on redox potential measurements have also been made in an attempt to obtain valid results. Studies to date have indicated gold or silver as the most suitable metals for this purpose.

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