Biochemical Pharmacology, 1963, Vol. 12, pp. 1265–1278. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF PHENAZINE METHOSULPHATE, PYOCYANINE AND EDTA ON MITOCHONDRIAL SUCCINIC DEHYDROGENASE

JOHN W. HARMAN and MONICA C. MACBRINN

Department of Pathology,
Medical School, University College, Dublin, 2, Ireland

(*Received* 5 *April* 1963; *accepted* 24 *June* 1963)

Abstract—An inhibition of succinic dehydrogenase during prolonged assay has been observed when PMS is used as the electron transfer system. This is caused by formation during assay of an intermediate which has been identified as pyocyanine. The inhibitory action so observed has been altered in relation to the PMS/protein ratio of the system under study, when EDTA is present during preparation or assay. With high ratios the enzyme is inhibited and in general with low ratios there is stimulation.

It is suggested that pyocyanine effects inhibition by partial blockage of one of the active sites on the enzyme, whilst leaving intact a second operative site.

THE introduction of PMS* as an electron carrier for assay of succinic dehydrogenase considerably facilitated more precise estimation of this enzyme. Its selective primary reaction with the enzyme enabled isolation and purification of the enzyme and determination of its composition[1]. Notwithstanding these advantages, it has been customary to utilize reactions between the enzyme and the acceptor which are confined to the briefest intervals acceptable for establishment of the enzyme activity. When pressed beyond this initial reaction time the system manifests irregularity in behaviour which stultifies its value for enzyme assay. In the course of our experiments on the enzyme, as confined within the mitochondrion, it was of importance to continue the reaction for longer periods than recommended by Singer and Kearney[2]. In attempting to achieve this prolongation regular alterations in the reaction were encountered. Because they are germane to precise interpretation of the interaction between the enzyme and the PMS electron acceptor, the deviation from initial reaction was more closely analysed. It was determined that the PMS exerts an action upon the enzyme with concomitant alteration in its functional properties. The experiments concerning this behaviour of the enzyme under the influence of the PMS are presented in this report and include some observations relevant to the mode of action and the properties of the derivative which is the most likely agent responsible for the phenomenon.

EXPERIMENTAL

Preparation of mitochondria

Mitochondria were prepared from livers of male Wistar rats, weighing 200 g, and fed ad libitum. They were killed by a sharp blow on the head, and decapitated. The

* Abbreviations: PMS, phenazine methosulphate; EDTA, ethylenediaminetetraacetic acid magnesium disodium complex; SDH, succinic dehydrogenase.

livers, rapidly removed intact and immersed in 0·44 M sucrose ice slush, were cut into small fragments and homogenized by a teflon homogenizer in 0·44 M sucrose at pH 7·2. All sucrose solutions contained 1×10^{-3} M *Tris* and the 0·44 M sucrose contained in addition 1×10^{-3} M EDTA with exceptions as indicated in the text. The homogenate, made up to a 10 per cent suspension, was centrifuged differentially[3, 4] by spinning for 10 min at 500 × *g*, then increasing speed rapidly to 900 × *g* for 7 min in a No. 6875 swing out head of an MSE "refrigerator" centrifuge model "p". The supernate (S_1) removed with automatic pipets (Alfred Bicknell Associates, Cambridge, Massachusetts, 5 ml capacity) and divided between four 50 ml lusteroid tubes for centrifugation in the Phywee ice "pirouette" model, rotor 1 at 9,000 × *g* for 8 min. The supernate (S_2) was decanted and the loose fluffy layer swirled up in a small volume of 0·44 M sucrose and discarded. The residue (R_2) dispersed in a small volume of 0·30 M sucrose, was suspended in about 90 ml, divided between two lusteroid tubes and centrifuged at 9,000 × *g* for 10 min Supernate (S_3) and fluffy microsomal layer were removed as before. Residues (R_3) were suspended in a small volume of 0·30 M sucrose and dispersed by a few strokes of the teflon homogenizer. All manipulations were performed between 0–4°.

Assay of succinic dehydrogenase

The PMS method of Kearney and Singer[1] was modified slightly to suit requirements of particular experiments. Conventional Warburg manometric technique was used for measurement of oxygen uptake, with air as gas phase at a temperature of 30°. Cyanide (or azide), phosphate buffer and mitochondrial suspension were placed in the main chamber of the flasks; PMS and succinate occupied the side arm. The centre well contained 0·2 ml aliquots of distilled water and fluted filter paper, and total fluid volume was 2·2 ml. Equilibration at 30° was for 6 min before tipping in the contents of the side arm and readings of oxygen uptake was observed for one hour. After the assay, pH of the contents of each flask was determined with a Radiometer type 22 pH meter.

Paper chromatography

Decomposition products of PMS were separated by the ascending technique on Whatman No. 1 paper, using amyl alcohol: ammonia (120 ml water saturated amyl alcohol, B.D.H. milk-testing grade, and 20 ml ammonia 0·91) or water saturated ethyl acetate as solvents[5]. The chromatographed material was eluted with ethyl acetate solvent, chloroform or water. The eluates were evaporated to dryness in vacuo. With aqueous extracts the freeze-dry technique was employed using the Saunders–Edwards freeze-dry apparatus. Dry residues were dissolved in the minimum of solvent and analysed in the Beckman DK_2 ratio-recording spectrophotometer.

Protein estimation

The method used was described by Layne[6] and is a modification of the Warburg and Christian technique.[7]

Chemicals

All solutions were prepared from A.R. grade chemicals in twice distilled water. When not in use solutions were stored in the refrigerator or deep freeze. PMS (Sigma

Chemicals) was prepared frequently, and stored at −20°, covered with silver foil to minimize decomposition. Phosphate buffer was prepared using the dibasic and monobasic potassium salts. Except where indicated all solutions were neutralized before dilution to final volume.

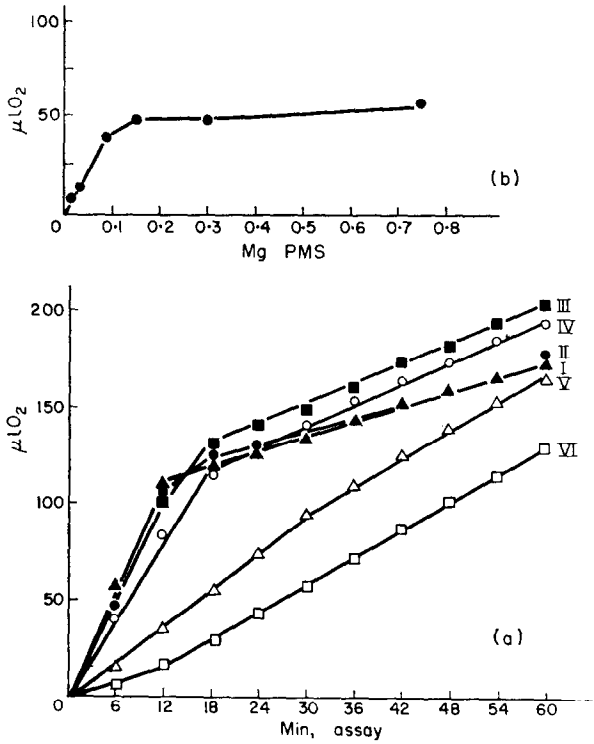

FIG. 1. Effect of PMS concentration on oxygen uptake.

Each flask contained 72·5 μmoles succinate in the side arm and 1·5 μmoles succinate in the main chamber during equilibration, 40 μmoles phosphate buffer pH 7·3; 1×10^{-3} M sodium cyanide; 4·8 mg standard mitochondrial protein in a final volume of 2·0 ml. Centre well contained 0·2 ml distilled water. The PMS was in the side arm.

(a)

I	0·75 mg PMS
II	0·3 mg PMS
III	0·15 mg PMS
IV	0·075 mg PMS
V	0·025 mg PMS
VI	0·001 mg PMS

Results are expressed as μl O_2 per flask. (b). Results are expressed as μO_2 for 6 min.

RESULTS

PMS assay

The concentration of PMS utilized to saturate the system is related to the duration of the experiment. When the assay proceeds for 6 min or slightly longer it ranges between 0·075 mg and 0·15 mg per flask if the mitochondrial protein is approximately 4–6 mg per flask (Fig. 1(b)), whereas when the period of observation is prolonged smaller concentrations suffice. This difference is related to the abrupt alteration in oxygen uptake, which is characteristic with higher levels of PMS. This difference is demonstrated in Fig. 1(a), which depicts the tremendously rapid acceleration of uptake with

higher levels of acceptor up to 12 min, at which time a new and restricted phase of uptake supervenes. On the contrary, when limited amounts of PMS are employed the uptake remains linear and may closely appproximate the final oxygen consumption obtained with higher levels of PMS, evident from comparison of curves III, IV and V. Brief assay accentuates the discrepancy in PMS saturation levels, whereas prolonged survey eliminates initial wide differences which are related to the PMS concentration.

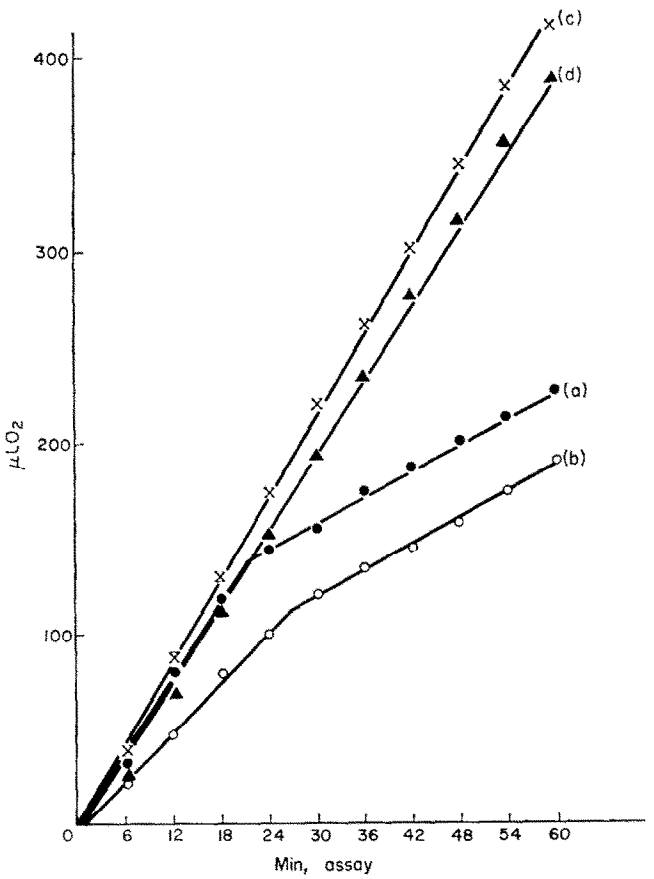

FIG. 2. Effect of different cytochrome oxidase inhibitors on PMS assay of succinic dehydrogenase. Each flask contained 40 μmoles phosphate buffer pH 7·3; 73·5 μmoles succinate and in addition
(a). contained 5×10^{-3} M sodium azide; 0·15 mg PMS; 4 mg mitochondrial suspension.
(b). 5×10^{-3} M sodium cyanide; 0·1 mg PMS; 4 mg mitochondrial suspension.
(c). 5×10^{-3} M sodium azide; 5×10^{-3} M sodium cyanide pH 7·0; 0·15 mg PMS; 4 mg mitochondrial suspension.
(d). 4·6 mg mitochondrial suspension.
All flasks had a total fluid volume of 2·2 ml. The centre well contained 0·2 ml distilled water, which was replaced with 6 N NaOH in D. PMS and 72 μmoles succinate were placed in the side arm and tipped in after 6 min equilibration. 1·5 μmoles succinate were placed in the main chamber to protect the mitochondria during equilibration. Different mitochondrial preparations were used for each curve. Results are expressed as μl O_2 uptake per flask.

There is a problem therefore as to the acceptable level of PMS suited for determination of V_{max} for the system, since the PMS itself may alter the system. In the present instance it has been decided to utilize a PMS concentration of 0·15 mg and an assay period of 6 min. From the reciprocal graphs calculated at 6 min a K_m (Michaelis–Menten constant) value of $1{\cdot}8 \times 10^{-3}$ M is obtained for succinate. From the estimation it would be calculated, if the reaction were linear, that for 60 min enzyme studies 36×10^{-3} M are required to follow the reaction for that time. But as seen from Fig. 1(a) a PMS level of 0·15 mg evokes an inhibition, which appears at 12 min and retards the electron transport to a distinctly different rate. This consequently imposes a choice of PMS levels, depending on whether brief or prolonged intervals of observation are satisfactory and whether the scrutiny of the biphasic system is germane. In so far as the biphasic system was under investigation and a period of 60 min activity was intended it was decided to use the 36×10^{-3} M succinate throughout further experiments. If the linear rate is to be studied (as curve V Fig. 1(a)) the determination of V_{max} is essential in each experiment.

Comparison of Succinoxidase and PMS systems

In the system defined above, which permits manifestation of inhibition, action of cytochrome oxidase is excluded by incorporation of cyanide. When this is replaced by azide, some acceleration is obtained in the earlier phase of the experiment, although the appearance of the typical inhibition is not curtailed, so that it is not a property of cytochrome oxidase inhibitor. This is further demonstrated by the remarkable and regularly observed result of including the two cytochrome oxidase inhibitors together. When both are conjoined (Fig. 2) the reaction is initially accelerated, as compared with that using cyanide alone, and the PMS inhibition is eliminated entirely so that the oxygen consumption parallels that of the intact succinoxidase system. It is unlikely that cytochrome oxidase inhibition contributes to the appearance of the biphasic uptake of oxygen. On the other hand the specific ability of both inhibitors in precluding inhibition by PMS has no ready explanation, unless they combine to protect the enzyme from deleterious influence of the electron acceptor or of one of its by-products. This problem is more comprehensively analysed in a separate report.

The comparison of the succinoxidase system of liver mitochondria with the PMS transport system of SDH as seen in Fig. 2 invites further examination of these enzymatic organizations. The salient difference manifested is an inhibition by the PMS. To elucidate this difference both systems have been submitted to graded levels of substrate. As demonstrated in Table 1 the succinoxidase system requires considerable substrate for saturation, and is linear for most of the levels employed up to the maximum concentration applied, beyond which further acceleration is not observed and some substrate inhibition may occur. With the PMS system the lower concentrations of substrate allow the rapid appearance of inhibition, most frequently developed by 12 min incubation. At higher concentrations the inhibition is minimized and at the highest used is least perceptible. The substrate provides some protection against the PMS inhibition whether azide or cyanide are included. With the least substrate concentration the inhibition may be as high as 80 per cent and falls as low as 30 per cent with the highest substrate level, although in most instances the oxygen uptake for the first 6 min is identical for all levels of substrate. Since both the PMS and substrate are

contained in the side arm and are brought into contact with the enzyme simultaneously the preferential action of the succinate is obvious so that its site of action is operative from its attachment site on the enzyme. The effect of the substrate suggests that the PMS and succinate influence each other by mutual attachment with the enzyme protein, flavin or iron moieties. Current evidence does not distinguish the exact site of action.

TABLE 1. GRADED LEVELS OF SUCCINATE IN (A) THE SUCCINOXIDASE SYSTEM AND (B) THE PMS SYSTEM

	μl O_2 uptake per flask	
A. Succinoxidase system		
Succinate	0–12 min	24–36 min
$1{\cdot}5 \times 10^{-3}$ M	0	3
$2{\cdot}55 \times 10^{-3}$ M	13	7
$8{\cdot}25 \times 10^{-3}$ M	32	33
$15{\cdot}75 \times 10^{-3}$ M	48	57
$18{\cdot}75 \times 10^{-3}$ M	46	61
$36{\cdot}75 \times 10^{-3}$ M	64	65
B. PMS system		
$1{\cdot}8 \times 10^{-3}$ M	19	4
$3{\cdot}75 \times 10^{-3}$ M	53	8
$7{\cdot}5 \times 10^{-3}$ M	78	22
$15{\cdot}0 \times 10^{-3}$ M	92	37
$18{\cdot}0 \times 10^{-3}$ M	109	53

(a) Each flask contained 40 μmoles phosphate buffer pH 7·3; 4·6 mg standard mitochondrial protein and succinate as indicated in a final volume of 2 ml. Centre well contained 0·2 ml 6 N NaOH.
(b) Each flask contained 40 μmoles phosphate buffer pH 7·2; sodium azide 5×10^{-3} M; PMS, 0·15 mg; 4·6 mg standard mitochondrial suspension and succinate as indicated in a final volume of 2 ml. Centre well contained 0·2 ml distilled water.
Different mitochondrial preparations were used in (a) and (b).
Results are expressed as μl O_2 uptake per flask.

Influence of pH on PMS system

It is of further considerable interest that pH has a profound influence on the inhibitory action of the PMS system in so far as it is altered by substrate concentration. Table 2A demonstrates, with cyanide for exclusion of cytochrome oxidase, how the elevated levels of substrate removes the inhibition at pH 7·5. With slightly increased pH at 7·8 the levels of substrate effective previously are unable to eliminate the PMS inhibition (Table 2B). Available evidence is too scanty to permit interpretation of the pH effect, although some experimental studies indicate that a rise of pH encourages the decomposition of PMS[8, 9] and the possibility exists that such a product produced by higher pH in greater amount may be specifically inhibitory to the enzyme.

In regard to substrate protection it may be of some importance that a particular level of substrate is uniform in its effect. Reference to Fig. 1(a) again illustrates that at a constant substrate concentration the degree of shielding provided is almost identical

regardless of the amounts of PMS utilized over a wide range. At whatever site the substrate defends the enzyme, it operates against all levels of inhibitor to the same extent. This suggests that the pH effect is unlikely to work through manufacture of more inhibitor but rather alters the relative affinities of the substrate and inhibitor for some active site.

TABLE 2. EFFECT OF pH ON SUCCINIC DEHYDROGENASE ACTIVITY WITH GRADED SUCCINATE LEVELS

	μl O_2 uptake per flask	
	A	
Succinate	0–12 min	24–36 min
16·5 μmoles	47	21
37·5 μmoles	54	38
73·5 μmoles	46	71
	B	
16·5 μmoles	54	11
37·5 μmoles	58	27
73·5 μmoles	64	31

Each flask contained 40 μmoles phosphate buffer pH 7·3, 1×10^{-3} M KCN, 0·1 mg PMS, 7·3 mg *Tris*-mitochondrial protein and succinate as indicated. A, had in addition 0·1 ml phosphate buffer pH 10·4, and B, 0·3 ml phosphate buffer pH 10·4. All flasks had a final volume of 2·0 ml. Centre well contained 0·2 ml distilled water. The pHs after assay were 7·5 and 7·8 in A and B respectively.

Results are expressed as μl O_2 uptake per flask.

Influence of EDTA on PMS systems

During consideration of factors capable of influencing the inhibitory activity of PMS, attention has been directed to preparation of the mitochondria. It has been found that the incorporation of EDTA in standard preparations assumes considerable importance. Experiments have been therefore devised to assess participation of chelation in the PMS assay system. Since other workers [10, 11] have explored the activity of this enzyme in preparations which did not contain chelating agents, a comparison has been made between mitochondrial suspensions including and omitting EDTA. Total liver homogenates were performed in 0·44 M sucrose containing 1×10^{-3} M *Tris*. After initial centrifugation, the supernatant (S_1) was divided, mitochondria from one part were suspended in 0·30 M sucrose containing 1×10^{-3} M *Tris* and 1×10^{-3} M EDTA, those from the second part in 0·30 M sucrose containing 1×10^{-3} M *Tris*. *Tris* mitochondria and EDTA mitochondria (R_3) were finally suspended in *Tris* sucrose (0·30 M sucrose with 1×10^{-3} M *Tris*) and EDTA sucrose (0·30 M sucrose containing 1×10^{-3} M *Tris* and 1×10^{-3} M EDTA) respectively. With such suspensions a decisive difference has been established between an enzyme fortified with EDTA and the control. In most instances the control suspension manifests unbroken linear oxygen uptake over the duration of the experiment irrespective of PMS concentration although the rate is related to the concentration. On the other hand when EDTA is included it effectively stimulates oxygen uptake and initiates the biphasic "inhibition" curve when

the PMS concentration is sufficient. Whilst this points to an important influence of EDTA it does not eliminate some relationship between the PMS concentration and mitochondrial protein concentration as a factor or that under other conditions the biphasic curve may not be evoked without EDTA. In order to evaluate this the protein concentration has been so varied that the ratio of PMS to protein is considerably increased and under these conditions it has been observed that the relative excess of PMS influences biphasic "inhibitory" activity independently of EDTA (Fig. 3).

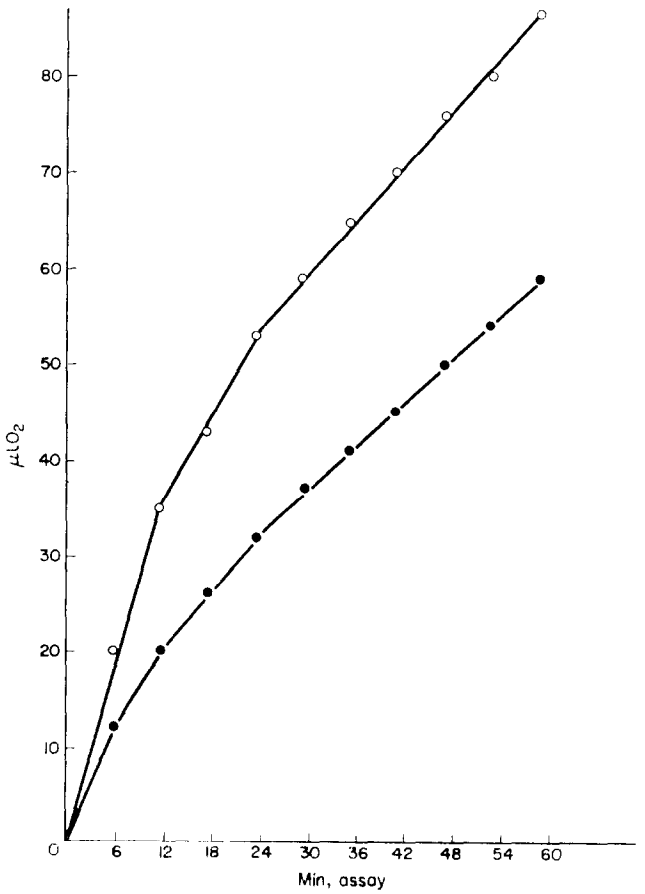

FIG. 3. Succinic dehydrogenase activity of *Tris* and EDTA mitochondrial preparations of low protein concentration.

Each flask contained 73·5 μmoles succinate; 40 μmoles phosphate buffer pH 7·3; 1×10^{-3} M KCN pH 7·0; 0·4 ml *Tris*–mitochondrial preparation (○——○) or 0·4 ml EDTA–mitochondrial preparation (●——●), in a final volume of 2·0 ml. Results are expressed as $\mu l.$ O_2/mg protein. The graph represents the mean of four experiments. The mean of the ratios of PMS to protein concentration per flask was 0·28 (range 0·21–0·34). The average protein concentration per flask was 2·8 mg.

When the protein concentration on the other hand is considerably increased, neither EDTA nor *Tris* preparations manifest biphasic inhibitory activity (Fig. 4). Whilst it might be considered that the PMS to protein relative proportions exert some influence, it is apparent from Table 3 that the total protein content also is an agent and when in

gross excess or deficiency it eliminates or facilitates appearance of the curve independently of EDTA, whereas with EDTA the absolute ratio assumes some added significance. It is probable that the bulk of enzyme determines the proportion of reactant site available to both the substrate and the inhibitor, whether PMS or one of its products. Since the substrate has some advantage of attachment, as shown above, its

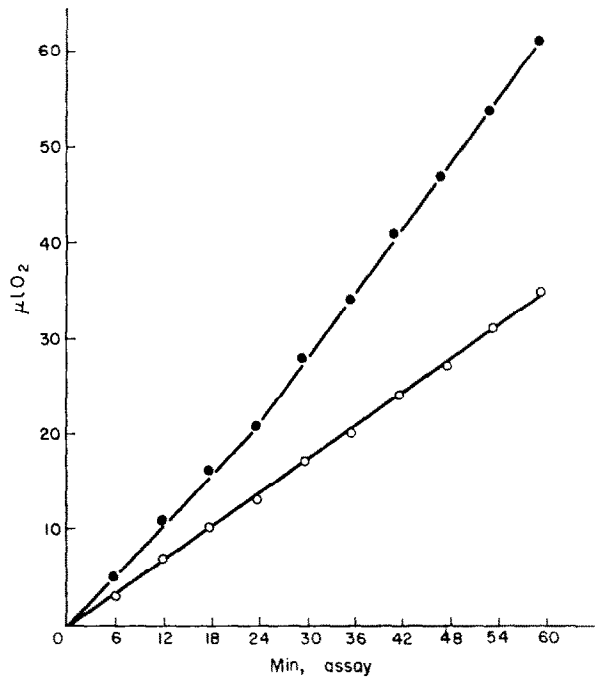

FIG. 4. Succinic dehydrogenase activity of *Tris* and EDTA mitochondrial preparations of high protein concentration.

Each flask contained 73·5 μmoles succinate; 40 μmoles phosphate buffer pH 7·3; 1×10^{-3} M KCN pH 7·0; 0·4 ml *Tris*-mitochondrial preparation (○——○) or 0·4 ml EDTA-mitochondrial preparation (●——●), in a final volume of 2·0 ml. Results are expressed as μl O_2/mg protein. The graph represents the mean of six experiments. The mean protein concentration per flask was 9·3 mg, and the mean PMS to protein ratio was 0·065 (range 0·04–0·075).

TABLE 3. EFFECT OF EDTA ON SUCCINIC DEHYDROGENASE ACTIVITY

	*PMS/Pr	Protein mg/flask	EDTA effect
A	0·065	9·3	Stimulation
B	0·015	6·9	Stimulation
C	0·045	6·9	Stimulation
D	0·38	6·9	Initial stimulation followed by inhibition
E	0·28	2·8	Inhibition

For A and E see legends for Figs. 4 and 3 respectively. B, C and D are the means of the results from five separate experiments, where the PMS levels per flask were 0·1 mg, 0·3 mg and 2·5 mg in B, C and D respectively. Other conditions as for A and E.

* PMS/Pr = PMS to protein ratio per flask.

protective influence will be more appreciable with a greater amount of enzyme, whereas with less enzyme competition with it by the inhibitor will be more apparent. In addition the lag in the appearance of the inhibition suggests that it may not be caused directly by the PMS but by some derivative formed during the course of the system's operation. In view of the known structure of succinic dehydrogenase[12, 13] which contains non-haem inorganic iron bound as an essential constituent, it is important to obviate the influence of EDTA at this site. To achieve this, experiments have been organized to include iron so as to mitigate chelation of this compound which is attached to the enzyme and is susceptible to binding by haptoglobin.[14] The results of this approach are depicted in Fig. 5 in which the incorporation of iron alone

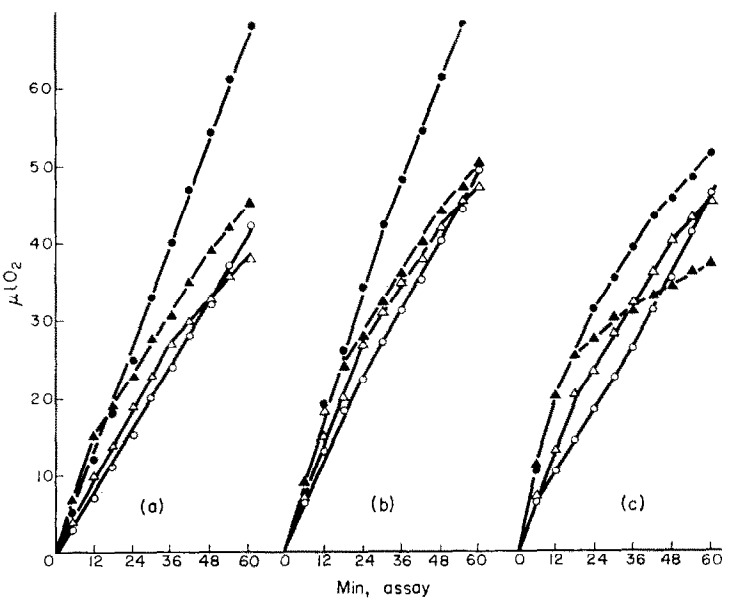

FIG. 5. Succinic dehydrogenase activity after incorporation of iron during mitochondrial preparation. The 2 ml assay mixture consisted of 73·5 μmoles succinate; 40 μmoles phosphate buffer pH 7·3; 1×10^{-3} M KCN pH 7·0; 0·1, 0·3 and 2·5 mg PMS per flask in A, B and C respectively and 0·4 ml mitochondrial preparation. After initial centrifugation, the supernatant (S_1) was divided in four (I, II, III, IV). Mitochondria from parts I and II were suspended in 0·30 M sucrose, containing 1×10^{-3} M *Tris*, while parts III and IV were suspended in 0·30 M sucrose containing 1×10^{-3} M *Tris* and 1×10^{-3} M EDTA. II and IV had in addition 1×10^{-3} M $FeCl_3$. On the graph I, II, III and IV are referred to as *Tris* (○——○), *Tris* + Fe (△——△), EDTA (●——●), and EDTA + Fe (▲——▲) respectively.

Results are the mean of three experiments and are expressed as μl O_2/mg protein. Mean protein concentration per flask is 6·4 mg and PMS to protein ratios are 0·015, 0·045, and 0·39 in A, B and C respectively.

allows slight effect and induces a minimal tendency to inhibit activity. Incorporation of EDTA has considerable stimulating activity which has the expected relationship to the level of PMS used, and as previously demonstrated the appearance of the biphasic activity is a characteristic of the PMS concentration. With inclusion of iron, either alone or in addition to EDTA, the stimulation is maintained and the inhibition curve

is rendered independent of the PMS concentration used. It is obvious that whatever action EDTA has in abettting the appearance of the inhibitory activity, it is not exerted directly by chelating enzymatic iron. These results suggest that the incorporation of iron alone facilitates some alteration in the PMS reaction and the appearance of the biphasic curve. The incorporation of EDTA alone stimulates the oxygen uptake and only at the highest levels of PMS allows a suggestion of the biphasic curve indicating that stimulation and biphasic activity are not directly connected. Finally the combination of EDTA and iron produces both initial stimulation and rapid appearance of inhibition of oxygen uptake. This indicates that the inorganic iron either free or chelated may be directly concerned in a reaction with PMS, which alters the electron transfer system.

The effect of several other metal ions has been tested for comparison with iron. It is noteworthy that Ni^{2+} and Co^{2+}, have a more dramatic effect in stimulating and in rapid induction of biphasic activity. On the other hand Sr^{2+} and V^{2+} are without effect. This is explicable on the basis that the electron transfer involved with iron is stimulated by Co^{2+} and Ni^{2+}.

Pyocyanine and the PMS assay

The experiments in the foregoing studies are unable to elucidate the manner in which PMS alters the rate of oxygen uptake. Its contribution to the inhibitory activity may be either by direct action of the compound or by intervention of some derivative formed from it in the assay procedure. In order to determine which of these explanations is pertinent experiments have been tailored. As a point of departure in these studies it has been observed that during the assay technique with PMS a green colour is invariably produced. It has been further determined that the break in oxygen uptake coincides with the manifestation of this green colour. The green substance has been extracted and its influence on the assay system determined. Assay systems, allowed to proceed with accumulation of considerable quantities of the pigment, have been extracted with chloroform. After evaporation *in vacuo* the pigment is redissolved in chloroform to give a 3·4% solution. Aliquots are added to the flasks. The chloroform is evaporated leaving dry green residues, which dissolves on addition of the assay mixture. After a slight initial inhibition, the extent of which depends upon the concentration of green substance, the oxygen uptake resumes for a short time a rate linearly similar to the control. At approximately 12–15 min, as in assay systems containing EDTA there occurs the typical reduced phase of oxygen uptake. The slope of this phase is similar at all concentrations indicating that the system had been saturated.

It has been deemed necessary to establish that the material formed during the reaction is derived from PMS and whether the enzyme system must be present for its development. A mixture of PMS and phosphate buffer was allowed to incubate at 20° for 2 days, during which time abundant green substance develops and is processed as above. On incorporation in assay mixtures it manifests behaviour which is identical with that of the green substance procured from assay systems. This establishes its non-enzymatic origin from PMS. The purity of the substance, however, remains in question. Multiple pigmented compounds form during the decomposition of PMS.[15] The uncertainity as to which of these is responsible for the action on the mitochondrial enzyme necessitates further purification. This has been accomplished by paper

chromatography which permits the separation of distinct brown, red and blue pigments. These are the three main bands, situated as follows—the brown near the solvent front, the blue closest to the point of application and the red between these two. The central portion of each band was eluted off, dried and redissolved in a small volume of solvent which is used to test the effect of each pigment on the enzyme complex. As seen in Fig. 6 neither the brown nor the red pigment produces significant alterations in oxygen uptake, whereas the blue pigment reproduces the effects observed with the crude green substance.

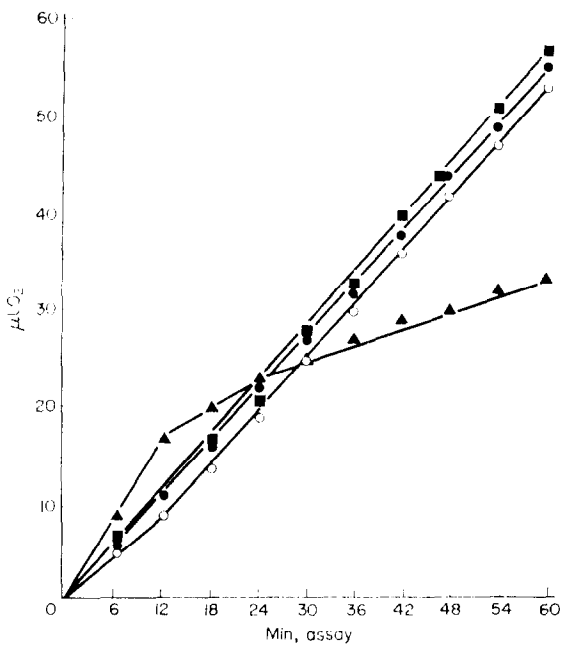

FIG. 6. Oxygen uptake in presence of three pigments isolated from green substance.
Each flask contained 73·5 μmoles succinate; 40 μmoles phosphate buffer pH 7·3; 1×10^{-3} M KCN pH 7·0; 2·5 mg PMS; 6·8 mg *Tris*–mitochondrial preparation and pigments as indicated in a final volume of 2·0 ml. ○——○, control; ●——●, 1 mg brown pigment; ■——■, 1·71 mg red pigment; and ▲——▲, 1·15 mg blue pigment.
Results are expressed as μl O_2/mg protein.

Identification of the blue pigment

An alkaline aqueous solution of the blue pigment, submitted to analysis on the Beckman DK_2 ratio recording spectrophotometer shows a characteristic spectrum. The maxima are 237 mμ, 310 mμ, and 376 mμ in the ultraviolet and a broad peak between 680–700 mμ in the visible region. On addition of hydrochloric acid, the solution turns red and gives a spectrum with maxima at 279 mμ, 385 mμ, a shoulder at 370 mμ in the ultraviolet, and a broad peak from 500–515 mμ. These spectra agree well with those obtained by Marchall, Solek, Grandjean-Radowach and Botte[16] for pyocyanine. A sample of pyocyanine which was received from Professor Marchall and re-extracted with chloroform to conform with our procedure has been shown to give identical spectra with those obtained with our sample. It has been concluded,

therefore, that the blue compound is pyocyanine. The similarity in trend between the effects of pyocyanine and EDTA on oxygen uptake, suggests that possibly EDTA catalyses decomposition of PMS. A solution of PMS treated with EDTA at final concentrations ranging from $3 \times 10^{-4} - 3 \times 10^{-2}$ M, changes in colour on standing in direct sunlight. The usual green colour appears more rapidly in the tubes containing EDTA and the rate is increased proportionately to increasing EDTA concentration.

DISCUSSION

It is clearly established in the present investigation that partial inhibition of succinic dehydrogenase occurs as a feature of the assay system itself. This is caused by decomposition of PMS electron acceptor into products, one of which is pyocyanine and this is the only one which duplicates the effect observed in the total assay system. Other derivatives are inert. The production of the pyocyanine is catalysed by EDTA and presumably by iron, a combination of EDTA and iron evidently in chelation is even more effective as a catalyst. This action of EDTA readily explains the property of the chelator in producing a rapid inhibition in the assay system. While the inhibition observed is explicable therefore on the basis of pyocyanine production the capacity of EDTA to stimulate may depend on the rapid production of pyocyanine in its presence. Other workers [11, 17, 18] using particulate systems have observed that EDTA is capable of altering the oxygen uptake of succinoxidase. On the other hand the manner in which pyocyanine acted also is not clarified. It was postulated by Guiditta and Singer[19] that PMS has two reaction sites, one on the flavoprotein and the other not further from the succinic dehydrogenase than cytochrome b. It is possible that PMS is replaced at one of these sites by pyocyanine totally and partially at the other to 50 per cent or more inhibition of activity as found in current studies. The present investigation with purified pyocyanine suggests that the system is readily saturated to a maximum which supports the idea that one reaction site is eliminated. Whereas the above observations may support the view that one reactive site is eliminated, consideration must also be directed to the importance of substrate competition. Our results in contrast with those of Marcus and Feeley[20] on L-amino acid oxidase show that substrate concentration significantly interferes with the inhibition by pyocyanine. This necessitates the inclusion of the site of attachment of the substrate and enzyme within the purview of the specific action of pyocyanine. Recent studies on the mechanism of the succinic dehydrogenase reaction render this interpretation feasible. It has been demonstrated[21, 22] that the succinic dehydrogenase acts in a stereospecific manner so that separate binding sites for paired hydrogens are involved in the exchange between the substrate and carrier system. It is possible that pyocyanine may interfere with one of these sites. In this context the profound influence of the pH of the system on the alteration of inhibition indicates that the pyocyanine may indeed act on the stereospecific site, since increase in hydrogen ion concentration facilitates the preferential attachment of the substrate.

Acknowledgements—One of us (M. MacB.) was in receipt of a Research Fellowship from the Medical Research Council of Ireland.

REFERENCES

1. E. B. KEARNEY and T. P. SINGER, *J. biol. Chem.* **219**, 963 (1956).
2. T. P. SINGER and E. B. KEARNEY, *Methods of Biochemical Analysis* (Edited by D. GLICK) Vol. 4, p. 307. Interscience, New York (1957).

3. W. C. SCHNEIDER and G. M. HOGEBOOM, *J. biol. Chem.* **183**, 123 (1950).
4. J. C. KALTENBACK and J. W. HARMAN, *Exp. Cell Res.* **8**, 435 (1955).
5. W. S. MOOS and J. W. ROWEN, *Arch. Biochem. Biophys.* **43**, 88 (1953).
6. E. LAYNE, *Methods of Enzymology* (Edited by S. P. COLOWICK and N. O. KAPLAN) Vol. 3, p. 447. Academic Press, New York (1957).
7. O. WARBURG and W. CHRISTIAN, *Biochem. Z.* **310**, 384 (1941).
8. F. KEHRMANN and E. HAVAS, *Ber.* **46**, 341 (1913).
9. H. MCILWAIN, *J. Chem. Soc.* 1701 (1937).
10. O. ARRIGONI and T. P. SINGER, *Nature, Lond.* **193**, 1256 (1962).
11. M. B. THORN, *Biochem. J.* **85**, 166 (1962).
12. T. P. SINGER, E. B. KEARNEY and P. BERNATH, *J. biol. Chem.* **223**, 599 (1956).
13. T. Y. WANG, C. L. TSOU and Y. L. WANG, *Scientia Sinica* **5**, 73 (1956).
14. E. B. KEARNEY and T. P. SINGER, *Biochem. Biophys. Acta* **17**, 596 (1955).
15. G. A. SWAN and D. G. FELTON, *Chemistry of Heterocyclic Compounds*, Vol. XI, Interscience, New York (1957).
16. J. G. MARCHAL, J. SOLEIL, C. GRANDJEAN-RADOWITCH and J. M. BOTTE, *Bull. Ass. Dipl. Microb., Nancy* **63**, 1 (1956).
17. W. D. BONNER, *Biochem. J.* **56**, 274 (1954).
18. E. C. SLATER and K. W. CLELAND, *Biochem. J.* **55**, 566 (1953).
19. A. GUIDITTA and T. P. SINGER, *J. biol. Chem.* **234**, 666 (1959).
20. A. MARCOS and J. FEELEY, *Biochim. Biophys. Acta* **59**, 398 (1962).
21. O. GAWRON, A. J. GLAID, T. P. FONDY and M. M. BECHTOLD, *Nature, Lond.* **189**, 1004 (1961).
22. O. GAWRON, A. J. GLAID, J. FRANCISCO and T. P. FONDY, *Nature, Lond.* **197**, 1270 (1962).