CCX. PHENAZINE COMPOUNDS AS CARRIERS IN THE HEXOSEMONOPHOSPHATE SYSTEM

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Certain phenazine compounds resemble the flavins in that they can readily form semiquinones, to which property, generally very rare in dyestuffs, Michaelis *et al.* [1936, 1, 2] attach much importance in determining the catalytic activities of the flavin enzyme and vitamin B_2.

Phenazine methiodide [Dickens, 1936, 1] like pyocyanine [Friedheim, 1934] and certain flavin compounds [Laser, 1934] has an action on tissue metabolism which is quite different from that of most of the usual oxidation-reduction dyestuffs [Dickens, 1936, 1]. In tissues exhibiting strong aerobic glycolysis, particularly tumour tissue, the respiration is increased and the aerobic glycolysis falls in the presence of these compounds which thus appear to exert a control of the Pasteur reaction [cf. Dickens, 1936, 2]. While the action of the simpler phenazines is rapid, enabling its detection in short experiments by the tissue-slice method, that of the flavins can only be demonstrated in tissue culture experiments, presumably owing to slow absorption or slow transformation into the active carrier. On the other hand the easily diffusible phenazines cause some inhibition of the anaerobic glycolysis also [Dickens, 1936, 1], so that this is not a simple augmentation of the Pasteur effect. This group of substances produces such unusual effects that an attempt has been made to elucidate the mechanism of their action by a study of the catalytic role of the phenazines in an isolated enzyme system. Weil-Malherbe [1937, 2] has studied the action of phenazine methochloride and pyocyanine in the α-hydroxyglutaric system.

In the experiments described below we have prepared some new phenazine compounds and used them as substitutes for the flavin enzyme of Warburg in the hexosemonophosphate system, comparing their catalytic activities with those of other carriers. We have also measured the oxidation-reduction potentials of a number of phenazines for correlating our results. The experiments reveal new aspects of the common features of the phenazines and flavin compounds, and it is shown that in the hexosemonophosphate system the catalytic activity of some of these compounds much surpasses that of the ordinary oxidation-reduction dyestuffs studied.

Green *et al.* [1934] used pyocyanine as carrier in this system in their studies on carrier-linked reactions. Later Ogston & Green [1935] stated their objections to the description of the flavin-phosphate-protein complex as an enzyme. In the following we have retained the term flavin enzyme, since the question cannot be regarded as settled.

Enzyme and substrate preparations

Enzyme preparations. Many of the experiments were made on preparations of "Zwischenferment" [Negelein & Gerischer, 1936] and coenzyme II of purity 1, generously supplied by Prof. Warburg, who also gave us a sample of flavin

enzyme purified by the $CHCl_3$ method [Warburg & Christian, 1933] containing in the 10% solution $4{\cdot}1 \times 10^{-8}$ g.mol. pigment per ml. as estimated by Prof. Warburg. For use, 0·1 mg. dry Zwischenferment per vessel was dissolved in 2 ml. *M*/100 phosphate buffer. For other experiments we used a simple enzyme preparation made from Lebedew fluid prepared from Löwenbräu, Munich, bottom beer yeast as follows. The finely ground yeast, air-dried, was incubated for 2½ hr. with 3 vol. water at 35°. After centrifuging, the clear fluid was diluted with 5 vol. distilled water and 0·15 *N* acetic acid was added with stirring and ice cooling, to give *p*H 4·6 (the volume of acetic acid required is about half the vol. of Lebedew fluid taken). The white precipitate was collected at the centrifuge, washed with 0·02 *M* acetate buffer *p*H 4·6, and dried quickly *in vacuo* over P_2O_5. The yield was about 2·5 g. per 250 g. dried yeast. If necessary, the enzyme may be dissolved in 0·02 *N* ice cold NaOH and, after centrifuging from some insoluble residue, reprecipitated by acetic acid as before. It may be kept in the refrigerator for several weeks in the dry state without loss of activity. For use it was dissolved (10–20 mg. per vessel) in ice cold *N*/100 NaOH or *M*/100 phosphate buffer with addition of NaOH to give a final solution of the required *p*H, and centrifuged. Although this enzyme is much cruder than the Warburg material, with the quantity taken the blank was zero or very small and the rates of O_2 uptake were equal with the two preparations.

Coenzyme II of purity 0·2 was prepared from horse blood corpuscles by the method of Warburg & Christian [1936], "1 and 2 Schritte". Of this 0·1 mg. per vessel used instead of 0·02 mg. of the preparation of purity 1 gave the same rate of O_2 uptake.

Substrate. Ba hexosemonophosphate was prepared from the action of dialysed muscle extract on glycogen [Ostern *et al.* 1936]. It was found essential to allow full digestion to reduce the glycogen content to a minimum, but traces nevertheless remained. Prof. Robison very kindly analysed a specimen as follows. Found: total P 7·4%; inorg. P 0·1%; H. and J. reduction 32·9; I.V. 32·3; Seliwanoff 5·6; $[\alpha]_{5461}$ 23·4°; glycogen+. Calc. for $C_6H_{11}O_9PBa$: P 7·85%. The analysis is that of a mixed hexosemonophosphate [cf. Robison, 1932] containing a little glycogen.

For the preparation of the solution of the Na salt, 0·145 g. Ba salt was ground with 0·354 g. Na_2SO_4, $10H_2O$ and diluted to 5 or 10 ml. after the addition of 0·125 ml. *N* HCl. The centrifuged solutions contained respectively 0·18 or 0·09 *M* Na hexosemonophosphate (P analysis).

Phenazine compounds

Methylphenazonium salts. Phenazine methosulphate was prepared by the addition of an equivalent of dimethyl sulphate, freshly distilled *in vacuo*, to phenazine in nitrobenzene solution [Kehrmann, 1913; Hillemann, 1938] and crystallized quickly from alcohol, M.P. 167°. Methylphenazonium chloride was obtained as yellow needles by repeated crystallization of the methosulphate in presence of KCl (found: Cl 15·1; calc. for $C_{13}H_{11}N_2Cl$ 15·4%). This salt has previously been described as green by Browning *et al.* [1922] who prepared it by dissolving in HCl the product obtained by action of alkalis on phenazine methosulphate. This must be regarded as impure, for methylphenazonium salts decompose in alkaline solutions [McIlwain, 1937, 2; Hillemann, 1938].

Methylphenazonium phosphate was prepared by the addition of excess Na_2HPO_4 to the methosulphate, each in the minimum amount of water; the salt separated as brown prisms.

Semiquinoid methylphenazonium phosphate was obtained in fine green needles by warming the methosulphate in alcohol with excess phosphoric acid: I_2 equiv. [McIlwain, 1937, 2] 299; calc. for $C_{13}H_{11}N_2PO_4$ 292. Partial reduction of the methylphenazonium salts under warm acid conditions has been previously recorded [Kehrmann, 1914].

2-*Aminophenazine methosulphate* was prepared according to Kehrmann [1913] and the remainder of the compounds by the methods previously described [McIlwain, 1937, 1, 2].

Potentiometric titrations

The potentials were determined by titration of *M*/2000–4000 solutions in phosphate buffer with *c.* *M*/200 chromous acetate, prepared by diluting the satd. aqueous soln. with 2 vol. de-aerated water in a storage burette of the type described by Cohen & Phillips [1929]. The electrode vessel was kept at $30 \pm 0{\cdot}1°$ and de-aerated and stirred with N_2 freed from O_2 by hot Cu. Potentials were read from 3 gold-plated electrodes which normally agreed within 1 mv. Quinhydrone in 0·1 *N* HCl was used as the standard half-cell.

The titration curves showed no evidence of 2-stage oxidation-reduction processes over the *p*H range investigated, but slight intermediate colours were in some cases observed. The initially yellow solutions of the alkyl phenazonium salts become slightly yellow-green before separation of the colourless dihydro-compound on complete reduction. The reduced keto compound was pale cream coloured and insoluble; the reduced sulphonates and aminophenazine were soluble and almost colourless.

$Na_2S_2O_4$ was initially used as the reducing agent but though this gave normal titration curves, the results were not considered reliable as the titration could not be repeated after oxidation of the reduced solution. Progressively more positive values were obtained on repetition. A secondary reaction had evidently occurred as the solution showed a green fluorescence and the reduced compounds were no longer insoluble. In the case of phenazine methosulphate the values approached those of the sulphonates. It is understandable that such sulphonates should be produced from the sulphite formed during reduction, as their preparation from phenazonium salts and sulphite has been described [McIlwain, 1937, 2]. The initial values in titration with $Na_2S_2O_4$ were however substantially correct; Preisler & Hempelmann [1937] have used $Na_2S_2O_4$ in such titrations.

The compounds investigated considerably increase the range of oxidation-reduction potentials observed in the phenazine series (Table I). The simple

Table I

Compound	E_h, *p*H 7 30°	Authority
Na *N*-methylphenazoniumdisulphonate betaine	+0·230	Present investigations
N-Methylphenazonium sulphonic acid betaine	+0·130	,,
Phenazine methosulphate	+0·080	,,
Phenazine ethosulphate	+0·055	,,
4-Keto-*N*-methylphenazine (pyocyanine)	−0·034	[Friedheim & Michaelis, 1931]
4-Keto-*N*-ethylphenazine	−0·055	Present investigations
Phenazine-1-carboxylic acid amide (chlororaphin)	−0·115	[Elema, 1933]
2-Amino-*N*-methylphenazine methosulphate	−0·145	Present investigations
2-Keto-*N*-methylphenazine	−0·165	[Preisler & Hempelmann, 1937]
1-Hydroxyphenazine	−0·173	[Michaelis, 1931]
2-Hydroxyphenazine	−0·214	[Preisler & Hempelmann, 1937]
2:7-Diamino-*N*-phenylphenazonium chloride (phenosafranine)	−0·252	[Stiehler *et al.* 1933]
2-Amino-7-dimethylamino-2-methylphenazine (neutral red)	−0·325	[Clark & Perkins, 1932]

phenazine quaternary salts and sulphonic acids are much more positive than previously studied compounds, some examples of which are quoted. Table I also affords an excellent example of correlation of E_h with the electronic properties of the substituent groups, electron-attracting groups producing more positive and electron-repelling groups more negative potentials.

Phenazines as carriers

Methods. The O_2 uptake was measured in Warburg manometers at 37·5°. Each vessel contained in the main part 2 ml. enzyme solution, 0·1 mg. coenzyme of purity 0·2 (in some experiments 0·02 mg. of purity 1), 0·3 ml. $M/10$ hexose-monophosphate. The side bulb held the solution of carrier in 0·2 ml. The inner cup contained 0·2 ml. N NaOH and the gas space was filled with pure O_2. Usually these conditions were adhered to, but sometimes slight variations were made. The O_2 uptake during the first hr. was measured; it continued regularly for a longer period provided that the substrate concentration did not fall too much. Readings were corrected for a blank without substrate. In the absence of added substrate the blank was small, a few μl. per hr., except when large quantities of autoxidizable carrier were present. This autoxidation is much reduced by working at a slightly acid reaction, *p*H *c.* 6·5, without affecting the catalytic activity, and this was done in later experiments; other experiments were at *p*H 7–7·5. The rates of O_2 uptakes were the same with the various combinations of enzyme and coenzyme used, within fairly close limits, since both these components were present in excess and the amount of carrier was ordinarily the limiting factor. The carriers were dissolved in water and where necessary neutralized. A few (Table II, Nos. 12, 19) were sparingly soluble and a suspension containing the wt. shown was placed in the side bulb. Flavin enzyme was dissolved in 10 vol. of water and centrifuged to remove a trace of denatured protein. Amounts of carrier are expressed in mg. and μl. (1 mM = 22400 μl.). In the case of flavin enzyme 1 mM prosthetic group, photometrically determined, is taken as 22400 μl.

Results. These are shown in Table II.

The results may be grouped according to the rate of O_2 uptake.

Group 1. Maximum activity (*c.* 200 μl./hr.) is given by only a few substances. These were: phenazine methosulphate, methochloride and ethosulphate (amount of carrier *c.* 20 μl.) and flavin enzyme (Nos. 3, 4, 5 & 10).

Group 2. Of less but still marked activity were the mono- and di-sulphonic acid derivatives of N-methylphenazine (Nos. 1 and 2), pyocyanine (No. 8) and its ethyl homologue (No. 9) whose activity exceeds that of pyocyanine, the 2-amino derivative of N-methylphenazine methosulphate (No. 14) and the red oxidation product of N-methylphenazine quaternary salts (No. 15) and its ethyl homologue (No. 16). In amounts of from 22–50 μl. this group caused O_2 uptakes of from 40–80 μl./hr. It is noteworthy that all the compounds of Groups 1 and 2, with the exception of the flavin enzyme, are derivatives of N-alkylphenazines.

Group 3. Among non-phenazine compounds investigated none approached in activity the simple phenazine quaternary salts. Brilliant cresyl blue and methylene blue showed most activity in this group, being comparable with the lower members of Group 2 (50 μl. dye caused *c.* 30–45 μl. O_2/hr.).

Group 4. The remaining compounds studied were of low or zero activity (with chlororaphin and dimethylalloxazine the lack of activity might have been due to their insolubility). Methyl Capri blue, though in the same region of potential as active carriers, belongs to this group. Acridine methochloride (No. 22, Table II) was quite inactive as carrier, though chemically it differs from phenazine methochloride only in the substitution of a CH group for a N atom.

Table II

No.	E_h, pH 7	Substance	Amount per vessel: mg.	Amount per vessel: μl.	O_2 uptake* μl. (1st hr.)	Turnover no. (Average over 1st hr.) Mol. O_2/min. / Mol. catalyst × 0·5
1	+0·230	Sodium *N*-methylphenazonium disulphonate betaine	0·5	30	79	0·088
2	+0·130	*N*-Methylphenazonium sulphonic acid betaine	0·5	37	68	0·061
3	+0·080	Phenazine methosulphate	0·3	22	204	0·310
4	—	Phenazine methochloride†	0·3	27	206	0·260
5	+0·055	Phenazine ethosulphate	0·3	19	190	0·330
6	+0·045	Brilliant cresyl blue	1·5	106	71·5	0·022
			0·75	53	47	0·029
			0·5	35	36	0·034
7	+0·011	Methylene blue	1·5	90	34	0·013
			1·0	60	28	0·016
			0·75	45	27	0·021
			0·5	30	27	0·030
			0·25	15	26	0·057
8	−0·011	Pyocyanine hydrochloride† (4-keto-*N*-methylphenazine)	0·45	45	47	0·030
9	−0·055	4-Keto-*N*-ethylphenazine	0·3	30	80	0·090
10	−0·060	Flavin enzyme	(20)‡	0·18	215	39·600
			(5)‡	0·045	110	81·400
11	−0·060	Methyl Capri blue	0·3	22	13	0·020
12	−0·115	Chlororaphin (satd.)	<0·3	<30	15	<0·020
13	−0·142	Gallophenin	0·5	37	4	0·003
14	−0·145	2-Amino-*N*-methylphenazine methosulphate	0·3	21	40	0·063
15	−0·165	2-Keto-*N*-methylphenazine	0·5	50	69	0·047
			0·14	14	35	0·083
16	−0·180	2-Keto-*N*-ethylphenazine	0·3	28	45	0·053
17	−0·180	Lactoflavin	0·3	20	0·5	0·001
18	−0·252	2:7-Diamino-*N*-phenylphenazonium chloride (phenosafranine)	0·3	21	0	0·000
Other compounds:						
19	—	6:7-Dimethyl alloxazine (satd.)	<0·1	< 7	0	0·000
20	—	Nicotinamide methiodide	0·5	42	1	0·000
21	—	Phenazine-1-carboxylic acid amide	0·5	49	4	0·003
22	—	Acridine methochloride	0·3	29	0	0·000
23	—	1:2:3:4-Tetrahydrophenazine methosulphate	0·3	20	25	0·040
24	—	Aneurin	2·0	133	0	0·000
25	—	Thiochrome	0·6	51	0	0·000

* Corrected for blank, if any. † See Fig. 1.
‡ Wt. of crude substance taken; flavin content photometrically determined.

The catalytic effect is dependent on the concentration of carrier. This is clearly shown by the data of Table II for brilliant cresyl blue and methylene blue. The turnover no. in the last column shows the decreasing activity with increasing concentration of these dyes. Since this is not here due to the limiting capacity of the enzyme system, both being feeble catalysts for this system, it is apparently due to a poisoning effect of these dyes when present in high concentration.

A different effect is seen with the *N*-alkylphenazines. Here, owing to their greater activity, the O_2 uptake is limited by the enzyme system when the amount of these catalysts is much increased. Fig. 1 shows the activity-concentration curves of phenazine methochloride and pyocyanine. (The preparation of enzyme was actually made to study the oxidation of phosphohexonate, the curves for

which are also included for comparison in Fig. 1. The rather high concentration, $M/30$, of phosphate buffer has inhibited the hexosemonophosphate oxidation more than that of phosphohexonate [see Dickens, 1938].)

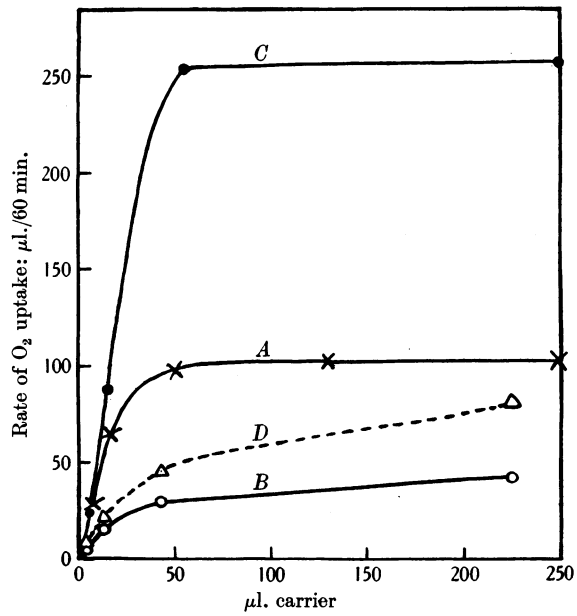

Fig. 1. Comparison of phenazine methochloride and pyocyanine as carriers. Curve A: phenazine methochloride, hexosemonophosphate. Curve B: pyocyanine, hexosemonophosphate. Curve C: phenazine methochloride, phosphohexonate. Curve D: pyocyanine, phosphohexonate. 20 mg. acetate-precipitated enzyme, 2 ml. $M/30$ phosphate buffer, 0·1 mg. coenzyme (purity 0·2), 0·3 ml. substrate.

Activity relative to flavin enzyme

While the absolute values of O_2 uptake with the hexosemonophosphate system when phenazines are added attain those given by flavin enzyme as carrier, the turnover numbers (T)[1] in Table II show clearly that their efficiency is very much less. For the flavin enzyme, $T = 40$–80 mol. O_2/0·5 mol. prosthetic group min. with our enzyme preparation. Warburg & Christian [1933] give the values as 25–50 mol. O_2/mol. prosthetic group min. In contrast with this high activity, the non-colloidal carriers listed in Table II give much lower T-values, the most active phenazonium salts having only about 1/250th of that of the flavin enzyme, while pyocyanine in similar concentration is about ten times less active still.

E_h *relationships*: "*specificity*"

The E_h of the simple N-alkylphenazonium salts varies from 0·08 to 0·06. That of the flavin enzyme is recorded by Kuhn & Boulanger [1936] as −0·06 V. (i.e. 0·12 V. more positive than lactoflavin). Although intermediate between

[1] For the calculation of the turnover no. it is important to know if the O_2 taken up is partly lost in side reactions (e.g. H_2O_2 formation or coupled oxidation) or if it is wholly reduced by the H transported by the catalyst concerned. Only in the latter case is the turnover no. given by the ratio: mol. O_2/0·5 mol. catalyst min. With 100% yield of H_2O_2 the equation becomes T = mol. O_2/mol. catalyst min., as used by Warburg & Christian [1933].

these in potential methylene blue and brilliant cresyl blue are inferior catalysts for this reaction. Hence thermodynamic considerations alone do not govern the efficiency of the carriers tested in this system. It is also noteworthy that other phenazines (Nos. 1, 2, 14, 16, Table II) whose potentials lie outside those of the alkylphenazonium salts and flavin enzyme in both positive and negative directions, are somewhat more efficient catalysts than the dyes methylene blue and cresyl blue, although according to their potentials these dyes should have activities between that of the most active alkylphenazines and flavin enzyme. There is thus a certain limited "specificity" in the chemical nature of the active carriers for this system. It may be possible to explain this structural specificity in terms of semiquinone formation [Michaelis *et al.* 1936, 1, 2]. The activity may be determined by the concentration of semiquinone in the half-reduced form at the reaction of the system.

Catalytic activities of phenazines added as semiquinones

Semiquinonoid methylphenazonium phosphate and 1:2:3:4-tetrahydrophenazine methosulphate [McIlwain, 1937, 1] were tested. The activity of the semiquinonoid and ordinary forms of the compounds was the same, but as the bright green solution of semiquinones rapidly changed to yellow on addition to the enzyme system, it is evident that only a trace of semiquinonoid compound could have been present, and this of course is no evidence against the importance of semiquinone formation.

Qualitative differences in effect of phenazines and flavin enzyme

Whereas the enzyme system used attacked Neuberg & Robison esters at about the same rate in presence of flavin enzyme as carrier, the Neuberg ester was much

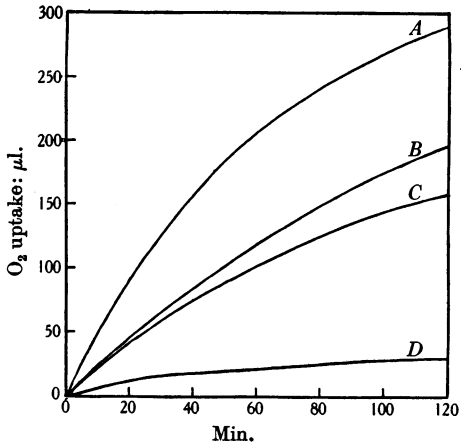

Fig. 2. Curve A: Robison ester + 0·3 mg. phenazine methochloride, 0·3 mg. phenazine methosulphate, or 10 mg. (crude) flavin enzyme. Curve B: Neuberg ester + 5 mg. flavin enzyme. Curve C: Robison ester + 5 mg. flavin enzyme. Curve D: Neuberg ester + 0·3 mg. phenazine methochloride. 0·1 mg. Zwischenferment in 2·3 ml. *M*/100 phosphate buffer, + 0·02 mg. coenzyme II of purity 1, + 0·4 ml. *M*/6 phosphoric ester.

less rapidly attacked than the Robison ester, when phenazine methochloride was used instead of flavin enzyme (Fig. 2).

Neuberg ester was prepared from Ca hexosediphosphate (B.D.H.) by hydrolysis with oxalic acid [Neuberg, 1918]. The Ca salt thus obtained was decomposed by the calc. amount of oxalic acid and after removal of Ca oxalate neutralized with NaOH.

As yet the mechanism of the oxidation of Neuberg ester has not been examined. It is therefore difficult to explain this difference, which may be accounted for either by the presence of some other enzyme than the flavin one in the crude preparation of flavin enzyme, or less probably by some inhibitory action of the phenazine compound on a component of the fructosemonophosphate-oxidizing system. That phenazine compounds can exert an inhibitory action on enzymes is readily shown. Weil-Malherbe [1937, 1] showed that succinic dehydrogenase is inhibited by pyocyanine and phenosafranine. The action of phenazine methochloride on carboxylase and succinic dehydrogenase is shown in Table III.

Table III. *Inhibition of carboxylase and succinic dehydrogenase*

Carboxylase. 50 mg. yeast carboxylase [Axmacher & Bergstermann, 1934] in 0·9 ml. water per vessel, with 0·3 ml. phosphate buffer *p*H 6·5 and 1 ml. addition of dye (or water). Side bulb contained 0·2 ml. *M*/10 Na pyruvate. Gas space, N_2. Temp. 25°.

Addition:	Water	2 mg. pyocyanine	2 mg. phenazine methochloride	2·6 mg. phenosafranine	2·5 mg. cresyl blue	1·7 mg. 2-keto-*N*-methylphenazine
			CO_2 evolved (μl.)			
30 min.	195	192	95	154	182	192
60 ,,	228	223	112·5	198·5	216·5	235
120 ,,	250	242	121·5	232	242	259

Inhibition by *M*/300 phenazine methochloride = 50%; others, none.

Succinic dehydrogenase. 50 mg. muscle enzyme, suspended in 1·5 ml. *M*/10 succinate in *M*/50 phosphate buffer *p*H 7·4; 0·5 ml. cytochrome. Side bulb contained dyes 0·4 ml. *M*/50. Air, 37·5°; 0·2 *N* NaOH in inner cup. Phenazine methochloride readings corrected for autoxidation of carrier.

Addition	Water	Phenazine methochloride	Pyocyanine	Phenosafranine
O_2 uptake μl. (60–120 min.)	98	76	32	28
Inhibition %	—	23	68	70

In these two cases very different actions are seen; phenazine methochloride in *M*/300 concentration inhibits carboxylase by 50% but has little action on succinic dehydrogenase. On the other hand pyocyanine and phenosafranine, which strongly inhibit succinic dehydrogenase [Weil-Malherbe, 1937, 1] have little effect on carboxylase. Michaelis & Smythe [1936] have already drawn attention to the poisoning by a similar concentration of pyocyanine of glucose fermentation in Lebedew fluid and its restoration by addition of a carboxylase preparation.

Action of iodide ion

In the earlier experiments with this system we used phenazine methiodide as in the earlier tissue experiments [Dickens, 1936, 1]. It was found with the hexosemonophosphate system that, using the methiodide as carrier, the reaction came to a standstill after 20–40 min. This was due to the I^- of the quaternary salt, since it did not occur with phenazine methochloride and the action of phenazine methochloride and flavin enzyme is inhibited by small quantities of NaI (Fig. 3).

The inhibition is perhaps due to an interaction either of I^- or of I_2 with the coenzyme, for it appeared to be partially removed by addition of fresh coenzyme to the system. The inhibition by I^- does not appear to have been observed before.

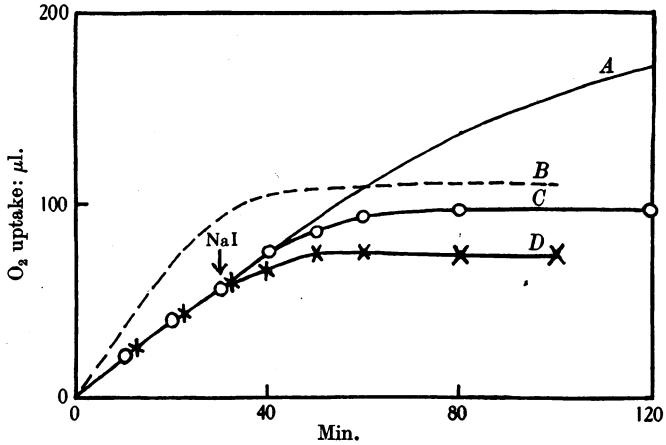

Fig. 3. Hexosemonophosphate oxidation. Curve A: 0·15 mg. methylphenazonium salt* or 5 mg. flavin. Curve B: 0·16 mg. phenazine methiodide. Curve C: 5 mg. flavin enzyme +0·1 mg. NaI, $2H_2O$ tipped in at 30 min. Curve D: 0·15 mg. methylphenazonium salt* +0·3 mg. NaI, $2H_2O$ tipped in at 30 min.

Action of phenazine methochloride on tissue metabolism

In view of the above results the action of the methochloride on tissue slices was tested. The results do not differ greatly from those seen with the methiodide [Dickens, 1936, 1], but as the fall of anaerobic glycolysis is less with the methochloride, the apparent partial restoration of the Pasteur mechanism is better seen (Table IV). The results with the red oxidation product of *N*-methylphenazines (2-keto-*N*-methylphenazine) were obtained with a pure specimen made synthetically [Kehrmann, 1924], and not by autoxidation, since free phenazine always results by the latter method; the specimen used was freely soluble in

Table IV. *Action of phenazine methochloride and 2-keto-*N*-methylphenazine on metabolism of Jensen sarcoma slices*

Warburg two-vessel technique, $NaHCO_3$-glucose Ringer. Temp. 37·5°. $CrCl_2$ in anaerobic vessels.

	Min.	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$	Q_{O_2}	$Q_G^{O_2}$	$Q_G^{N_2}$
		Control			Phenazine methochloride (10^{-3} *M*)		
Exp. 1	0–60	−10·8	+22·2	+40·8	−13·5	+ 8·6	+45·6 (yellow)
	60–120	—	—	+36·4	—	—	+31·9 (colourless)
	120–180	—	—	+35·0	—	—	+25·0 (colourless)
Exp. 2	0–30	−14·3	+21·4	—	−28·0	+12·1	—
	30–60	−10·1	+18·6	—	−10·3	+ 4·1	—
	60–90	− 9·1	+18·0	—	− 9·6	+ 3·1	—
		Control			2-Keto-*N*-methylphenazine (10^{-3} *M*)		
Exp. 3	0–60	−16·8	+20·4	+28·2	−18·3	+13·8	+16·6 (red)
	60–120	−14·7	+20·0	+17·5	−15·7	+14·0	+13·5 (v. pale pink)
	120–180	−13·1	+15·5	+15·9	−15·1	+13·8	+13·7 (colourless, re-oxidized to red on admitting air)

* Not the iodide.

salt solution unlike that previously employed [Dickens, 1936, 1] which was therefore impure. In aerobic experiments with tissue the phenazonium salts become partly oxidized to a red compound, presumably this one. This compound inhibited anaerobic glycolysis more than phenazine methochloride, but did not reduce the aerobic glycolysis as much in Exp. 3 of Table IV, though it had a pyocyanine-like action on aerobic glycolysis.

Autoxidation. Measured in $NaHCO_3$-glucose Ringer solution, by the two-vessel method of Warburg under the same conditions as in the tissue experiments, the autoxidation of 10^{-3} *M* phenazine methochloride in 95 % O_2 + 5 % CO_2 was slow, -3μl. O_2, $+4\mu$l. CO_2 per 4 ml. per hr. When tissue is present the oxidation is probably greater, but it is difficult to allow for it accurately. Calculations showed that with the quantities used, it could not result in any serious error, and that the measurements were substantially those of tissue metabolism and not of autoxidation of the carrier. When in experiments without tissue, in which autoxidation was allowed to proceed in a more alkaline medium, the O_2 consumption was measured and the acid production was titrated, it was found that for 1 mol. phenazine methochloride 0·5 mol. O_2 is consumed and 1 equiv. acid (1 mol. HCl) is set free. Hence autoxidation would be expected to increase aerobic acid production, whereas the addition of phenazine methochloride to tissue slices produces a fall of aerobic glycolysis and it is in fact probable that part of the residual acid production in presence of phenazine methochloride arises from this. On the other hand reduction anaerobically may have given values for glycolysis which were a little too high, but calculation showed a max. error of *c.* 10 %.

4·6 mg. phenazine methochloride were added from the side bulb of a Warburg manometer vessel to 4 ml. *N*/100 NaOH contained in the main part. Air, 37·5°. The initially rapid oxidation became very slow after 50 min. when 199 μl. O_2 had been consumed and the equiv. of 1·80 ml. *N*/100 acid liberated = 403 μl. Calc. for $\frac{1}{2}O_2$ and 1HCl; 224 μl., 448 μl. respectively. A bright red $CHCl_3$-soluble compound resulted. Indophenoloxidase preparation from heart muscle brings about oxidation of phenazine methochloride at neutral reaction to form a red compound soluble in $CHCl_3$. The quantitative investigation of this has not been made. Free phenazine in addition to the red compound results from alkaline oxidation of the methochloride [McIlwain, 1937, 2].

Summary

The E_h of a series of phenazine derivatives, determined by potentiometric titration, have been correlated with the electronic properties of the substituents. The activity of the phenazines and some non-phenazine dyestuffs as carriers in the hexosemonophosphate system has been determined. According to their activity, the substances fall into four groups: (1) most active: phenazine methosulphate, methochloride and ethosulphate, E_h + 0·055 to + 0·080; (2) moderately active: mono- and di-sulphonic acids derived from *N*-methylphenazine, E_h + 0·13, + 0·23; pyocyanine, E_h − 0·011 and its ethyl homologue; (3) less active: brilliant cresyl blue and methylene blue; (4) others inactive: the most active of the above compounds had only about 1/250th of the turnover no. of the flavin enzyme, E_h − 0·06. Structure is evidently more important than E_h in determining the activity of carriers for this system. A property common to the phenazines and flavin is the ability to form semiquinones, and the peculiar activity of these compounds in this system may be due to this property.

When phenazine methochloride is used instead of flavin enzyme, the Neuberg ester is attacked much less readily than the Robison ester; with flavin enzyme as carrier both are attacked at about the same rate. Carboxylase is inhibited by *M*/300 phenazine methochloride, not by pyocyanine or phenosafranine; succinic

dehydrogenase is inhibited by phenosafranine, but much less by phenazine methochloride in the concentration used. The hexosemonophosphate system is inhibited by iodide in low concentration. The action of phenazine methochloride on the metabolism of tumour tissue is to increase respiration and diminish aerobic glycolysis.

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