JOURNAL OF PHARMACY AND PHARMACOLOGY

VOLUME XI, 1959

WITH THE

TRANSACTIONS

OF THE

BRITISH PHARMACEUTICAL CONFERENCE

HELD AT

BOURNEMOUTH

SEPTEMBER 21 to 25, 1959

LONDON:
THE PHARMACEUTICAL PRESS
17 BLOOMSBURY SQUARE, W.C.1

JOURNAL OF PHARMACY AND PHARMACOLOGY

Editor: George Brownlee, D.Sc., Ph.D., F.P.S.

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Annual Subscription **63s.** Single Copies **7s. 6d.**

17 BLOOMSBURY SQUARE, LONDON, W.C.1

Telegrams: Pharmakon, Westcent, London *Telephone:* HOLborn 8967

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Telegrams: Pharmakon, Westcent, London *Telephone:* HOLborn 8967

Vol. XI. No. 4 **April, 1959**

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Telegrams: Pharmakon, Westcent, London *Telephone:* HOLborn 8967

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Telegrams: Pharmakon, Westcent, London *Telephone:* HOLborn 8967

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Telegrams: Pharmakon, Westcent, London *Telephone:* HOLborn 8967

Vol. XI. No. 8 **August, 1959**

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Telegrams: Pharmakon, Westcent, London *Telephone:* HOLborn 8967

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JOURNAL OF PHARMACY AND PHARMACOLOGY

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Telegrams: Pharmakon, Westcent, London *Telephone:* HOLborn 8967

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REVIEW ARTICLE

OXIDATIVE PHOSPHORYLATION

By J. D. Judah, B.M., M.A., M.R.C.P.

Department of Morbid Anatomy, University College Hospital Medical School, London

It must be made clear that this review is in no sense comprehensive. The writer has made no attempt to cover the entire literature, even of the past few years. Rather, it is intended to give the general reader some idea of the present state of the problem, and will to that extent reflect the personal interests of the author.

Oxidative phosphorylation: P:O Ratios

The process of oxidative phosphorylation may be defined as the synthesis of adenosine triphosphate (ATP) from inorganic phosphate and adenosine diphosphate (ADP) during the passage of electrons along the respiratory chain of enzymes and coenzymes. It does not include glycolytic phosphorylations, such as the one observed in the oxidation of triosephosphate. Nevertheless, this phosphorylation serves as a very useful starting point for our discussion. In the oxidation of triosephosphate, 1 mol. of substrate is oxidised to 1:3-diphosphoglyceric acid, with the addition of 1 mol. of inorganic phosphate from the medium. Under the influence of the appropriate phosphokinase, this phosphate is transferred to ADP to make ATP. During the oxidation, 1 mol. of diphosphopyridine nucleotide (DPN) is reduced to DPNH. Thus 1 mol. of phosphate is transferred per mol. of hydrogen (or more exactly per pair of electrons) transferred. If now, the DPNH formed were to be oxidised by means of an artificial carrier system with the uptake of oxygen, it would be found that 1 atom of oxygen would be consumed per mol. of phosphate taken up, and the efficiency of the process could be expressed in this way, by taking the ratio of phosphate to oxygen, which in this instance would be 1·0.

If, however, one found that the ratio exceeded 1, when the oxidation was conducted by some system other than the artificial one, one would be forced to conclude that the oxidation of DPNH was itself yielding further phosphorylations, and in fact that a process of oxidative phosphorylation was taking place.

It is some time since Belitzer and Tsibakowa[1] and Ochoa[2], using systems oxidising substrates of the tricarboxylic acid cycle, were able to show that P:O ratios substantially above 1 were obtainable, and that participation of the electron transport chain in phosphorylation reactions was certain. Further studies have been concerned with the location of these phosphorylations along the chain, and to determining the number of phosphorylations which occur when a pair of electrons are transferred from pyridine nucleotide to oxygen.

Table I shows the transport chain together with the potentials of each couple, and may be used for reference. In Ochoa's early experiments, though he had been able to obtain P:O ratios above 1·0, it was found that the direct oxidation of reduced DPN would not itself give rise to phosphorylations, and this raised serious problems, especially since he also found that the oxidation of citrate, which is also coupled to a pyridine

TABLE I

ENERGY SCALE

	E_o m.volts
Pyridine nucleotide	−320
Flavoprotein	− 60
Cytochrome *b*	0
Cytochrome *c*	+260
Cytochrome *a*	+290
Oxygen	+815

nucleotide, in this case triphosphopyridine nucleotide (TPN), also failed to give rise to phosphorylations. It might therefore have been concluded that the phosphorylation did not occur during oxidation of the reduced coenzyme, but during the oxidation of the substrate by the coenzyme. Ogston and Smithies[3] examined this problem theoretically and pointed out that the potential of the DPN:DPNH couple would be adequate for this purpose only at ratios of DPN to DPNH so high that vanishingly small amounts of DPNH would be present and that oxygen uptake would be effectively zero, since the further oxidation of DPNH, catalysed by flavoprotein, requires a certain concentration of this substance. Clearly this result did not fit the observed facts, and subsequent investigation has shown why.

The work which is now to be considered began in 1947–48. At that time, Green and his co-workers[4,5] began to study the particulate "cyclophorase" system, obtained from a variety of tissues, and Lehninger[6] studied oxidations by particles derived from liver. At the same time, Hogeboom, Schneider and Pallade[7] devised methods for the isolation of cellular components, with the separation of intact mitochondria as probably their most significant contribution.

TABLE II

P:O RATIO OBTAINED WITH THREE SUBSTANCES DURING OXIDATION BY MITOCHONDRIA. RAT LIVER MITOCHONDRIA. BATH TEMPERATURE 15°

Substrate	O_2 uptake μ atoms	Phosphate uptake μ moles	P:O ratio
L-Glutamate	6·8	26	3·8
β(OH) butyrate	8·1	23·4	2·9
Succinate	12	21·9	1·8

All these preparations had the property in common that they were able to oxidise intermediates of the tricarboxylic acid cycle with the simultaneous esterification of inorganic phosphate, provided that Mg ions, ADP and inorganic phosphate were present. It was early demonstrated that the mitochondria were in fact the active units in the different preparations.

Table II shows the P:O ratios obtained with several substrates during oxidation by mitochondria. They are all high, are in substantial agreement with figures obtained by other workers, and show that electron transport chain participation must be extensive. Not only is the efficiency great, but the rates of oxidation are also rapid. The first significant step toward localisation in the transport chain was taken by Lehninger[8] who, repeating Ochoa's experiments with DPNH, was able to demonstrate a clear-cut phosphorylation associated with its oxidation.

TABLE III

SPAN DPNH TO OXYGEN

Substrate	DPNH disappearing μ moles	Phosphate uptake μ moles	P:O ratio
DPNH	3·68	3·98	1·08

The reason for this contrary finding lies in the mitochondrial structure. Lehninger correctly supposed that two pathways for DPNH oxidation must exist: one external, in which there was electron transport without phosphorylation, and the other internal, which was coupled to the phosphorylation mechanism. Hence the high P:O ratios with substrates of the cycle, and none with added DPNH. He surmounted the difficulty by exposing isolated mitochondria to hypotonic conditions, which increased their permeability to added DPNH. Table III shows that a well-marked phosphorylation resulted.

Now in the span from DPNH to oxygen, at least three phosphorylations can be accommodated, energetically speaking, and in this laboratory[9] we were able to show that at least one of these occurred during the oxidation of reduced cytochrome *c*. The P:O ratio for this step was about 1. Though at the time there was some doubt about the significance of these results, later work by Lehninger[10] and by Maley and Lardy[11] has extended and confirmed the earlier work (Table IV). Finally,

TABLE IV

PHOSPHORYLATION COUPLED TO OXIDATION OF REDUCED CYTOCHROME *C*

Oxygen uptake μ atoms	Phosphate uptake μ moles	P:O ratio
8·0	7·3	0·91
11·0	6·1	0·56

Lehninger[12] was able to demonstrate that two phosphorylations occurred in the span between DPNH and cytochrome *c*. (Table V).

Thus, a total of three phosphate molecules are esterified per pair of electrons passing between DPNH and oxygen, and the P:O ratio for substrates linked with pyridine nucleotide is therefore 3, since we have already seen that no phosphorylation is possible during the passage of electrons between substrate and nucleotide.

With one substrate, α-oxoglutarate, the ratio is 4, the reason for this being a substrate-linked phosphorylation, which occurs during the

oxidative decarboxylation, and which is entirely different from the electron chain phosphorylations. These high figures have given rise to much discussion about the overall efficiency of the whole process. It is clear that this calculation may only be made if one knows the potential span of the electron transport chain and the free energy of the terminal phosphate group of ATP. The former figure may be obtained readily from the

TABLE V

SPAN β(OH) BUTYRATE TO FERRICYTOCHROME

Substrate	½ Ferricytochrome *c* mμ moles	Phosphate uptake mμ moles	P:O ratio
β(OH) butyrate	13·2	21·2	1·61

published data, but it must be borne in mind that the significance of the value is in doubt until something is known of the relative concentrations of the reduced and oxidised form of each couple. Thus, we have already quoted figures of Ogston and Smithies[3] which show that phosphorylation could occur during electron transport between substrate and DPN, but only when the relative concentration of DPNH is so low that respiration could not take place at all. It is necessary, therefore, to determine whether the different components of the respiratory chain are in states which may be compared to those for which the data in the literature for their potentials were calculated; that is, for 50 per cent reduction, and a ratio of oxidised to reduced forms of 1·0.

The work of Chance[13] gives some solid ground for the determination of the necessary quantities. Using extremely sensitive methods of spectrophotometry, he has been able to observe the major components of the chain in isolated mitochondria under a variety of experimental conditions, and to determine their relative concentrations. It can be shown that in mitochondria catalysing active oxidative phosphorylation, the ratios of reduced to oxidised forms do not vary sufficiently from unity to make any correction necessary and that the standard values may be used without fear of too great error. This therefore gives us a total span of about 1,100 mv., and if it is taken as a rough rule that 250 mv. is equivalent to 12 kcal., then we get a figure of 54 kcal. for the whole span. The free energy change on hydrolysis of the terminal phosphate group of ATP must also be known in order to calculate the efficiency of the oxidative process, and here there has been much discussion over figures. Lipmann[14] in his now classical review, gave a calculated figure of 9 to 11 kcal. Since then, the figure has first risen to 12 kcal. and fallen to as low as 5·7 kcal. Burton[15] in a discussion of these figures gives a value of 8·4 kcal. for specified standard conditions, which when corrected for the conditions under which experimental determinations of oxidative phosphorylation are conducted, gives a value of about 12·5 kcal. With a P:O ratio of 3, this gives an efficiency of nearly 70 per cent. Whether these figures may be transferred to whole cells is a question which cannot be answered as yet.

Regulatory Function of Oxidative Phosphorylation

During the discussion on the sites of phosphorylation, it was mentioned that isolated mitochondria will oxidise substrates rapidly in the presence of inorganic phosphate and ADP. It has long been known that the concentration of these substances is critical in determining the rate of respiration, and that when either falls to a low level respiration is reduced by a factor of about 10 at the limit.

Considering ADP first, Lardy and Wellman[16] were the first to show that as ADP was phosphorylated to ATP, so the rate of respiration declined to the limiting value, when nearly all the ADP had been converted. The addition of a system which withdraws the terminal phosphate group (for example, glucose and hexokinase; creatine and creatine phosphokinase) will cause an immediate acceleration of the respiratory rate. These results immediately suggest a mechanism whereby the rate of respiration may be regulated. Since the best figures for tissue analysis of adenine nucleotide suggest that ATP is the major, if not the only form present, it follows that mechanisms which permit loss of the terminal group will bring about acceleration of respiration. Thus hormonal regulation (for example, by thyroxine) may centre about this point.

Chance[13] has also studied this point in some detail. He was able to study the steady states of the respiratory carriers in isolated mitochondria under conditions in which active oxidative phosphorylation was taking place, with high levels of ADP present, and in others where ADP was almost exhausted, being converted to ATP. He found that in these two extreme conditions, the steady state levels of oxidised and reduced forms of the respiratory carriers showed large differences. Thus, when ADP was low, increasing reduction was observed throughout the chain, being maximal at the level of DPN, which was 90 per cent in the reduced form, and hardly noticeable at the level of cytochrome a_3 (cytochrome oxidase), the intermediate components fell into intermediate categories. Upon making ADP available, this state of affairs reversed itself, and increased oxidation was observed, the DPN now being about 50 per cent in the reduced form.

These observations are of the utmost importance, for they illustrate the phenomenon of coupling of respiration and phosphorylation in a most unambiguous way, in terms of the respiratory carriers. Incidentally, they also support the direct experiments on the localisation of phosphorylations, and show that one of these occurs at flavoprotein level, a fact which has so far escaped direct experimental proof. To return, however, to the coupling phenomenon, this interdependence of respiration and phosphorylation may only be observed in native mitochondrial preparations. Where, for example, the mitochondria have been damaged, as by exposure to detergents, or to hypotonic conditions, then the rate of respiration will be independent of nucleotide acceptor concentration and the phosphorylation mechanism itself may be destroyed. The mechanism by which this coupling occurs has been the subject of considerable discussion. Numerous schemes have been drawn up, the salient features of which are summarised below.

Mechanism and Coupling of Respiration and Phosphorylation

All the schemes have to account for the inhibition of respiratory rate when ADP becomes limiting, and therefore have to postulate a reaction during the process of dehydrogenation in which the respiratory carriers are phosphorylated or in which these carriers react with some other molecule which is thereby activated, and which may therefore react with inorganic phosphate in a phosphorolytic reaction. The subsequent step is transfer of phosphate to ADP to form ATP. It is easy to see that if the initial reaction, either of phosphate or some other molecule, is with reduced carrier, then the complex has to be split if further electron transport is to occur. And if we postulate that the transfer reaction to form ATP is rate-limiting, then the regulation of the rate of respiration by availability of ADP is readily explained.

Lipmann[17] formulated a scheme in which the respiratory carriers were phosphorylated, while Slater[18] suggested a reaction between the carriers and another compound which was then phosphorylated after dehydrogenation of the complex.

Chance[13] believes that the process is more complex. His views may be summarised as follows, where A and B are respiratory carriers and C and X are unknown compounds, C being an inhibitor of the respiratory chain, which is responsible for the slow rate of respiration in a coupled system when ADP concentration is limiting.

The oxidised form of B is in combination with C, thus

$$B + C \leftrightharpoons B.C$$

The reduced form of A, AH_2 then reacts with B.C

$$AH_2 + B\,C \leftrightharpoons A + BH_2{\sim}C$$

then

$$BH_2{\sim}C + X \leftrightharpoons BH_2 + X{\sim}C$$

and X∼C undergoes phosphorolysis and subsequent transfer of phosphate to ATP occurs,

$$X{\sim}C + ADP + \text{inorg. } P \leftrightharpoons X + C + ATP$$

It is plain that these are all paper schemes, but they do draw attention to two main points. Firstly, they can explain the existence of "uncoupled" respiration, for example in Chance's scheme, if the compound X∼C were able to react rapidly with water rather than inorganic phosphate, it is clear that the rate-limiting transfer reaction would be by-passed, that oxidation would become independent of ADP, and that no phosphorylation would be observed. This situation might be met where the mitochondria have been damaged. Secondly, they can be used to explain the mechanism of action of certain agents which are known to inhibit phosphorylation without affecting respiration.

It should also be noted that the reaction mechanism involves several steps, the minimum being two, first a phosphorolysis, and second a transfer. The phosphorolysis may itself proceed in two stages. All these steps are presumably catalysed by enzymes, but it is not impossible that the phosphorolytic step is spontaneous and non-enzymatic. For example, Stadtman and White[19] have shown that *N*-acetyl imidazole will

react reversibly with inorganic phosphate to give acetyl phosphate, and it might well be that some similar mechanism is involved in the early stages of the oxidative phosphorylation reaction.

We have up to now talked of the coupling phenomenon as though the dependence of respiration on ADP were absolute, and it might be assumed from this that the rate of respiration is stoichiometrically related to the concentration of the nucleotide. This is not the case, for Chance has shown that respiration may proceed linearly until the ADP is nearly exhausted, and that the rate is essentially the same within a 10,000 fold variation in the ratio of ATP to ADP. Nevertheless, this should not be taken to mean that the respiratory rate is not governed by acceptor availability *in vivo*, for as we have already noted, it is probable that in whole cells, the major part of the adenine nucleotide is present as ATP.

The dependence of respiration on the presence of a sufficiency of inorganic phosphate has been demonstrated many times[9], and it is clear that its mechanism may be treated in the same way that has been applied to ADP. Thus in the reaction scheme which was given above, if there were inadequate inorganic phosphate present, the phosphorolysis of X∼C would be reduced in rate, and hence the electron transport would also be slowed. That these schemes may have basis in fact is demonstrated by their affording satisfactory explanations for the phenomena of uncoupling by chemical reagents, which will now be considered.

Action of Uncouplers of Oxidative Phosphorylation

In 1948, Loomis and Lipmann[20] showed that low concentrations of 2:4-dinitrophenol completely inhibited the phosphate uptake of a mitochondrial preparation while leaving its respiration unaltered. Since that time, a vast amount of work has been done on the subject[5,8,9,21] and numerous other compounds have been found to do the same thing.

However, dinitrophenol is an excellent example to consider here, and the discussion will centre around this substance. Loomis and Lipmann[20] were also able to show that when their enzyme preparations were incubated in a medium deficient in inorganic phosphate, the respiration rate fell to low levels, and that uncoupling concentrations of dinitrophenol stimulated this rate to normal figures (Table VI). They concluded that dinitrophenol might "replace" inorganic phosphate in their system, perhaps in a similar way to arsenate, which actually does replace orthophosphate in the glycolytic phosphorylation.

That this is not the case is shown clearly by the experiments of Judah[9] who demonstrated that the "replacement" only took place when small amounts of inorganic phosphate were present in the medium. This implied that dinitrophenol was actually sparing inorganic phosphate by preventing its uptake into ATP. Judah and Williams-Ashman[21] also made similar observations with respect to adeninenucleotide (Table VI). They found that when ADP was omitted from the medium the respiration rate was low and that addition of dinitrophenol greatly stimulated the rate of respiration under these conditions. However, if the mitochondrial preparations were well washed, then dinitrophenol was no longer effective,

suggesting that as with inorganic phosphate, a certain small amount of nucleotide is required, and that once again this is a sparing effect of dinitrophenol. Now if this is so, then something is clearly wrong. For in our consideration of the mechanism by which inorganic phosphate is esterified it became apparent that a phosphorolysis followed by a transfer reaction must occur. Dinitrophenol is presumably inhibiting one or both of these steps, and if so should make the system independent of

TABLE VI

"REPLACEMENT" EFFECT OF DINITROPHENOL (DNP). RAT LIVER MITOCHONDRIA OXIDISING GLUTAMATE

	μl. O_2 respiration	
	No DNP	+DNP
Complete system	255	258
No phosphate	80	288
No nucleotide	99	180

either inorganic phosphate, adenine nucleotide or both. The fact that it does not do any of these things is a puzzle which is largely ignored by those considering the mechanism by which it works. It may be that there is some other reason for the failure of dinitrophenol to replace adenine nucleotide. For example, the mitochondrial system may require small amounts of adenine nucleotide not solely for the respiratory function, but also for maintenance of structure, in which case it might well be that it would be difficult to demonstrate complete independence of the former.

A clue to the nature of dinitrophenol action was provided by the observation of Lardy and Wellman[22] that it greatly increases the ATP-ase activity of intact mitochondria. However, this cannot be the sole explanation for its uncoupling action because (1) Slater[23,24] has shown that the ATP-ase action may be anulled by increasing the activity of the hexokinase normally added to the reaction mixture, which thus successfully competes for ATP. (2) While dinitrophenol reduces the phosphorylation coupled to DPN-linked substrate oxidation, it leaves untouched the esterification of inorganic phosphate which is coupled to the anaerobic dismutation of α-oxoglutarate with ammonia[9]. (3) Reduction of incubation temperature reduces the activity of the ATP-ase, but has no effect on the dinitrophenol inhibition of oxidative phosphorylation. Lardy and Wellman[22] suggest that this apparent ATP-ase activity is in fact due to interaction of dinitrophenol with an intermediate in the phosphorylation process (see the schemes suggested by various authors and described earlier). In other words, the ATP-ase activity is due to a reversal of the oxidative phosphorylation, and inorganic phosphate is liberated in the presence of dinitrophenol because of the hydrolysis of an unknown phosphorylated intermediate. Thus,

$$ATP + X \leftrightharpoons ADP + X\sim P$$
$$X\sim P + DNP \leftrightharpoons X + iP$$

or

$$X\sim P + DNP \leftrightharpoons DNP\sim P$$
$$DNP\sim P \rightarrow DNP + iP$$

Other suggestions including that of Chance[13] have been put forward. The latter authors suggest that dinitrophenol interacts with X∼C in their scheme, rather than with a phosphorylated intermediate. A compound dinitrophenol∼C is then formed, and this accounts for the acceleration of respiration by dinitrophenol in systems which are limited by ADP concentration, since C is inhibitory to the respiratory chain and the formation of the compound with dinitrophenol lifts this inhibition. These ingenious ideas explain the findings very well, and are supported by the experiments of Slater and Lewis[25], who found that insect sarcosomes oxidised α-oxoglutarate with a P:O ratio of around 1·0. They were also able to show that addition of dinitrophenol accelerated the respiration by a factor of 3, despite the fact that hexokinase was present and that ADP concentration could not be limiting; not only this, but there was also an *increase* in the amount of phosphate esterified, a most unusual finding. It is suggested that the insect system possesses a very slow transfer reaction from intermediate to ADP, compared with the usual mammalian systems, and that the effect of dintrophenol may be explained by its interaction with an intermediate, as shown above. However, there is one fact which cannot be explained by these theories, and it is that low bath temperatures will prevent the loss of inorganic phosphate from ATP in the presence of dinitrophenol while the effect of the phenol does not vary. It seems hard to get round this point.

Though a great deal of work has been done on dinitrophenol, other substances which uncouple phosphorylation from respiration are also known[8,21], though not so much attention has been paid to them. A few words concerning some of these substances would not be out of place here. Azide is best known for its inhibitory effect on cytochrome oxidase, and it is not generally appreciated that in low concentrations it is a powerful inhibitor of oxidative phosphorylation[9,26]. Experiments based on its function as a cytochrome oxidase inhibitor are liable to be complicated by this other property. Azide evidently acts differently from dinitrophenol. For instance, it will not accelerate the respiration of phosphate-deficient mitochondrial systems, but little else is known about it.

Dicoumarol, the antagonist of vitamin K, has been shown by Martius and Nitz-Litzow[27,28] to be a powerful uncoupler of oxidative phosphorylation. They suggested that this inhibition was related to the antagonism to vitamin K and that naphthoquinones were playing a part in electron transport and phosphorylation. In support of this view, they were able to show that mitochondria isolated from vitamin K deficient birds gave low P:O ratios, which were elevated by addition of vitamin K. Recently, Lehninger [29–31] has been able to show that dicoumarol inhibits phosphorylation at every step in the electron transport chain and it therefore seems that naphthoquinones must either operate at every step in the process as a component of the phosphorylation system, or that dicoumarol has another action separate from its antagonism to vitamin K. In any event, the hypothesis put forward by Martius to explain his results is no longer tenable. He suggested that vitamin K was actually part of the electron-transport chain, acting between DPN and cytochrome *c*, and

that dicoumarol caused a diversion of electron flow to a non-phosphorylating path. These experiments are all extremely interesting and thought-provoking and further developments are to be hoped for.

The last compound which is to be considered here is the hormone, thyroxine. In view of the similarities in effect on animals (increased basal metabolic rate with wasting), produced by dinitrophenol and thyroxine, and the fact that both are substituted phenols, it is not surprising that many workers have tried to show similar actions of the two substances *in vitro*, The early work was hampered by an inability of the thyroxine to reach the active centres in the mitrochondria, but Martius and Hess[32] found that pre-incubation of the particles with low concentrations of thyroxine produced typical uncoupling effects. Hoch and Lipmann[33] made the fascinating observation that mitochondria from hamster liver are normally more permeable than those from rat liver, and that on these former, thyroxine worked readily without pre-incubation to give low P:O ratios without significant effect on respiration. Unfortunately, the interpretation of these results is placed in doubt by the recent work of Lehninger[34] who has shown that a particle derived from mitochondria (which will be referred to later) is capable of a brisk oxidative phosphorylation which is uncoupled by dinitrophenol, but which is quite insensitive to thyroxine. He has also shown that the action of thyroxine on intact mitochondria is probably due to the swelling brought about in these particles when they are incubated with the hormone. The question, of course, is whether this action may in any way be related to the physiological effect of the hormone. At the present time the writer is quite unable to see any answer.

Exchange Reactions and Oxidative Phosphorylation

In the foregoing sections, we have dealt with phenomena which are undoubtedly coupled to electron flow, but here it is intended to deal with exchange reactions, in which by the use of a label, either ^{32}P or ^{18}O, it is possible to show that phosphate groups or the oxygen of phosphate are transferred, in some cases in the absence of a net oxygen uptake.

Cohn[35] was the first to show that ^{18}O-labelled phosphate exchanged its oxygen with water during the process of oxidative phosphorylation. The most significant part of her work lay in the demonstration that some 6 cycles of oxygen exchange took place for each mol. of inorganic phosphate esterified during the passage of electrons along the respiratory chain, and she was also able to show that dinitrophenol inhibited the exchange phenomenon.

It is clear that a P–O link is being repeatedly formed and broken, and the inference is that some compound is being formed with phosphate which can bring its oxygen into equilibrium with the oxygen of water. Boyer, Falcone and Harrison[36] have also observed that this oxygen exchange may occur in the absence of a net oxygen uptake. Using mitochondrial preparations, they found that providing ATP were present, a rapid exchange took place between inorganic P and water and that this exchange was independent of substrate and took place in anaerobic

conditions. If ^{32}P were the label, then a group transfer was found, and the label appeared in ATP. The rate of the phosphate exchange was about a seventh of the oxygen exchange. Dinitrophenol and azide block these exchanges. If ADP and not ATP is present, the exchange, at least of ^{32}P is negligible. Boyer and his colleagues suggest that these reactions represent the reversal of oxidative phosphorylation. Since the electron transport chain in these mitochondria will be in a variety of oxidation states, representing the steady state for an inactive system, that is, with DPN almost completely reduced, and the intermediate components between DPNH and oxygen being in intermediate conditions, it is not hard to see that electron flux might well take place and might cause the observed reactions. It might also be that these reactions depend on the existence of intermediates of oxidative phosphorylation. Thus if an active intermediate existed, and this has been postulated in the scheme, it is not hard to see how it could be in equilibrium with both inorganic phosphate and the terminal phosphate of ATP. Such an intermediate could also react with water to give the oxygen exchange.

The available evidence does not give much support to either theory at the moment. Thus it can be shown that antimycin A, which is an inhibitor of the electron transport chain which acts between cytochrome *b* and cytochrome *c*, will not affect the ^{32}P exchange. This might mean either that the bulk of the electron flux occurs between DPNH and flavoprotein, or that the electron flux is of no importance in the exchange reaction. Quite recently, Cohn and Drysdale[37] reported that during oxidative phosphorylation, not only was there a marked oxygen exchange between inorganic phosphate and water, but that a further exchange took place before the phosphate was taken up into ATP. This could only mean that an intermediate existed which was itself rapidly exchanging oxygen with water, and was the first evidence of such a compound. Unfortunately, Boyer and his colleagues[38] have shown that the concentration of intermediate, calculated from the figures of Cohn and Drysdale, would have to be very large indeed to satisfy the theory of an intermediate, and suggest an alternate mechanism for the findings. This is that the substrate, β-hydroxybutyrate, was being converted to the coenzyme A derivative. As the similar acetate activation process is accompanied by oxygen transfer from acetate carboxyl to the phosphate of AMP, derived from ATP, it seems plausible that the exchange observed by Cohn is in fact not related to oxidative phosphorylation.

We are therefore left with these interesting observations. They may prove valuable in the elucidation of the problems of oxidative phosphorylation, but at the moment there would appear to be no certainty as to interpretation.

Mitochondrial Preparations: Fractionation of the Phosphorylation System

It seems worth while to consider the variety of preparations which are available for the study of oxidative phosphorylation. Numerous attempts have been made to break the mitochondrial system down to simpler

units, and in addition a variety of tissues have been used. A summary of the present state is therefore attempted here.

Mitochondria

The classical preparation is a modification of that of Hogeboom, Schneider and Pallade[7], who used 0·88 M sucrose for homogenising rat liver, and isolated intracellular particles by differential centrifugation at low temperatures. The modification consists in using 0·25M sucrose, and is due to Schneider[39]. Better activities are obtained with the lower concentration of sucrose and lower centrifugal speeds are required, though the mitochondria tend to lose their normal elongated shape. Rat liver has been the material of choice for most studies, but Slater[23] has used particles isolated from heart muscle. These preparations have yielded much information in Slater's hands, but they have the drawback that they are relatively difficult to set free from the muscle cells and that yield is limited. Furthermore, the heart contains large amounts of calcium,

TABLE VII

COBALT EFFECT ON BRAIN MITOCHONDRIA PRE-TREATED WITH 1:10 PHENANTHROLINE

	No cobalt	Cobalt $3{\cdot}3 \times 10^{-6}$M
Respiration μ atoms O	8·7	11·2
Phosphate uptake μ moles ..	5·2	14·0
P:O ratio	0·6	1·3

which during the isolation procedure becomes attached to the mitochondria, and which acts as a powerful inhibitor of oxidative phosphorylation. In order to obviate this, the homogenate must be made in relatively high concentrations of ethylenediamine tetra-acetic acid (edetic acid) in order to bind the calcium. The effect of calcium has itself been used to study the phosphorylation system and will be considered later. Mitochondria have also been isolated from brain[40]. These particles are very similar to those from liver. They need edetic acid, however, in much the same way that heart sarcosomes do, and also show a peculiar inability to oxidise added citrate, despite the fact that added pyruvate is oxidised rapidly without accumulation of citrate in the flasks[40]. These workers also found that incubation of brain mitochondria with a variety of chelating agents resulted in powerful inhibition of respiration. For example, *o*-phenanthroline when added to mitochondria and then removed by washing on the centrifuge, resulted in preparations which rapidly lost their respiratory activity. At the same time, the P:O ratio fell to low figures. Reversal could be brought about by cobaltous salts in low concentration; nickel salts also had the same effect, and it is probable that there is no specificity in this result, despite the fact that a range of other ions had no effect (Table VII).

The whole problem of metal requirements for oxidative phosphorylation is exceedingly complicated. Thus, Lindberg and Ernster[41,42] have shown that calcium ions when added to rat liver mitochondria bring about a

requirement for manganese. If the particles are pre-incubated with calcium, oxidative phosphorylation falls to low levels. The addition of Mn will bring about some reversal when added later, and will prevent the inhibition if added at the same time. The presence of ATP together with Mn gives the best protection. Cleland and Slater[43] have shown that calcium ions disrupt the structure of the mitochondria, and this finding, together with those of Christie and colleagues[40] make one wonder whether these metal effects do not represent some structural effect on the particles rather than a fundamental one on the phosphorylating system? The position of magnesium in this matter was at one time unassailable. It is required for phosphorylation by all mitochondrial systems, and its known function in other phosphokinase systems (for example, hexokinase) made it seem all the more likely that this was a true requirement. The work of Lehninger now to be considered casts doubt on this belief.

Mitochondrial Fractionation

The breakdown of the mitochondrial structure invariably results in almost total loss of activity, with the exception of one process. Thus, the suspension of the particles in water which brings about gross swelling and the escape of soluble protein, nucleotides, and other substances, reduces the P:O ratio to small figures, increases the ATP-ase activity to maximum and damages the oxidative mechanism. The addition of surface active agents has similar effects. The exposure of the mitochondria to solvents such as ethanol or acetone in any great concentration destroys them utterly. In face of these facts, it is not surprising that attempts to reduce the particle system to simpler units have had little success. Cooper and Lehninger[29] have, however, succeeded in obtaining minute particles from rat-liver mitochondria which will oxidise β-hydroxybutrate and succinate with a coupled phosphorylation. They used digitonin to split the mitochondria, and followed this with differential centrifugation at high speed. The final centrifugation at 105,000 $\times$ g gave a small pellet of active particles, the particle weight of which is roughly calculated at about 1/3000th that of the parent mitochondrion.

These submitochondrial particles require only substrate, inorganic orthophosphate and ADP for their activity. Magnesium is not required, and is indeed somewhat inhibitory in the usual concentration. Calcium has no effect on the phosphorylation. We must therefore bear in mind the possibility that the effect of both these ions on intact mitochondria is related to their action on mitochondrial structure. That this is probably the case with calcium has already been mentioned, but the observations of Lehninger are the first good evidence that Mg requirement is in the same general category, and fits in with the remarks made about Mn and Co earlier in this review.

Lehninger and his colleagues have studied this preparation in some detail. Amongst their important findings is that ADP is the sole acceptor of phosphate. Other nucleoside diphosphates are inactive in this system. This point could never be settled with intact mitochondria, for it is known that they contain a variety of nucleotides, and while ADP has been used

constantly with such systems, there was always the chance that the initial phosphorylation involved some other compound (for example, inosine diphosphate, which is known to be a primary acceptor in the substrate-linked phosphorylation coupled to the oxidation of α-ketoglutarate to succinate). They have also shown that phosphorylation occurs in the span substrate to cytochrome *c* and in the span from reduced cytochrome *c* to oxygen. Thus the whole chain is complete and present. Their observations on dicumarol have already been referred to.

Since the publication of Lehninger's work, Hatefi and Lester[44] have described successful fractionation of beef-heart mitochondria. The investigation of their particles has not been reported with the same detail as has Lehninger's, and further work is awaited. At least one major difference is known, however. Whereas the digitonin preparation gives particles which will oxidise only succinate and β-hydroxybutyrate, the beef-heart particles appear to contain all the enzymes of the tricarboxylic acid cycle, together with the phosphorylating enzymes (see Table VIII for summary).

TABLE VIII

ATTEMPTS AT FRACTIONATION

	Lehninger preparation	Hatefi and Lester preparation
Method	1 per cent digitonin	15 per cent ethanol
Source	Rat liver mitochondria	Beef heat mitochondria
Yield	0·5 per cent	7·5 per cent
Substrate specificity	β(OH) butyrate and succinate (low)	All T.C.A. intermediates
Activity	Same as starting material or better	Variable
Stability	Unstable	Stable to freezing

The last type of preparation which is to be considered is of some interest. Hunter[45] found that when isolated mitochondria were incubated with 0·02M inorganic orthophosphate for 10 minutes at 30°, they were unable to oxidise β-hydroxybutyrate. The addition of DPN to such preparations stimulated the oxygen uptake, and also resulted in a coupled phosphorylation. It appears that the treatment renders the particles DPN-less, without interfering with the phosphorylation system. This is in marked contrast to other situations where DPN is required by mitochondria. For example, ageing of the particles causes loss of nucleotides into the medium, and a strong DPN requirement is then observed, but there is no coupled phosphorylation. Christie and Judah[46], investigating the action of carbon tetrachloride on the liver, found that mitochondria isolated from such livers required the addition of DPN for respiration, and that this respiration was associated with normal P:O ratios. The same result can be obtained by soaking isolated mitochondria in CCl_4 *in vitro*, but the degradative process is not readily controlled and may proceed too far.

Finally, a word or two about respiratory pathways in mitochondria. It would appear that there are at least two pathways for electron transport, one "internal" and coupled to the phosphorylating system, the other

"external" and non-phosphorylating. Thus, we have seen that in the case of DPNH, the reduced nucleotide is rapidly oxidised by intact mitochondria without a coupled phosphorylation unless access is assured to the internal pathway by hypotonic treatment. But the problem is not so simple as that. Thus, in Lehninger's submitochondrial particles, β-hydroxybutyrate is oxidised with a coupled phosphorylation, whereas added DPNH is oxidised without phosphorylation, as in the case of intact mitochondria. These particles do not, however, have the same permeability characteristics as intact mitochondria. They are immune to hypotonic conditions, they do not respond to calcium or to Hunter's phosphate treatment. Why, therefore, do they not show a coupled phosphorylation with added DPNH?

Lehninger[29] thinks that electron-transport in this case is probably mediated by a DPN-cytochrome *c* reductase which is not coupled to a phosphorylating system, and which is also insensitive to antimycin A, and which is similar to that described by Vernon, Mahler and Sarkar[47].

It follows that the mitochondrial system is a sort of maze of pathways, that aside from the two well-defined "internal" and "external" ones, there may be systems which normally do not function in electron transport, but which may come into action upon disturbance of the mitochondrial structure.

Finally, it must be emphasised that all attempts to fractionate or degrade the mitochondrial system have only succeeded in producing smaller structures. In every case there is a considerable organisation left intact. No one has yet succeeded in obtaining a preparation in which the oxidative phosphorylation system can be reconstructed from any of its parts. It is either complete or it does not appear to exist.

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RESEARCH PAPERS

THE EFFECT OF SPLENECTOMY ON THE PRODUCTION OF ANAPHYLACTIC SHOCK IN THE GUINEA PIG AND THE RAT

BY R. K. SANYAL AND G. B WEST

From the Department of Pharmacology, School of Pharmacy, Brunswick Square, London, W.C.1.

Received September 3, 1958

Splenectomy in the guinea pig affords protection against active anaphylactic shock, if the operation is performed a short time before the challenge with antigen. When the challenge is made under ether anaesthesia, the maximal protective action is present 24 hours after splenectomy but is lost during the next 48 hours. If the challenge is made under urethane anaesthesia, the protective action is present 72 hours after the operation. The mechanism of the protective action of splenectomy is not clear, although it may be due to a loss of tissue antibodies or of complement since passive anaphylaxis is not altered by the operation. In contrast, removal of the spleen in the rat fails to modify the symptoms of anaphylactic shock.

THE spleen is claimed to be a site of antibody formation[1–5], and its removal may modify the production of anaphylactic shock. In this paper, the role of the spleen in anaphylaxis has been studied in the guinea pig and the rat.

METHODS

Female guinea pigs weighing about 400 g. and albino rats weighing about 150 g. were used in this study. Adult rabbits provided the anti-serum for the passive sensitisation experiments. The rats were fed on cubes (No. 41, Associated London Flour Millers Ltd.), the guinea pigs and rabbits on diet No. 18B. Drinking water was allowed *ad lib.*

Sensitisation, challenge and assessment of shock. Guinea pigs were actively sensitised by an intraperitoneal injection of 0·5 ml. of horse serum and challenged under ether or urethane anaesthesia with an intravenous dose of 1 ml. of horse serum either three or ten weeks later. Other guinea pigs were passively sensitised by an intraperitoneal injection of 2 ml. of anti-serum obtained from rabbits bled 10 days after the last of a series of 6 daily intraperitoneal injections each of 1 ml. of horse serum. The recipient guinea pigs were then challenged 24 hours later with an intravenous dose of 1 ml. of horse serum. Shock was assessed as follows; (i) mild shock, consisting of prolongation of anaesthesia and occasional sneezing; (ii) moderate shock, consisting of retching, sneezing, coughing and hurried respiration; (iii) severe shock, consisting of dyspnoea, periodic cessation of respiration and occasional violent respiratory efforts together with opening of the mouth at each inspiration.

Rats were sensitised by an intraperitoneal injection of either horse serum or diluted eggwhite and shock assessed on challenge, as previously described[4].

Collection of guinea pig lung. Guinea pig lung was collected before and after challenge in each animal. On the day of challenge with antigen, the guinea pig was anaesthetised with urethane (1·5 g./kg. intraperitoneally) and blood pressure recordings were taken from the carotid artery. Artificial respiration was applied through a tracheal cannula and a piece of right lung was removed for the extraction of histamine. A portion of the right phrenic nerve was also removed before challenge for studying tissue mast cells in the attached pleura. Twenty to thirty minutes after the challenging dose of antigen had been given, similar pieces of left lung and phrenic nerve were removed from each animal. The lung tissues were weighed, extracted with trichloroacetic acid and assayed on the isolated guinea pig ileum[5]. The phrenic nerves were mounted on microscope slides, fixed in alcohol, stained with toluidine blue and mounted.

Splenectomy. The spleen was removed from guinea pigs under ether anaesthesia. Through a left lateral incision in the abdominal cavity, the spleen was mobilised by tearing the lienophrenic ligament with the finger and then removed after ligaturing the pedicle. The abdominal cavity was closed with atraumatic ophthalmic catgut, and the skin incision joined with suture clips. In other guinea pigs, the spleen was exposed but not removed, and these mock-splenectomised animals served as controls. Groups of rats were similarly splenectomised or mock-splenectomised, though the operation in this species is easier, there being no lieno-phrenic ligament.

Bleeding in all operated animals was minimal. After the operation, they were always placed in sterilised cages and allowed food and water. A few guinea pigs died in the ensuing weeks, but most recovered rapidly from the operation.

Results

Experiments with Guinea Pigs

Splenectomy before sensitisation. Splenectomy in the guinea pig failed to modify the process of sensitisation to foreign protein. When the operation was performed 24 hours before sensitisation and the animals were challenged under ether anaesthesia 3 weeks later, 4 out of 5 guinea pigs suffered fatal anaphylactic shock, the other exhibiting severe shock. A similar result was also obtained when 5 mock-splenectomised animals were similarly challenged.

Splenectomy before challenge. Splenectomy in the guinea pig a short time before challenge considerably reduced the severity of anaphylactic shock. When the challenge was made under ether anaesthesia, the maximal effect occurred 24 hours after splenectomy. At this time interval, only 1 out of 18 animals had severe shock, 1 moderate shock, 12 mild shock whilst 4 failed to exhibit any shock. In contrast, all 17 mock-splenectomised animals at this time interval had severe shock, 15 dying within 30 minutes of the challenge. These results are shown in Table I. When the interval between operation and challenge was extended to 72 hours, the severity of the shock was not reduced, mortality rates exceeding 75 per cent in both splenectomised and mock-splenectomised groups.

When the challenge was made under urethane anaesthesia, the protective effect of splenectomy on anaphylactic shock was demonstrable 72 hours after the operation. Whereas the mortality rate of mock-splenectomised guinea pigs (4 out of 10) was similar to that of unoperated sensitised animals (5 out of 12) given the challenging dose of antigen, there were no deaths and only mild shock in 10 splenectomised animals. Several of the

TABLE I

THE INFLUENCE OF SPLENECTOMY OR MOCK-SPLENECTOMY, PERFORMED AT VARYING TIME INTERVALS BEFORE INTRAVENOUS CHALLENGE BY HORSE SERUM, ON THE PRODUCTION OF ANAPHYLACTIC SHOCK IN SENSITISED GUINEA PIGS. CHALLENGE WAS GIVEN UNDER ETHER ANAESTHESIA

Time interval (hours)	Splenectomy				Mock-splenectomy			
	Degree of shock			Mortality rate (per cent)	Degree of shock			Mortality rate (per cent)
	Mild	Moderate	Severe		Mild	Moderate	Severe	
0	1	4	16	72	0	5	19	75
6	3	1	1	40	0	1	3	75
24	12	1	1	11*	0	0	17	94
48	0	2	7	66	0	0	8	75
72	0	3	5	75	0	1	7	87

* Four animals in this group failed to exhibit any degree of shock.

surviving guinea pigs in each group were killed 1 hour after the challenge and examined. In both the unoperated and mock-splenectomised animals, there were haemorrhagic patches and consolidation in the lungs, which microscopically showed collapse with oedematous inter-alveolar septa (Fig. 1A and B). On the other hand, the microscopic appearance of the lungs of the splenectomised animals after challenge was normal (Fig. 1C).

TABLE II

THE INFLUENCE OF SPLENECTOMY OR MOCK-SPLENECTOMY, PERFORMED THREE DAYS BEFORE INTRAVENOUS CHALLENGE BY HORSE SERUM, ON THE HISTAMINE CONTENT (μG./G.) OF LUNGS OF SENSITISED GUINEA PIGS. CHALLENGE WAS GIVEN UNDER URETHANE ANAESTHESIA

Treatment	Histamine content		Change (per cent)
	Pre-shock value	Post-shock value	
Splenectomy	20·0	20·0	0
	22·4	22·7	+1
	44·0	45·0	+2
	50·0	50·0	0
	80·0	75·0	−6
	85·0	80·0	−6
	180·0	186·0	+3
Mock-splenectomy	12·6	10·1	−19
	13·4	11·2	−16
	28·6	18·0	−37
	30·0	21·2	−29
	30·3	10·4	−66
	36·0	28·0	−22
	120·0	66·0	−45

Pleural mast cells of unoperated guinea pigs undergoing anaphylactic shock showed marked distortion, degranulation and disruption (Fig. 2A) and similar changes were found in mock-splenectomised animals given the challenge either under ether anaesthesia 24 hours after the operation or

under urethane anaesthesia 72 hours after the operation (Fig. 2B). On the other hand, pleural mast cells from animals similarly treated after splenectomy were normal in appearance (Fig. 2C).

The intravenous injection of horse serum into unoperated sensitised guinea pigs under urethane anaesthesia resulted in a rise of the arterial blood pressure which sometimes lasted for 30 minutes. A similar

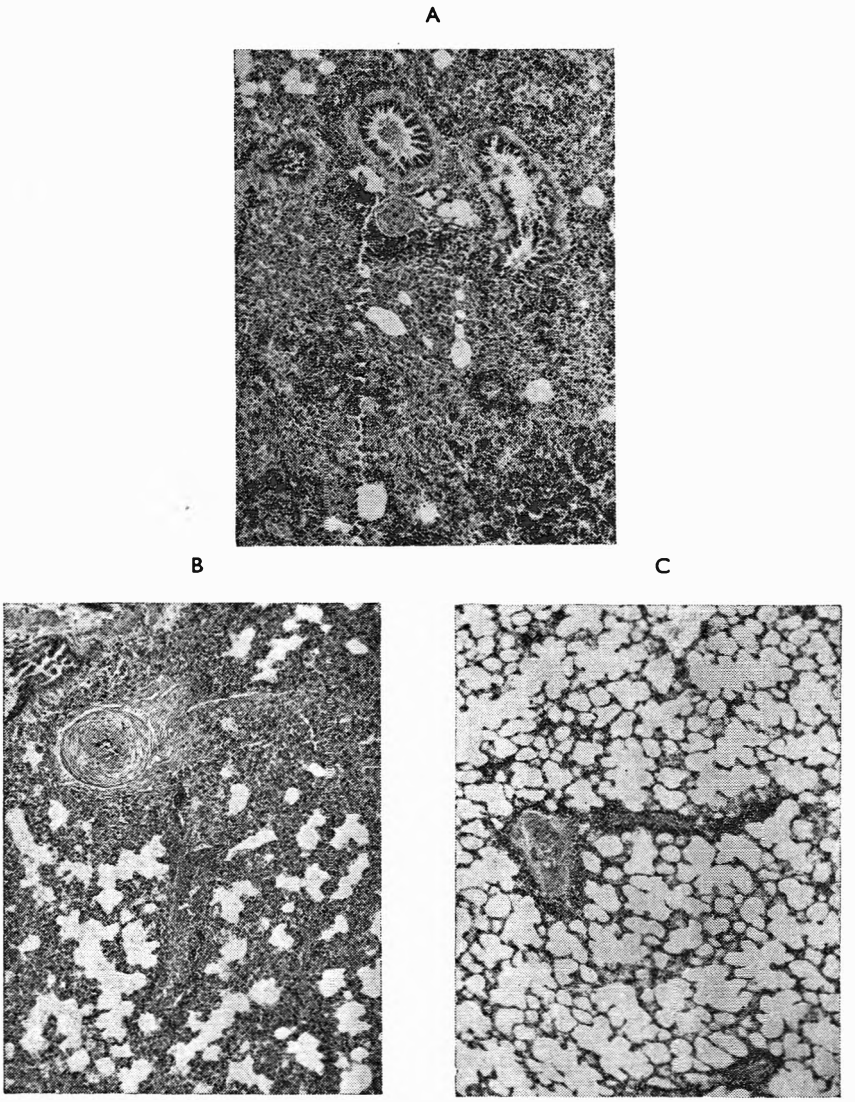

FIG. 1. Guinea pig lung, 1 hour after anaphylaxis. H. and E. × 130. Antigen is horse serum. Challenge performed under urethane anaesthesia. A, unoperated; B, mock-splenectomised 3 days before challenge; C, splenectomised 3 days before challenge. Note that splenectomy before challenge protects the lungs from damage by antigen.

pressor response was obtained when mock-splenectomised sensitised animals were given the antigen 72 hours after the operation. In sharp contrast, however, the injection of horse serum into splenectomized sensitised animals at this time interval failed to alter the blood pressure.

The histamine content of the lungs of mock-splenectomised guinea pigs was reduced by about 36 per cent (range 16–66) after anaphylaxis under urethane anaesthesia 72 hours after the operation. On the other hand,

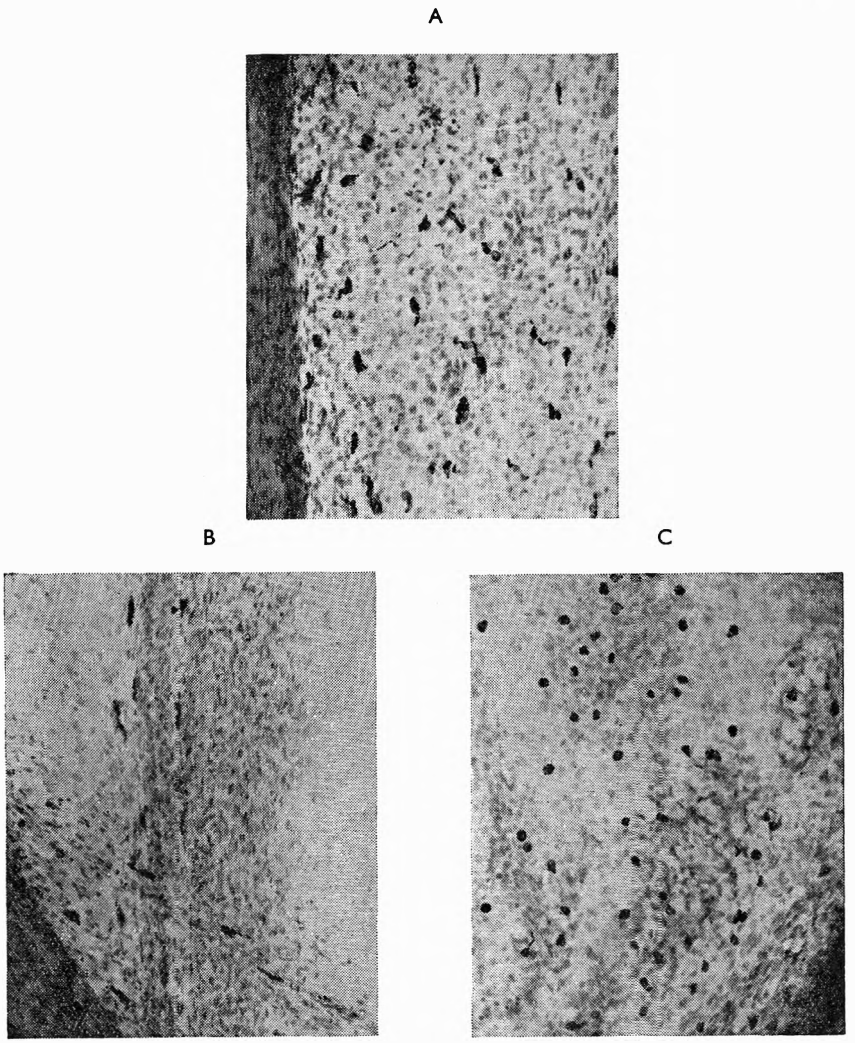

FIG. 2. Guinea pig pleural mast cells, 1 hour after anaphylaxis. Toluidine blue × 200. Antigen is horse serum. Challenge performed under urethane anaesthesia. A, unoperated; B, mock-splenectomised 3 days before challenge; C, splenectomised 3 days before challenge. Note that splenectomy before challenge protects the pleural mast cells from damage by antigen.

the histamine content of the lungs of splenectomised animals similarly challenged was not altered. These results are shown in Table II and also illustrate the wide range of histamine-content of guinea pig lung before anaphylaxis.

The protective action of splenectomy on anaphylactic shock was also noted after a period of sensitisation of 10 weeks. When the operation was performed under ether anaesthesia 24 hours before the challenging dose, 6 mock-splenectomised guinea pigs died within 30 minutes whereas none of the 6 splenectomised animals showed more than mild shock. When the challenge was delayed till 72 hours after the operation, there was no significant difference between the splenectomised and mock-splenectomised animals, 5 out of 6 in each group dying within 1 hour.

When the time course of the return of anaphylactic sensitivity was studied in splenectomised guinea pigs which had survived anaphylactic shock, it was found that a period of 10 days was necessary for the full recovery. Mock-splenectomised animals similarly treated also needed about 10 days for recovery of anaphylactic sensitivity.

When the uterus or intestine of a sensitised guinea pig which had been mock-splenectomised 72 hours previously was suspended in Tyrode's solution in an organ bath, the addition of the specific antigen resulted in a contraction and the tissue exhibited desensitisation. On the other hand, uteri or intestine of splenectomised sensitised animals generally showed no response on addition of antigen.

Splenectomy and passive anaphylaxis. Splenectomy did not protect the guinea pig against passive anaphylaxis. When rabbit anti-serum having a precipitin titre of 1/400 was injected into groups of 6–8 guinea pigs which had been splenectomised or mock-splenectomised 1 or 48 hours previously, and the animals were challenged with the antigen 24 hours after sensitisation, the shock value of both groups was similar, about half in each group dying within 1 hour.

In another experiment, two groups of 6 guinea pigs 1 hour after splenectomy were injected with rabbit anti-serum, the complement of which had been destroyed by heat at 56° for 30 minutes. On the next day, one group was given 2 ml. of fresh guinea pig serum containing complement whilst the other group was injected with 2 ml. of normal saline. All animals were challenged 3 hours later. There were no fatal reactions in either group but the shock was more severe in those animals which had received guinea pig complement.

Splenectomy and amine sensitivity. The influence of splenectomy on the sensitivity of guinea pigs to histamine and 5-hydroxytryptamine (5-HT) was studied by determining their preconvulsion times when exposed to aerosols of these amines[6]. In general, splenectomy did not alter the preconvulsion time to histamine but considerably increased that to 5-HT. For example, the preconvulsion time to aerosols of histamine (0·4 per cent w/v) of groups of 8 splenectomised, mock-splenectomised or unoperated animals was the same (about 50 sec.). Likewise, intravenous doses of 0·8 mg./kg. histamine killed groups of 4 splenectomised, mock-splenectomised or unoperated guinea pigs in 2–3 minutes. But

the average preconvulsion time to aerosols of 5-HT (0·5 per cent w/v) of groups of 8 mock-splenectomised or unoperated animals was 67 seconds whereas that of splenectomised animals was 174 seconds.

Experiments with Rats

Splenectomy performed either before sensitization or before challenge failed to modify the production of anaphylactic shock in the rat. Shock values were similar in splenectomised rats to those in mock-splenectomised animals.

Discussion

The present results show that if splenectomy in guinea pigs is performed a short time before the challenge with the antigen there is a considerable reduction in the intensity of the anaphylactic shock. This is manifest by (i) a reduced mortality rate, (ii) no macroscopic or microscopic changes in the lung, (iii) no alteration in the appearance of pleural mast cells, (iv) no change in the arterial blood pressure, and (v) no reduction in the lung histamine. These effects cannot be attributed to the stress of the operation, since the symptoms noted in mock-splenectomised animals undergoing anaphylactic shock resemble those of unoperated animals receiving similar treatment. The maximal protective effect occurs 24 hours after splenectomy when the challenge is made under ether anaesthesia but is lost by 72 hours. If the challenge is made under urethane anaesthesia, the effect is still demonstrable on the 3rd post-operative day. This difference in duration of effect may be accounted for by the fact that urethane itself possesses a partial anti-anaphylactic property[7,8].

The removal of the spleen protects the guinea pig from active, but not passive, anaphylaxis. To explain this action, the following possibilities exist: (i) the tissues for a short period of time become resistant to the substances released during anaphylaxis, (ii) the union of antigen and antibody is prevented, or (iii) subsequent steps of the antigen-antibody union are inhibited.

It has been shown that splenectomised guinea pigs are more resistant to the action of 5-HT than are mock-splenectomised animals. But histamine is the major toxic substance released during anaphylactic shock in this species and its toxicity is not reduced, and so it is unlikely that the protective action of splenectomy can be solely explained on the basis of increased resistance to 5-HT. As the Dale–Schultz reaction is prevented and pleural mast cells do not show any major change, the union of antigen and antibody and its subsequent steps are more likely to be involved. If the spleen is the major source of antibody formation, then its removal may temporarily prevent their replenishment to the tissues, which in consequence will lose their sensitivity to antigen. This sensitivity will return when non-splenic sources take up the function of the spleen. Since the maximal protective effect is obtained within 24 hours of the operation, a very rapid turn-over of antibodies in the tissues is indicated. Such a possibility however is unlikely since anaphylactic shock can be induced up to at least 7 days after passive sensitisation[3].

It is possible that splenectomy removes temporarily a tissue constituent essential for anaphylaxis. Rice[10] has recently confirmed that there is a lowering of complement titre after anaphylaxis and showed that the return of the titre to pre-shock levels only occupies 24 hours. The transient protective effect of splenectomy may therefore be explained in terms of a diminution of the complement titre. However, the injection of fresh guinea pig serum (containing complement) only slightly aggravated the shock in splenectomised animals, passively sensitised with antiserum heated to 56°. Direct estimations of complement titre have not been made in the present experiments and it is doubtful if a sufficient reduction occurs to modify the anaphylactic reaction.

Ungar[11] postulated in 1953 that a proteolytic enzyme, namely fibrinolysin, is activated during anaphylaxis. He suggested that fibrinolysin is neutralised by antifibrinolysin, the activity of which is stimulated by a constituent of the spleen termed "Splenin-A" and depressed by another constituent termed "Splenin-B". Normal spleen is said to form more of "Splenin-A" than of "Splenin-B" (Ungar and Damgaard[12]) so that splenectomy would be expected to aggravate, rather than alleviate, anaphylactic shock. This is contrary to the results reported in this paper.

If circulating antibodies protect against anaphylaxis[13], the absence of the symptoms when the challenge is given after splenectomy may be due to the antigen-antibody reaction occurring in the blood. Guinea pigs once sensitised maintain the sensitised state for many years[14] and the present results show that the protective effect of splenectomy can be demonstrated in animals which have reached a steady state of anaphylactic sensitisation (after 10 weeks of sensitisation). This state is possible only if antibodies are constantly being produced to replace those lost from the tissues. If a delicate balance exists between the antibodies in the tissues and those in the blood, and if the blood antibody titre is controlled by the spleen, then its removal will upset this balance and the tissues will rapidly give up their antibodies. On challenge, the reaction will occur mostly in the blood and the animal will be temporarily immune. As a result of the high blood antibody content, some of the antibodies will return to those tissues with the highest avidity. The lungs, for example, acquire partial anaphylactic sensitivity 3 days after splenectomy, whereas the uterus at that time is still in a comparatively sensitised state.

It is thus impossible to name a single factor to account for the protective effect of splenectomy on anaphylaxis in the guinea pig. The loss of antibodies and of complement from the tissues as well as the increased resistance of the animal to released 5-HT may all contribute to this action.

The rat, unlike the guinea pig, remains sensitised to foreign protein for only a short time after sensitisation[4]. When the spleen is removed in this species before sensitisation and the animal is challenged 12–14 days later, the production of anaphylactic shock is unaltered. Further, when the spleen is removed a short time before challenge, anaphylaxis is again unaltered, and it appears that the spleen in the rat is not such an important site of antibody formation as it is in the guinea pig.

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STABILITY OF VITAMIN B_{12}.

PART II. PROTECTION BY AN IRON SALT AGAINST DESTRUCTION BY ANEURINE AND NICOTINAMIDE

BY S. L. MUKHERJEE AND S. P. SEN

From the Microbiological Section, Albert David (Laboratories) Ltd., Calcutta

Received July 14, 1958

The decomposition products of aneurine in solution at pH 8·0 and aneurine and nicotinamide at pH 4–4·5 destroy vitamin B_{12} activity. Ferric chloride has a protective effect. The decomposition products have reducing properties, and it is thought that these are responsible for the loss of B_{12} activity. Solutions containing the reducing substances heated to dryness and redissolved lost the ability to destroy the vitamin. Cysteine and H_2S are shown to destroy the activity of vitamin B_{12}, and again ferric chloride offers protection.

PREVIOUSLY[1] we observed that vitamin B_{12} deteriorates progressively in association with aneurine and nicotinamide in solution at a pH range between 4 to 4·5 when stored at room temperature or subjected to accelerated test conditions; the deterioration can be satisfactorily prevented by the use of iron salts. The total loss of aneurine was shown to be the same when aneurine alone in solution and aneurine together with nicotinamide in solution are subjected to accelerated test conditions at a pH range between 4 and 4·5. The decomposition product or products of aneurine alone have no destructive action on vitamin B_{12}, while those of aneurine and nicotinamide together have.

This led us to suppose that under the test conditions the decomposition products of aneurine and aneurine plus nicotinamide were not the same. The effect of the decomposition products was further investigated and the protective effect of an iron salt, $FeCl_3$, assessed on vitamin B_{12} in their presence. Therefore the pH of the solutions was varied, and as vitamin B_{12} activity can be destroyed by reducing substances the reducing effect of the decomposition products was investigated. Feller and Macek[2] suggested the thiazole moiety of aneurine to be responsible for the destruction of B_{12}, and polarographic studies[3,4] have shown that aneurine in alkali releases an –SH group and in higher concentrations of alkali finally decomposes releasing H_2S[5]. We therefore investigated the reducing effect of both cysteine and H_2S on vitamin B_{12}, and also the protective effect of $FeCl_3$ in these solutions.

EXPERIMENTAL

Solutions. Aneurine and nicotinamide were dissolved in 5 ml. of distilled water and the pH adjusted with HCl or NaOH; no buffer was added. When aneurine (150 mg.) and nicotinamide (1 g.) were dissolved in 5 ml. of water the pH of about 4·3 was not adjusted. The solutions tested in Table II were heated and then cooled and after acidification titrated with 0·01N iodine solution. Some, after adjusting the pH to

4·5, had 50 μg. of vitamin B_{12} added and the solution was reheated for 4 hours at 100°, after which the B_{12} was assayed.

Cysteine and H_2S solutions in amounts shown in the Tables were freshly prepared.

The "test" solution used in Table IV was made as follows.

Aneurine (150 mg.) was dissolved in 5 ml. of water and the pH adjusted to 8·0. The solution was heated in an open test tube on a water bath at 100° for 15 minutes and the pH, now about 7, was adjusted to 4·5. The solution was either diluted to 10 ml. and vitamin B_{12} 50 μg. (or the B_{12} plus ferric chloride, 5 mg.) added and heated for 4 hours at 100° and the B_{12} assayed, or the solution was evaporated to dryness and the residue dissolved in 10 ml. of water. The solution so formed was then titrated with iodine or 5 μg./ml. of B_{12} added and the solution heated for 4 hours at 100° and the B_{12} assayed.

Iodimetric titration. The usual procedure of iodimetric titration was followed by titrating aneurine or aneurine and nicotinamide solution, after acidification with HCl, with N/100 iodine solution using mucilage of starch as internal indicator. To the 10 ml. of solution described in Tables II and III, was added 1 ml. of concentrated HCl in the cold and 1 ml. of 1 per cent starch solution. To this, N/100 iodine solution was added dropwise with stirring. The end point occurred when the entire solution became blue for about 2 seconds. Due to the transient character of the blue colour, difficulties were experienced with the end point, particularly with the cysteine solution and with the aneurine and nicotinamide solution. Hence the end point chosen was the first appearance of the blue colour throughout the solution and which lasted for about 2 seconds.

Tests for Mercaptans. Colour tests for mercaptans were made with $FeCl_3$, $CuSO_4$ and nitroprusside solutions, and although cysteine solution gave characteristic colour reactions with all three, the decomposition products of aneurine and aneurine and nicotinamide did not. They reacted only with alkaline nitroprusside solution to give a pink colour.

Assays. The microbiological potency of vitamin B_{12} was determined by the "Cup Plate Assay Method" using *Escherichia coli* mutant M200 as test organism, developed by Bessel and others[6] and Cuthbertson and others[7]. The accuracy of this microbiological method is $\pm$ 10 per cent. Aneurine was assayed fluorimetrically by the thiochrome method of the U.S.P. XV.

Results

Table I shows the effect of aneurine in solution and aneurine with nicotinamide in solution at different ranges of pH on the stability of vitamin B_{12} when subjected to heating for 4 hours at 100° in 10 ml. rubber capped vials, and also shows the protection offered by $FeCl_3$.

It seems that aneurine solution subjected to an elevated temperature test with vitamin B_{12} at pH ranges between 4 to 6·5 causes no destruction of the B_{12} although there is substantial destruction of aneurine. But aneurine solution at pH 7·5 and 8 under similar conditions of heating

destroys B_{12}, and $FeCl_3$ offers significant protection. Also aneurine with nicotinamide in the concentrations indicated in the Table destroy vitamin B_{12} at pH ranges between 4 to 8, $FeCl_3$ offers nearly full protection at pH 4 and significant protection at higher pH ranges. The total loss of aneurine in aneurine and nicotinamide solution at various pH ranges is more or less equivalent to the total loss of aneurine when aneurine solution alone was heated at similar pH ranges.

TABLE I

EFFECT OF ANEURINE (15 MG./ML.) IN SOLUTION AND ANEURINE (15 MG./ML.) WITH NICOTINAMIDE IN SOLUTION, AT DIFFERENT RANGES OF pH, ON THE STABILITY OF VITAMIN B_{12} (5 μG./ML.) WHEN SUBJECTED TO HEATING FOR 4 HOURS AT 100° IN 10 ML. RUBBER CAPPED VIALS. ALSO THE PROTECTION OFFERED BY $FeCl_3$

Initial nicotinamide mg./ml.	$FeCl_3$ mg./ml.	Initial pH	Potency after test		pH after test
			Aneurine mg./ml.	Vitamin B_{12} μg./ml.	
—	—	4·0	11·0	4·5	3·9
—	—	5·5	10·5	4·5	5·1
—	—	6·5	10·2	4·5	5·1
—	—	7·5	8·4	0·05	5·6
—	0·5	7·5	7·8	1·25	5·8
—	—	8·0	7·5	0·5	5·6
—	0·5	8·0	6·2	2·5	6·0
100	—	4·0	10·6	Nil	3·9
100	0·5	4·0	10·6	4·5	3·9
100	—	6·0	11·0	Nil	5·4
100	0·5	6·2	11·0	3·0	5·5
100	—	7·9	7·0	0·5	5·4
100	0·5	8·1	5·1	2·5	6·0

TABLE II

THE IODINE TITRE VALUES OF SOLUTIONS OF ANEURINE, NICOTINAMIDE, ANEURINE AND NICOTINAMIDE, CYSTEINE AND H_2S AND THEIR EFFECT ON STABILITY OF VITAMIN B_{12} (5 μG./ML.) AFTER HEATING AT 100° FOR 4 HOURS

Substance in solution in open test tube	pH adjusted to	Time of heating min.	Iodine solutions ml. of N/100	Vitamin B_{12} potency after elevated temperature test μg./ml. approx.
Aneurine 150 mg./5 ml.	4·5	30	0·1	4·5
"	4·5	60	0·1	4·5
"	4·5	120	0·1	4·5
"	4·5	240	0·1	4·5
"	6·5	30	0·5	4·5
"	6·5	60	0·3	4·5
"	6·5	120	0·2	4·5
"	6·5	240	0·1	4·5
"	8·0	15	5·0	Nil
"	8·0	30	4·0	Nil
"	8·0	60	2·4	0·5
Nicotinamide 1 g./5 ml.	4·5	60	0·1	4·5
"	4·5	240	0·1	4·5
"	8·0	60	0·1	4·5
"	8·0	240	0·1	4·5
Aneurine 150 mg. and nicotinamide 1 g./5 ml.	4·3	60	1·6	2·0
"	4·3	120	3·3	Nil
"	4·3	240	4·2	Nil
Cysteine 2 mg./10 ml.	—	—	3·8	Nil
H_2S solution 0·5 mg./10 ml.	—	—	3·0	Nil

The iodine titre values of solutions of aneurine, nicotinamide and aneurine and nicotinamide and their effect on stability of vitamin B_{12} after heating at 100° for 4 hours, and also the effect of cysteine and H_2S on vitamin B_{12} stability, are shown in Table II.

Results in Table II indicate that aneurine solution heated for 4 hours at pH 4·5 to 6·5 does not produce any appreciable iodine titration value and also does not destroy vitamin B_{12}. Similarly, nicotinamide solution heated for 4 hours at a pH between 4·5 and 8·0 does not produce any

TABLE III

THE IODINE TITRE VALUES AND VITAMIN B_{12} POTENCY OF VITAMIN B-COMPLEX INJECTION* WHEN STORED AT ROOM TEMPERATURE (27–37°) IN 10 ML. VIALS

pH	Time stored months	Iodine in ml. of N/100	Vitamin B_{12} potency
4·5	16	3·0	Nil
4·5	12	2·7	Nil
4·5	8	2·6	Nil
4·5	6	2·4	Nil
4·5	3	2·0	3·5
4·5	Fresh	0·8	4·5

* Composition of B-Complex Injection in mg./10 ml. in rubber capped vials—aneurine 150; riboflavine 15; pyridoxine 50; nicotinamide 1000; panthenol 50; choline HCl 50; benzyl alcohol 150 and also vitamin B_{12} 50 μg.

TABLE IV

CHANGES IN IODINE TITRATION VALUE OF THE "TEST" SOLUTION BEFORE AND AFTER EVAPORATION AND THE EFFECT OF THIS ON THE STABILITY OF VITAMIN B_{12} (5 μG./ML.) HEATED AT 100° FOR 4 HOURS IN COMPARISON WITH THE EFFECTS OF CYSTEINE AND H_2S SOLUTION. ALSO THE PROTECTION OFFERED BY $FeCl_3$

Substance in solution	Potency of aneurine before heating at 100° mg./ml.	Iodine titre ml. N/100	$FeCl_3$ mg./ml.	Potency of aneurine after test mg./ml.	Potency of vitamin B_{12} after test μg./ml.
"Test" solution diluted to 10 ml. with added vitamin B_{12} 50 μg.	10·6	5·0	—	5·2	Nil
"	10·6	5·0	0·5	8·0	4·5
"Test" solution evaporated to dryness. Residue in 10 ml. water with vitamin B_{12} 50 μg.	10·0	0·9	—	7·0	4·5
Cysteine 2 mg. in 10 ml. with vitamin B_{12} 50 μg.	—	3·8	—	—	Nil
"	—	3·8	0·5	—	4·5
H_2S solution, 0·5 mg. in 10 ml. with vitamin B_{12} 50 μg.	—	3·0	—	—	Nil
"	—	3·0	0·5	—	4·5

appreciable iodine titration value nor has any effect on vitamin B_{12}. Cysteine and H_2S solution cause destruction of vitamin B_{12} and give appreciable iodine titre; also aneurine at pH 8 and aneurine and nicotinamide solution at pH 4 to 4·5 when heated, produce significant iodine titration values and destroy vitamin B_{12}. It is suggested that the iodine titre of the solution gives an indication of the presence of reducing substances, perhaps containing an –SH group or H_2S, which are likely to bring about B_{12} destruction.

Table III gives the iodine titre values and vitamin B_{12} potency of a vitamin B-complex injection when stored at room temperature (27–37°) in

10 ml. vials. The results indicate that the iodine titre of the vitamin B-complex injection increases with time of storage, and is paralleled by the progressive destruction of vitamin B_{12}.

Table IV shows the changes in iodine titration value of the "test" solution before and after evaporation and the effect of this on the stability of vitamin B_{12} heated at 100° for 4 hours in comparison with cysteine and H_2S solution, and also the protection offered by $FeCl_3$. The results indicate that on heating aneurine solution at pH 8 for 15 minutes, decomposition products are produced which possess properties which destroy vitamin B_{12}. $FeCl_3$, however, offers protection to the B_{12}. The decomposition products were volatile in nature, for when the solution containing them is evaporated to dryness the residue in solution gives much lower iodine titre and also loses its vitamin B_{12}-destroying property. 0·2 mg./ml. of freshly prepared cysteine solution and 0·05 mg./ml. of H_2S solution also possess vitamin B_{12}-destroying properties, when subjected to elevated temperature, which can be satisfactorily protected by 0·5 mg./ml. of $FeCl_3$.

Discussion

Although the thermal decomposition product or products of aneurine alone under the conditions of the experiment between pH ranges of 4 to 6·5 were not detrimental to vitamin B_{12} stability, the decomposition product or products of aneurine alone at pH 7·5 and 8 did destroy vitamin B_{12} activity. This was prevented significantly by ferric chloride. Heating aneurine solution alone for short intervals at pH 8·0 resulted in significant loss of aneurine, and the solution adjusted to pH 4 to 4·5 for better stability and subjected to heating at 100° for 4 hours after adding vitamin B_{12} can destroy the B_{12} activity (see Table IV). Other workers[8] have shown that vitamin B_{12} can be reduced by a variety of reducing substances, and our studies on the thermal decomposition product or products of aneurine in solution at pH 8·0 revealed the presence of certain titratable reducing substances which from the changes in pH appear to be of an acidic nature. When aneurine and nicotinamide are heated in solution at pH 4 to 4·5, similar titratable reducing substances are formed and both solutions can destroy B_{12} activity. (See Table II.) The protective effect of ferric chloride in the presence of aneurine and nicotinamide is interesting, and the mode of action is perhaps by sparing the vitamin B_{12} from the reductive influence of the decomposition product or products. When a solution containing decomposition product or products derived from heating aneurine solution alone at pH 8·0 is evaporated on water bath to dryness, and the residue taken into solution, its titratable reducing properties are reduced and so also is its ability to destroy B_{12} activity. During the evaporation the solution was found to liberate H_2S as evidenced by blackening of the moist lead acetate paper. Feller and Macek[3] suggested that the thiazole moiety of aneurine is responsible for destruction of vitamin B_{12}, and it has also been shown[4,5] that aneurine in alkali releases an –SH group and with higher concentration of alkali finally decomposes into H_2S[5]. The initial decomposition product, therefore, is perhaps of a thiol nature, which on further heating decomposes into H_2S.

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POLYHYDROXY (CATECHOLIC) PHENOLIC ACIDS—THE FORMATION OF *m*-HYDROXY- AND METHOXY-DERIVATIVES IN MAN

By S. L. Tompsett

From the Biochemical Laboratory, Northern General Hospital, Edinburgh

Received September 15, 1958

A method has been described for the determination of the methoxy group in phenolic compounds. Tannic and 3:4-dihydroxybenzoic acids when administered orally to man are excreted in appreciable quantities as 4-hydroxy 3-methoxybenzoic and *m*-hydroxybenzoic acids.

The present communication is an extension of the studies reported previously[1]. Human urine contains appreciable quantities of *m*-hydroxy- and 4-hydroxy-3-methoxyphenolic acids[2–8]. The origin of the *m*-hydroxy acids has been obscure since they do not occur in nature nor is their source dietary. Paper chromatographic studies suggest that 3:4-dihydroxyphenolic derivatives may be dehydroxylated to produce *m*-hydroxyphenyl compounds and also methylated to produce 4-hydroxy 3-methoxyphenyl[5,6,9,10].

The present paper is concerned with quantitative studies involving the determination of the methoxy group and the colorimetric determination of *m*-hydroxybenzoic and vanillic acids.

Methods

Preparation of Urine Extracts

10 ml. of urine is placed in a test tube together with 1 ml. of 10 N hydrochloric acid and the whole placed in a boiling water bath for 1 hour. After cooling the hydrolysed urine is extracted three times with 40 ml. quantities of re-distilled ether in a measuring cylinder[1]. The combined ether extracts are evaporated to dryness. This process does not appear to have any effect upon the parent substances under investigation.

The Colorimetric Determination of m-*Hydroxybenzoic Acid and Vanillic Acid* (4-*Hydroxy*-3-*methoxybenzoic Acid*

The technique in principle has been described previously[1,8]. Initially a separation is made using paper chromatography and the Kawerau Unit with benzene : acetic acid : water as the solvent.

An ethanolic solution of the urinary extract (≡ 2·5 ml. urine) is applied to the paper. After development, *m*-hydroxybenzoic acid is eluted with ethanol from strips 1 to 4 and vanillic acid from strips 6 to 8. After removal of the ethanol, the two phenolic acids are determined by means of 2:6-dichloroquinone chloroimide (the Gibbs Reaction). The colour is allowed to develop for 30 minutes after which the mixture is shaken with *n*-butanol.

With *m*-hydroxybenzoic acid, the aqueous phase is used for colorimetric determination while with vanillic acid, the *n*-butanol extract is used. Standards of 10, 20 and 40 μg., are set up at the same time.

The Determination of the Methoxy (Phenolic) Group

The procedure consists of hydrolysis by acid and the collection of the liberated methanol, oxidation of the methanol to formaldehyde, and the determination of the formaldehyde with chromotropic acid.

Hydrolysis. Hydrolysis and distillation is carried out in all glass equipment consisting of a 100 ml. round bottomed flask attached to a water cooled condenser. The material under examination is contained in the flask and to this are added 10 ml. of water and 5 ml. of concentrated sulphuric acid. The mixture is heated and the distillate collected. The heating is continued until the sulphuric acid reaches the fuming stage. The mixture is allowed to cool, when the heating is continued after the addition of 5 ml. of water, the sulphuric acid being again allowed to reach the fuming stage. This part of the procedure is repeated again so that three distillates in all are collected.

About $\frac{1}{2}$ to 1 g. of sodium bicarbonate is added to the combined distillate which is heated in apparatus similar to that used above; 10 ml. of distillate containing the methanol is collected. Less drastic forms of hydrolysis have been found to be quite ineffective.

Oxidation of methanol to formaldehyde[11]. *Reagents.* 5 per cent v/v ethanol in water; 1 per cent w/v potassium permanganate; 25 per cent v/v phosphoric acid; hydrogen peroxide, 5 volumes.

Technique. To the 10 ml. of distillate are added 1 ml. of 5 per cent ethanol, 5 ml. of 1 per cent potassium permanganate and 1 ml. of 25 per cent phosphoric acid. The mixture is allowed to stand at room temperature with frequent shaking for 1 hour, after which excess of permanganate is removed by the addition of hydrogen peroxide. The mixture is transferred to an all-glass still and heated to boiling; 10 ml. of distillate is collected.

Colorimetric determination of formaldehyde[12]. *Reagents.* Chromotropic acid reagent (prepared fresh before use. 0·2 g. of purified chromotropic acid is dissolved in 2 ml. of water to which 48 ml. of 13 M sulphuric acid is added). 9 M sulphuric acid.

Technique. Into a test tube is measured 1 to 3 ml. of distillate. Water is added to 3 ml. 5 ml. of chromotropic acid reagent is then added and after mixing, the tube is placed in a boiling water bath for 30 minutes. After cooling the mixture is diluted to 10 ml. with 9 M sulphuric acid. Readings are made against a blank at 570 mμ.

Blank. With each set of determinations a blank is set up using 10 ml. of water, the complete procedure being carried out except for the initial acid hydrolysis.

Standards. Initially standards of methanol (0·2, 0·5 and 1·0 mg.) were set up, the complete procedure being carried out except for the initial acid hydrolysis. Later standards of vanillic acid (0·5, 1·0 and 2·0 mg.) were set up, the complete procedure being carried out.

The Determination of Polyhydroxy (Catecholic) Phenolic Acids

The method has been described in detail elsewhere[1].

Results and Discussion

A number of compounds possessing the methoxy group have been examined using the complete procedure. Methanol was used as the standard. The results are shown in Table I. The methyl group of such

TABLE I

The determination of the methoxy group
(Quantities employed—0·5, 1·0, 2·5, 5 and 10 mg.)

Compound	Recovery expressed as per cent of theoretical
4-Hydroxy-3-methoxybenzoic acid	101 to 109
4-Hydroxy-3-methoxy cinnamic acid	98 to 108
Codeine	89 to 99
4-Hydroxy-3-methoxy phenylacetic acid	84 to 97
p-Methoxyphenylacetic acid	81 to 93
m-Methoxyphenylacetic acid	83 to 95

compounds as methionine, *N*-methylnicotinamide, and choline is not determined by the procedure. In the case of codeine the procedure measures the methoxyl but not the *N*-methyl group.

The urinary excretion of phenolic methoxy compounds after the oral ingestion of 1 g. of tannic or 3:4-dihydroxybenzoic acids has been studied in man. Catecholic phenolic acids were measured at the same

TABLE II

The urinary excretion by man of phenolic methoxy compounds after the oral ingestion of 1 g. of tannic or 3:4-dihydroxybenzoic acids

		3:4-Dihydroxy-phenolic compounds	Phenolic methoxy compounds	
		(as 3:4-dihydroxy-benzoic acid) (mg.)	(as methanol) (mg.)	(as vanillic acid) (mg.)
Tannic acid				
1.	A.	18·5	4·5	22·5
	B.	49·5	20·5	102·5
2.	A.	16·5	3·2	16·0
	B.	46·5	25·6	128·0
3.	A.	14·5	3·8	19·0
	B.	38·5	23·8	119·0
3:4-Dihydroxybenzoic acid				
1.	A.	16·5	3·8	19·0
	B.	198·0	33·3	167·0
2.	A.	18·5	4·0	20·0
	B.	185·0	36·8	184·0
3.	A.	14·5	3·2	16·0
	B.	210·0	38·0	190·0

A—control. B—after administration

time. In these experiments the night urine (10 p.m. to 7 a.m.) was used to reduce the effect of any changes in dietary intake. The results are shown in Table II and it will be seen that a marked proportion of the ingested acids are excreted in a methylated condition.

The experiments with 3:4-dihydroxybenzoic acid were repeated but "vanillic" and "*m*-hydroxybenzoic" acids were estimated. The results are shown in Table III. It will be noted that a marked increase in the

TABLE III

THE URINARY EXCRETION BY MAN OF "*m*-HYDROXYBENZOIC ACID" AND "VANILLIC ACID" AFTER THE ORAL INGESTION OF 1 G. OF 3:4-DIHYDROXYBENZOIC ACID

	3:4-Dihydroxybenzoic acid (mg.)	*m*-Hydroxybenzoic acid (mg.)	Vanillic acid (mg.)
1. A.	14·5	25·5	4·8
B.	185·0	145·0	96·5
2. A.	21·5	14·5	6·8
B.	165·0	125·0	135·0
3. A.	18·5	18·5	14·5
B.	175·0	165·0	145·0

A—control. B—after administration.

Total accountable recovery per cent of 3:4-dihydroxybenzoic acid

1.	43
2.	53
3.	49

excretion of 4-hydroxy-3-methoxybenzoic (vanillic) acid is accompanied by a marked increase in the excretion of *m*-hydroxybenzoic acid. It would appear that the metabolism of 3:4-dihydroxyphenolic acids includes methylation to produce a 4-hydroxy 3-methoxy compound and dehydroxylation to produce a *m*-hydroxy compound.

For 3:4-dihydroxybenzoic acid, known metabolites account for approximately 50 per cent of the intake during the short period of observation.

Acknowledgements. I am indebted to the Institute of Paper Chemistry, Appleton, Wisconsin, U.S.A., for a gift of homovanillic acid.

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SOME 3-ARYLPROPANE-1:2-DIOLS

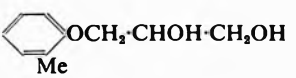
By (Miss) Y. M. Beasley, V. Petrow, O. Stephenson and A. M. Wild

From the Chemical Research Laboratories, The British Drug Houses Ltd., London, N.1

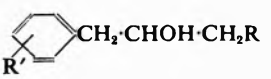
Received August 12, 1958

Some 3-arylpropane-1:2-diols formally related to mephenesin (I) were synthesised for examination as muscle relaxants. Their pharmacological study failed to reveal advantages over (I).

Although numerous structural variants of 1-*o*-toloxypropane-2:3-diol[1–3] (mephenesin) (I) have been prepared in this and other laboratories, no information is available on the biological properties of the related 3-arylpropane-1:2-diols (II; R = OH), which may be regarded as "deoxa" analogues of (I). We have, therefore, prepared some glycols of this type for pharmacological study.



(I)



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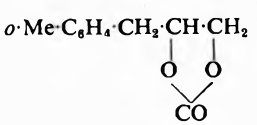

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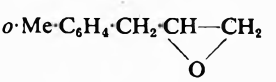
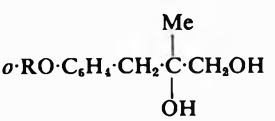


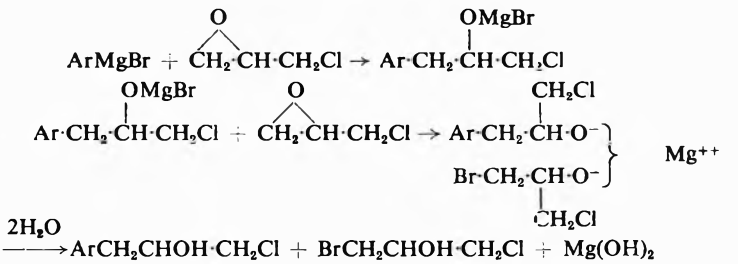
(IV)



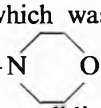
(V)

The syntheses of 3-aryl-2-hydroxypropyl chlorides (II; R = Cl) by reaction of 2:3-epoxypropyl chloride with aryl Grignard reagents is discussed in detail in a review by Gaylord and Becker[4]. Their production in somewhat better yield (> 60 per cent) by use of lithium aryls at *ca.* −78° has been reported by Gilman and others[5,6]. 3-*o*-Tolyl-2-hydroxypropyl chloride (II; R = Cl, R′ = *o*-Me), which formed the starting point of our investigation, was obtained in only moderate yield by Fourneau and Tiffeneau[7] and subsequently by Read and others[8] employing the Grignard technique.

We now find that by careful addition of two molar equivalents of 2:3-epoxypropyl chloride to a cooled ethereal solution of *o*-tolyl magnesium bromide, followed by heating the reaction mixture for two hours under reflux, the yield of 3-*o*-tolyl-2-hydroxypropyl chloride may be raised to > 70 per cent. 1-Bromo-3-chloro*iso*propanol (cf. 4), identified by conversion to 1:3-diphenoxy*iso*propanol, was a by-product. The yield was not improved by further increase in the quantity of 2:3-epoxypropyl chloride used, but fell markedly when less than two molar equivalents were employed. These results indicate the reaction mechanism:

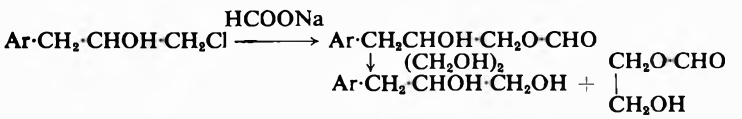


Conversion of the chlorohydrin (II; R = Cl, R′ = *o*-Me) into the required glycol (II; R = OH, R′ = *o*-Me) was achieved in excellent yield by heating for several hours with a slight excess of aqueous sodium carbonate (cf. Read and others[5]). The structure of the compound so obtained was confirmed by its alternate synthesis from *o*-allyl toluene by oxidation with peracetic acid followed by mild hydrolysis of the resulting glycol acetates (cf. Hershberg[9]). It reacted with phosgene in benzene solution in the presence of a tertiary base to give the dioxolone (III), which passed into 3-*o*-tolylpropane-1:2-diol 1-carbamate on reaction with concentrated aqueous ammonium hydroxide.

Short treatment of the chlorohydrin (II; R = Cl, R′ = *o*-Me) with one equivalent of cold methanolic potash gave 1:2-epoxy-3-*o*-tolylpropane (IV), which was converted into 1-morpholino-3-*o*-tolylpropan-2-ol (II; R =  , R′ = *o*-Me) on reaction with morpholine. 1-Piperidino- and 1-pyrrolidino-3-*o*-tolylpropan-2-ol were similarly prepared. Reaction of the epoxide with succinimide in boiling ethanol in the presence of a basic catalyst[10] gave the succinimido-derivative which was converted into 2-hydroxy-3-*o*-tolylpropylamine hydrochloride on hydrolysis with concentrated hydrochloric acid.

3-*o*-Alkoxyphenyl-, 3-*p*-chlorophenyl-, 3-*p*-tolyl- and 3-*p*-*n*-butoxyphenylpropane-1:2-diol were readily prepared by hydroxylation of the corresponding allyl-derivatives, and subsequently, by hydrolysis of the appropriate chlorohydrins. In contrast to 2-hydroxy-3-*o*-tolylpropyl chloride (above), attempted conversion of the chlorohydrins into the glycols with boiling aqueous sodium carbonate led to the formation of the corresponding epoxides. The diols were ultimately obtained, however,

by heating the chlorohydrins with a slight excess of sodium formate or acetate in ethane-1 : 2-diol (cf. Brooks and Humphrey[11]) when ester interchange occurred as indicated below:



Epoxide formation surprisingly did not take place under these experimental conditions as 6-aryl-3-oxahexane-1 : 5-diols, which would certainly be present in the reaction products through reaction of the epoxides with ethane-1 : 2-diol[12], were not isolated.

Two β-methyl analogues (V) were prepared by hydroxylation of the corresponding methallyl ethers.

Pharmacological study of the above compounds was kindly undertaken by Dr. A. David and his colleagues (Biological Laboratories, Godalming, Surrey).

Experimental

2-*Hydroxy*-3-o-*tolylpropyl chloride*[7,8] (II; R = Cl, R′ = *o*-Me). To a stirred solution of *o*-tolylmagnesium bromide (prepared from magnesium (14·6 g.) and *o*-bromotoluene (102·6 g.) in ether (700 ml.)), 1 : 2-epoxypropylchloride (111 g.) was added at such a rate that the mixture was kept refluxing gently. After addition was complete the mixture was heated under reflux for 2 hours, cooled in ice, and decomposed by careful addition of a solution of ammonium chloride (37 g.) in water (250 ml.) followed by just sufficient concentrated hydrochloric acid to break the resultant emulsion. The ether layer was separated, washed and dried and the ether removed by distillation. The residual oil was fractionated at 0·3 to 0·4 mm. to yield two main fractions (i) b.p. 42 to 49°, and (ii) b.p. 82 to 86° Fraction (i) was redistilled. It had b.p. 95° at 15 mm. and proved to be 1-bromo-3-chloro-propan-2-ol. It formed a *phenyl urethane* which separated from light petroleum (b.p. 80 to 100°) in shining plates, m.p. 76 to 77°. Found: C, 41·5; H, 4·0; N, 4·5; Halogen, 39·5. $C_{10}H_{11}O_2NClBr$ requires C, 41·0; H, 3·8; N, 4·8; Halogen, 39·5 per cent. Fraction (ii) (80 g., 72 per cent yield) had b.p. 84 to 85° at 0·4 mm. on refractionation, and proved to be 2-*hydroxy*-3-o-*tolylpropyl chloride*. Found: C, 65·1; H, 7·0; Cl, 19·2. Calc. for $C_{10}H_{13}OCl$: C, 65·0; H, 7·1; Cl, 19·2 per cent.

3-o-*Tolyl*-1 : 2-*epoxypropane* (IV). A solution of 2-hydroxy-3-*o*-tolylpropyl chloride (18·45 g.) in methanol (50 ml.) was treated with a solution of potassium hydroxide (5·6 g.) in methanol (75 ml.). The mixture was warmed on the steam bath for 10 minutes, the potassium chloride collected and the filtrate concentrated under reduced pressure. The residual oil was distilled at 0·1 mm. to yield the *product*, b.p. 49 to 51°. Found: C, 80·7; H, 8·3. $C_{10}H_{12}O$ requires C, 81·0; H, 8·1 per cent.

N-(2-*Hydroxy*-3-o-*tolyl*)-*propyl succinimide.* A mixture of the foregoing epoxide (3·1 g.) and succinimide (1·4 g.) in the minimum of hot ethanol containing pyridine (3 drops) was heated on the steam bath for 8 hours. The *product* which separated on cooling had m.p. 148 to 149° after crystallisation from ethanol. Found: C, 67·8; H, 6·9; N, 5·8. $C_{14}H_{17}O_3N$ requires C, 68·0; H, 6·9; N, 5·7 per cent. Hydrolysis of the succinimide derivative with 6N hydrochloric acid (cf. [10]) yielded 2-*hydroxy*-3-o-*tolylpropylamine hydrochloride* which had m.p. 183 to 184° after crystallisation from ethanol/ether. Found: C, 59·4; H, 7·9. $C_{10}H_{16}ONCl$ requires C, 59·5; H, 8·0 per cent.

3-o-*Tolylpropane*-1:2-*diol* (II; R = OH, R′ = *o*-Me). (*a*) A suspension of 2-hydroxy-3-*o*-tolylpropylchloride (50 g.) in water (500 ml.) containing sodium carbonate (29 g.) was heated under reflux for 10 hours. The *product* (33g.), isolated with chloroform, had m.p. 77 to 78° after crystallisation from chloroform/light petroleum (b.p. 60 to 80°). Found: C, 72·1; H, 8·5. $C_{10}H_{14}O_2$ requires C, 72·3; H, 8·5 per cent.

(*b*) To a solution of peracetic acid prepared from 30 per cent hydrogen peroxide (56 g.) and glacial acetic acid (150 ml.), *o*-allyl toluene (33 g.) was added with stirring over 30 minutes. Reaction was completed by heating at 80 to 85° for 2 hours. After cooling and dilution with water, the resultant oil was extracted with chloroform, the extracts washed with aqueous sodium carbonate and with water and the chloroform distilled off.

The residual oil after hydrolysis with methanolic potash and isolation with chloroform was distilled at 0·4 mm. to yield unchanged *o*-allyl toluene, b.p. 40°, and the *product* (20 g.) as an oil b.p. 120° which solidified rapidly. It had m.p. 76 to 78° after crystallisation from chloroform/light petroleum (b.p. 60 to 80°) and was identical with the compound prepared in experiment (*a*).

3-o-*Tolylpropane*-1:2-*diol monocarbamate* (II; R = $O{\cdot}CO{\cdot}NH_2$, R′ = *o*-Me). To a solution of the foregoing diol (32·8 g.) in benzene (200 ml.) was added with stirring and water cooling, a solution of phosgene (20 g.) in benzene (200 ml.), followed by a solution of phenazone (37·6 g.) in the minimum volume of chloroform. After allowing to stand overnight, the phenazone hydrochloride was collected and the filtrate washed with iced water until neutral.

A portion of the filtrate was concentrated. The resultant solid, after crystallisation from benzene/light petroleum (b.p. 40 to 60°), yielded 4-o-*tolylmethyldioxol*-2-*one*, m.p. 48 to 49°. Found: C, 68·4; H, 6·2. $C_{11}H_{12}O_3$ requires C, 68·7; H, 6·3 per cent.

The bulk of the original filtrate was stirred at room temperature with aqueous ammonia (300 ml., d = 0·880) for 6 hours, ammonia gas being passed into the mixture at intervals. The solid *carbamate* (22·7 g.) which separated was collected. It crystallised from ethyl acetate/light petroleum (b.p. 60 to 80°) in needles m.p. 97 to 100°. Found: C, 63·1; H, 7·4; N, 6·6. $C_{11}H_{15}O_3N$ requires C, 63·1; H, 7·2; N, 6·7 per cent.

1-*Pyrrolidino*-3-o-*tolylpropan*-2-*ol* was prepared by condensation of 2-hydroxy-3-*o*-tolylpropyl chloride with pyrrolidine (1 mole) in methanolic

potash (1 mole). It had b.p. 108 to 110° at 0·3 mm. Found: C, 76·3; H, 9·6; N, 5·9. $C_{14}H_{21}ON$ requires C, 76·7; H, 9·7; N, 6·4 per cent. The *hydrochloride*, m.p. 110° separated from ethyl acetate as a hygroscopic solid. Found: N, 5·5; Cl, 14·3. $C_{14}H_{22}ONCl$ requires N, 5·5; Cl, 13·9 per cent. The *salicylate* had m.p. 138 to 139° after crystallisation from ethyl acetate/ether. Found: C, 70·5; H, 7·7; N, 3·7. $C_{21}H_{27}O_4N$ requires C, 70·6; H, 7·6; N, 3·9 per cent.

1-*Piperidino*-3-o-*tolylpropan*-2-*ol* was prepared as for the pyrrolidino analogue. The *hydrochloride*, after crystallisation from ethyl acetate/methanol, had m.p. 190 to 191°. Found: C, 66·3; H, 8·9; N, 4·8. $C_{15}H_{24}ONCl$ requires C, 66·8; H, 9·0; N, 5·2 per cent.

1-*Morpholino*-3-o-*tolylpropan*-2-*ol* had m.p. 138 to 139° after crystallisation from ethyl acetate/ether. Found: C, 61·6; H, 8·1; N, 4·8; Cl, 13·4. $C_{14}H_{22}O_2NCl$ requires C, 61·8; H, 8·2; N, 5·2; Cl, 13·1 per cent.

3-p-*Tolylpropane*-1 : 2-*diol* (II; R = OH, R′ = *p*-Me), prepared by heating *p*-allyl toluene with peracetic acid at 70° for 7 hours followed by hydrolysis of the mixed acetate esters with methanolic potash, had b.p. 120 to 125° at 0·4 mm. and m.p. 45°. Found: C, 72·5; H, 8·3. Calc. for $C_{10}H_{14}O_2$, C, 72·3; H, 8·5 per cent.

3-p-*Chlorophenylpropane*-1 : 2-*diol* (II; R = OH, R′ = *p*-Cl), prepared by hydroxylation of *p*-chloroallylbenzene with peracetic acid, had b.p. 145° at 0·5 mm. and m.p. 45°. Found: C, 57·5; H, 6·0; Cl, 19·0. $C_9H_{11}O_2Cl$ requires C, 57·9; H, 5·9; Cl, 19·0 per cent.

2-*Methyl*-3-o-*tolylpropane*-1 : 2-*diol*, obtained by the action of peracetic acid on 2-β-methallyl toluene, had m.p. 58 to 60° after crystallisation from light petroleum (b.p. 60 to 80°). Found: C, 73·1; H, 8·6. $C_{11}H_{16}O_2$ requires C, 73·3; H, 8·9 per cent.

2-*Hydroxy*-3-(o-*methoxyphenyl*)*propyl chloride* (II; R = Cl, R′ = *o*-MeO) prepared by reaction of 2 : 3-epoxypropyl chloride with *o*-methoxyphenyl magnesium bromide in ethereal solution, had b.p. 106 to 108° at 0·4 mm. Found: C, 60·2; H, 6·7; Cl, 18·1. $C_{10}H_{13}O_2Cl$ requires C, 59·8; H, 6·5; Cl, 17·7 per cent.

3-o-*Methoxyphenylpropane*-1 : 2-*diol* (II; R = OH, R′ = *o*-MeO). The foregoing chlorohydrin (16 g.) was dissolved in ethane diol (35 ml.) and sodium formate (8·25 g.) added. The mixture was stirred and heated under gentle reflux for 6 hours when it was cooled, diluted with water and the separated oil extracted with ethylene dichloride. After washing with salt solution the solvent was removed and the residual oil distilled at 0·2 mm. Unchanged chlorohydrin (2 g.) was obtained followed by the *product*, b.p. 126 to 128° at 0·2 mm. (9·7 g., 76 per cent yield allowing for unchanged material). Found: C, 65·9; H, 8·2. $C_{10}H_{14}O_3$ requires C, 65·9; H, 7·7 per cent.

2-*Hydroxy*-3-(o-*ethoxyphenyl*)-*propylchloride* (II; R = Cl, R′ = *o*-EtO), prepared in 60 per cent yield by reaction of *o*-ethoxyphenyl magnesium bromide with 2 mole equivalents of 2 : 3-epoxypropyl chloride in ethereal solution, had b.p. 109 to 110° at 0·4 mm. and solidified in hexagonal plates on standing. Found: C, 61·8; H, 7·1; Cl, 16·3. $C_{11}H_{15}O_2Cl$ requires C, 61·5; H, 7·0; Cl, 16·5 per cent.

3-o-*Ethoxyphenylpropane*-1:2-*diol* (II; R = OH, R′ = *o*-EtO). The foregoing chlorohydrin (16·7 g.) was dissolved in ethane diol (27 g.), sodium formate (7·9 g.) added and the mixture heated with stirring to gentle reflux for 5 hours. It was then cooled, diluted with water, and the product isolated with chloroform. After removal of the chloroform the residual oil was distilled at 0·4 mm. to yield the *product* (60 per cent) as an oil, b.p. 136 to 138°. Found: C, 67·4; H, 7·9. $C_{11}H_{16}O_3$ requires C, 67·3; H, 8·2 per cent.

The same product was obtained, but only in 35 per cent yield, by the action of peracetic acid (2 mole) on *o*-ethoxyallylbenzene, followed by hydrolysis of the resultant acetate esters with a suspension of sodium carbonate in boiling ethanol.

2-*Hydroxy*-3-o-*propoxyphenylpropyl chloride* (II; R = Cl; R′ = *o*-PrO) had b.p. 102° at 0·1 mm. and solidified on standing. Found: C, 62·6; H, 7·1. $C_{12}H_{17}O_2Cl$ requires C, 63·0; H, 7·5 per cent.

3-o-*Propoxyphenylpropane*-1:2-*diol* (II; R = OH, R′ = *o*-PrO), prepared by heating the foregoing chlorohydrin (19·5 g) with sodium formate (8·84 g.) in ethane diol (30 g.) for 7 hours, had b.p. 131 to 134° at 0·2 mm. Found: C, 68·5; H, 8·5. $C_{12}H_{18}O_3$ requires C, 68·5; H, 8·6 per cent.

o-*Propoxybromobenzene*, prepared by reaction of *n*-propyl bromide with the sodium salt of *o*-bromophenol in *n*-propanol, had b.p. 72° at 0·5 mm. Found: C, 50·6; H, 5·4; Br, 37·0. $C_9H_{11}OBr$ requires C, 50·3; H, 5·2; Br, 37·2 per cent.

o-*Propoxyallylbenzene* had b.p. 84 to 85° at 1·4 mm. Reaction with peracetic acid and subsequent hydrolysis yielded 3-*o*-propoxyphenylpropane-1:2-diol, identical with the product described earlier.

o-*Butoxybromobenzene* had b.p. 75° at 0·5 mm. Found: C, 52·0; H, 5·8; Br, 34·6. $C_{10}H_{13}OBr$ requires C, 52·4; H, 5·7; Br, 34·9 per cent. Reaction of its Grignard reagent with 2:3-epoxypropyl chloride yielded 2-*hydroxy*-3-o-*butoxyphenylpropylchloride* (II; R = Cl, R′ = *o*-BuO), b.p. 124° at 0·4 mm.

3-o-*Butoxyphenyl*-1:2-*epoxypropane*, b.p. 90° at 0·1 mm. was obtained when the foregoing chlorohydrin was heated under reflux for several hours with aqueous sodium carbonate in an attempt to prepare the diol. Found: C, 75·9; H, 8·6. $C_{13}H_{18}O_2$ requires C, 75·7; H, 8·8 per cent.

3-o-*Butoxyphenylpropane*-1:2-*diol* (II; R = OH, R′ = *o*-BuO) was obtained (*a*) in small yield by hydrolysis of the foregoing epoxide (5 g.) in water (50 ml.) containing concentrated sulphuric acid (1 drop) at reflux temperature for 4 hours.

(*b*) In 76 per cent yield by heating the chlorohydrin (24·2 g.) with sodium formate (10·2 g.) in ethane diol (35 g.) for 6 hours. The diol crystallised from light petroleum (b.p. 60 to 80°) in needles, m.p. 67 to 69°. Found: C, 69·6; H, 9·0. $C_{13}H_{20}O_3$ requires C, 69·6; H, 9·0 per cent.

(*c*) In 40 per cent yield by the action of peracetic acid on o-*butoxyallylbenzene*. The latter compound had b.p. 64 to 66° at 0·2 mm. Found: C, 82·5; H, 9·7. $C_{13}H_{18}O$ requires C, 82·0; H, 9·5 per cent.

3-p-*Butoxyphenylpropane*-1:2-*diol* (II; R = OH, R′ = *p*-BuO,) obtained by the action of peracetic acid on *p*-butoxyallyl benzene, had b.p. 155° at 0·5 mm. and m.p. 53° after crystallisation from light petroleum (b.p. 60 to 80°). Found: C, 69·8; H, 9·0. $C_{13}H_{20}O_3$ requires C, 69·6; H, 9·0 per cent.

o-*Amyloxyallyl benzene* had b.p. 92 to 93° at 0·8 mm. Found: C, 82·2; H, 9·5. $C_{14}H_{20}O$ requires C, 82·3; H, 9·9 per cent.

3-o-*Amyloxyphenylpropane*-1:2-*diol* (II; R = OH, R′ = *o*-AmO) was obtained as an oil, b.p. 160° at 0·7 mm. by the action of peracetic acid on the foregoing allyl compound. Found: C, 70·8; H, 9·0. $C_{14}H_{22}O_3$ requires C, 70·5; H, 9·3 per cent.

o-*Propoxymethallylbenzene* prepared from *o*-methallylphenol had b.p. 74 to 75° at 0·3 mm. Found: C, 82·2; H, 9·7. $C_{13}H_{18}O$ requires C, 82·0; H, 9·5 per cent. On reaction with peracetic acid it was converted into 3-o-*propoxyphenyl*-2-*methylpropane*-1:2-*diol* (V; R = Pr) (in 40 per cent yield), b.p. 118 to 119° at 0·3 mm. Found: C, 69·8; H, 9·0. $C_{13}H_{20}O_3$ requires C, 69·6; H, 9·0 per cent.

o-*Butoxymethallylbenzene* had b.p. 92 to 93° at 0·6 mm. Found: C, 82·7; H, 9·9. $C_{14}H_{20}O$ requires C, 82·3; H, 9·9 per cent. On treatment with peracetic acid at 70° for 3 hours it yielded 3-o-*butoxyphenyl*-2-*methylpropane*-1:2-*diol* (V; R = Bu), obtained as a viscous oil, b.p. 132 to 134° at 0·4 mm. Found: C, 70·1; H, 9·1. $C_{14}H_{22}O_3$ requires C, 70·5; H, 9·3 per cent.

3-cyclo*Hexylpropane*-1:2-*diol*, prepared from allyl*cyclo*hexane, had b.p. 120° at 0·5 mm. Found: C, 68·6; H, 11·5. $C_9H_{18}O_2$ requires C, 68·3; H, 11·5 per cent.

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THE ACTION OF MORPHINE AND MORPHINE-LIKE ANALGESICS APPLIED ON THE INTRALUMINALLY PERISTALTIC REFLEX OF THE ISOLATED GUINEA PIG ILEUM

By M. Medaković

From the Department of Pharmacology, Medical Faculty, University of Belgrade, Yugoslavia

Received July 18, 1958

Morphine and morphine-like analgesics inhibit peristalsis of the isolated guinea pig ileum, when introduced into its lumen. Their effects were parallel to their relative analgesic potencies. Since suitable doses of morphine did not inhibit the responses to nicotine and to 5-HT, it is thought that the inhibitory action of morphine and related drugs, involves the afferent part of the peristaltic reflex. Peristalsis, previously abolished by intraluminal morphine, can be restored by 5-HT by the same route. That the inhibitory action of the intraluminal morphine upon the peristalsis may be caused, at least partly, by its antagonism towards intrinsic 5-HT and may take place on the same mucosal receptors, is discussed.

Trendelenburg[1] has described the inhibitory action of morphine on the peristaltic reflex of the isolated guinea pig ileum. Since then the action of morphine upon the isolated guinea pig ileum has been analysed by several authors [2–9]. When morphine was added to the bath in which the segment was suspended, the results indicated that it acted on the postganglionic fibres of the intramural nervous system, inhibiting the neuromuscular transmission[6–9], or the synaptic transmission of impulses[2].

Recently the role of 5-hydroxytryptamine (5-HT) in the initiation of peristalsis has been described by Bülbring and Lin[10,11]. Introduced into the lumen of the isolated guinea pig ileum, 5-HT was found to stimulate peristaltic movement while morphine[5] and morphine-like analgesics[12] have been described as potent inhibitors of 5-HT action upon the guinea pig ileum when added to the bath. It seemed, therefore, attractive to study in the bath the action of morphine and some related analgesics upon peristalsis under conditions used by Bülbring and Lin[10,11].

Method

A modification of Trendelenburg's original method by Beleslin and Varagić[13], which allowed the introduction of drugs into the lumen and also the washing of the lumen, was used throughout the present experiments. The segments of the guinea pig ileum were suspended in a 20 ml. bath, containing Tyrode solution kept at 36° and aerated with O_2.

The following drugs were used: morphine hydrochloride, dihydromorphinone hydrochloride, codeine phosphate, pethidine hydrochloride, 5-hydroxytryptamine creatinine sulphate and nicotine hydrogen tartrate. All the drugs, except nicotine and 5-HT in some experiments, were injected into the lumen of the isolated segments, in 0·1 ml. volume,

diluted with Tyrode solution. All the doses and concentrations refer to the salts used.

Results

The Action of Morphine in the Lumen

The introduction of 0·1 to 5μg. of morphine into the lumen was found to inhibit the peristalsis elicited by raising the intraluminal pressure. If the volume of the suspended segment is estimated to be approximately 0·5 to 1 ml., then the actual effective concentration of morphine might be estimated to be 10^{-7} to 10^{-5} g./ml.

As in experiments described by Kosterlitz and others[14], two separate phases of the longitudinal muscle contraction during peristalsis were distinguished. A slow increase in tone of the longitudinal muscle in response to the raised intraluminal pressure was followed by a secondary, rapid contraction, which was immediately followed by the emptying of the segment. When injected in threshold doses, morphine inhibited first the rapid, secondary contraction and the emptying phase to the same degree. The inhibition of the primary, slow longitudinal contraction became evident only when the dose of morphine was increased. By increasing the dose sufficiently it was abolished completely.

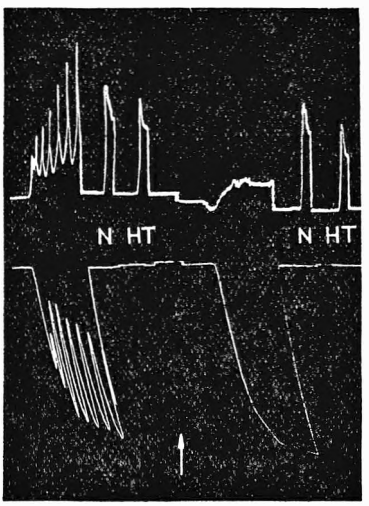

FIG. 1. The inhibition of the persitaltic reflex by the intraluminal application of morphine, without the inhibition of the response to nicotine and to 5-HT. At N, 40 μg. of nicotine, and at HT, 4 μg. of 5-HT was added to the bath and allowed to act for 30 seconds. At arrow, 1 μg. of morphine was injected into the intestinal lumen. Upper tracing, longitudinal muscle; Lower tracing, circular muscle.

Morphine inhibition of the peristaltic reflex was reversible and lasted for 10 to 60 minutes after washing out, depending on the dose used and on the duration of the contact. In some experiments the inhibitory effect of morphine wore off in spite of its continuous presence in the lumen of the isolated segment.

To obtain some evidence on the possible site of action of morphine in the lumen, its inhibitory action on peristalsis was compared with its action on the response to nicotine and to 5-HT added to the bath. Thus, between two records of the peristalsis, nicotine, 15 to 20 μg., and 5-HT, 2 to 5 μg., were added to the bath and the intraluminal pressure raised again, causing contractions of the longitudinal muscle comparable to the peristaltic contractions of the longitudinal muscle. By adjusting the dose of morphine, the peristalsis could be inhibited or abolished, while the action of nicotine and that of 5-HT remained unchanged (Fig. 1).

However, with higher doses of morphine, the effects of nicotine and of 5-HT were also inhibited or abolished.

The Action of Morphine-like Analgesics

Dihydromorphinone, pethidine and codeine inhibited peristalsis when introduced into the lumen of the isolated segment in suitable doses (Fig. 2). Dihydromorphinone was found to be approximately five times

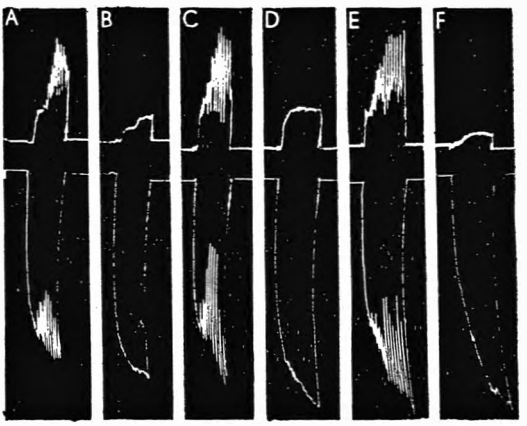

FIG. 2. Comparison of inhibitory activities of morphine and some related drugs on the peristaltic reflex. At A, C and E control records. Between A and B, 1 μg. of morphine; between C and D, 20 μg. of codeine; and between E and F, 0·2 μg. of dihydromorphinone was introduced into the lumen of the intestine. After B, D and F the drugs were washed out. Upper tracing, logitudinal muscle; Lower tracing, circular muscle.

as potent as morphine, and the inhibitory activity of pethidine and codeine was 1/10 and 1/50 to 1/20 respectively (morphine = 1). These data indicated a parallelism of inhibitory action with relative analgesic potencies.

The Influence of 5-HT on the Inhibitory Action of Morphine

It has been shown that 5-HT added to the lumen of the isolated guinea pig ileum stimulated peristalsis under normal conditions[10,11], as well as when the reflex had been depressed by cooling[13]. In the present experiments, peristalsis previously inhibited by morphine in the lumen was restored by 5-HT by the same route. As can be seen from Figure 3, both the preparatory and the emptying phase of the peristaltic reflex were stimulated immediately after 5-HT was introduced into the lumen. However, with a higher dose of morphine, peristalsis was abolished, and could not be restored by the addition of a higher dose of 5-HT.

Figure 4 shows that 5-HT in the lumen, caused a contraction of the longitudinal muscle of the segment which was previously paralysed by morphine, and the intraluminal pressure of which had been raised before the addition of the 5-HT. This contraction disappeared as soon as the pressure was decreased, in spite of the presence of 5-HT in the lumen.

Discussion

We have shown that morphine and morphine-like analgesics introduced intraluminally into the isolated guinea pig ileum inhibit peristalsis. That the addition of morphine to the bath abolishes this reflex has been known since Trendelenburg's demonstration[1]. Schaumann[6] has shown that morphine, added to the bath abolished not only peristalsis, but also the contraction of the longitudinal muscle caused by nicotine. This led him to suggest that morphine acted on the efferent part of the peristaltic reflex. Later, morphine was found to inhibit acetylcholine formation from cholinergic nerve endings of the autonomic intestinal nervous system[7–9].

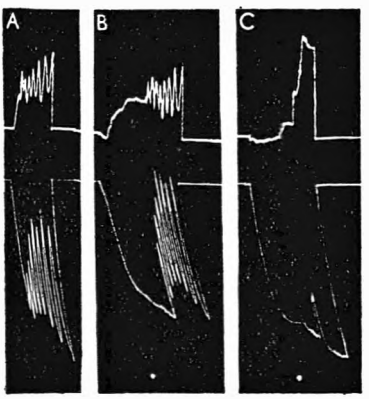

Fig. 3. Effect of intraluminal injection of 5-HT on the inhibitory action of intraluminal morphine upon the peristalsis. At A control record. Between A and B, 1 μg. and between B and C, 10 μg. of morphine was introduced into the intestinal lumen. At white dot in B, 5 μg., and in C, 50 μg. of 5-HT was applied into the lumen.

The present experiments have shown that morphine can inhibit peristalsis without any depression of the response to nicotine or to 5-HT. The response to nicotine is due to the stimulation of intramural ganglia, and according to Gaddum and Hameed[15] the effect of 5-HT upon the isolated guinea pig ileum is also exerted upon intestinal ganglia. But it is supposed that these drugs act upon different ganglionic receptors[15]. Trendelenburg[16] has found that morphine inhibited the stimulating effect of 5-HT upon the superior cervical ganglion of the cat, leaving the effect of nicotine intact. Therefore, both the nicotine and 5-HT were used as controls in the present experiments. The failure of morphine to inhibit the action of either nicotine or 5-HT upon the gut, while abolishing the peristaltic reflex, suggests that this drug, applied into the lumen of the intestine, was acting on afferent nervous structures of the peristaltic reflex. However, higher doses of morphine, applied by the same route, inhibited not only the peristalsis, but also the effects of both nicotine and 5-HT. This finding may be explained on the assumption, that if higher doses of morphine were applied into the lumen, the drug diffused from the lumen to the outside and affected either the ganglia or the postganglionic fibres of the intestinal cholinergic nervous system.

The experiment presented in Figure 4 indicates that the intestinal ganglia were not involved either in the inhibitory action of intraluminal morphine or in the stimulatory action of 5-HT. It also shows that the longitudinal contraction of the segment, which is normally elicited by raising the intraluminal pressure, can be abolished by morphine in the lumen and restored by intraluminal 5-HT. This contraction, produced by raising the intraluminal pressure, has been shown not to involve intramural intestinal ganglia[6,17]. Thus, the experiment presented in Figure 4 could be interpreted as meaning that the restoring action, which 5-HT exerted upon peristalsis in the present experiments, was not caused by facilitating ganglionic transmission, in the sense of findings made by Trendelenburg[16]. Thus, it seems plausible to suppose that this action of 5-HT is exerted somewhere in the afferent part of the peristaltic reflex, probably by sensitising to intraluminal pressure the receptors of the intestinal mucosa, which are involved in the initiation of the peristaltic reflex. The antagonism between morphine and 5-HT might take place on the same 5-HT sensitive receptors, which have been shown to be involved in the peristaltic reflex[11].

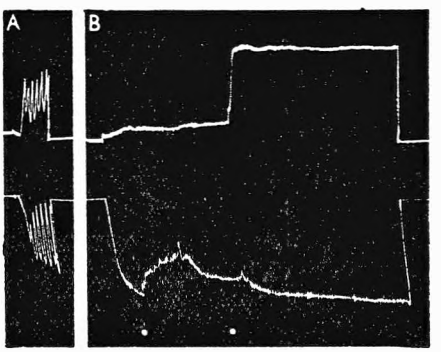

FIG. 4. Sensitation of the intestinal segment paralysed by morphine, to the raised intraluminal pressure, by the intraluminal introduction of 5-HT. At A, control record. Between A and B, 1 μg. of morphine was introduced into the intestinal lumen. At first white dot 1 μg. and at the second 5 μg. of 5-HT was introduced by the same route. Note the reappearance of the contraction of the longitudinal muscle after the addition of 5-HT and its disappearance on the decrease of the intraluminal pressure.

Bülbring and colleagues[11,18] have found that the intraluminal introduction of cocaine (10^{-4}) and procaine (10^{-3}), abolished the peristaltic reflex but it does not seem probable that the inhibitory action of morphine on peristalsis is due to a local anaesthetic effect, since the inhibitory concentrations of morphine used were below those of cocaine[18].

The question arises whether the inhibition of the peristalsis caused by morphine, can be ascribed, at least partly, to the antagonistic action of this drug towards the intrinsic 5-HT. The latter substance has been shown to sensitise the pressure receptors of the intestinal mucosa[11]. Morphine-like analgesics in the bath have been shown to inhibit the effect of 5-HT upon the isolated guinea pig ileum, their activity being parallel to their analgesic potencies[12]. Also when the 5-HT antagonists, LSD and BOL, are introduced into the lumen the opposite effects to those of 5-HT are produced[10,11].

Acknowledgements. The author is indebted to Dr. V. Varagić for criticising this paper. 5-Hydroxytryptamine was kindly supplied by Farmitalia, Milano.

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ASSAY OF PROMAZINE AND ITS SEPARATION FROM CHLORPROMAZINE AND PROMETHAZINE

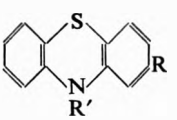
By L. Cavatorta

From the Analytical Department, Lepetit S.p.A., Milan, Italy.

Received June 30, 1958

A colorimetric method is described to identify and quantitatively determine promazine, in pharmaceutical preparations. The method is applicable to promazine and promethazine mixtures, and promazine and chlorpromazine in mixtures. With a second colorimetric method promethazine or promethazine in the presence of the other two substances can be determined.

Among phenothiazine derivatives chlorpromazine (I) and promazine (II) have been much used as tranquillising drugs. Both may be associated in pharmaceutical preparations with the antihistamine drug promethazine (III), an isomer of promazine.



(I) R = Cl; R′ = $CH_2CH_2CH_2NMe_2HCl$
(II) R = H; R′ = $CH_2CH_2CH_2NMe_2HCl$
(III) R = H; R′ = $CH_2—CHNMe_2HCl$ (with CH_3 branch)

Promazine may be assayed by methods similar to those for chlorpromazine and promethazine. The more important of these are as follows.

(1) Colorimetric assay based on the colour developed by oxidizing the phenothiazine ring to thionyl-like derivatives[1–6]. Among the oxidizing agents, bromine water, nitric acid, sulphuric acid, potassium persulphate, iodic acid and ferrous salts are mentioned; but difficulty in stabilising the colour is encountered. Calò and colleagues[6] find sulphuric acid poorly specific, while the colour developed by potassium persulphate with chlorpromazine has a poor stability. Stable and reproducible colours for chlorpromazine were obtained with both iodic and phosphoric acids by these authors.

(2) Ultra-violet spectrophotometric assay after dissolving in dilute hydrochloric acid.

(3) Assay in non-aqueous medium: the dimethylamino group is titrated with perchloric acid[7] in dioxane.

(4) Gravimetric assay, by precipitating the bases in the form of picrates[8].

For methods 2, 3 and 4, high purity is required; the presence of only traces of basic compounds may alter the ultra-violet absorption curve and may also produce erroneous results with both the perchloric acid titration and the picrate determination.

The separation of promazine from chlorpromazine or promethazine has not been described, while the separation of chlorpromazine from promethazine has been described by Berti[9], who used chromatographic methods, and recently by Calò with an ionophoretic method[6].

Principle of the Proposed Method

The method described for the assay of promazine, chlorpromazine and promethazine mixtures is based on the method described by Overholser and Yoe[10] for the assay of phenothiazine by the formation of a complex with palladium chloride and also on the fact that promethazine differs from chlorpromazine in having a substituted *iso*propyl instead of a propyl group. Promethazine may be differentiated from the two other products using mercuric sulphate as reagent, which besides acting as oxidizing agent is also used to detect *iso*propanol in the presence of propanol[11].

Complex with Palladium Chloride

According to Overholser and Yoe[10] palladium chloride reacts with phenothiazine giving blue coloured solutions or a precipitate which is not an oxidation product but a complex having the formula

$$Pd(C_{12}H_9NS_2)Cl_2$$

For the reaction, 1 ml. of an aqueous solution of 0·03 mg./ml. of $PdCl_2$ and a solution of phenothiazine in an amount of acetone which must not exceed 20 per cent by volume of the total solution, are added to 5 ml. of a buffer at pH 2·9. The reading must be made and compared with a standard immediately as the colour is not stable. The optimum amount of phenothiazine is 40 μg.; with amounts greater than 100 μg. the colour is very unstable. The colour obtained for pure phenothiazine ranges between blue and purple. The coloured complex may be extracted with ethyl acetate or chloroform yielding a red coloured extract which is less stable than the blue colour of the buffered solution. The buffer is necessary, as small quantities of salts or acids may alter the intensity of colour.

To avoid the use of acetone as solvent the phenothiazines are assayed as hydrochlorides, but colours obtained with a concentration of 0·03 mg./ml. of palladium chloride are too faint, and 1 mg./ml. is necessary. We also found that buffer at pH $2 \pm 0{\cdot}1$ gave a more stable colour. With this modification a bright red colour is obtained for promazine, chlorpromazine and promethazine; the calibration curve is the same for the three products. The Lambert-Beer law is closely observed; the colour rises to a maximum intensity after 10 minutes and is stable for about 2 hours having a maximum absorption at about 500 mμ. The optimum amount of compound for the assay is between 50 and 250 μg. The colour can be extracted by stirring the solutions with ethyl acetate, which extracts the colour due to chlorpromazine and promethazine but does not extract that due to promazine. The gold-yellow colour of the organic phase is stable for an hour and has the absorption maximum at 440 mμ. The optimum concentration ranges between 500 and 1,000 μg./ml.

Reaction with Mercuric Sulphate Reagent

Promethazine heated with this reagent gives either a red colouration or a red precipitate. Under the conditions described the red colour is stable for one hour and has an absorption maximum at 500 mμ, while promazine and chlorpromazine develop no colour.

Experimental

Determination of Promazine, Chlorpromazine and Promethazine with Palladium Chloride

Reagents: (1) Buffered solution at pH = 2 ± 0·1: (10 g. of sodium acetate trihydrate are dissolved in 50 ml. of water: to the solution 80 ml. of N hydrochloric acid and water are added to a final volume of 200 ml.); (2) palladium chloride solution: (to 50 mg. of $PdCl_2$, hydrochloric acid is added to 50 ml.); (3) standard solutions of promazine hydrochloride in water, chlorpromazine hydrochloride in water, promethazine hydrochloride in water (99 per cent pure potentiometrically assayed).

Procedure: 0·5 ml. of $PdCl_2$ reagent is added to a mixture of 5 ml. of buffer solution and 1 ml. of aqueous solution containing from 50 to 150 μg. of the product to be assayed. Water is added to make 7 ml. The mixture is stirred, and after 15 minutes the red colour developed read a 500 mμ with a Beckman D.U. spectrophotometer using a 1 cm. cell, against a blank prepared in the same way without addition of the active substance.

TABLE I

Colour stability as obtained in the colorimetric assay of promazine, promethazine and chlorpromazine (200 μg./ml.) with $PdCl_2$

	E after minutes					
	5	10	20	30	60	120
Promazine	0·295	0·305	0·305	0·305	0·305	0·305
Chlorpromazine ..	0·295	0·295	0·300	0·300	0·300	0·300
Promethazine ..	0·290	0·290	0·295	0·295	0·295	0·295

TABLE II

Absorption values (*E*) at different wavelengths for promazine, chlorpromazine and promethazine (200 μg./ml.)

mμ	450	470	480	490	500	510	530	550
Promazine	0·16	0·25	0·295	0·300	0·305	0·293	0·265	0·210
Chlorpromazine	—	0·26	0·290	0·295	0·300	0·290	0·270	0·200
Promethazine	0·15	0·25	0·280	0·290	0·295	0·290	0·270	0·200

From the calibration curve, the concentration in μg./ml. of promazine, chlorpromazine and promethazine is obtained. At the same concentration two or three phenothiazine derivatives yield practically the same extinction as a single member of the mixture. Table I showing the values obtained with 200 μg./ml. after different times, confirms the stability of the colour. Table II gives the values of the absorption obtained from different wavelengths with 200 μg./ml. of each of the three substances.

Assay of Chlorpromazine and Promethazine by Extraction with Ethyl Acetate of Colour Developed with Palladium Chloride

The first reaction is repeated with solutions containing about 500 μg./ml. of either chlorpromazine or promethazine or a total of 500 μg./ml. of both. More compound is required in this reaction because the extinction of the gold-yellow solution obtained by extraction with ethyl acetate is lower than the red colour obtained in buffered solutions working at the same concentrations.

TABLE III

EXTINCTION VALUES AT 440 mμ OF ETHYL ACETATE SOLUTIONS OF PROMAZINE, CHLORPROMAZINE AND PROMETHAZINE (500 μG./ML.)
(3 assays)

Promazine ..	0	0	0
Chlorpromazine ..	0·135	0·135	0·140
Promethazine ..	0·07	0·07	0·07

To a 100 ml. separating funnel is added 5 ml. of buffer solution, 0·5 ml. of palladium chloride reagent, 1 ml. of a solution of the compounds at the above mentioned concentration and 1 ml. of water. The mixture is stirred ten times and 10 ml. of ethyl acetate added. The mixture is again stirred ten times, the aqueous layer is removed and the organic layer is washed with 5 ml. of N HCl. After 5 minutes the colour extracted into the organic phase is read at 440 mμ against a blank and against standard solutions prepared in the same way using 500 μg. of promethazine or chlorpromazine or a mixture of both, the proportions varying according to the composition of the mixture to be assayed. The results are reported in Table III.

TABLE IV

COLOUR STABILITY AS OBTAINED IN THE COLORIMETRIC ASSAY OF CHLORPROMAZINE AND PROMETHAZINE WITH $PdCl_2$ AFTER EXTRACTION WITH ETHYL ACETATE (500 μG./ML.)

	E after minutes					
	5	10	20	30	60	90
Chlorpromazine ..	0·130	0·135	0·135	0·135	0·135	0·120
Promethazine ..	0·07	0·07	0·07	0·07	0·07	0·065

TABLE V

ABSORPTION VALUES (*E*) AT DIFFERENT WAVELENGTHS FOR ETHYL ACETATE SOLUTION OF CHLORPROMAZINE AND PROMETHAZINE (500 μG./ML.)

mμ	400	420	430	440	450	460	480
Chlorpromazine	0·11	0·120	0·125	0·135	0·130	0·120	—
Promethazine	0·06	0·065	0·065	0·07	0·06	0·05	—

Table IV reports the values of reading with 500 μg./ml. of chlorpromazine obtained after different times showing the stability of the colour in ethyl acetate. Table V presents the absorption values at different wavelengths obtained with 500 μg./ml. of these substances.

Promethazine Assay with Mercury Sulphate

Reagent: 5 g. of yellow mercury oxide is dissolved in a mixture of 80 ml. of concentrated sulphuric acid and 20 ml. of water.

Procedure: to 1 ml. of reagent is added 1 ml. of an aqueous solution containing from 50 to 100 μg./ml. of promethazine. The mixture is heated on a boiling water bath for ten minutes, cooled, and water added to 5 ml. The colour is read after ten minutes at 500 mμ in a 1 cm. cell with a Beckman D.U. against a blank prepared from 1 ml. of reagent and 4 ml. of water. The concentration of promethazine is read from the calibration curve.

Table VI gives the values of readings with 100 μg./ml. of promethazine obtained after different times, showing the stability of colour.

TABLE VI

COLOUR STABILITY AS OBTAINED IN THE COLORIMETRIC ASSAY OF PROMETHAZINE WITH $HgSO_4$ (100 μG./ML.)

E after minutes					
3	5	10	20	30	60
0·270	0·280	0·280	0·280	0·280	0·278

TABLE VII

ABSORPTION VALUES (*E*) AT DIFFERENT WAVELENGTHS OBTAINED WITH 100 μg./ml. OF PROMETHAZINE

mμ	450	475	500	510	520	530
	0·235	0·250	0·275	0·270	0·220	0·185

Table VII gives the values of absorption with the different wavelengths obtained with 100 μg. of promethazine. No colour developed with the conditions described, on proportional quantities of promazine and chlorpromazine. With mixtures of promethazine and promazine or chlorpromazine, the calibration curves so obtained were equal to that already obtained with pure promethazine.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

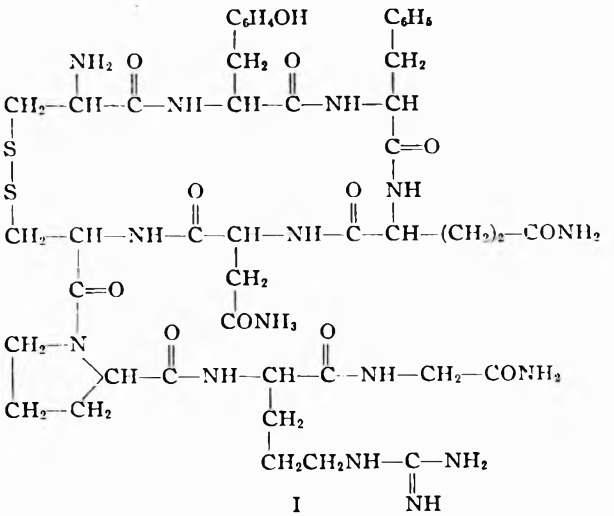
Glyceryl Trinitrate, Determination of, by Nitrite Method. G. Hansen. (*Arch. Pharm. Chem.*, 1958, **65**, 541.) Nitrite is produced when glyceryl trinitrate is decomposed with sodium hydroxide, the quantity obtained being constant even when experimental conditions are varied. Heating 25 ml. of glyceryl trinitrate solution (10 per cent) with 3 ml. of 2N sodium hydroxide in a water bath for 10 minutes, or allowing to stand at room temperature for 1 hour, yields nitrite equivalent to 63·2 per cent of the nitrogen present. The quantity of nitrite in the reaction mixture may be determined by diluting and adding a reagent consisting of ethyl-α-naphthylamine hydrobromide 8 mg., procaine hydrochloride 250 mg., 2N acetic acid 100 ml. The solution is allowed to stand for 30 minutes, and the light absorption measured at 525 mμ. The colour intensity is proportional to the quantity of nitrite in the sample. A correction is made for the nitrite content of the sample, determined separately. This method is stated to be sensitive, reproducible, and specific for alkyl nitrates. Some excipients, such as agar, interfere in the determination, and the alkaline solution must not be heated if lactose is present. G. B.

Papaverine, Colorimetric and Fluorimetric Tests for. H. Wachsmuth and K. Cornelis. (*J. Pharm. belg.*, 1958, **13**, 130.) For colorimetric analysis a sample containing 2 to 15 mg. of papaverine is dissolved in ethanol and diluted to 1·5 ml. 5 ml. of a 0·45 per cent w/v solution of sodium β-naphthaquinone sulphonate in methanol and 1 ml. of pyridine are added and the tube sealed and heated for 2 hours in a boiling water bath. After cooling and extracting with chloroform, an aliquot quantity of the chloroform layer is diluted to 10 ml., dried over anhydrous sodium sulphate, and its optical density at 530 mμ determined against a reagent blank. The quantity of papaverine is calculated from the optical density. Results are satisfactory, and are not affected by the presence of atropine, codeine or phenobarbitone. The suggested fluorimetric method is more sensitive, but the presence of codeine or phenobarbitone may give rise to errors. A sample containing 10 to 100 μg. of papaverine is dissolved in 1 ml. of hydrochloric acid and heated in a stoppered tube for 1 hour in a boiling water bath, cooled in ice, carefully neutralised with ammonium hydroxide solution and diluted to 10 ml. The quantity of papaverine is calculated from the intensity of the strong green fluorescence, measured with a spectrophotometer. G. B.

Quinine from Quinidine, Rapid Chemical Method for Distinguishing. M. Petković. (*Acta Pharm. Jug.*, 1958, **8**, 7.) About 10 mg. of base or salt is placed in a watchglass, and about 1 ml. of ethanol (96 per cent) added. After the solid has dissolved, one drop of dilute sulphuric acid is added and mixed thoroughly with the solution. A drop of solution is placed on filter paper and treated with iodine vapour for 30 seconds. Spots due to quinine appear grey-blue to grey-purple with a dark yellow edge, and those due to quinidine dark yellow, changing to yellow on allowing to stand in the air. G. B.

ORGANIC CHEMISTRY

Arginine-Vasopressin, Synthesis of the Pressor-Antidiuretic Hormone. V. du Vigneaud D. T. Gish, P. G. Katsoyannis and G. P. Hess. (*J. Amer. chem. Soc.*, 1958, **80**, 3355.) Arginine-vasopressin (I) the principal pressor and antidiuretic hormone of the posterior pituitary gland of beef has been



I

synthesised from the protected hexapeptide amide, carbobenzoxy-L-glutaminyl-L-asparaginyl-S-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide. Treatment of this with hydrobromic-acetic acids yielded L-glutaminyl-L-asparaginyl-*S*-benzyl-L-cysteinyl-L-prolyl-L-arginylglycinamide dihydrobromide. This was converted to the monohydrobromide with triethylamine and coupled with the azide of *S*-benzyl-*N*-tosyl-L-cysteinyl-L-tyrosyl-L-phenylalanine to give a crude protected nonapeptide hydrobromide, which on treatment with sodium and liquid ammonia (to remove the protecting groups), and oxidation of the resulting sulphydryl nonapeptide by aeration in dilute aqueous solution pH 6·7, gave the required product. Purification was effected by countercurrent distribution and electrophoresis. The activity of the synthetic product (360–400 units/mg.) is comparable with that of natural arginine-vasopressin. Both natural and synthetic products behaved similarly on countercurrent distribution, paper electrophoresis at two different pH's and on ion exchange chromatography with IRC50. J. B. S.

Sulphanilyl- and Sulphonylcarbamic Acid Derivatives and their Blood-sugar Lowering Activity. E. Haack. (*Arzneimitt.-Forsch.*, 1958, **8**, 444.) The main methods of synthesising these compounds are reviewed and their chemotherapeutic relationships correlating structure with activity, toxicity, length and intensity of effect and metabolic effects. In 13 tables, 133 compounds are characterised, most of which are new. D. B. C.

PHARMACY

Amphetamine Sulphate, *In Vitro* Method for the Determination of the Rate of Release of, from Sustained Release Medication. J. Royal. (*Drug Standards*, 1958, **26**, 41.) The following method was applied to sustained-release capsules

containing amphetamine sulphate in the form of small spherical pellets. A weighed quantity of the pellets was placed in a modified U.S.P. tablet disintegration apparatus, which was operated with 150 ml. of simulated gastric fluid at 37°. Samples were taken at intervals up to 60 minutes, after which the gastric fluid was replaced by simulated intestinal fluid, and samples taken up to 6 hours. Sodium hydroxide was added to the samples, which were then distilled to separate the amphetamine. The amphetamine obtained from gastric fluid samples was determined spectrophotometrically at 257 mμ applying a correction for the absorption due to other substances. With the intestinal fluid samples it was found necessary to remove interfering substances by extraction with ether before determination of the absorption at 257 mμ. The residue remaining on the screen of the disintegration testing apparatus was also distilled with sodium hydroxide and assayed spectrophotometrically at 257 mμ. G. B.

Bactericide: Leukocide Ratio: A Technique for the Evaluation of Disinfectants. L. Greenberg and J. W. Ingalls. (*J. Amer. pharm. Ass., Sci. Ed.*, 1958, **47**, 531.) To determine the toxicity of disinfectants to leukocytes, 0·2 ml. of fresh defibrinated rat blood was mixed with 1 ml. of diluted disinfectant in a centrifuge tube, and allowed to stand at 37° for 10 minutes. About 50 ml. of warm sterile isotonic saline was added and the suspension centrifuged, the supernatant fluid being discarded. A suspension of *Micrococcus pyogenes* var. *aureus* in rat serum was used to test the residue, 0·1 ml. being mixed with the residue and incubated at 37° for 1 hour. A loopful was removed, stained with Wright's stain, buffered with Wright's buffer, washed and examined microscopically for evidence of phagocytosis. The disinfectants were also examined for bactericidal effect, and the ratio of bactericidal to leukocidal concentrations calculated in each case. Under the conditions of the tests, only gentian violet, "Clorax" (a combination of sodium chlorate and sodium metaborate) and povidone-iodine were less toxic to leukocytes than to bacteria. G. B.

Polyvinyl Chloride or Rubber Caps, Stability of Aqueous Solutions in Ampoules and Multiple-dose Containers, Closed by means of. A. B. Nielsen. (*Dansk. Tidsskr. Farm.*, 1958, **32**, 109.) A number of solutions were stored in ampoules, vials closed with rubber caps, and vials closed with "resistant caps" made from polyvinyl chloride backed with rubber. Solutions of sodium aurichloride and potassium permanganate were shown to be most rapidly reduced in the vials closed with rubber caps, and during six months' storage ascorbic acid solutions decomposed to the greatest extent in contact with the rubber caps. In experiments with solutions of adrenaline and sodium nitrite little difference was observed between rubber and polyvinyl chloride caps. "Resistant" caps absorb sodium metabisulphite to a smaller extent than rubber caps, and seem preferable for many solutions. However, they absorb phenol and chlorocresol more readily than rubber caps, and so cannot be recommended for use in the presence of phenolic bacteriostatics. G. B.

Vitamin Preparation, Liquid, An Investigation of the Relative Stability of. J. N. Delgado, F. V. Lofgren and H. M. Burlage. (*Drug Standards*, 1958, **26**, 51.) Multivitamin solutions containing aneurine, ascorbic acid, nicotinamide, riboflavine-5-phosphate sodium, panthenol, pyridoxine hydrochloride, vitamin B_{12}, folic acid and vitamins A and D were prepared in a vehicle containing water 20 per cent, with either propylene glycol 80 per cent or

propylene glycol 40 per cent and glycerin 40 per cent. Some solutions containing a greater proportion of water were made, and in some cases aneurine nitrate was used instead of the hydrochloride. The solutions were assayed for aneurine, ascorbic acid, riboflavine, pyridoxine, nicotinamide and vitamin A, stored at 47° for 30 days, and again assayed. It was shown that increasing the proportion of water decreased the stability of aneurine, both hydrochloride and nitrate. Aneurine nitrate was more stable in the vehicle containing 80 per cent of propylene glycol, and the hydrochloride was more stable in that containing 40 per cent each of glycerin and propylene glycol. The addition of disodium calcium edetate and ethyl hydrocaffeate appeared to improve the stability or ascorbic acid. A vehicle containing disodium calciumedetate 0·01, ethyl hydrocaffeate 0·05, water 20 and propylene glycol 80 was found to be the most satisfactory. The method of preparation of the multivitamin solution is described in detail, and the following percentages of excess should be added to provide for a shelf-life of 2 years; aneurine nitrate 55, riboflavine-5-phosphate sodium 33, ascorbic acid 23, pyridoxine hydrochloride 16, nicotinamide 20, and vitamin A 16. G. B.

PHARMACOLOGY AND THERAPEUTICS

Captodiame, Investigation of the Mechanism of its Potentiating Effect on Hexobarbitone. I. Eberholst, I. Huus and R. Kopf. (*Arzneimitt.-Forsch.*, 1958, **8**, 379.) Captodiame, like iproniazid, appears to potentiate hexobarbitone by inhibiting its metabolism. Thus if mice were given captodiame (20 mg./kg.) followed in 30 minutes by hexobarbitone, and the latter analysed after a further 10, 15, 30 and 60 minutes, the concentration of hexobarbitone was significantly higher than that in mice which had not received the captodiame. At the time of awakening from anaesthesia, however, the concentration was the same in the two groups. *In vitro* experiments with liver slices suggested that captodiame had an inhibitory effect on the metabolism of hexobarbitone. Chlorpromazine was also tested for its effect and was found to be a much more powerful potentiator of hexobarbitone. It appeared to have no effect on the metabolism of hexobarbitone, and thus acted by a different mechanism. D. B. C.

Cascara, Chronic Potassium Depletion due to. B. J. Houghton and M. A. Pears. (*Brit. med. J.*, 1958, **1**, 1328.) This is a report of a case of chronic potassium depletion in a woman of 55. In addition to anorexia, dryness of the mouth, thirst, and general weakness, her legs became so weak that she was unable to lift them or to flex her hips. For at least five years previous to admission she had habitually taken 10–15 grains of cascara, two or three times a week. Replacement of potassium by intravenous drip, using 4 g. of potassium chloride in a litre of 5 per cent dextrose in water, produced a dramatic improvement within 24 hours, and she made an uneventful recovery. The results of balance studies carried out over a nine-day period are reported. Over the 9 days from the beginning of treatment the patient gained a total of 796 mEq. of potassium and lost 376 mEq. of sodium, without concomitant changes in weight.

S. L. W.

Chlorothiazide: Clinical and Laboratory Studies. W. C. Watson, T. J. Thomson and J. M. Buchanan (*Lancet*, 1958, **1**, 1199.) A study of the clinical and biochemical effects of a single oral dose of chlorothiazide 2 g., given to 22 patients with various "water-retaining" conditions, showed it to be an

effective and safe oral diuretic, which acts principally by promoting excretion of sodium and chloride, but in certain circumstances by promoting excretion of potassium and bicarbonate. In general, the best diuretic response occurred in the type of patient who responds well to mersalyl. In patients with pulmonary heart-disease the response was disappointing. It is doubtful whether a regimen of divided doses (i.e., 1 g. twice daily) has any clinical advantages; unless there are urgent reasons for obtaining a maximal diuresis, single doses are preferable, as they avoid the fatigue of unnecessary nocturia. S. L. W.

Chlorothiazide, Diuretic Action. N. A. Matheson and T. N. Morgan (*Lancet*, 1958, **1**, 1195.) In trials on 10 healthy men, chlorothiazide, in a dose of 2 g. by mouth was shown to be an effective diuretic. The diuretic effect appeared within 2 hours, was well maintained over the first 8 hours, and then subsided but was not quite complete by 12 hours. During the second 12-hour period the excretion-rates returned to control values. With intravenous injection of 500 mg. of the sodium salt, the changes were maximal within the first hour, and the effect lasted about 4 hours. Compared with acetazolamide 0·5 g., chlorothiazide 2 g. is a less potent carbonic-anhydrase inhibitor, hence recovery of base is more rapid. The action of chlorothiazide is likely to be maintained and because of this it may induce hypopotassaemia. S. L. W.

5-Hydroxytryptamine in Tissues, Turnover of. S. Udenfriend and H. Weissbach. (*Proc. Soc. exp. Biol. N.Y.*, 1958, **97**, 748.) Although the bulk of 5-hydroxytryptamine is present in the mucosa of the gastrointestinal tract, significant amounts are also found in blood platelets, brain, spleen and lung. Enzymes involved in 5-hydroxytryptamine formation and destruction are present in all depots except blood platelets, indicating that the amine is apparently made, released and destroyed for specific functions in these depots. In this paper a study is made of the rate of turnover of 5-hydroxytryptamine in various tissues in order to help in the elucidation of these functions. Using tryptophan-^{14}C and 5-hydroxytryptophan-^{14}C it was possible to measure the turnover of 5-hydroxytryptamine in a number of tissues of the rabbit. The half-lives were found to be: platelets and spleen, 33–48 hours; stomach, 17 hours; intestine, 11 hours. Although amounts of 5-hydroxytryptamine in the brain were too small to permit isotopic measurement, it was possible to estimate its turnover from the increase following the administration of harmaline, an inhibitor of 5-hydroxytryptamine metabolism. Such estimates indicated a half-life of the order of minutes. M. B.

Iproniazid, Effect of, on Brain Levels of Noradrenaline and Serotonin. S. Spector, D. Prockop, P. A. Shore and B. B. Brodie. (*Science*, 1958, **127**, 704.) It is known that iproniazid inhibits monoamine oxidase, an enzyme which can inactivate noradrenaline and serotonin—substances present in the brain. Monoamine oxidase plays a major role in the physiological inactivation of both these amines in the brain. The data presented in this paper show that repeated doses of iproniazid induce a marked rise in the brain levels of both noradrenaline and serotonin, together with signs of central stimulation. However, it is not possible to conclude that the central stimulant effect of iproniazid is causally related to the increase in brain amines. Nevertheless it is significant that the administration of large doses of 3:4-dihydroxyphenylalanine, a noradrenaline precursor, or of 5-hydroxytryptophan, a serotonin precursor, causes central excitation which is enhanced by pretreatment with iproniazid. M. B.

(+)-Lysergic Acid *cyclo*Alkylamides. Pharmacology of. Z. Votava, I. Podvalová and M. Semonský. (*Arch. int. Pharmacodyn.*, 1958, **115**, 114.) Five (+)-lysergic acid *cyclo*alkylamides, i.e. *cyclo*propyl-, *cyclo*butyl-, *cyclo*pentyl-, *cyclo*hexyl- and *cyclo*heptylamide were synthesised and their pharmacological activity was compared with that of (+)-lysergic acid diethylamide and ergometrine. The *cyclo*butyl and *cyclo*pentyl derivatives showed marked oxytocic, antiserotonic and mydriatic effects, while the others were less active. All of the drugs were equipotent in producing a rise in the body temperature of rabbits and a rise followed by a prolonged fall in mice. Blood pressure was hardly affected in anaesthetised rabbits and dogs and in unanaesthetised dogs. Toxic doses produced central effects similar to those of amphetamine. Preliminary clinical tests confirmed that the substances produced oxytocic effects without any hallucinogenic action. W. C. B.

Methocarbamol in Neuromuscular Disease. H. W. Park. (*J. Amer. med. Ass.*, 1958, **167**, 168.) Methocarbamol was evaluated in 42 patients with a variety of disorders manifesting an increase in involuntary muscle tone. In 30 patients with pyramidal tract and acute myalgic disorders use of the drug resulted in a significant improvement in 27 (90 per cent), questionable improvement in 2, and none in 1. There was no change in 12 patients with chronic arthritic, extrapyramidal and myalgic disorders. Good results were obtained in patients with acute low back pain and acute torticollis. All patients who had a demonstrable effect to the medication attained the effect within 30 to 45 minutes after intravenous administration (0·5 to 1 g.), or within 72 hours on oral medication (1 to 2 g. four times daily). Side-effects were negligible and were reversed on slight reduction of dosage. S. L. W.

Normorphine, Human Pharmacology and Addiction Liability of. H. F. Fraser, A. Wikler, G. D. Horn, A. J. Eisenman and H. Isbell. (*J. Pharmacol.*, 1958, **122**, 359.) Other workers have suggested that normorphine, a possible metabolite of morphine, may be an effective analgesic but showing little evidence of physical dependence. Therefore a study has been made of its pharmacology and its addiction liability. It was found that in single doses normorphine caused less sedation, less depression of temperature, less respiratory depression and less pupillary constriction than did equal doses of morphine. Administration of 9 to 10 mg. of normorphine every six hours for seven doses caused less, but longer lasting, pupillary constriction than did equal doses of morphine. Cumulation of the sedative effects of normorphine occurred in this experiment. When normorphine was substituted for morphine in addicted patients it completely supressed the morphine abstinence syndrome. The intensity of abstinence observed after withdrawal of normorphine was far less than the intensity of abstinence from morphine. Marked cumulation of sedative effects occurred during direct addiction to normorphine and prevented raising the dose to the level which could easily have been attained with morphine. Partial tolerance to the sedative effects developed. Nalorphine precipitated definite abstinence syndromes in patients addicted to normorphine. The intensity of abstinence after the withdrawal of the normorphine was slow in onset and milder than abstinence from morphine, methadone or codeine. The urinary excretion of 17-hydroxycorticosteroids was depressed during chronic administration of normorphine and elevated transiently after the normorphine was discontinued. These results suggest that the practical addiction liability of normorphine is low. M. B.

Pempidine in the Treatment of Hypertension. M. Harington, P. Kincaid-Smith and M. D. Milne. (*Lancet*, 1958, **2**, 6.) Pempidine is a ganglion-blocking agent closely resembling mecamylamine in its properties. It may be used orally as the bitartrate, which contains about 50 per cent of the active base, or intravenously as the hydrochloride, which contains about 80 per cent. As it is freely absorbed from the gut, the oral form will usually be preferred. Both drugs are excreted more rapidly in acid than in alkaline urine, are concentrated in tissue containing a high proportion of cell nuclei, and easily cross the blood-brain barrier. There are, however, important differences which may make pempidine a more useful antihypertensive agent than mecamylamine. The excretion of pempidine is more rapid and is less affected by variation in acid-base balance than that of mecamylamine and allows the dosage of pempidine to be quickly increased; the hypotensive effect of pempidine is less variable. The effective dosage of pempidine in hypertension in man is usually lower than that of mecamylamine; side-effects will probably be less severe and briefer. From a clinical trial on 27 patients with hypertension who received continuous treatment with pempidine over periods varying from 2–20 weeks it was shown that a steady hypotensive effect can be maintained throughout the day when pempidine is given orally at 5-hourly intervals. The initial dose was usually 2·5 mg. given four times daily, and this was raised rapidly by increments of 2·5 mg. daily until an adequate hypotensive effect was achieved. The effective dose varied widely but the average total given as maintenance treatment was 32·5 mg. bitartrate (16 mg. base). There was no evidence of the development of tolerance. Most of the patients experienced some side-effects similar to those observed with other ganglion-blocking agents. The most frequent symptoms were constipation, dryness of the mouth, and blurring of vision. All side-effects (including a syndrome of early paralytic ileus in 3 patients) disappeared rapidly on stopping the drug. S. L. W.

Pyridine-2-aldoxime Methiodide Therapy for Alkylphosphate Poisoning. T. Namba and K. Hiraki. (*J. Amer. med. Ass.*, 1958, **166**, 1834.) Pyridine-2-aldoxime methiodide (PAM) is a specific reactivator of alkylphosphate-inhibited acetylcholinesterase and produces a prompt and complete relief of symptoms due to poisoning with insecticides such as parathion. For this purpose it completely replaces atropine. Cholinesterase activity of red blood cells is restored instantly and completely recovers; that of the serum recovers only transiently. It has no influence on the cholinesterase activity of normal blood. One intravenous injection of 1 g. of PAM is usually sufficient, but the dose may be repeated or increased if necessary. No serious side-effects have been encountered even with large doses. If given in sufficient dosage the effect, even of a single injection, is dramatic, with prompt recovery of clear consciousness, disappearance of muscular fasciculations, and improvement of respiratory function. The successful treatment of five serious cases of parathion poisoning due to spraying is reported. S. L. W.

Pyrogen, Endogenous, Role of, in the Genesis of Fever. W. B. Wood, Jr. (*Lancet*, 1958, **2**, 53.) Evidence is presented that endogenous pyrogen derived from polymorphonuclear leucocytes in inflammatory exudates is one of the factors responsible for many kinds of fever. Leucocytes have been shown to be the principal source of endogenous pyrogen in granulocytic exudates, and to discharge pyrogen into the surrounding medium while still motile and functionally active. The pyrogen acts directly on the thermoregulatory centres

of the brain. Its chemical constitution has not been elucidated, but it differs from bacterial pyrogens, pyrexin, and pyrogenic polysaccharides in being inactivated on heating to 90° for 30 minutes, non-dialysable through Cellophane, resistant to the action of trypsin, chymotrypsin, and ribonuclease, and unaffected by changes in pH from 2·0–10·5. It is possible that cells other than polymorphonuclear leucocytes may be able to produce similar pyrogenic factors. G. B.

Piperazine, Action of, on *Necator, Trichuris* and *Strongyloides*. J. A. McFadzean and S. R. Smithers. (*Trans. R. Soc. trop. Med. Hyg.*, 1958, **52**, 235.) Five cases each of infestation with *Necator americanus*, *Trichuris trichiura* and *Strongyloides stercoralis* were treated with piperazine adipate. For *Trichuris* infestation a single dose of 3 g. was given, followed by 0·6 g. thrice daily for 5 days, while for *Strongyloides* 0·6 g. was given 5 times daily for 5 days. The drug appeared to have no action on either *Trichuris* and *Strongyloides*. Three patients with *Necator* infestation were given a single dose of 2·4 to 3 g., a fourth patient was given 3 g., followed after 2 days by 3·6 g., and a fifth patient was given 3 g., followed on the 3rd and 5th days by 3·6 g. It appeared that piperazine had some effect on the infestation, but that repeated doses were necessary to achieve significant diminution in the number of parasites.

G. B.

Polybactrin Aerosol in Prophylaxis of Surgical Sepsis. R. M. Gibson. (*Brit. med. J.*, 1958, **1**, 1326.) A clinical trial is reported in which Polybactrin Aerosol applied locally to wound surfaces was used in 250 consecutive neurosurgical operations. Polybactrin is a mixture of polymyxin, bacitracin and neomycin for local use. The difficulties associated with its relative insolubility and instability in aqueous solutions have been overcome by suspending the mixture in an inert, highly volatile anhydrous liquid "dichloro-tetrafluoroethane". The wound was sprayed at each wound layer encountered on opening and closure. The results were contrasted with a comparable series in which the antibiotic technique was not used. The analysis showed a reduction in the incidence of major sepsis from 7·2 to 0·4 per cent, and in minor sepsis from 1·2 to 0·8 per cent. S. L. W.

Quinalbarbitone in Pre-anaesthetic Medication. J. E. Eckenhoff and M. Helrich. (*J. Amer. med. Ass.*, 1958, **167**, 415.) A blind study of morphine, pethidine, alphaprodine, quinalbarbitone, and saline solution as pre-anaesthetic medicaments was made in 1400 surgical patients. The solutions of narcotics and of quinalbarbitone were prepared so as to provide equipotent doses of each of the drugs per ml. (in the case of quinalbarbitone the dose was 75 mg./ml.). All the injections were given intramuscularly and all were given in combination with atropine sulphate. The results showed that the use of quinalbarbitone led to a higher proportion of alert and awake patients, free from apprehension, than did the narcotics. The narcotics produced more drowsy or sleepy patients, but also a higher incidence of apprehension; they also produced more undesirable side-effects than quinalbarbitone. Respiratory depression was more common with the narcotics. When narcotics had been used pre-operatively the patients remained narcotised longer after anaesthesia than did those who received quinalbarbitone or saline solution, but they did not complain so much of pain or appear so restless as did the latter group. The authors suggest that the continued routine use of narcotics for pre-anaesthetic medication is unwise and unwarranted. S. L. W.

Triamcinolone in the Treatment of Psoriasis. W. B. Shelley, J. S. Harun and D. M. Pillsbury. (*J. Amer. med. Ass.*, 1958, **167**, 959.) Sixty patients with psoriasis were treated with triamcinolone in doses of 12–16 mg. daily by mouth. In 36 of the patients there was a prompt and undoubted response; within a week the scaling and erythema diminished significantly, and within two to four weeks of continued treatment the psoriasis was in some patients completely cleared up, though on cessation of treatment or reduction of dosage the lesions regularly returned. The remaining 24 patients failed to show any response. A wide variety of reversible side-effects were observed. Some were favourable, such as the stimulation of hair-growth in alopecia areata, and many were unfavourable, such as flushing, hyperhidrosis, facial hirsutism and facial contour changes. The treatment should be reserved for acute extending psoriasis not controllable by other means or for very extensive and severe chronic psoriasis. Topical triamcinolone therapy was found to be without effect. S. L. W.

Triamcinolone in the Treatment of Rheumatoid Arthritis. E. F. Hartung. (*J. Amer. med. Ass.*, 1958, **167**, 973.) Sixty-seven patients with rheumatoid arthritis were treated with triamcinolone (free alcohol form). The initial dose was usually 24 mg. a day, given in four divided doses, the average maintenance dose being 10 mg. daily in four divided doses. In 23 of the patients the drug was stopped within six months owing to side-effects. Of 23 treated for six months up to 11 months the therapeutic results were good in 16. In the treatment of rheumatoid arthritis the drug appears to have four important advantages over other steroids: (1) lack of production of oedema; (2) fewer gastrointestinal symptoms, especially the peptic ulcer syndrome; (3) less psychic irritation; and (4) no effect on arterial blood pressure. Nevertheless, it retains many of the disturbing side-effects of the other corticosteroids, particularly the masculinising effects, facial rounding, cutaneous purpura or ecchymosis, and acne. Increased sweating and leg cramps were prominent. S. L. W.

Warfarin as an Anticoagulant. S. Baer, M. W. Yarrow, C. Kravitz and V. Markson. *J. Amer. med. Ass.*, 1958, **167**, 704.) The anticoagulant effects of warfarin and dicoumarol were compared in 200 patients, suffering from acute myocardial infarction or severe coronary insufficiency. Warfarin was given orally to 164 patients, the prothrombin times being determined daily. Individual dosages were adjusted until the daily dose (usually 5–7·5 mg.) needed to maintain the prothrombin level between 15 and 30 per cent was found. Dicoumarol was given to 19 patients and dosages similarly adjusted until the maintenance dose (between 20 and 100 mg. daily) was found. An additional group of 17 patients, started on dicoumarol therapy, were later transferred to warfarin. Of all patients receiving warfarin, 87 per cent reached true therapeutic levels within 48 hours, and 83·5 per cent were within therapeutic range 70 per cent of the time or more. With dicoumarol, only 33·3 per cent of the patients were within therapeutic range for 70 per cent of the time. Warfarin was found easy to use, and prothrombin times were smoothly and easily controlled with small daily maintenance doses. There were no important side-effects. The authors conclude that warfarin is far superior to dicoumarol as an anticoagulant, and most closely approaches the "ideal anticoagulant." S. L. W.

BOOK REVIEW

METHODEN DER ORGANISCHEN CHEMIE (*Houben-Weyl*). Fourth Edition. Edited by Eugen Müller. Volume 1, Part I. Allgemeine Laboratoriumpraxis. Pp. xlii + 1048 (including Index) and 517 illustrations. Georg Thieme Verlag, Stuttgart, 1958. Moleskin, DM.198.00.

This book, as volume I of the sixteen which are to comprise this edition, provides an introduction to the series which is fundamental in its approach to the subject of methods in organic chemistry. The opening chapters on the science of materials used for the construction of laboratory apparatus provides useful information not always readily accessible elsewhere, on subjects such as the physical and chemical properties of glass, plastics and ceramics for special purposes, the structure and mechanical properties of metals, and electrolytic and non-electrolytic corrosion of metals. The information on the storage of chemicals is somewhat limited in its scope, and its inclusion seems to be of doubtful value. The same may also be said of the chapter dealing with apparatus supports. The remaining, and by far largest, section of the book is devoted to the discussion of theoretical and practical aspects of all the major handling techniques of the chemical laboratory. They range in subject matter from the simple and everyday techniques of filtration, centrifugation, crystallisation, drying and distillation to the most modern and complex as exemplified by countercurrent distribution and preparative electrophoresis. Particularly useful are small sections dealing with procedures for breaking emulsions and salting out, and the chapters on redox resins, inclusion compounds and the isolation of compounds by complex formation. An extensive and particularly well written chapter covers the theory and practice of extraction methods, with special reference to the techniques of countercurrent distribution and partition chromatography. A special feature of the latter section is a useful guide to the choice of appropriate solvent systems. As with all volumes of this series, information is clearly and concisely presented, and extensively referenced throughout.

J. B. STENLAKE.

LETTER TO THE EDITOR

The Paper Chromatographic Separation of Hyoscyamine and Norhyoscyamine

SIR,—Numerous combinations of paper and developing solvent have been proposed for the separation of tropane alkaloids by paper chromatography[1,2], but no system has been described so far for the separation of hyoscyamine and norhyoscyamine, which occur together in *Duboisia myoporoides*[3], *Duboisia leichhardtii*[4,5], and certain other species[6]. It has now been found that these alkaloids may be separated on Whatman's No. 1 paper which has been treated with an aqueous solution of oxalic acid and blotted between sheets of filter paper. Suitable developing solvents are water-saturated *n*-butanol and *iso*-butanol; the alkaloids are run as salts. After development the chromatograms are dried at room temperature for not less than four hours and the spots revealed by immersion in an aqueous tartaric acid solution of potassium iodobismuthate[7], or by spraying with solution of potassium iodoplatinate[8].

The best results were obtained with paper impregnated with M/40 oxalic acid solution. Representative R_F values by the descending technique for a number of tropane alkaloids are as follows.

	n-Butanol	*iso*Butanol
Oscine	0·11	0·10
Hyoscine	0·48	0·39
Meteloidine	0·57	0·52
Hyoscyamine	0·63	0·60
Valeroidine	0·69	0·68
Norhyoscyamine	0·70	0·69
Tigloidine	0·78	0·75
apo-Atropine	0·86	0·83

By this means it is possible to detect the presence of 5 per cent of norhyoscyamine in hyoscyamine.

The above combinations of paper and developing solvent also afford a separation of meteloidine from other alkaloids, a separation which has not been reported previously.

R. E. A. DREY.

Wellcome Chemical Works,
Dartford, Kent.
November 7, 1958.

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