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# A New Method for Preparing Flavin-adenine Dinucleotide

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Flavin-adenine dinucleotide (FAD) was first prepared by Warburg & Christian (1938*a*, *b*), and their method has since been used by many workers, some of whom recorded the purity of their products (e.g. Klein & Kohn, 1940; Hellerman, Lindsay & Bovarnick, 1946; Bessey, Lowry & Love, 1949; Schrecker & Kornberg, 1950); only Hellerman *et al.* (1946) claimed that they had prepared pure FAD. Burton (1951) prepared FAD by chromatography on alumina, and Sanadi & Littlefield (1951) by chromatography on Florisil, but in neither case was a pure product obtained.

This paper describes a convenient and simplified method of isolating FAD, making use of chromatography on powdered cellulose; by this method, products containing 90% FAD have been obtained. The absorption spectra of FAD and related compounds are discussed.

## EXPERIMENTAL

*Spectrophotometric measurements.* These were made in quartz cells, 1 cm. thickness, in a Beckman model DU spectrophotometer, which had been allotted the number B9 in a collaborative test (Gridgeman, 1951). The performance of the instrument was satisfactory, except at 313 m$\mu$. where $K_2Cr_2O_7$, the standard substance used in the test, has a trough in its spectrum; at 313 m$\mu$., B9 differed from the mean value in its estimate of the strength of solution C by 5%. The molecular extinction coefficients ($\epsilon$) of riboflavin (Table 2) were confirmed, using another Beckman (B13 of Gridgeman, 1951) and a Hilger-Watts Uvispek spectrophotometer (number unavailable).

For measuring the absorption spectra, readings were made every 2·5 m$\mu$. in the region of the maxima, and every 5 m$\mu$. elsewhere. The slit widths at 450, 375 and 260 m$\mu$. were 0·04, 0·1 and 1·3 mm., respectively; using the standard curve supplied by the Beckman manufacturers, these slit widths corresponded to band widths of 0·8, 0·9 and 3·4 m$\mu$., respectively. The wavelength scale of the instrument had recently been checked, and the results in the collaborative test were satisfactory (Gridgeman, 1951). Measurements were made in 0·1 M-phosphate buffer, pH 7·0, except where stated to the contrary.

The content of FAD was estimated by the light absorption at 450 m$\mu$., using $\epsilon = 11{\cdot}3 \times 10^3$ l. mole$^{-1}$ cm.$^{-1}$ (Warburg & Christian, 1938*b*; present work). Impurities which absorbed light at 450 m$\mu$. were detected by the addition of $Na_2S_2O_4$: the ratios in Table 1, stage *A*, have been corrected for the presence of 4% of an impurity which absorbed light at 450 m$\mu$.; this impurity was removed by the later stages of the purification.

*Preparation of FAD.* Baker's yeast (28 lb.; United Yeast Co., Cambridge) was extracted by the method of Warburg & Christian (1938*b*) as far as the precipitation of the crude Ag salt of FAD. The precipitate was suspended in 140 ml. water and dissolved by adding excess saturated KCl; a few drops of 2N-$HNO_3$ were added to precipitate AgCl, the

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Table 1. *Stages in the preparation of FAD*

| Stage* | Ratios of light absorption at different wavelengths: 375 mμ./450 mμ. | 305 mμ./450 mμ. | 275 mμ./450 mμ. | 266 mμ./450 mμ. | 260 mμ./450 mμ. | Yields from 28 lb. yeast (mg. FAD) | Purity on dry weight basis |
|---|---|---|---|---|---|---|---|
| *A* | 1·02 | 0·84 | 6·3 | 8·8 | 9·3 | 70 | 6% |
| $B_1$ | 0·82 | 0·16 | 3·4 | 5·7 | 5·8 | 8 | |
| $B_2$ | 0·82 | 0·15 | 2·6 | 3·75 | 3·7 | 10 | |
| $B_3$ | 0·82 | 0·13 | 2·5 | 3·55 | 3·45 | 30 | Not determined |
| $B_4$ | 0·82 | 0·13 | 2·75 | 4·05 | 3·95 | 8 | |
| $B_5$ | 0·82 | 0·19 | 3·2 | 5·0 | 5·1 | 3 | |
| *C* | 0·82 | 0·14 | 2·8 | 4·05 | 3·95 | 15 | |
| *D* | 0·82 | 0·14 | 2·45 | 3·35 | 3·25 | 28 | 90% |

* For the explanation of these different stages, see text.

suspension centrifuged, the precipitate washed with a little water, and the washings combined with the supernatant (Dixon, unpublished method). $(NH_4)_2SO_4$ was added (1 g./2 ml. solution) and the FAD extracted into 20 ml. molten *p*-cresol; this solution was washed and the FAD re-extracted into 40 ml. water as described by Warburg & Christian (1938*b*). The aqueous solution (Table 1, *A*) was shown by paper chromatography (Crammer, 1948; solvent: *n*-butanol:acetic acid:water (4:1:5, v/v)) to contain FAD as its main fluorescent component, together with a small amount of flavin mononucleotide (FMN) and three compounds which had a bright blue fluorescence in ultraviolet light. The purity was determined by estimating the content of FAD spectrophotometrically (1·75 mg. FAD/ml.) and by drying 0·5 ml. solution *A in vacuo*, first at room temperature over $P_2O_5$ and then at 100° (residue weighed 14·2 mg.); the purity was 6%.

Whatman ashless powdered cellulose (250 g.) was suspended in 1200 ml. of a solvent mixture composed of *n*-butanol (2 vol.), *n*-propanol (2 vol.) and water (1 vol.) and the slurry poured into a glass tube; only redistilled solvents were used. The aqueous solution of FAD (40 ml.) was slowly diluted with 80 ml. *n*-propanol and then with 80 ml. *n*-butanol and applied to the column at a rate of 15 ml./hr.; the FAD was adsorbed as a narrow band at the top of the column. It was essential to develop the column slowly (15 ml. solvent/hr.), and in complete darkness to avoid photodecomposition. The band of FAD reached the bottom of the column on the seventh day, and fractions were then collected hourly, dried at 35° under reduced pressure, dissolved in water, dried again, dissolved in water and a sample examined in the spectrophotometer; fractions with similar absorption characteristics were combined. The front and tail of the FAD band were contaminated with impurities which had $R_F$ values on paper chromatograms differing little from FAD ($R_F = 0{\cdot}01$ in this solvent), but paper chromatography showed that FAD was the only fluorescent compound in the band. The characteristics of the middle fractions are shown (Table 1, $B_1$–$B_5$). The solvent which was used to develop the column was recovered by distillation and was then suitable for re-use, but the column itself was discarded after all the FAD had been eluted.

The less pure fractions (20 mg.) having a ratio of light absorption at 260 mμ.:450 mμ. greater than 4·0 were dissolved in water (5 ml.) and chromatographed on a second column of powdered cellulose (400 g.) using water, saturated with *iso*amyl alcohol, as solvent. It is inadvisable to chromatograph these impure fractions a second time in the solvent *n*-butanol:*n*-propanol:water (2:2:1, v/v). If the impure fractions contain more than 20 mg. FAD, it is best to increase the size of the second column proportionately. The powdered cellulose (400 g.) was suspended in 2 l. solvent, made into a column, and the FAD chromatographed at a flow rate of 10 ml./hr. Fractions were collected, dried as before, dissolved in water and examined in the spectrophotometer; the main fraction (Table 1, *C*) was added to fractions $B_2$–$B_4$ (Table 1).

The solution of FAD was evaporated under reduced pressure at 35°, a current of air being made to impinge upon the surface; when the volume of the solution was about 2 ml., orange needles appeared and the evaporation was stopped. The flask was warmed to 60° for 1 min., cooled slowly and stored at 2° overnight. The solid was collected on a microfilter, washed quickly with 2 ml. ice-cold water, 4 ml. ethanol and 4 ml. ether and dried immediately by heating *in vacuo* over $P_2O_5$ at 100° for 1 hr. (Table 1, *D*).

*Determination of purity.* A sample of FAD (5 μg.) was chromatographed on paper, and no other fluorescent spot could be detected, using several different solvent systems (Whitby, 1950, 1952); this method would have detected 5 mμg. riboflavin or FMN, if these impurities had been present.

A sample was weighed (0·106 ± 0·001 mg.), dissolved in 10 ml. phosphate buffer and $\ln(I_0/I)$ at 450 mμ. found to be 0·137; taking $\epsilon = 11{\cdot}3 \times 10^3$, the purity was calculated to be 90%. Preparations with this degree of purity have been obtained three times by the method described, but attempts to remove the impurities by crystallization, or by precipitation from aqueous solution with organic solvents have been unsuccessful, and have usually resulted in partial breakdown to FMN; it is thought that the impurities are mainly inorganic.

Addition of $Na_2S_2O_4$ reduced the light absorption of FAD at 450 mμ. by 91%, compared with 95% for riboflavin; although this difference suggests the presence of an impurity which absorbs light at 450 mμ., this explanation is not favoured in view of the results of acid hydrolysis (see below).

The value of the apparent $K_m$ (Stadie & Zapp, 1943; Hellerman *et al.* 1946) for the recombination of the apoenzyme of D-amino-acid oxidase (Burton, 1951) with FAD was found to be $2{\cdot}3 \times 10^{-7}$ M; under similar conditions, Warburg & Christian (1938*b*) obtained $2{\cdot}5 \times 10^{-7}$ M and Singer & Kearney (1950) obtained $2{\cdot}0 \times 10^{-7}$ M.

*Yield.* 31 mg. of a bright-orange powder containing 90% of FAD was isolated from 28 lb. baker's yeast. This yield (1 mg. FAD/lb. yeast) is consistently obtained, and is almost identical with the yield of FAD obtained by

Warburg & Christian (1938*b*) from baker's yeast.* Further quantities of FAD can be prepared either by continuing the evaporation of the mother liquor from which preparation *D* (Table 1) was obtained, or by diluting the mother liquor slowly with 2 vol. ethanol and then with 2 vol. ether; these preparations are less pure, on a dry-weight basis, than preparation *D*, and differ from it in their absorption spectrum, but FAD is the only fluorescent compound in them.

## RESULTS

### *Molecular extinction coefficients ($\epsilon$) of flavins*

Warburg & Christian (1938*b*) reported that riboflavin and FAD both had $\epsilon = 11{\cdot}3 \times 10^3$ at 450 m$\mu$., but riboflavin has since been shown to have $\epsilon = 12{\cdot}2 \times 10^3$ at this wavelength (Singer & Kearney, 1950; Whitby, 1952). Since solutions of FAD are frequently estimated by their light absorption at 450 m$\mu$., the molecular extinction coefficients of FAD have been redetermined; this was done indirectly, by comparison with FMN, since 100 % pure FAD was not available.

*FMN.* A sample of FMN was kindly provided by Dr T. P. Singer; this was examined by paper chromatography and found to contain a small amount (approx. 1 %) of riboflavin. (Found: N, 11·2; P, 6·4. Calc. for $C_{17}H_{19}O_9N_4PNa_2$: N, 11·2; P, 6·2 %.) After drying *in vacuo* over $P_2O_5$ at 100° for 1 hr., a specimen was weighed, dissolved and the intensity of absorption measured; it gave $\epsilon = 12{\cdot}2 \times 10^3$ at 450 m$\mu$.

*FAD.* The value of $\epsilon$ at 450 m$\mu$. for FAD was determined by hydrolysing 1 ml. of an aqueous solution (150 $\mu$g./ml.) with 1 ml. 0·1 N-HCl at 100° for 1 hr.: Abraham (1939) used similar conditions to hydrolyse FAD to FMN; the completeness of hydrolysis in the present case was confirmed by paper chromatography. The solution was neutralized with 0·1 N-NaOH, 0·25 ml. 0·1 M-phosphate buffer (pH 7·0) added, and the volume made up to 5 ml. with water. A control solution was prepared in which 1 ml. FAD was acidified and immediately neutralized; this solution was not heated. Both adenosine-5′-phosphate (AMP) and FMN are stable under these conditions (Michelson & Todd, 1949; Abraham, 1939; Forrest & Todd, 1950).

The spectrum of hydrolysed FAD was compared with the sum of the individual spectra of pure samples of AMP and FMN (all at pH 7·0), and found to be almost identical; this showed that the preparation of FAD was uncontaminated by impurities which absorbed light in the region of the spectrum investigated, and enabled $\epsilon$ at 450 m$\mu$. for FAD to be calculated in terms of the known value of $\epsilon$ for FMN. Hydrolysis of FAD to FMN was found to increase the intensity of absorption at 450 m$\mu$. by 8 %; from this result, which was confirmed twice, $\epsilon$ for FAD at 450 m$\mu$. was calculated to be $11{\cdot}3 \times 10^3$. This experiment confirms the value obtained by Warburg & Christian (1938*b*).

The AMP used in this experiment was kindly provided by Dr D. M. Brown, to whom I am indebted for permission to quote the following analytical results. (Found: C, 34·6; H, 4·7; N, 20·1. Calc. for $C_{10}H_{14}O_7N_5P$: C, 34·6; H, 4·1; N, 20·2 %.)

### *Absorption spectra of flavins*

In Fig. 1, the spectrum of riboflavin (for criteria of purity see Whitby, 1952) is compared with the spectrum of FAD. Although the FAD is only 90 % pure, the comparison is valid because the FAD does not contain impurities which absorb light in this region of the spectrum. These curves differ from the spectra obtained by Warburg & Christian (1938*b*), where riboflavin and FAD have almost identical spectra between 390 and 450 m$\mu$.

The spectrum of FMN is very similar to the spectrum of riboflavinyl glucoside (Whitby, 1952), but both spectra differ slightly from the spectrum of riboflavin in the region 330–420 m$\mu$. (Fig. 1). In this region, the spectrum of FMN is consistently lower than riboflavin, the difference being very small at the extremes of the range, but gradually increasing to a difference of 3 % at 400 m$\mu$. and of 2 % at 375 m$\mu$. It is not thought that these differences are due to spectrophotometric errors, as they depend upon the ratios of readings obtained with the same instrument, and the ratios are consistently found to differ; nor are the differences peculiar to the particular instrument, as they have been confirmed with other spectrophotometers. Further, when a solution of FMN was hydrolysed with N-$H_2SO_4$ at 100° for 4 hr., cooled and neutralized to pH 7·0, the spectrum of the final solution was found to be identical with the spectrum of riboflavin; paper chromatography showed that riboflavin was the only flavin present in the final solution.

Table 2 compares the molecular extinction coefficients, obtained for flavins and AMP in the present work, with the values in the literature. The wavelengths chosen are the same as Warburg & Christian (1938*b*) selected, although riboflavin, FMN and riboflavinyl glucoside have maxima at

* Since this paper was received, Dimant, Sanadi & Huennekens (1952) published a method for preparing FAD of 30 % purity (5–6 mg. FAD/lb. pig liver or baker's yeast); further purification is described, giving FAD of 50–60 % purity, but is attended by relatively high losses and yields are not given.

The properties of the FAD prepared by Dimant *et al.* (1952) differ from those of the preparation described here, and from the FAD of Warburg & Christian (1938*b*), both in the absorption spectrum and in the D-amino-acid oxidase test. It is possible that the FAD of Dimant *et al.* (1952) still contained derivatives of adenine which would account for the differences.

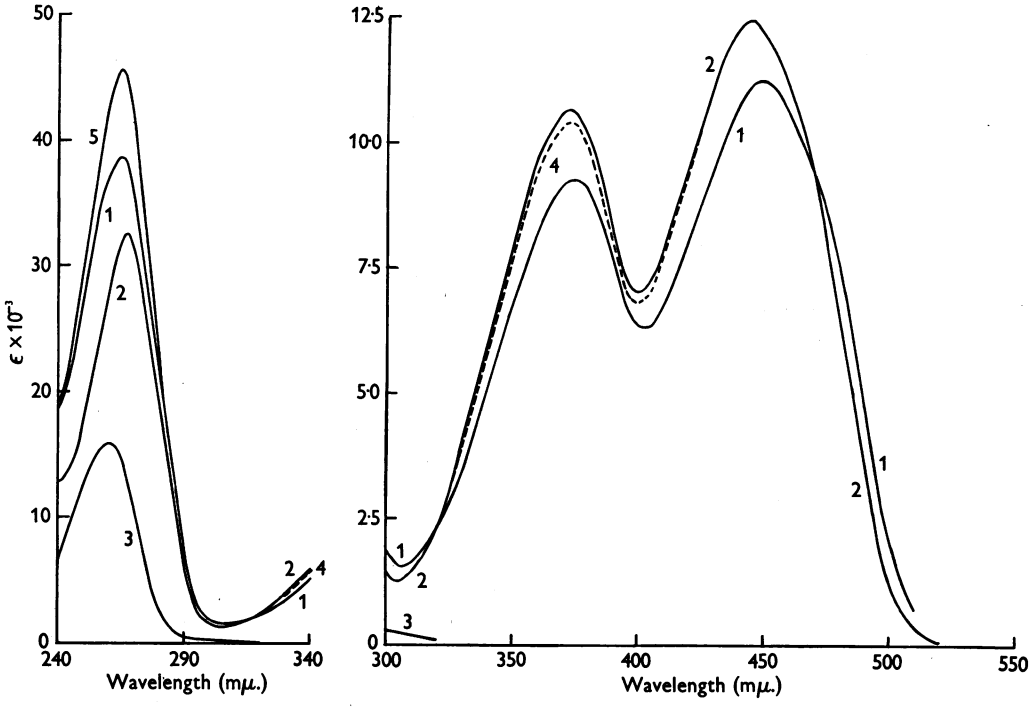


Fig. 1. Absorption spectra of flavins and of adenosine-5′-phosphate (AMP). 1, flavin-adenine dinucleotide (FAD); 2, riboflavin; 3, AMP; 4 (interrupted line), flavin mononucleotide (FMN) and riboflavinyl glucoside (above 420 mμ. this curve coincides with curve 2, and below 330 mμ. it is similar to curve 2); 5, theoretical spectrum of AMP + FMN between 240 and 280 mμ; between 290 and 510 mμ. the theoretical spectrum is the same as the spectrum of FMN. For frequency of experimental points, see text.

Table 2. *Molecular extinction coefficients of various riboflavin and adenine derivatives (measured in 0·1 M-phosphate buffer, pH 7·0, unless otherwise stated)*

| | $\epsilon$ (l. mole$^{-1}$ cm.$^{-1}$ × 10$^{-3}$) | | | | |
|---|---|---|---|---|---|
| | 510 mμ. | 450 mμ. | 375 mμ. | 260 mμ. | Reference |
| Riboflavin | 0·4 | 12·2 | 10·6 | 27·7 | Present work |
| Riboflavinyl glucoside | 0·4 | 12·2 | 10·4 | 27·4 | |
| Flavin mononucleotide (FMN) | 0·4 | 12·2 | 10·4 | 27·1 | |
| Adenosine-5′-phosphate (AMP) | — | — | — | 15·9 | Present work and Kalckar (1947) |
| Flavin-adenine dinucleotide (FAD) | 0·8 | 11·3 | 9·3 | 37·0 | Present work |
| Hydrolysed FAD | 0·4 | 12·2 | 10·4 | 42·2 | |
| FMN + AMP (calculated) | 0·4 | 12·2 | 10·4 | 43·0 | |
| FAD, pH 2·7 | 0·7 | 11·8 | 9·8 | 40·0 | |
| Riboflavin | 0·4 | 11·3 | 9·8 | 25·7 | Warburg & Christian (1938*b*) |
| FAD | 0·8 | 11·3 | 9·0 | 37·0 | |
| Riboflavin + adenosine (calculated) | 0·4 | 11·3 | 9·8 | 39·8 | |
| FAD | — | 11·3* | 9·4 | 41–42 | Singer & Kearney (1950) |

* From Warburg & Christian (1938*b*).

445, 373 and 266 mμ., and FAD at 450, 375 and 263 mμ. From Fig. 1 it will be seen that at pH 7·0 the spectrum of FAD is lower in the region of 260 mμ. than the theoretical curve obtained by adding the spectra of FMN and AMP; at pH 2·7 the spectra of FMN and AMP are closely similar to their spectra at pH 7·0, but the spectrum of FAD is altered and at pH 2·7 it more closely resembles the theoretical curve (Table 2).

## DISCUSSION

It is probably true to say that pure FAD has not yet been prepared. Warburg & Christian (1938*a*, *b*) obtained products which gave nearly correct

analytical values, but later workers have not been as successful with this method. Although Hellerman *et al.* (1946) believed that their FAD was pure, they determined its purity spectrographically by comparison with riboflavin, and it has now been shown that riboflavin and FAD do not have the same molecular extinction coefficient at 450 mμ.; the FAD used by Hellerman *et al.* (1946) therefore would not have been more than 92 % pure. Singer & Kearney (1950) suggested that the L-amino-acid oxidase of moccasin venom was a convenient source of small amounts of pure FAD; they did not obtain any absolute criteria of purity because of the small amounts of FAD prepared, but the absorption spectrum of their FAD shows that it contained impurities which absorbed light in the region of 260 mμ.

The present isolation procedure is, with one modification, the same as that of Warburg & Christian (1938*b*) in its initial stages. The later stages of the method of Warburg & Christian (1938*b*) involve fractionation of the barium salts of nucleotides, for which considerable skill is required; these stages have been replaced by a chromatographic procedure which requires little supervision, and which gives a product free from contamination by other compounds which absorb light in the region of the spectrum investigated. The advantages of this method are considered to outweigh the disadvantages that large volumes of organic solvents are required, with the accompanying danger of rapid photodecomposition.

Warburg & Christian (1938*b*) observed a difference between the molecular extinction coefficient of FAD at 260 mμ. and the sum of the molecular extinction coefficients of riboflavin and adenosine at this wavelength; the difference between the observed and calculated values is now shown to be greater than Warburg & Christian (1938*b*) believed (Table 2). After hydrolysis of FAD, the spectrum is almost identical with the sum of the individual spectra of FMN and AMP; it is suggested that the diminution of absorption, which occurs on combination of FMN with AMP to form FAD, is analogous to the quenching of fluorescence of aqueous solutions of FAD at pH 7·0 relative to riboflavin (Weber, 1950). This diminution of absorption is less at pH 2·7 (Table 2), and a similar dependence on pH was observed by Bessey *et al.* (1949) and Weber (1950) for the quenching of the fluorescence of FAD. Weber (1950) suggested that the quenching of fluorescence was due to the formation of an internal complex between the adenine and flavin components of FAD, and this internal complex may be the cause of the differences between the spectrum of FAD and the sum of the spectra of FMN and AMP. In the present work it has been found that the three maxima in the spectrum of FAD all show diminution in absorption relative to the sum of absorptions for FMN and AMP (Fig. 1), but the percentage diminution is largest in the region of 260 mμ. (Table 2). This may well be due to the fact that both the *iso*alloxazine and purine portions of FAD individually show an absorption maximum in this region.

The spectrum of riboflavinyl glucoside was shown to differ slightly from the spectrum of riboflavin (Whitby, 1952), and has now been found to resemble that of FMN. It is suggested that substitution of certain groups in the terminal position of the ribityl side chain of riboflavin causes a small, but significant, diminution of the spectrum in the 330–420 mμ. region, relative to the spectrum of riboflavin.

## SUMMARY

1. Chromatography on powdered cellulose has been used to prepare flavin-adenine dinucleotide (FAD) of 90 % purity, free from contamination by other flavins and from contamination by compounds which absorb light between 240 and 510 mμ.

2. The molecular extinction coefficients of FAD have been reinvestigated.

3. The possibility is discussed that the diminution in the absorption of FAD, at pH 7·0, relative to the combined absorption of its components (flavin mononucleotide and adenosine-5′-phosphate), is analogous to the quenching of fluorescence of FAD under similar conditions, and is due to the formation of an internal complex.

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# The Inhibition of Erythrocyte Cholinesterase by Tri-esters of Phosphoric Acid

## 3. THE NATURE OF THE INHIBITORY PROCESS

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The inhibition of cholinesterase by organophosphorus compounds has been called irreversible inhibition. However, it has become clear that *in vivo* there are differences in the rate of return of cholinesterase after tetraethyl pyrophosphate (TEPP), di*iso*propyl phosphonofluoridate (di*iso*propyl fluorophosphonate, DFP) (Dayrit, Manry & Seevens, 1948; Hobbiger, 1951) and after *NN'*-di*iso*propylphosphorodiamidic fluoride (bis*iso*propylaminofluorophosphine oxide; Isopestox) (Callaway, Davies & Risley, 1952). By experiments *in vitro* it has been demonstrated that the inhibition of cholinesterase by TEPP is slowly reversible (Hobbiger, 1951; Wilson, 1951). If the inhibitory process is a phosphorylation of the enzyme then the stability of the enzyme phosphate should be dependent upon the other groups attached to the phosphorus. On this basis the enzyme inhibited by dimethyl *p*-nitrophenyl phosphate should be more unstable than that inhibited by the diethyl analogue (E 600). In this paper this is confirmed and the process of reversal of inhibition after dimethyl *p*-nitrophenyl phosphate has been shown to be consistent with a reaction involving a dephosphorylation and not with a simple reversal of the inhibitory process. Burgen & Hobbiger (1951) have also shown that the inhibition of cholinesterase by *m*-(dimethylphosphate)*N*-trimethylanilinium methyl phosphate is reversible but attributed this to a failure of the inhibitor to phosphorylate the enzyme.

An examination has also been made of the effect of temperature on the rate of inhibition of cholinesterase by E 600. These results show that the inhibitory process has a high activation energy and is not a simple adsorption process. These observations and the work of others on the reactions of organophosphorus compounds are discussed and a mechanism is put forward which would explain both inhibition and reversal. This is similar to that postulated by Wilson, Bergmann & Nachmansohn (1950) for the hydrolysis of acetylcholine which is presumably the natural substrate for this enzyme.

## EXPERIMENTAL

### *Methods and Materials*

Cholinesterase has been determined as previously described in detail (Aldridge & Davison, 1952*a*). Normally the buffer contains 0·0357 M-$NaHCO_3$, 0·164 M-NaCl and 0·040 M-$MgCl_2$ but in some experiments the $MgCl_2$ has been omitted. Rabbit blood was usually obtained from an ether-anaesthetized rabbit by cannulation of the carotid artery and bleeding out. Citrate was the anti-coagulant and the blood was stored at 5°.

A preparation of A-esterase (Aldridge, 1953*a*), which will hydrolyse dialkyl *p*-nitrophenyl phosphate inhibitors but which is free from cholinesterase activity, may be prepared by adding 1 ml. of approx. $10^{-4}$ M-DFP to 10 ml. citrated rabbit plasma or serum. The solution is then incubated for 1 hr. at 37° and stored at +5°. The excess DFP is rapidly removed by rabbit serum (Mazur, 1946; also cf. Table 2) and the inhibition of the cholinesterase by DFP is not measurably reversible for many weeks under these conditions.

It should be noted that after inhibition of rabbit erythrocyte cholinesterase by dimethyl *p*-nitrophenyl phosphate the inhibition is stopped upon the addition of substrate and the enzyme activity is returning throughout its determination. Although in these circumstances the output of $CO_2$ is not linear with respect to time, the best straight line through these points (Aldridge, Berry & Davies, 1949) has been calculated and the slope used as a measure of the activity over the period of determination.