The Nitration of Benzoic and Phenylacetic Acids and Phenylalanine: the Chemical Basis of the Mohler and Kapeller-Adler Reactions

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While developing a method for the estimation of benzoic acid (Dickens & Pearson, 1951), it was found that the main colour-forming substance responsible for the Mohler (1890) test for benzoic acid was not 3:5-dinitrobenzoic acid, the main nitration product, but another compound formed in small quantity during the nitration as had been previously suggested on indirect evidence by Kapeller-Adler (1932). Quantitative estimation of the intensity of the red colour given on reduction in alkaline solution by the various dinitrobenzoic acids has shown, as reported in the present paper, that in contrast to the weak reaction of 3:5-dinitrobenzoic acid, 2:5-dinitrobenzoic acid gives more than one hundred times as strong a colour test when equimolar amounts of these two substances are directly reduced with hydroxylamine in ammoniacal solution by the method described by Grossfeld (1927). This observation called for a re-investigation of the basis of the Mohler test for benzoic acid, the result of which is reported in this paper.

The hot nitration of phenylalanine was thought by Kapeller-Adler (1932) to yield 3:4-dinitrobenzoic acid via its para nitration and oxidative removal of the side chain giving *p*-nitrobenzoic acid followed by the introduction of a second nitro group in the ortho-position to the first. The basis of this interpretation was the fact that the colour given on alkaline reduction of the nitrated phenylalanine was violet, like that of nitrated *p*-nitrobenzoic acid, and not cherry red as it is in the Mohler reaction for benzoic acid. Block & Bolling (1939), who studied the nitration of phenylalanine in some detail, and slightly modified Kapeller-Adler's quantitative method for the micro-estimation of this substance (see Block, 1938), confirmed that a violet colour was given by phenylalanine in contrast to the red colour given by benzoic acid. They also showed, in agreement with Meisenheimer (1903) and Meisenheimer & Patzig (1906), that a number of aromatic compounds containing two nitro groups in the ortho-position to one another also gave a violet, while those having two nitro groups in a para relationship gave a red colour test. The *m*-dinitro compounds gave no colour or only a very weak one. On this evidence they considered that phenylalanine and phenylacetic acid were nitrated to give first the *p*-nitro compounds

which were then further nitrated to the 3:4-dinitro compounds, whereas benzoic acid they suggested, as Kapeller-Adler (1932) had previously thought probable, might form 2:5-dinitrobenzoic acid which could be responsible for the cherry-red colour. They were unable, however, to detect 2:5-dinitrobenzoic acid as a nitration product of either benzoic acid or *m*-nitrobenzoic acid under the conditions employed in this test, but they stated that 2:5-dinitrobenzoic acid was formed by nitration of *o*-nitrobenzoic acid. All these statements are based on the differences of colour (red or violet) given by *p*- and *o*-dinitro substituents on reduction in alkaline solution, and we have thought it necessary to examine quantitatively the amounts of these various substances formed on nitration and where possible to check their formation by isolation.

Block & Bolling (1939) also produce evidence which they consider supports the view that they have isolated from the nitration of L-phenylalanine (A) 3:4-dinitrophenylalanine. $\frac{1}{2}\text{C}_9\text{H}_9\text{OH}$, m.p. 155°, decomp. 182°. They also describe the isolation after reduction of the nitration mixture by hydrogen sulphide of: (B) 'diaci-3:4-dinitrodihydrophenylalanine', m.p. 182–183°, and (C) a further impure substance which may have been '3:4-dinitrodihydrobenzoic acid', softening at 65–70°, decomp. 220–250°. (The nomenclature of these reduced dinitro-compounds is mentioned in the Discussion below.) The colours given with ammonia by B and C, and by A after reduction, were violet. The intensities were (A) 'over 160 %', (B) 140 % of the colour given by nitration of an equimolar amount of phenylalanine and subsequent treatment by hydroxylamine and ammonia. Substances A and B gave no ninhydrin reaction for the α -amino group. The evidence for the position of the nitro groups was again based solely on the colour reaction. The analyses for carbon, hydrogen and nitrogen do not support very convincingly the proposed constitutions of A and B, and no analysis of C is given.

Evidently these observations, useful as they are, leave a number of uncertainties, especially the actual amount of colour-forming substance produced, as well as its chemical nature. Some of these points have been studied by us in more detail and are described below.

EXPERIMENTAL

All m.p.'s are uncorrected.

The preparation of 2:5-dinitrobenzoic acid by hot nitration of o-nitrobenzoic acid. *o*-Nitrobenzoic acid (5 g.), potassium nitrate (10 g.) and conc. H_2SO_4 (100 ml., sp.gr. 1.84) were mixed in a flask, closed with a rubber bung and heated for 1.5 hr. at 100° . The cooled mixture was poured into 200 ml. water, and extracted for 16 hr. with ether. The mixed acids were shaken out from the ethereal extract with about 400 ml. ice-cold N-NaOH . The alkaline solution was acidified by the addition of conc. HNO_3 (5–10 ml.), extracted with ether, and the ether evaporated. The solid residue was dissolved in 40 ml. boiling water, and to the boiling solution a solution of 5 g. lead acetate in 10 ml. water was added. On cooling, a precipitate formed which did not dissolve on re-heating. It was filtered from the hot suspension, boiled with 50 ml. water, again filtered hot and washed on the filter with a further 50 ml. boiling water. The precipitate was dissolved in hot dil. HNO_3 and from the cooled solution the nitro compound separated. Yield 0.67 g. (10% of theoretical), m.p. $174\text{--}176^\circ$, mixed m.p. 176° with authentic 2:5-dinitrobenzoic acid (see below) of m.p. 177° . After a further crystallization from dil. HCl , it had m.p. 177° , alone or mixed with authentic material. (Found: C, 39.6; H, 2.1, N, 12.8. Calc. for $\text{C}_7\text{H}_4\text{O}_6\text{N}_2$: C, 39.6; H, 1.9; N, 13.2%.) Methyl ester, m.p. 94° , mixed m.p. with authentic specimen (see below) 94° . (A specimen of 2:4-dinitrobenzoic acid of m.p. 180° gave mixed m.p. 162° ; a specimen of 2:6-dinitrobenzoic acid of m.p. 200° gave mixed m.p. 168° with the above analysis specimen of m.p. 177° .)

The comparison specimens of 2:5-dinitrobenzoic acid, m.p. 177° , and its methyl ester, m.p. 94° , were prepared from a sample of 2:5-dinitrotoluene, kindly supplied by Dr H. H. Hodgson, by dichromate oxidation following the method described in *Organic Syntheses* (Gilman, 1932). (The other dinitrobenzoic acids used were prepared by similar methods.)

The basis of the Mohler reaction. The above evidence shows that under conditions closely similar to those used by Mohler (1890) and Waelsch & Klepetar (1935), for the hot nitration of benzoic acid in their respective colorimetric tests for benzoic acid, *o*-nitrobenzoic acid first formed would be further nitrated to give 2:5-dinitrobenzoic acid. Cold

nitration of benzoic acid yields some 20% *o*-nitrobenzoic acid mixed with nearly 80% *m*-nitrobenzoic acid; if the former yields 2:5-dinitrobenzoic acid in the hot solution, the 3:5-dinitrobenzoic acid, previously assumed to be the basis of these authors' colour reaction, will be contaminated with 2:5-dinitrobenzoic acid.

Measurements of colour intensity in the Waelsch & Klepetar test as performed on various nitrobenzoic acids is shown in Table 1. It is evident that 3:5-dinitrobenzoic acid—the main product in the hot nitration of benzoic acid—is a very weak chromogen in this test. The fact proved above, that *o*-nitrobenzoic acid is converted to 2:5-dinitrobenzoic acid under the conditions used by Mohler and by Waelsch & Klepetar, and the very high chromogenic value recorded for this substance in Table 1, make it very probable that the Waelsch & Klepetar reaction is really mainly due to the formation of 2:5-dinitrobenzoic acid. A yield of $100 \times 4.7/57$, or 8.2% of this compound from nitration of benzoic acid would be necessary if all the colour were due to it. However, part of the colour development is due to the other nitration products. 3:5-Dinitrobenzoic acid, although it represents about 80% of the nitration product under these conditions, would yield only about 7% of the total colour. On the other hand, the yield of 2:5-dinitrobenzoic acid actually isolated by us (10% of theoretical from *o*-nitrobenzoic acid) would account for 30% of the colour given by benzoic acid, and the isolation from the complex nitration mixture was far from quantitative. The above calculation is based on the formation of 20% of the *o*-nitro compound in the mononitro stage of the hot nitration, 10% of which appears finally as 2:5-dinitrobenzoic acid, or 2% of the amount of benzoic acid taken.

In view of the fact that the true chromogen in the Mohler and Waelsch & Klepetar tests is thus shown to be quantitatively a very minor product, formed to the extent of only a few per cent by hot nitration of benzoic acid, it seems unsuitable as the basis of a satisfactory quantitative reaction, as indeed this test has proved to be in our hands (see Dickens & Pearson, 1951).

Nitration of phenylalanine. DL-Phenylalanine (0.825 g.) was nitrated and subsequently reduced with H_2S as described by Block & Bolling (1939). The brown solid (0.74 g.) which separated from the concentrated butanol extract was

Table 1. Intensity of colour given in the Waelsch & Klepetar test by benzoic acid (24.4 mg. = 0.2 mmol.) and equivalent amounts of its nitro derivatives

(Benzoic, *o*-, *m*- and *p*-nitrobenzoic acids were nitrated hot, as in the method of Waelsch & Klepetar (1935), followed by reduction of the mixture with hydroxylamine in ammonia. The other compounds were directly reduced by hydroxylamine in ammonia. The optical density ($E_{1\text{ cm.}}$) of the solution (diluted as required) was measured in a 1 cm. cell with a green filter (transmission 5000–5400 Å.) and is calculated for the undiluted solution in each instance.)

Acid	$E_{1\text{ cm.}}$	Colour	Mols. of the acid required to give same colour ($E_{1\text{ cm.}}$) as 1 mol. benzoic acid
Benzoic	4.7	Red	1.00
<i>o</i> -Nitrobenzoic	19.6	Red	0.24
2:5-Dinitrobenzoic	57.0	Red	0.082
<i>m</i> -Nitrobenzoic	1.0	Red	4.7
3:5-Dinitrobenzoic	0.41	Red	11.5
2:3-Dinitrobenzoic	2.03	Violet	2.32
3:4-Dinitrobenzoic	30.9	Violet	0.152
<i>p</i> -Nitrobenzoic	6.6	Greenish → violet	—
2:4-Dinitrobenzoic	0.6	Olive	—
2:6-Dinitrobenzoic	0.2	Olive	—
Styphnic acid (2:4:6-trinitroresorcinol)	—	Bright yellow, no trace of red	—

recrystallized from hot water, m.p. 182–184° (decomp.). Found: (1) dried over CaCl_2 : C, 41.5; H, 4.0; N, 16.3%; (2) dried *in vacuo* at 60° over P_2O_5 : C, 41.9; H, 3.8; N, 16.2%. Dinitrophenylalanine ($\text{C}_9\text{H}_9\text{O}_6\text{N}_2$) requires: C, 42.4; H, 3.5; N, 16.5%. 'Dinitrodihydrophenylalanine' ($\text{C}_9\text{H}_{11}\text{O}_6\text{N}_2$) requires: C, 42.0; H, 4.3; N, 16.3%. Block & Bolling stated that the m.p. of their crop 2 oven-dried at 110° ('presumably diaci-3:4-dinitrodihydrophenylalanine') was 182–183° and found C, 43.6; H, 4.1; N, 16.2%. Unlike the Block & Bolling compound, neither the crude nor the recrystallized material gave a violet colour with ammonia either alone or in the presence of hydroxylamine.

H_2S was passed into neutral solutions of 3:4- and 2:5-dinitrobenzoic acids under the same conditions as used above, and the excess H_2S was blown off with N_2 . No colour was developed with NH_3 alone, but after reduction with hydroxylamine the 3:4-compound gave a violet, and the 2:5-compound a red colour with NH_3 . It therefore appears that no dihydro compound was formed by passing H_2S into the neutral solution of these dinitro compounds under the above conditions. There is no evidence other than their colour test to support Block & Bolling's statement that the nitration product, m.p. 182–183°, from phenylalanine is the 3:4-isomer. Presumably the greater part of the nitration product consists of other isomers which, since they give no colour test, may be the 3:5- and 2:4-dinitro derivatives.

Nitration of phenylacetic acid. Borsche (1909) pointed out that earlier workers had reported the m.p. of 2:4-dinitrophenylacetic acid as 160°, whereas by nitration of phenylacetic acid below 60° in mixed H_2SO_4 - HNO_3 pure 2:4-dinitrophenylacetic acid, m.p. 179–180° (decomp.), is readily obtained after recrystallization. This observation suggested the simultaneous formation of isomeric dinitro compounds, which we investigated by nitration of phenylacetic acid exactly as described by Borsche (1909). The crude solid which separated as pale-yellow crystals on pouring the crude nitration mixture into ice-cold water had m.p. 179–180° (decomp.), 73% yield. It gave on treatment with hydroxylamine and NH_3 a red colour which rapidly changed to violet, the intensity of the latter colour corresponding to an estimated yield of mixed 3:4- and 2:5-dinitrophenylacetic acids amounting to about 8% of the weight of phenylacetic acid taken, on the assumption that the colour intensities for these apparently unknown dinitro compounds are the same as those of the corresponding dinitrobenzoic acids (see below).

On recrystallizing the above crude product from hot water the m.p. of the pure 2:4-dinitrophenylacetic acid was unchanged (179–180°), but the colour test became progressively weaker with recrystallization and after the third recrystallization was negative, showing that the chromogenic component had been completely eliminated, although little material was lost. (Found: C, 42.6; H, 2.7; N, 12.2. Calc. for $\text{C}_9\text{H}_7\text{O}_6\text{N}_2$: C, 42.5; H, 2.7; N, 12.4%). This evidence shows also that with phenylacetic acid the colour-forming nitration products are present only as very minor components of the nitration mixture, and that the main product, 2:4-dinitrophenylacetic acid, when pure does not give this colour test.

The absorption curve of the reduced nitration products derived from phenylalanine and phenylacetic acid. The nitration of weighed amounts of phenylalanine was performed by heating for 20 min. at 100° with conc. H_2SO_4 containing 20% (w/v) KNO_3 , followed by reduction of the ice-cold diluted nitration mixture with hydroxylamine and NH_3 (Block, 1938).

Phenylacetic acid was nitrated at 100° as described by Waelsch & Klepetar (1935), followed by heating for 1 min. with hydroxylamine and NH_3 as in the Waelsch & Klepetar method for estimation of benzoic acid.

The light absorption of the coloured solutions thus obtained was determined in the Hilger Uvispek photoelectric spectrophotometer. The resulting curves are shown in Fig. 1, in which the log extinction coefficients are plotted per $\mu\text{g.mol.}$ of substance taken for nitration.

In Fig. 1 are also included absorption curves for the coloured solutions from 3:4- and 2:5-dinitrobenzoic acids, which were directly reduced in ammoniacal solution as in the Waelsch & Klepetar procedure; in these instances the extinction is given per $\mu\text{g.mol.}$ of the dinitro compound actually taken for reduction. From inspection of Fig. 1 it appears that the chromogenic substances arising from the nitration of both phenylalanine and phenylacetic acid are likely to be mixtures of the respective 3:4- and 2:5-dinitro compounds. In the case of phenylacetic acid the colour which develops is very unstable, changing in type from curve *E* (about 0.5 hr. after the reduction) to curve *D* (1.5 hr.); this is accompanied by an obvious shift of colour from red to violet. The other compounds also gave somewhat unstable colours of which the general characteristics are shown in curves *A*, *B* and *C* of Fig. 1.

Fig. 1. Absorption spectra of solution obtained on reduction by ammoniacal hydroxylamine of the following: curve *A*, 3:4-dinitrobenzoic acid; curve *B*, 2:5-dinitrobenzoic acid. Curve *C*, nitration and reduction of phenylalanine. Curves *D* and *E*, reduction of crude dinitrophenylacetic acid obtained from phenylacetic acid (see text); curve *E*=0.5 hr., *D*=1.5 hr. after reduction. All extinction coefficients are expressed on the basis of 1 g.mol. of the substance taken for nitration and/or reduction.

As has been mentioned above, the maximum absorption of the phenylalanine compound (at 540 $\text{m}\mu$) lies between those of 3:4- and 2:5-dinitrobenzoic acids (560 and 515 $\text{m}\mu$, respectively). If it be assumed that the corresponding dinitro derivatives obtained from phenylalanine and phenylacetic acid give the same molar extinctions as these two dinitrobenzoic acids, the proportion of mixed 3:4- and 2:5-dinitro derivatives arising on nitration are seen to be (Fig. 1) approximately 8 and 15%, respectively, for phenylacetic acid and phenylalanine.

DISCUSSION

This work has shown that complex mixtures of substances are formed in the hot nitration of benzoic acid and phenylalanine under conditions similar to those used for the quantitative estimation of these two substances by the modifications of the Mohler reaction devised by Waelsch & Klepetar (1935) and Kapeller-Adler (1932). These authors' colorimetric reactions depend largely on the formation of substances which are not the main nitration product.

In the case of benzoic acid this was previously suspected to be so. The hot nitration of benzoic acid yields primarily 3:5-dinitrobenzoic acid, which we find contributes less than 8 % of the final (red) colour reaction, obtained on reduction of the dinitro compound in alkaline solution. On the other hand, pure 2:5-dinitrobenzoic acid gives molecule for molecule about 140 times as intense a colour reaction as the 3:5-dinitro compound. We have shown by isolation of the pure compound from the nitration of *o*-nitrobenzoic acid that at least 10 % of 2:5-dinitrobenzoic acid is formed in this reaction, and the losses during this isolation must have been considerable. The formation during the direct nitration of benzoic acid of a 7 % yield of 2:5-dinitrobenzoic acid would be required if this substance accounts for all the colour developed. Since *o*-nitrobenzoic acid on nitration gives about one-third of the colour intensity given by 2:5-dinitrobenzoic acid in this test, it appears that a yield of the 2:5-derivative up to 30 % of theoretical might be possible in this step, although some of this colour may be due to other nitration products. These include 2:3-, 2:4- and 2:6-dinitrobenzoic acids, none of which is a strong colour-former, and also styphnic acid (Griess, 1874) which, however, gives no red colour at all (Table 1). Hence it appears likely that the Mohler reaction is probably mainly due to the formation of 2:5-dinitrobenzoic acid in a yield approximating to 7 % of the benzoic acid taken.

Nitration of phenylacetic acid according to our experiments also yields a mixture of nitro compounds, comprising at least 73 % 2:4-dinitrophenylacetic acid (by isolation), and a mixture, probably of the 2:5- and 3:4-dinitro derivatives, which are together equivalent to about 8 % of the total. The 2:5-compound is probably responsible for the initially formed red colour on reduction of the crude alkaline nitration product, which fairly rapidly passes into a violet colour presumably due to the 3:4-dinitro compound. The pure 2:4-dinitrophenylacetic acid, which is the main product, gives no colour reaction.

These results are of value in considering the behaviour of phenylalanine under the same conditions. As pointed out by Kapeller-Adler (1932) the violet reaction given by this substance is probably due to a 3:4-dinitro compound. This, however, does

not constitute a main product, for on attempting the isolation of the reduced dinitro compound prepared as described by Block & Bolling (1939) we obtained as the main crop a substance shown by analysis to be a dinitrophenylalanine which no longer gave this reaction, although it had the same melting point as that given by these authors for the so-called 'diacidihydrodinitro-3:4-phenylalanine'. By analogy with the behaviour of phenylacetic acid it seems probable that the main component may have been 2:4-dinitrophenylalanine.

The absorption spectrum of the crude nitration product from phenylalanine after reduction in ammonia solution resembles that of a mixture of 2:5- and 3:4-dinitrobenzoic acids after the same treatment. This suggests that the chromogenic dinitro compounds produced from phenylalanine have their nitro groups in the same positions. Their total amount is estimated at about 15 % of the total nitration product. Hence, for phenylalanine also, the test depends on the formation of a quantitatively minor product of nitration, and this cannot be regarded as an entirely satisfactory basis for such a micro-estimation.

The nature of the products formed by mild reduction of the *o*- and *p*-dinitrobenzene derivatives has been much studied (cf. Meisenheimer, 1903; Meisenheimer & Patzig, 1906), but their chemical structure remains uncertain. They yield respectively blue-violet and red salts with alkalis which may be formulated as quinonoid structures of the type I. The *m*-dinitro compounds could not form structures of this type, and give no appreciable colour with this test. Block & Bolling (1939) consider that the colourless pseudo-acids from which these coloured salts are derived may be represented as shown at II—the so-called 'diacidihydrodinitro' compound. However, even in the case of the alkali salts of the simpler dinitro aromatic compounds no really satisfactory structure can be considered to be established (Sidgwick, 1942), and we have therefore placed these names in quotation marks in the text.

SUMMARY

1. The mechanism of the Mohler colour reaction for benzoic acid, and the Kapeller-Adler reaction for phenylalanine, have been investigated by a study of the possible dinitro compounds formed and the colours which they give on reduction with hydroxylamine in alkaline solution.

2. In the case of benzoic acid, 2:5-dinitrobenzoic acid appears to be mainly responsible for the development of the red colour on reduction. The preparation of this substance by nitration of o-nitrobenzoic acid is described.

3. The colour formed with phenylacetic acid is considered to be due to a mixture of the 2:5- and 3:4-dinitro compounds.

4. The violet colour given by nitration, followed by alkaline reduction, of phenylalanine appears also to be due to a mixture of products, which probably include 2:5- and 3:4-dinitro derivatives of phenyl-

alanine. The main nitration product was shown by analysis to be a non-chromogenic dinitrophenyl-alanine.

5. All of the above chromogens are relatively minor products of nitration, and as such do not appear to provide a really satisfactory basis for quantitative estimation of these aromatic substances.

We wish to thank Dr H. H. Hodgson for his kindness in supplying samples of 2:5- and 2:3-dinitrotoluene and Dr O. L. Brady for samples of 2:6- and 3:4-dinitrotoluene, from which the corresponding dinitrobenzoic acids were prepared. All analyses were made by Drs Weiler and Strauss.

REFERENCES

- Block, R. J. (1938). *The Determination of the Amino-acids*, p. 75. Minneapolis: Burgess Publishing Co.
 Block, R. J. & Bolling, D. (1939). *J. biol. Chem.* **129**, 1.
 Borsche, W. (1909). *Ber. dtsh. chem. Ges.* **42**, 1313.
 Dickens, F. & Pearson, J. (1951). *Biochem. J.* **48**, 216.
 Gilman, H. (1932). *Organic Synth.* Collective Vol. 2, p. 385.
 Griess, P. (1874). *Ber. dtsh. chem. Ges.* **7**, 1225.
 Grossfeld, G. (1927). *Z. Untersuch. Nahr.- u. Genussm.* **53**, 467.
 Kapeller-Adler, R. (1932). *Biochem. Z.* **252**, 185.
 Meisenheimer, J. (1903). *Ber. dtsh. chem. Ges.* **36**, 4174.
 Meisenheimer, J. & Patzig, E. (1906). *Ber. dtsh. chem. Ges.* **39**, 2526.
 Mohler, M. E. (1890). *Bull. Soc. Chim., Paris*, **3**, 414.
 Sidgwick, N. V. (1942). (Revised by Taylor, T. W. J. & Baker, W.) *The Organic Chemistry of Nitrogen*. Oxford: Clarendon Press.
 Waelsch, H. & Klepetar, G. (1935). *Hoppe-Seyl. Z.* **236**, 92.

The Action of Sulphydryl Inhibitors upon *iso*Citric Dehydrogenase with Especial Reference to the Behaviour of Some Trivalent Arsenicals*

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The —SH nature of *isocitric* dehydrogenase is still in some doubt because Adler, Euler, Günther & Plass (1939) stated that the enzyme from pig heart was sensitive to iodoacetic acid, whereas Barron & Singer (1945) said that the enzyme from liver was not affected by —SH reagents. It seemed important to examine the effect of arsenical substances on this enzyme in relation to other arsenical studies in this laboratory (Peters, Sinclair & Thompson, 1946). This paper records some observations of the effect of several —SH and other inhibitors upon the enzymes prepared from pig heart and from kidney tissue. A new difference between the action of mono- or di-substituted arsenical compounds has been found, which is thought to be of theoretical interest. Observations upon this enzyme are also related to the recent work in this laboratory (Buffa, Lotspeich,

Peters & Wakelin, 1950), in which fluoroacetate was found to inhibit the tricarboxylic acid cycle at the citrate stage.

EXPERIMENTAL

*iso*Citric dehydrogenase. The preparation used in most experiments was a dialysed phosphate extract of acetone dried powder of pig heart. In making the acetone powder of pig heart certain modifications of previous methods (Straub, 1942; Ochoa & Weisz-Tabori, 1948) shortened the procedure and increased the enzymic activity of the resulting phosphate extract; the procedure was as follows. A pig heart, obtained as soon as possible after the death of the animal, was immediately freed of fat and gross connective tissue, and cut into pieces which were placed in ice for transport back to the laboratory. All further operations were carried out in the cold room. The pieces of chilled heart muscle were placed in a Waring blender with three times their volume of acetone which had been previously chilled to -10° . After blending for 30 sec. the pulp was pressed out through muslin. These procedures were repeated twice more. The resulting heart

* A preliminary account of these experiments was given to the XVIIIth International Congress of Physiology, Copenhagen, in August, 1950 (Lotspeich & Peters, 1950).