126. DETERMINATION OF FUMARATE AND MALATE IN ANIMAL TISSUES

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Previous workers [see Straub, 1936; Jacobsohn *et al.*, 1932] have determined *l*(−)-malate in animal tissues by the polarimetric method which is based on the high rotation of the uranyl and molybdenum compounds of *l*(−)-malate. Since fumarate forms a well-defined equilibrium with *l*(−)-malate in animal tissues its concentration can be calculated when the concentration of malate is known. The polarimetric method, however, gives only approximate results since the uranyl and molybdenum compounds of other hydroxy-acids occurring in tissues, such as lactate [see Weil-Malherbe, 1937], *iso*citrate [Martius, 1938], phosphoglycerate [Green *et al.* 1937] and α-hydroxyglutarate [Weil-Malherbe, 1937] also show high rotations. The method moreover requires relatively large quantities of malate, 10 mg. in 20 ml. giving a reading of 0·5° in a 2 dm. tube.

In this paper a new method for the determination of *l*(−)-malate and fumarate is described. Fumarate is reduced to succinate in the presence of zinc and phosphoric acid and the succinate formed is determined manometrically with succinic dehydrogenase. The concentration of malate is calculated from the equilibrium constant. This method is more specific than the polarimetric one and it can be applied to quantities of fumarate from 0·05 mg. upwards.

Reagents

1. Metaphosphoric acid, 5% aqueous solution.
2. Zinc filings, 20–30 mesh.
3. $CuSO_4, 5H_2O$, 20% aqueous solution.
4. 10 *M* phosphoric acid solution; 100 ml. phosphoric acid B.P., sp. gr. 1·75, are made up with water to 158 ml.
5. Reagents and apparatus used for the manometric determination of succinic acid [see Krebs, 1937].

Procedure

The following procedure was found to effect the complete conversion of fumarate into succinate.

The tissue suspension, or similar material, is deproteinized at 40° by the addition of 1/5th of its volume of 5% metaphosphoric acid and filtered. An aliquot of the filtrate is transferred to a Kutscher-Steudel extractor, or a measuring cylinder, and 0·5 g. zinc filings, 2·3 ml. phosphoric acid and 0·25 ml. $CuSO_4$ solution for 10 ml. aliquot are added. After 60 min., when the greater part of the Zn has been decomposed, succinic acid is extracted with ether (without further addition of acid) and determined as previously described [see Krebs, 1937; Elsden, 1938; Cohen, 1939].

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Recoveries from pure solutions are shown in Table 1. They are within ±5% of the added fumarate. Recoveries from tissue suspensions in which fumarate and malate were in enzymic equilibrium depend on the temperature (Table 2) and on other factors (Table 3). The temperature effect is somewhat smaller than that reported by Jacobsohn [1934], but of the same order of magnitude.

Table 1. *Recovery of fumarate from pure solutions*

Amount of fumarate added (ml. 0·01 *M*)	μl. O_2 absorbed in presence of succinic dehydrogenase	μl. O_2 calc.	Yield (%)
4	442	448	98·6
2	217	224	97·0
1	116	112	103·3
0·5	57·6	56	103·0
0·25	29·4	28	105·0
4	456	448	102·0
4	439	448	98·1
4	486	448	95·6

Table 2. *Recovery of fumarate and l(−)malate added to muscle extract containing fumarase*

10 ml. 0·01 *M* fumarate or *l*(−)malate equivalent to 1120 μl. O_2; 5 ml. 0·1 *M* phosphate buffer *p*H 7·4, 5 ml. muscle extract, made by extracting 1 g. pigeon breast muscle with 50 ml. H_2O; the solutions were kept at different temperatures until the equilibrium was established (30–90 min.).

Temp. ° C.	Substrate used	O_2 absorbed in fumarate determination μl.	Ratio fumarate or malate added / fumarate recovered: Observed	Ratio fumarate or malate added / fumarate recovered: Average	Ratio *l*(−)malate / fumarate: Average
50	Fumarate	302	3·71		
50	,,	309	3·62		
50	,,	306	3·67	3·65	2·65
50	*l*(−)Malate	312	3·59		
50	,,	296	3·68		
40	Fumarate	263	4·25		
40	,,	266	4·21		
40	,,	271	4·13		
40	,,	264	4·24	4·17	3·17*
40	*l*(−)Malate	274	4·09		
40	,,	274	4·09		
30	Fumarate	253	4·42		
30	,,	248	4·50		
30	,,	249	4·49	4·54	3·54
30	,,	245	4·56		
30	,,	237	4·72		
20	Fumarate	195	5·74		
20	,,	196·5	5·70		
20	,,	205	5·46	5·57	4·57
20	,,	196	5·71		
20	*l*(−)Malate	214	5·24		

* In an earlier paper the figure 3·8 was given. This value was obtained at a time when the yield of fumarate was incomplete (about 90%), owing to the use of an excess of acid during the reduction of fumarate.

The ratio $\frac{\text{fumarate or malate added}}{\text{fumarate recovered}}$ (column 5) is the factor by which the fumarate or malate found in the presence of fumarase is to be multiplied in order to obtain the sum of fumarate and malate present. The value of this figure is not appreciably

Table 3. *Effect of tungstate on the equilibrium between fumarate and* l(−)*malate*

Conditions as in Table 2, 40°.

Concentration of Na tungstate %	Fumarate added (in terms of μl. O_2)	Fumarate recovered (in terms of μl. O_2)	Ratio $\frac{\text{fumarate added}}{\text{fumarate recovered}}$
0	1120	269	4·17
0·5	1120	235	4·76
2·0	1120	111	10·1

affected by *p*H within 6·8 and 7·8, or by the ions of the tissue and the saline media.

Specificity. Malate, tartrate, oxaloacetate, aspartate, glutamate, citrate, aconitate do not react to form succinate under the above conditions. Maleic acid behaves like fumaric acid, but since it is not present in biological material, it does not need to be considered. If the solution contains succinate, it must be determined separately in an aliquot before the treatment with Zn and acid, and the value obtained must be deducted from the succinate found in the fumarate determination.

Notes

1. *Deproteinization.* Tungstic acid (Folin-Wu) is not suitable as a deproteinizing agent. It was found that addition of sodium tungstate to the tissue suspension rapidly shifts the equilibrium fumarate ⇌ malate in favour of malate (Table 3). This is due to the formation of sodium tungstimalate $(NaC_4H_4O_5)_2WO_2$ [see Gernez, 1890; Henderson *et al.*, 1899]. Deproteinizing agents which are ether-soluble, e.g. trichloroacetic acid, are also to be avoided, as they interfere with the determination of succinic acid.

When fumarase is present, deproteinization must be carried out under defined conditions, since the equilibrium between fumarate and malate depends on the temperature (see Table 2) and in general it will be advisable to deproteinize at the temperature of the experiment in which the fumarate was formed.

There seems to be no appreciable loss of fumarate by adsorption on the precipitated protein.

2. *Stability of metaphosphoric acid solution.* HPO_3 in aqueous solution is gradually converted into H_3PO_4. At 0° the conversion is complete in 150 days, at 14° in 30 days, at 31° in 5 days, at 61° in 4½ hr. and at 95° in less than 1 hr. [Sabatier, 1888]. It follows that the solution must be prepared without heating and that it can be stored for a short time in the cold.

3. *Zinc preparations.* The commercial zinc preparations vary greatly in their readiness to react with phosphoric acid. These differences are largely eliminated by the addition of $CuSO_4$.

4. *Concentration of acid.* The yields of succinate are incomplete when the acidity during the reduction is high, for instance, when an excess of sulphuric acid is used. H_3PO_4 was chosen because it is weaker than H_2SO_4. A large excess of phosphoric acid is to be avoided.

5. *Frothing during the reduction of fumarate.* If excessive frothing occurs during the evolution of H_2, a drop of capryl alcohol may be added to the suspension. The alcohol later passes into the ethereal extract and is removed by evaporation on the steam bath together with the ether.

6. *Ether.* The ether used must be free from peroxide. It is kept over sodium and distilled freshly before use.

7. *Extraction of succinic acid.* The main cause of low yields is incomplete extraction of succinic acid and special attention must therefore be paid to the efficiency of the extractor. We use continuous extractors of the Kutscher-Steudel type, of the dimensions shown in Fig. 1. Each unit is provided with ground glass joint No. A 29 and with a set of 3 internal funnels of varying diameter (6 mm., 13 mm. and 16 mm.). The wider funnels are used when the volume of the aqueous solution is relatively small. A wide funnel increases the height of the aqueous layer, and thereby the efficiency of the extraction. If the lower end of the funnel is clean-cut, the ether bubbles passing through the aqueous solution are very small and no advantage is afforded by the use of sintered glass in the funnel bottom.

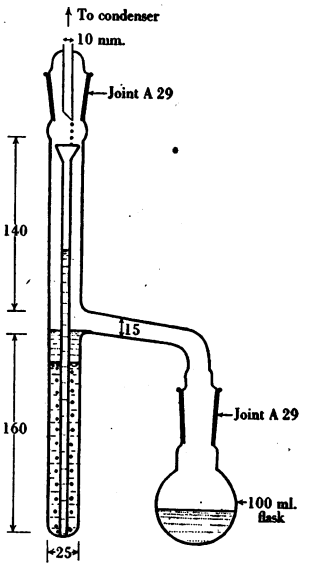

Fig. 1. *Extractor.* The dimensions are given in mm. and refer to internal diameters.

Since the rate of extraction depends on many factors, it is difficult to define standard conditions which ensure complete extraction. It is therefore advisable to test the extractor with known amounts of succinate. The following data may serve to give an approximate estimate of the rate of extraction. In an experiment where the volume of the aqueous phase was 50 ml. and the amount of ether passing through the solution about 16 ml. per min., 56 % of the succinic acid was extracted in 30 min., 73 % in 60 min., 96·5 % in 120 min., 99·3 % in 180 min. and 100 % in 240 min.

In the routine procedure the efficiency of the extraction can be tested by means of a dye of a suitable partition coefficient, the extraction of which can be easily followed with the eye. We have examined[1] a number of such "extraction indicators" and found that their rates of extraction fall in the following order:

cresol red > phenol red > rosolic acid > litmus.

Since the dye must not interfere with the later adjustment of *p*H of the extracted material, dyes were used which can also serve as *p*H indicators in the neutral range. Rosolic acid (1 ml. 0·1 % solution) was found to be the best indicator for the extraction of succinic acid. Its extraction is somewhat slower than that of succinic acid and the almost complete extraction of the indicator therefore ensures complete extraction of succinic acid.

When the extraction is complete, 1 ml. 0·1 *M* Na_2HPO_4 solution is added to the ether and the ether is evaporated on the steam bath. To remove the last traces of the ether, the flask is left on the bath until about 50 % of the aqueous solution has been evaporated. The residue is then transferred, with a 1 ml. pipette, to a 5 or 10 ml. measuring cylinder, neutralized to approximately *p*H 7·0 (rosolic acid or phenol red) and made up to a known volume (1–3 ml.) with the washings from the extraction flasks.

8. *Succinic dehydrogenase.* Succinic dehydrogenase is prepared by washing minced pigeon breast muscle or mammalian heart three times with water. For

[1] Preliminary experiments on the use of extraction indicators were carried out in this department by Dr P. P. Cohen.

storage, the washed muscle is frozen in the freezing box of the refrigerator; the preparation keeps for several weeks in the frozen form (P. P. Cohen, personal communication).

SUMMARY

A method for the determination of fumarate and *l*(−)malate is described. Fumarate is reduced to succinate in the presence of zinc and phosphoric acid and the succinate formed is determined manometrically with succinic dehydrogenase. The concentration of *l*(−)malate is calculated from the equilibrium constant. At *p*H 7·4 the ratio *l*(−)malate/fumarate was found to be 2·65 at 50°, 3·17 at 40°, 3·54 at 30° and 4·57 at 20°.

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