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Chimica biologica. — *A spectrophotometric method for fumaric acid estimation by Fürth and Herrmann reaction* (*). Nota di VINCENZO ZAPPIA, MARIE TIXIER (**) e FRANCESCO SALVATORE, presentata (***) dal Corrisp. F. CEDRANGOLO.

RIASSUNTO. — La reazione di Fürth e Herrmann è stata opportunamente modificata in modo da permettere il dosaggio dell'acido fumarico nei materiali biologici. Lo spettro di assorbimento del composto formato dall'acido fumarico in presenza di piridina ed anidride acetica presenta un massimo di assorbimento a 385 mμ. L'agente deproteinizzante più vantaggioso è risultato essere l'ac. tricloroacetico. Nei materiali biologici l'ac. ossalacetico è il composto che presenta la maggiore possibilità di interferire con la reazione; l'interferenza può essere valutata dall'analisi del rapporto delle densità ottiche a due lunghezze d'onda, 385 e 315 mμ.

The estimation of fumarate in biological materials is frequently required during the study of various enzymic reactions; the diacid plays, indeed, a primary role in cell metabolism. A number of methods for fumarate estimation is reported in the literature, the most used being: (i) the enzymatic method [1], (ii) the spectrophotometric method of Racker [2], (iii) chromatographic and electrophoretic methods of separation followed by titration or colorimetry [3–7]. The first one, based on manometric measurement of carbon dioxide formed by the action of malic enzyme, is laborious and time-consuming, and does not allow the use of inhibitors (e.g. malonate, etc.) of the enzymes that are utilised in fumarate estimation. Racker's [2] method, based upon absorption of fumarate at 240 mμ, can be used only in purified preparations, because of interference by proteins, nucleic acids and other u.v.-absorbing substances.

In 1935, Fürth and Herrmann [8] described a reaction based upon the formation of a color complex of either tartaric, citric or cis-aconitic acid in the presence of pyridine and acetic anhydride. The reaction has been recently used for the determination of itaconic and fumaric acids in pure solution [9], and has been developed for citric acid estimation in biological materials [10].

In this paper are presented results that show the advantages of some modifications of this reaction for the rapid quantitative estimation of fumarate in biological systems.

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MATERIALS.

All chemicals utilised were pure compounds for analysis. Tissue homogenates were performed in a Potter-Elvehjem apparatus (equipped with a teflon pestle). The spectrophotometric analyses were carried out with a recording spectrophotometer " Optica CF–4 ". Automatic pipets for delivering pyridine and acetic anhydride are necessary.

RESULTS.

Absorption spectrum and standard curve.—The absorption spectrum of the compound formed from the reaction of fumaric acid in presence of pyridine and acetic anhydride has been performed in the following assay: 1 ml aqueous solution containing 10 μmoles of fumarate, 1.3 ml of pyridine, and 5.7 ml of acetic anhydride. The test-tubes were shaken after the addition of each reagent and placed in a thermostatic bath at 32° C. After 30 min the spectrophotometric readings in 1 cm cell were taken and a spectrum was recorded ranging from 308 to 418 mμ (see fig. 1). The maximum of the absorbance

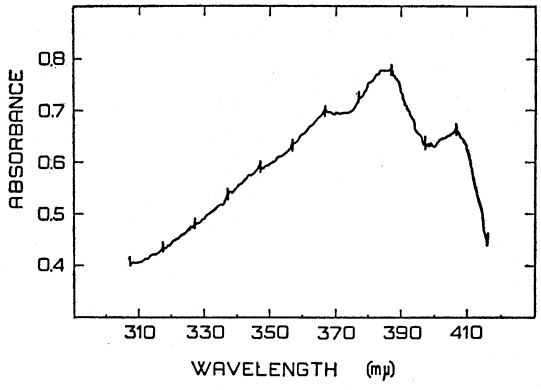

Fig. 1. – Absorption spectrum of the compound formed from fumarate after pyridine–acetic anhydride reaction (for experimental conditions see text).

was observed at 385 mμ, while a second peak was recorded at 408 mμ, and a shoulder at 368 mμ. A standard curve (fig. 2) is plotted at 385 mμ with different amounts of fumarate ranging from 0.5 to 12 μmoles; the curve follows Beer's law in the range used.

Deproteinization of the biological samples and modifications of Fürth and Herrmann reaction.—The problems of the deproteinization arises for the application of the method to biological samples. The deproteinizing agent was selected by the evaluation of its interference with Fürth and Herrmann

reaction and by evaluation of the effective degree of deproteinization. Table I shows data of experiments with different deproteinizing agents. The phosphotungstic acid, and to a lesser extent the acetic acid, gave some turbidity after

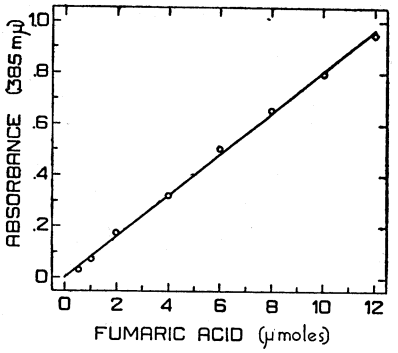

Fig. 2. – Standard curve for estimation of fumaric acid in the range 0.5–12 μmoles.

1 ml of sample containing fumaric acid was mixed with 1.3 ml of pyridine and 5.7 ml of acetic anhydride. Reaction time: 30 min at 32° C.

deproteinization, and therefore could not be utilised. The trichloracetic acid (TCA) as well as the perchloric acid (PCA), however, did not give any interference to the Fürth and Herrmann reaction.

TABLE I.

Influence of various deproteinizing agents on Fürth and Herrmann reaction.

The assay was prepared as follows: 1 ml of fumarate solution containing the deproteinizing agent in concentrations indicated below; the addition of 1.3 ml of pyridine and 5.7 ml of acetic anhydride for colour development was followed by the spectrophotometric readings 30 min thereafter.

ADDITIONS	Absorbance at 385 mμ
Fumarate (2 μmoles)	0.185
» » + TCA (a)	0.180
» » + PCA (b)	0.172
» » + acetic ac. (c)	0.205 (d)
» » + PTA (e)	0.340 (d)

(a) trichloroacetic acid (20% final concentration).
(b) perchloric acid (1.5 M final concentration).
(c) 25% final concentration.
(d) after the addition of the deproteinizing agent, a certain turbidity, causing higher extinction values, appeared.
(e) phosphotungstic acid (5% final concentration).

The perchloric acid used in the presence of tissue homogenate (chicken, fish and rat liver) allows only for a very low recovery of added fumarate; and furthermore, high amounts of interfering substances remained in the supernate after the deproteinization. With trichloroacetic acid in final concentration of 3% the first, but not the second, disadvantage was present.

TABLE II.

Recovery of added fumarate in the presence of varying concentrations of TCA.

10 ml of liver homogenate (30 % in phosphate buffer, 0.1 M, pH 7.4) were deproteinized with 10 ml of 6% or 4% TCA. After centrifugation at 9,000 × g for 15 min, 0.1 ml of fumarate were added to 0.9 ml of supernate (water was used in experiments of column 2). For the reaction mixture see fig. 2.

Added fumarate (μmoles)	E_{385} (no tissue present)	% RECOVERY OF ADDED FUMARATE			
		chicken liver		rat liver	
		3% TCA	2% TCA (a)	3% TCA	2% TCA(a)
1	0.073	38.3	36.9	34.2	37.2
2	0.150	44.3	51.8	40.5	49.5
3	0.240	43.5	62.0	43.0	47.2
4	0.330	52.4	76.8	49.8	54.0
5	0.420	55.4	77.4	51.2	54.5

(a) This TCA concentration gave rise to a resuspension of some precipitate a few minutes after the addition of the reagents.

In Table II data are given showing the recovery of added fumarate in the presence of different concentrations of trichloroacetic acid. The recovery obtained was between 30% and 80%: a greater recovery was always obtained with 2% TCA, but this concentration did not allow for a good deproteinization. Results of Table II would indicate a competitive action between TCA and acetic anhydride, in the reaction with pyridine. Therefore, the amounts of pyridine were increased while decreasing the acetic anhydride as indicated in fig. 3. The data shown in this figure suggest that, by increasing the pyridine up to 3 ml in each sample, the recovery of added fumarate was always greater; further increase in the amount of pyridine gave a lower recovery. Further evidence of the competitive effect between TCA and acetic anhydride or fumarate was given by experiments given as data in fig. 4: in fact, higher TCA concentration gave lower fumarate recovery.

The following procedure is suggested after the modifications discussed in the paragraph. 1.5 ml of the mixture containing the homogenate were

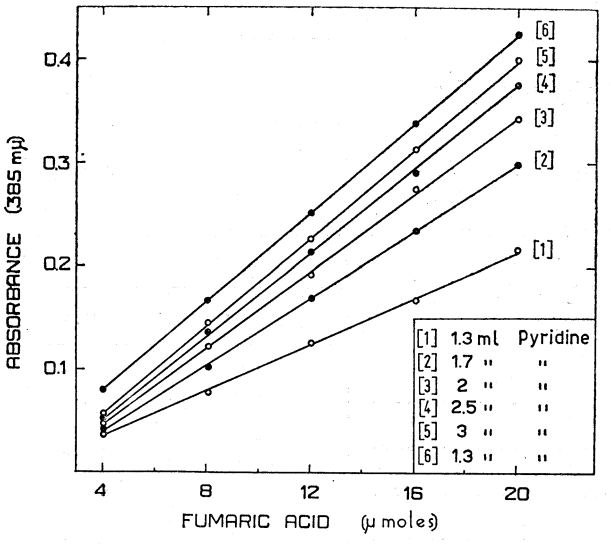

Fig. 3. – Correlation of fumarate concentration and optical density at 385 mμ in the presence of different amounts of pyridine.

Each sample was prepared as follows: 1 ml of homogenate (chicken liver, 30% in phosphate buffer, 0.1 M, pH 7.4), 1 ml of fumarate solution containing the amounts indicated, 2 ml of 6% TCA. Centrifugation was at 9,000×g for 15 min. To 1 ml of supernate were added varying amounts of pyridine as indicated, and then acetic anhydride to give a total volume of 8 ml. In curve (6) water was substituted for the homogenate.

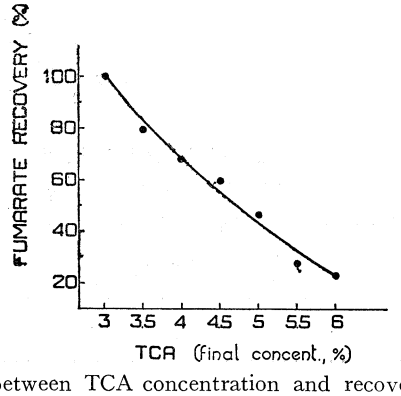

Fig. 4. – Relation between TCA concentration and recovery of fumaric acid added to tissue homogenate (100% has been assigned arbitrarily to the sample containing TCA in final concentration of 3%).

Each sample contained: 1 ml 30% homogenate (chicken liver) in phosphate buffer 0.1 M, pH 7.4, 1 ml of fumarate (20 μmoles), 2 ml of TCA to meet the final concentration indicated. After centrifugation (see fig. 3) 1 ml of supernate was mixed with 3 ml of pyridine and 4 ml of acetic anhydride.

added to 0.3 ml of TCA 20%. After appropriate centrifugation 1 ml of the supernate was added to 3 ml of pyridine and 4 ml of acetic anhydride. Incubation for 30 min at 32°C and spectrophotometric readings at 385 mμ were then performed. In the experiments for the standard curve the same conditions should be provided, using varying amounts of fumarate in the volume of 1.5 ml containing the homogenate, the addition of TCA being done at zero time.

TABLE III.

Absorbance at 385 mμ *of various organic acids after Fürth and Herrmann reaction.*

The assays were prepared as follows: 1 ml of organic acid was mixed with 3 ml of pyridine and 4 ml of acetic anhydride.

COMPOUNDS	E_{385}		
	5 μmoles	10 μmoles	25 μmoles
fumaric ac.	0.395	0.780	1.650
malic ac.	0.037	0.058	0.167
oxalacetic ac.	0.135	0.280	0.785
succinic ac.	0.002	0.005	0.013
oxoglutaric ac.	0.015	0.030	0.084
citric ac.	1.550	> 2.000	> 2.000
pyruvic ac.	0.002	0.004	0.010

TABLE IV.

Ratios E_{385}/E_{315} *in the presence of varying quantities of fumaric and oxalacetic acids.*

The assays contained 1 ml of oxalacetic and fumaric acids in the indicated molecular ratio (0 to 10 μmoles of each acid).

MOLECULAR RATIO OF FUMARIC TO OXALACETIC ACID	E_{385}/E_{315}
100 : 0	2.02
80 : 20	0.72
60 : 40	0.48
40 : 60	0.32
20 : 80	0.23
0 : 100	0.14

Specificity.—The problem of specificity is quite important when working on biological materials considering the peculiar metabolic position of fumarate in Krebs cycle, as well as its structural relationships with other components

of the cycle itself. Table III shows extinction values at 385 mμ of various organic acids in different concentrations. Succinate, α-oxoglutarate, pyruvate and malate did not give any appreciable extinction values in these conditions, or at the most very low ones (e.g. malate). The citric acid gave high interference, and its presence in a certain amount prevents the use of this method for fumarate estimation. The oxalacetate also gives a certain degree of interference, but this can be eliminated by the use of the values of a ratio of extinction readings at 385 and 315 mμ. The maximum of the absorption when oxalacetate was substituted for fumarate is, in fact, at the wavelength of 315 mμ. Table IV gives the values of this ratio when different amounts of the two acids are present. A correction is easily made by taking readings at 315 mμ besides those at 385 mμ.

CONCLUSIONS.

The modifications of the Fürth and Herrmann reaction, by increasing the amount of pyridine used, allow for the use of an acid deproteinizing agent, like trichloroacetic acid. This was used in the presence of tissue homogenate and in these conditions the recovery of added fumarate was very satisfactory. The maximum of the absorption spectrum was found to be at 385 mμ, and at this value the spectrophotometric readings were taken. It is suggested that experiments for the standard curve should always be performed in the presence of the tissue deproteinized at zero time. This allows for the exact evaluation of fumarate formed. A ratio of extinction values at 385 and 315 mμ is proposed for checking the presence of oxaloacetate, and for making appropriate correction. The application of this method to follow enzymatic reactions (e.g. bacterial aspartase) will be described in detail in a later paper.

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