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The Titration Curve of Gelatin

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Titration curves have played a considerable part in both the determination of the amino acid composition of proteins and in studying their behaviour as acids and bases (Cohn & Edsall, 1943). A serious discrepancy was known to exist for many years between the analytically determined dicarboxylic acid content of gelatin (or collagen) and the values derived from titration. The discrepancy persisted even after it became clear that some of the dicarboxylic acid residues were present, in collagen and in certain types of gelatin, as amides. Improved determinations of the dicarboxylic acids showed that there had been errors in previous analyses. There remained, however, a discrepancy (Ames, 1952) in the content of histidine, the titration curve giving too high a value. In spite of recent improvements of methods of amino acid analysis, titration remains a valuable tool for studying the peculiarities of dissociating groups, especially when these are chemically labile; for example, where ionizing groups are blocked or modified by treatment with reagents, or where carboxylic groups are present in part as amides.

The present paper describes the experimental method and its application to two types of gelatin: this has enabled four of the five most important reactive groups to be estimated, namely carboxyl, imidazole, α-amino, guanidino.

EXPERIMENTAL

Materials. Alkali-processed ox-hide gelatin (1), isoionic point pI = 4·92. The gelatin was prepared commercially from ox hide, which had been soaked for several months in a saturated solution of $Ca(OH)_2$ at about 15°. After neutralization, gelatin was extracted from the hide using warm water (*ca.* 60°).

Acid-processed pig skin gelatin (2), pI = 9·1. The gelatin was prepared commercially from pig skin by extraction at pH 3·5–4·0, with warm water. No alkaline pre-treatment was used.

De-ionization. 4–6% (w/v) solutions of each of the two gelatins were de-ionized by passage through a mixed bed of Amberlite resins IR–120 and IRA–400 (Rohm and Haas Co., Philadelphia 5, Pa.) as described by Janus, Kenchington & Ward (1951). The pH of the solution leaving the resin bed gave the pI of the gelatin. The de-ionized solutions were set, dried and ground to give a coarse powder. The pH of the solution of the acid-processed gelatin was brought, after de-ionization, from 9·1 to *ca.* 5·0 by the addition of HCl, before drying and grinding. The ash content of the gelatins was reduced to less than 0·1% (w/w) from 2·5% for gelatin (1) and 0·5% for gelatin (2).

Preparation of solutions for titration. A weighed quantity of the de-ionized gelatin, the water content of which had been determined by drying for 18 hr. at 105° in the form of a thin film, was allowed to swell and then dissolved by gentle warming to 40°. Concentrations of 4·5% (w/v) were used. The concentration was checked by drying a weighed quantity of solution at 105° for 18 hr.

pH *standardization.* All pH measurements were made with Doran 'Alkacid' glass electrodes (L.S.B. Components Ltd., Stroud, Glos.) with a modified Cambridge pH meter (Kenchington & Ward, 1954), which enabled several electrodes to be used in succession, without re-standardization, for the duration of a titration-curve determination. The following solutions were used for standardization, all pH values being for 40°. (I) 0·1 N-HCl, pH 1·08 (Hitchcock, 1931); (II) 0·05 N potassium hydrogen phthalate, pH 4·03 (Bates, 1948); (III) 0·1 N-KOH, pH 12·38.

The value for 0·1 N-KOH is the mean of measurements obtained in this laboratory.

Titration apparatus. Direct titration of gelatin solutions has been used, rather than the method of Hitchcock (1931) and Ames (1952), which involved addition of quantities of acid or alkali to a series of gelatin solutions. For each gelatin, the titrations with 0·2N-HCl and with 0·2N-KOH, were carried on side by side in two vessels (Fig. 1) at $40 \pm 0{\cdot}1°$, and were preceded by corresponding blank titrations of distilled water.

Each titration vessel carried two 'Alkacid' electrodes, (*A*), which were connected through a four-position selector switch to the pH meter. The saturated calomel electrodes (*B*) were connected in parallel to the same meter. Readings were taken with all four electrodes throughout the experiments; this prevented failure of one electrode from spoiling an experiment, or more usually, enabled the electrodes giving the more reproducible values, for the pH-standardization solutions, to be selected in calculating the results.

A stream of N_2 was passed through (*D*) to keep the solutions free of CO_2. The same tube was used in conjunction with the two-way tap (*E*) for withdrawing solutions or distilled water. The burette jet (*C*) was an extension fitted to a standard 10 ml. automatic burette by rubber tubing. A funnel replaced the burette when adding standardizing solutions or distilled water.

Titration procedure

Electrode standardization. The four electrodes were standardized using solution II (pH 4·03) and they were then checked by measuring the pH of the following cycle of solutions I, II, III, II, I. If any electrode showed irregular behaviour, it was removed and replaced by another electrode. After completing the titrations, the electrodes were finally checked with solution II and either I or III. The value obtained, if within 0·02 pH unit of the original value, was regarded as indicating correct functioning of an electrode throughout the titration.

Blank titration. 25 ml. of distilled water were added to each titration vessel. The stream of N_2 removed dissolved CO_2 in 10–15 min., before the commencement of the titration, raising the pH to $7{\cdot}0 \pm 0{\cdot}3$. Additions of 0·2N-HCl were then made to one titration vessel, and of 0·2N-NaOH to the second. The amounts were such as to make the total added follow the steps 1, 2, 3, 5, 10 and 25 ml. After each addition, 1 min. was allowed for stirring, and the pH was then measured. The last addition brought the concentration of the reagent in each vessel to 0·1N, and the pH was compared with that obtained during standardizing with solutions I and III. pH discrepancies of $\pm 0{\cdot}02$ with the acid, and $\pm 0{\cdot}05$ with the alkali were the maximum permitted divergences.

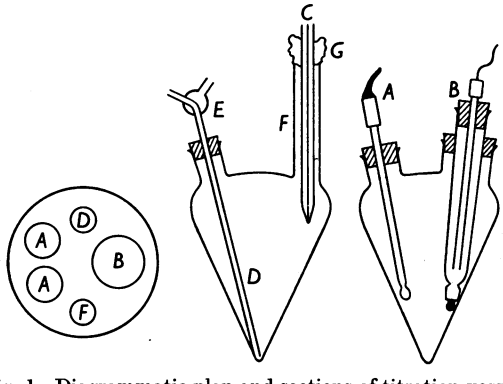

Fig. 1. Diagrammatic plan and sections of titration vessel. *A*, Doran 'Alkacid' glass electrode; *B*, Cambridge saturated calomel electrode; *C*, capillary tube extension to burette jet, surrounded by *G*, a loose plug of cotton wool; *D*, delivery tube; *E*, two-way tap; *F*, neck of vessel used for clamping.

Gelatin titration. The gelatin titrations were then performed using 25 ml. of the gelatin solution in each vessel. The volumes of acid or alkali added were regulated to produce increments of the order of 0·2–0·4 in the pH of the solutions. The limits of titration were set at pH's 1·5 and 12, since beyond these points the differences in pH between the solutions with and without gelatin were so small that the errors in the measurement of pH rendered the results inaccurate. Occasional difficulty with frothing caused by the N_2 stirring was overcome by the addition of minute amounts of *sec.*-octanol.

Back titration. After completion of the two gelatin titrations, the contents of each flask were back-titrated by addition of a volume of the complementary reagent equal to the volume of the titrating reagent already added. In the absence of gelatin decomposition, the pH values of the resulting solutions were within $\pm 0{\cdot}02$ pH unit of the original values. An added indication of decomposition during titration is given by drift of pH values under the extreme acid or alkaline conditions. No drift was observed with the gelatins used.

Calculation of results

The method of calculation of the combined hydrogen ions as the pH is lowered, and of hydroxyl ions to neutralize the hydrogen ions released by the protein when the pH is raised, is essentially that given by Cohn & Edsall (1943) where earlier work is referred to in detail. The assumption is made that the relation between pH and the concentrations of hydrogen or hydroxyl ions for the same total concentration of acid or alkali is not affected by the presence of protein. In a solution of HCl of C_1 equivalents/l., the hydrogen ion concentration $[H^+]$ will be C_1 moles/l. Then it is assumed that

$$\mathrm{pH} = -\log \alpha\,[H^+], \tag{1}$$

where α is a function of C_1 only (T, P constant throughout).

$$\mathrm{pH}_1 = -\log \alpha C_1. \tag{2}$$

In the presence of protein in this same solution, $[H^+]$ becomes C_2, $C_1 - C_2$ moles of H^+ having combined with protein. In these conditions it is assumed that

$$\mathrm{pH}_2 = -\log \alpha C_2. \tag{3}$$

From (2) and (3)

$$\log C_2/C_1 = \mathrm{pH}_1 - \mathrm{pH}_2. \tag{4}$$

Similarly, for (OH') ions,

$$\log C_2/C_1 = \mathrm{pH}_2 - \mathrm{pH}_1. \tag{5}$$

The particular application of this treatment to the data of the present method is given below.

First, from the blank titration on water alone, $\log [H^+]$ and $\log [OH']$ are plotted against pH, giving straight lines. From these graphs are read off the values of pH which would be obtained, if the quantities of reagents added to the gelatin solutions were added to the same volume of water.

The values for combined ions are obtained from equations (6) which are derived from equations (4) and (5), the units being m-moles/g. gelatin.

$$\left.\begin{aligned}&\text{Acid titration:}\\&\text{combined } H^+ = \frac{v}{5g}\{1-\text{antilog}\,(pH_w - pH_g)\};\\&\text{Alkali titration:}\\&\text{combined } OH' = \frac{v}{5g}\{1-\text{antilog}\,(pH_g - pH_w)\};\end{aligned}\right\} \quad (6)$$

where v is the volume of 0·2N reagent added to 25 ml. solution; g is the weight in g. of anhydrous ash-free gelatin in the original 25 ml. of solution; pH_g is the pH of the 25 ml. of solution after the addition of v ml. of reagent; pH_w is the pH of 25 ml. of water after addition of v ml. of reagent. The combined hydrogen and hydroxyl ions are then plotted as positive and negative ordinates, using pH values as abscissae.

RESULTS AND INTERPRETATION

The resultant curve for gelatin made by the alkaline process is given in Fig. 2, where the zero of the combined ion scale is set at the isoionic point of the gelatin. Since the gelatin used had been de-ionized it was isoelectric and isoionic at the start of the titration. The position of the zero point is of no significance, however, and may be determined by the arbitrary pH of the original solution, even when this does not represent the isoionic point. The combined ion scale may be considered, after Cohn & Edsall (1943), to show the number of protons lost with increase of pH from the state of maximum positive charge at pH 1·5 or less. In the curve for the acid-processed gelatin (Fig. 3), the zero of the combined ion scale is set near pH 4, the starting point of the titrations, whereas the isoelectric and isoionic points are at pH 9·1. The difference between these two curves has been shown by Ames (1952) to be due to the conversion, by alkaline processing, of non-ionizable amide groups attached to dicarboxylic acid residues, into ionizable carboxyl groups.

Cannan (1942) has analysed the titration curves of proteins into regions characteristic of the titration of the ionizable groups present. Cannan's criteria applied to the groups present in gelatin are as follows: (*a*) pH 1·5–6, carboxyl groups; (*b*) pH 6–8·5, α-amino and imidazole groups; (*c*) pH 8·5–11·5, ε-amino groups. The values in the first line of Table 2 are obtained by analysis of the curve of alkali-processed gelatin by these criteria. Since at the isoelectric point the net molecular charge is zero, it follows that the number of carboxyl groups titrating from pH 1·5 to this point must equal the total number of charged basic groups in the molecule (Cohn & Edsall, 1943). As the only basic group not estimated directly by the curve is the guanidino group, the amount of this is calculated by difference, and is included in Table 2. Also included are values for α-amino groups calculated on a basis of a linear molecule of molecular weight 60 000; and for residual amide groups calculated as follows. The isoelectric point, pI, for this gelatin is 4·92, which is 0·10 pH unit higher than the value of pH 4·82 which is the lowest found from observations on many alkali-processed gelatins, and which may be assumed to correspond to zero amide groups. This increase must be due to the inability of a number of the carboxyl groups to ionize in the region of pH 1·5–4·8, owing to blocking by amide groups.

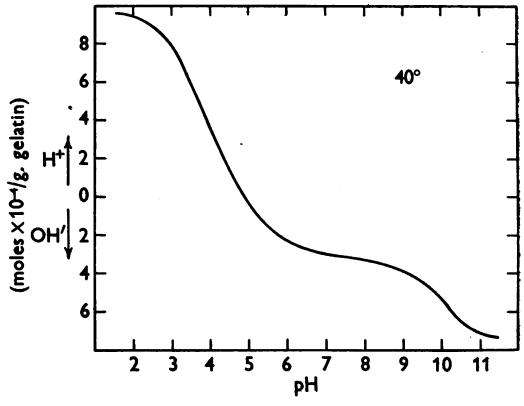

Fig. 2. Titration curve of alkali-processed gelatin, pI 4·92. This curve is based on the mean values from the five experiments summarized in Table 1. Individual points were too many to show, but cf. Fig. 3.

Table 1. *Analysis of five experimental titration curves of the same alkali-processed gelatin*

All values in m-moles/g. anhydrous ash-free gelatin.

Group	Maximum value	Minimum value	Range	Mean value
Carboxyl	1·29	1·20	0·09	1·23
α-Amino + imidazole	0·07	0·06	0·01	0·06
ε-Amino + phenolic hydroxyl	0·43	0·40	0·03	0·42

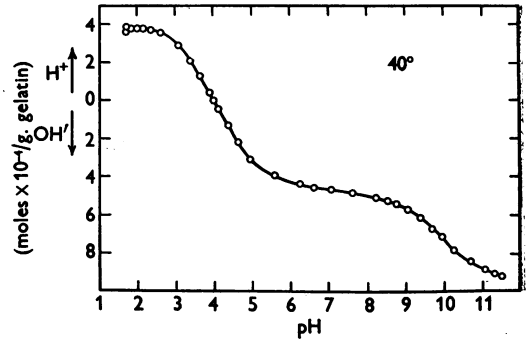

Fig. 3. Titration curve for acid-processed gelatin, pI 9·1.

Table 2. *Analysis of dissociable groups in gelatin*

All quantities in m-moles/g. of anhydrous ash-free gelatin.

Method of analysis	Ionizable groups: Free carboxyl + amide	Total carboxyl	ε-Amino including phenolic hydroxyl	Imidazole + α-amino	− α-Amino* = imidazole	Guanidino
From commercial alkali-processed gelatin						
Titration curve after Cannan (1942)	1·19 + 0·03†	1·22	0·39	0·12	− 0·02 = 0·10	0·44†
Chromatography after Moore & Stein by Eastoe (private communication)	1·246 + 0·052	1·298	0·41‡ + 0·015§ = 0·42(5)	.	. 0·05	0·48
Titration curve by revised criteria	1·23 + 0·03†	1·26	0·42	0·06	− 0·02 = 0·04	0·48†
Titration curve of Hitchcock (1931) by revised criteria	1·26 .	1·26	0·6 (0·48)‖	0·07	− 0·02 = 0·05	0·30† (0·41)‖
Titration curve of commercial acid-processed gelatin	0·85 + 0·35†	1·20	0·42	0·06	− 0·02 = 0·04	0·49†

* Calculated on the assumption of a linear polypeptide of molecular weight 60000 (Courts, 1953).
† Calculated from the titration curve, using p*I*.
‡ Lysine plus hydroxylysine plus ornithine.
§ Tyrosine.
‖ Values for Hitchcock's later batch.

As the rest of the molecule remains the same, the increase may be considered as equivalent to adding an amount of alkali sufficient to combine with the same number of carboxyl groups to the gelatin of p*I* 4·82 and thus to increase its pH from this figure to pH 4·92. This amount of alkali and hence the quantity of amide groups concerned can be determined from the titration curve of the gelatin of p*I* 4·82. The titration curve of this material in the region above pH 3 will be indistinguishable from that of the gelatin under consideration, of p*I* 4·92, and thus the curve in Fig. 2 may be used, giving the value 0·03 m-moles.

The second line of Table 2 consists of the comparable values derived by ion-exchange chromatography of hydrolysed gelatin by the method of Moore & Stein (1951) by Eastoe (private communication). It will be seen at once that there is, on the whole, fairly good agreement between the values obtained, except in the case of histidine. In order to resolve this discrepancy, it has been necessary to reconsider the criteria for the analysis of the titration curve.

First, the shape of the curve from pH 1·5 to 6 cannot be accounted for by assigning a single p*K* value to all the carboxyl groups. It is, however, possible to reproduce the experimental curve within the experimental accuracy by the use of two p*K* values, pK_1, pK_2, corresponding to groups present to the extent of A and $B \times 10^4$ mols/g. protein, and by use of the relation

$$\log [COO^-]/[COOH] = pH - pK,$$

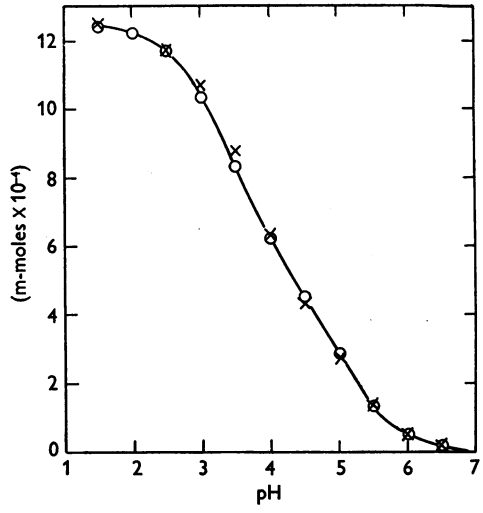

Fig. 4. Titration curve of carboxyl groups in alkali-processed gelatin. The points calculated for a mixture of 0·72 and 0·53 m-moles of acids of p*K* 3·4 and 5·0, respectively, are shown as ○, with a curve drawn through them. The experimental points for 1 g. of anhydrous ash-free gelatin are shown as ×.

which is sufficiently accurate for the present purpose. By inspection and trial the values of A, B, pK_1 and pK_2 may be adjusted to give a calculated curve in excellent agreement with the experimental one. This has been done in Fig. 4, where $pK_1 = 3{\cdot}4$, $pK_2 = 5{\cdot}0$, $A = 7{\cdot}2$, $B = 5{\cdot}3$. It is not suggested that these values are of more than empirical significance

since the curves could readily be fitted with three or more pK values. Whatever choice was made must, however, leave some 3 % of the total carboxyl titration to occur above pH 6. The use of a revised limit of pH 6·5 reduces this to less than 1 % giving a titration above pH 6·5 almost equal to that expected for α-amino and histidine below pH 6·5. The limit may not apply for other proteins unless the ratio of histidine to dicarboxylic acid residues is small, as in gelatin. Where the histidine content is comparable with that of dicarboxylic acids the 'tail' of the histidine titration below pH 6·5 would become significant.

Secondly, collagen and its derived protein gelatin are distinguished from other proteins in that they contain relatively large amounts of hydroxylysine. This has been shown by Van Slyke, Hiller, Macfadyen, Hastings & Klemperer (1940) to have pK 9·50 for its ε-amino group, compared with the figure of 10·3 for lysine. Also, whereas the heat of ionization ΔH, of the carboxyl group is practically zero, (Cohn & Edsall, 1943) and thus the pK is not temperature-dependent, the converse is true for the ε-amino groups. Cohn & Edsall (1943) give a value of $\Delta H = 10\text{–}12 \times 10^3$ cal./mole for the ε-amino groups. Using the relation

$$\Delta H = RT^2 \frac{\mathrm{d} \ln K}{\mathrm{d}T},$$

with $T = 300°$ K as a mean value for the range 20–40° C, (d/dT) pK = −0·02(6). For a temperature rise from 20 to 40°, the pK will decrease by 0·5 for both lysine and hydroxylysine residues, giving values of 9·8 and 9·0 at 40°. This difference in temperature dependence between the acidic and basic groups in amino acids was first recorded by Birch & Harris (1930).

While Cannan's range of pH 8·5–11·5 would exclude only about 10 % of the hydroxylysine at 20°, it excludes over 25 % at 40°, so that it is necessary to shift the pH range used according to the temperature. At 40°, the range will become pH 8·0–11·5, and this will mean that about 1 % of the lysine and 10 % of the hydroxylysine will titrate below this range corresponding to 0·01 m-mole/g., but this will in any case be compensated by *ca.* 15 % of the histidine and α-amino groups, titrating above pH 8·0.

Using these new criteria, namely carboxyl pH 1·5–6·5, α-amino plus imidazole pH 6·5–8·0, ε-amino pH 8·0–11·5, and the same methods as before for amide and guanidino groups, gives line three of Table 2. The maximum inaccuracy of the individual titration curve appears to be ± 5 % at the extreme ends; and for mean values from five separately determined curves, ± 2 % might be expected. This is illustrated in Table 1.

DISCUSSION

When allowance is made for the difference in temperature, the agreement between the results for the alkali-processed gelatin and those for a similar gelatin obtained by Ames (1952) is excellent. The difference between the present curve and that of Ames for acid-processed gelatins lies entirely in the estimated amount of carboxyl groups present. Ames does not specify the isoelectric point of his material more closely than 'about neutrality' whereas the present material had pI = 9·1; it is of interest that by applying the converse of the method used earlier to determine the amide content from the isoelectric point, the calculated values are pI = 7·5 for Ames' material, and pI = 9·1 for the present material. These comparisons can be justified only if the total amino acid composition of both materials is closely similar to that of the lime-processed hide gelatin used in the present work. This is substantiated by the titration values for the basic groups, and by the estimation of the amide groups and their addition to the titratable carboxyl groups to give the total value for dicarboxylic acids. The agreement with the values, calculated by the revised criteria, for the curve obtained by Hitchcock (1931) for an Eastman Kodak gelatin is excellent, except in that his ratio of ε-amino groups to guanidino groups is considerably different, as is apparent from the shape of the high pH portion of his curve. As Hitchcock's work appears to have been carefully performed, the most probable explanation is a difference in the composition of the gelatin used. Albanese (1940) recorded a high 'lysine'-value for an Eastman Kodak gelatin, although the arginine figure was not correspondingly reduced.

Since the total number of basic groups in Hitchcock's gelatin remained unchanged, as shown by the pI 4·84, a possible explanation is that considerable arginine → ornithine conversion had occurred. The conversion of a guanidino group into an amino group is known to occur to a slight extent during the alkaline pre-treatment of collagen (Bowes & Kenten, 1948), but a different order of change would be required to account for the results. Hitchcock noted the discrepancy between his work and earlier results reviewed by Cohn (1925). He recorded that another batch of gelatin from the same source gave results in better agreement with the earlier work. The values for this second batch, in so far as they are available, are given in brackets with the comparable figures for the main work in line 4 of Table 2.

Another possibility, suggested by Ames (1952), is that the anomalous gelatin used by Hitchcock was decomposing under alkaline titration conditions; such decomposition can produce this type of curve. The absence of a titration back to the zero point, as

used in the present method, leaves this point uncertain.

The agreement between the titration curve estimation by the new criteria (Table 2, line 3) and the chromatographic estimation (Table 2, line 2) for the alkali-processed gelatin is so close as to be within the experimental error of the former. It is possible that there is a real discrepancy in the values for lysine, but this point is being further investigated. The correction of the imidazole figure by a reduction of approximately 50% automatically adjusts the arginine figure by the same absolute, but proportionately smaller, amount, and brings this latter value into good agreement.

SUMMARY

1. A rapid method of determining the titration curve of gelatins is described and the accuracy indicated.

2. Revised criteria for the interpretation of the titration curve are presented.

3. The anomalous values for the histidine content of gelatin by titration curve analysis are brought into agreement with those from other methods.

4. The whole of the analytical data obtainable by use of the titration curve and isoelectric point are shown to be in agreement with those from chromatographic analysis.

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Oxidative Phosphorylation in Insect Sarcosomes

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The unique structure of the indirect flight muscles of certain insects belonging to the natural orders Hymenoptera and Diptera has been known for many years. Aubert (1853) was the first to describe the unusually large and abundant granules, later named sarcosomes by Retzius (1890), which are a characteristic feature of these muscles.

Keilin (1925) stated that 'among all the organisms examined the highest concentration of cytochrome is found in the thoracic muscles of flying insects'. On the other hand, little cytochrome was found in the corresponding muscles of wingless insects. Keilin suggested that the high concentration in the thoracic muscle of flying insects had a connexion with their unusually high activity. The wing muscle of the housefly, for example, is capable of producing more than 300 separate contractions per second (Marey, 1874).

Recently, Williams and his colleagues have shown that the cytochrome in the thoracic muscles of *Drosophila funebris* and the blowfly *Phormia regina* is contained in the sarcosomes (Watanabe & Williams, 1951, 1953; Levenbook, 1953). In this respect and in their chemical composition and enzymic content, the insect sarcosomes resemble mitochondria from vertebrate tissues. Nevertheless, until 1953, oxidative phosphorylation, which is a characteristic property of mitochondria and sarcosomes of vertebrate tissues, had not been demonstrated in insect sarcosomes. Sacktor (1953) reported that although sarcosomes isolated from the housefly *Musca domestica* L. oxidized α-ketoglutarate (α-oxoglutarate) rapidly, he was unable to show oxidative phosphorylation. He found that this was not due to the adenosinetriphosphatase (ATPase) activity of the sarcosomes, since the added hexokinase and glucose were able to compete successfully with the ATPase for added ATP. As already reported briefly (Lewis & Slater, 1953), we were able to demonstrate oxidative phosphorylation in sarcosomes isolated from the blowfly *Calliphora erythrocephala*. At about the same time, Watanabe & Williams (1953) quoted a personal communication from Sacktor stating that he had demonstrated

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