The Effect of Organic Acids, Inhibitors and Enzymes on the Respiration of *Chlorella*

By D. M. ENY

Cornell University, Ithaca, U.S.A.

(Received 31 May 1951)

In the course of an investigation on the respiratory mechanism of the green alga *Chlorella*, experiments were conducted to study the utilization for growth in the dark of several organic acids (Eny, 1950). The effects of these organic acids on the rate of oxygen uptake and carbon dioxide evolution were observed (Eny, 1951). To elucidate further the respiratory mechanism, the action of various enzymes and enzyme inhibitors known to influence the aerobic path of carbohydrate metabolism in animal tissues was studied. The present investigation included manometric determination of the influence of cytochrome oxidase, coenzyme I (CoI), and adenosinetriphosphate (ATP) on the respiratory rate of *Chlorella*, determination of adenosinetriphosphatase (ATPase) activity, and estimation of dehydrogenase activity.

MATERIALS AND METHODS

Nutrient solutions were prepared by standard methods (Mandels, 1943), and buffering action was provided by potassium phosphate and was increased by addition of the organic acids. The pH of the system was adjusted to 5.4–5.6. The organic acids used were lactic, pyruvic, acetic, *cis*-aconitic, citric, succinic, fumaric, malic, butyric, and propionic.

Chlorella cell homogenates were prepared by a technique described by Umbreit, Burris & Stauffer (1945), utilizing a glass homogenizer, essentially a piston rotating with a vertical motion in a closely fitting glass cylinder. Examination of the homogenate showed almost entire destruction of the cells. Only 3–8% of the original count consisted of intact cells.

The strain of *Chlorella* used was originally isolated from soil by Wann (1921) and has been maintained in the laboratories of Cornell University. The strain has not been classified taxonomically (Mandels, 1943).

The cytochrome oxidase preparation was obtained from ox heart by the method of Keilin & Hartree (1938). This preparation also contains cytochromes *a*, *b* and *c*, fumarase, and several dehydrogenases. CoI was prepared from baker's yeast by the Williamson & Green (1940) method. ATP was prepared from rabbit muscle by the DuBois, Albaum & Potter (1943) method.

The manometric experiments were conducted in the dark with the Warburg apparatus according to the method described by Umbreit *et al.* (1945). The Warburg flasks were of the two side arm, centre-well and venting-plug type. They

contained the cell homogenate with the necessary volume of water and nutrient solution, the organic acid intermediates, enzymes and/or inhibitors, and KOH or water in the centre well for the determination of oxygen and carbon dioxide respectively. A thermobarometer and two control flasks, one for CO₂ and one for O₂, contained all the components of the system studied. The enzymes and inhibitors were kept in the separate compartments. Results are expressed as q_{O_2} and q_{CO_2} which are $\mu\text{l./hr. O}_2$ uptake or CO₂ evolution for a volume of homogenate approximately equivalent to 10⁹ cells.

Determination of ATPase activity. The test system was prepared by the method developed by DuBois & Potter (1943). The materials to be tested consisted of cell suspensions, cell homogenates or cell extracts. The cells were washed six times by repeated stirring and centrifuging with 0.02M-KNO₃ solution (phosphate-free) before grinding or preparation of the final substrate. The ATP solution contained 565 $\mu\text{g. phosphorus/ml.}$ when tested with potato apyrase (Krishnan, 1949).

The test tubes containing the system were placed in a water bath at 30° for periods from 30 min. to 3 hr. The reaction was stopped by the addition of 2 ml. of 20% (w/v) trichloroacetic acid. The tubes were centrifuged and 2 ml. samples of supernatant were taken for the determination of hydrolysed phosphorus.

The phosphorus was determined by Sumner's (1944) method. Controls were run with each series, one containing all reagents except the ATP, the other containing all reagents except the cell homogenate. The final values were corrected by subtracting the two controls.

Dehydrogenase tests. The dehydrogenase activity was estimated by measuring the degree of methylene blue (MB) reduction according to a slightly modified Thunberg method (cf. Umbreit *et al.* 1945). After a few preliminary experiments with Thunberg tubes, they were rejected because of the difficulty of preventing air leakage or of keeping the cell homogenate isolated during evacuation. Test tubes (10 ml.) (Fisher photocolormeter) were used instead. Anaerobic conditions were maintained by placing a layer of mineral oil, 1.5 cm. thick, on top of the MB, thus isolating the system from contact with air. The cell homogenate was pipetted through the oil and the system left in the dark at 37° for varying periods of time. The degree of MB reduction was measured on the electrophotometer at 6500 Å. after centrifuging the cell fragments to the bottom of the tubes.

Although readings were taken at various intervals, only those obtained at 12, 24 and 36 hr. after the experiment was set up are reported. These readings were selected on the basis of significance and convenience of comparison.

Table 1. *Changes in the oxygen uptake of Chlorella homogenate when treated with various substances*

(All values of q_{O_2} and q_{CO_2} were obtained by Warburg manometric experiments conducted in the dark. The figures for q_{O_2} and q_{CO_2} refer to systems to which organic acids, but no enzymes or inhibitors, were added. q_{O_2} and q_{CO_2} indicate $\mu\text{l./hr.}$ of O_2 uptake and of CO_2 evolution respectively for homogenate equivalent to approximately 10^9 cells. The calculations were made on a 4 hr. basis. Each figure represents the average of three determinations with the same batch of homogenate. The percentage stimulation or inhibition varied within a range of $\pm 4\%$. Values were calculated on the O_2 uptake on a 2 hr. basis. + Indicates stimulation; - indicates inhibition; 0 indicates no effect. The ATP solution contained $565 \mu\text{g.}$ of phosphorus/ml. A 1% (w/v) CoI solution was used.)

Organic acid	Concn. (M)	q_{O_2}	q_{CO_2}	Percentage stimulation or inhibition with added substances					
				Cytochrome oxidase (0.4 ml.)	CoI (0.4 ml.)	ATP (0.4 ml.)	Sodium malonate (0.02 M)	KCN (0.004 M)	Sodium azide (0.001 M)
Lactate	0.05	632	987	+79	+72	0	+7	-73	-78
Pyruvate	0.05	676	178	-12	-18	0	+10	-59	-65
Acetate	0.05	952	611	-16	0	0	+13	-64	-68
Aconitate	0.03	810	496	0	0	0	+6	-50	-64
Citrate	0.02	335	121	-20	0	0	+10	-61	-64
Succinate	0.03	484	382	-8	0	0	+8	-46	-57
Fumarate	0.03	700	486	-28	0	0	+2	-54	-70
Malate	0.05	410	344	+74	+93	0	+6	-68	-72

RESULTS

Manometric experiments

Table 1 gives the q_{O_2} and q_{CO_2} for *Chlorella* homogenate with addition of individual organic acids and without enzyme or inhibitor, and the percentage

Fig. 1. Cumulative effects as studied in a Warburg manometric apparatus, of cytochrome oxidase, CoI, and 0.02 M-NaF (curves C and D) and of 0.004 M-KCN and cytochrome oxidase (curves E and F) on the respiration rate (O_2 uptake and CO_2 evolution) of *Chlorella* homogenate on malate. Curves A and B represent the controls with 0.05 M-malate. Each flask contained a total liquid volume of 5 ml. and the equivalent of 1.4×10^7 cells. The concentration of CoI was 0.04% (w/v). 0.2 ml. of cytochrome oxidase preparation was used.

stimulation or inhibition produced by the addition of several substances. Cytochrome oxidase and CoI both stimulated the respiration of the *Chlorella* homogenate in the presence of sodium lactate and sodium malate. Cytochrome oxidase did not affect

respiration in the presence of aconitate and slightly inhibited it with the other acids.

Potassium cyanide inhibited the oxygen uptake in varying degrees according to the organic acid present in the system. The largest inhibitions were produced in the presence of lactate and malate. Respiration was 70% inhibited by 0.001 M-azide and completely stopped by higher concentrations.

Fig. 2. CoI stimulation (0.04% w/v) and KCN inhibition (0.004 M) as studied in a Warburg apparatus, of the respiration of *Chlorella* homogenate on malate. Curves A and B represent the controls with 0.05 M-malate. Each flask contained a total liquid volume of 5 ml. and the equivalent of 1.4×10^7 cells.

Since malonate specifically inhibits succinic dehydrogenase, the use of malonate with succinate should stop the formation of fumarate and inhibit the respiration of *Chlorella* if the oxidation requires succinic dehydrogenase as the flavoprotein. Actually malonate produced some increase of oxygen uptake, not only with succinate but with all the acids used.

A fairly large concentration of fluoride (0.02M), was needed to produce inhibition but when reached, the inhibition was complete. Pyrophosphate inhibition was effective in the same general manner at 0.01M concn., whilst even at the very low concentration of 0.0001M, iodoacetate was completely toxic to the respiratory system.

No change in the rate of respiration was observed when quantities varying from 0.1 to 0.5 ml. of an ATP solution containing 565 μ g. of phosphorus/ml. were used.

Since the effects of the enzymes and inhibitors tested showed with malate as well as or better than with the other organic acids, they have been charted graphically to typify the results. Figs. 1 and 2 show the changes in respiratory rate produced by the addition of CoI, cytochrome oxidase and several inhibitors to *Chlorella* homogenate in dilute nutrient solution containing 0.05M-malate. In both figures, curves A and B represent respectively the oxygen uptake and carbon dioxide evolution without tipping enzymes or inhibitors from the side arms of the flask.

Determination of ATPase activity

To determine whether the results obtained with ATP were due to the absence of a hydrolysing enzyme or to the ineffectiveness of ATP in the metabolism of *Chlorella*, estimations of the hydrolytic enzyme of ATP were made using cell suspensions, cell extracts, or cell homogenates. The results obtained with the suspensions and extracts were not significant. Those obtained with the homogenates are shown in Table 2.

The recovery of phosphorus generally increased with the amount of homogenate in the test system and calcium chloride gave little noticeable activating effect. The time required by the *Chlorella* homogenate to produce detectable amounts of phosphorus through ATP hydrolysis was much longer than that

generally necessary for animal tissues. However erratic the phosphorus recovery, the results are positive and indicate the presence of an enzyme producing some hydrolysis of ATP. Several enzymes of this type have been isolated from plant material, one of the best sources being potato (Krishnan, 1949).

Dehydrogenase tests

In animal tissue, the presence of cyanide is necessary to obtain the test for lactic acid dehydrogenase (Straub, 1940), as cyanide binds the pyruvate which is formed. Otherwise the accumulation of pyruvate would reverse the direction of the reaction and prevent the reduction of MB. If pyruvate is added to the system after the MB is reduced, the blue colour returns, showing that the MB is re-oxidized and that the reaction goes toward lactate.

In one of the tests conducted with *Chlorella* homogenate, the cyanide was omitted. The reaction continued, however, and reduction was nevertheless obtained. Pyruvate was then added to the system, but the blue colour did not reappear. Apparently the system was different from that of animal tissues and it was decided to further utilize the MB technique to study the possible dehydrogenases involved. The method gives an approximate relative rate of oxidation of the acids by the dehydrogenase system.

The colour readings of the test system are given in Table 3. The times appear unduly long, but since duplicates gave closely similar results it would seem that the results are due to the cell homogenate processes rather than to bacterial contamination. All the acids decreased the reduction time of MB, which indicates that they produce a rise in dehydrogenase activity by stimulating the respiration rate.

Pyruvic acid reduced MB more quickly than any of the other acids, and addition of CoI and cyto-

Table 2. *Estimation of ATPase in Chlorella homogenate*

(The complete test system (DuBois & Potter, 1943) contained cell homogenate, 0.1M-acetate buffer pH 6.5, 0.04M-CaCl₂, as activator and ATP. 0.4 ml. of the ATP solution represents 226 μ g. of phosphorus. The phosphorus was analysed by the technique of Sumner (1944).)

Sample no.	Buffer (ml.)	Cell homogenate (ml.)	CaCl ₂ (ml.)	ATP (ml.)	Time (hr.)	Phosphorus recovery (μ g.)
1	0.05	2.0	0.25	0.4	1	40
2	0.05	2.0	0.25	0.4	1	24
3	0.05	2.0	0.00	0.4	1	28
4	1.00	1.0	0.25	0.4	1	19
5	1.00	1.0	0.25	0.4	1	21
6	1.00	1.0	0.00	0.4	1	15
7	0.20	3.0	0.25	0.4	3	58
8	0.20	3.0	0.25	0.4	3	43
9	0.20	3.0	0.00	0.4	3	32
10	0.50	2.0	0.25	0.4	3	57
11	0.50	2.0	0.25	0.4	3	17
12	0.50	2.0	0.00	0.4	3	36

chrome oxidase to the pyruvate system did not activate the MB reduction.

Table 3. *Dehydrogenase tests*

(The tests were conducted by a slightly modified Thunberg technique. Each tube contained 1 ml. of 0.02% MB, 1 ml. of 0.02M-phosphate buffer pH 6.5, organic acids to 0.05M final concentration, inhibitors to concentrations listed, or 0.5 ml. of 1.0% (w/v) CoI solution, or 0.5 ml. of cytochrome oxidase preparation, water to adjust to 6 ml., 1.5 cm. layer of mineral oil, 1 ml. of *Chlorella* homogenate pipetted through the oil.)

Substrate	Colorimeter readings at			
	0 hr.	12 hr.	24 hr.	36 hr.
Lactate	76	29	8	1.0
Pyruvate	76	10	4	1.2
Acetate	76	43	18	1.8
Aconitate	76	47	18	1.2
Citrate	76	55	28	1.6
Succinate	76	59	30	2.5
Fumarate	76	34	12	1.5
Malate	76	38	16	2.0
No acid	76	70	54	37.0
Lactate + cytochrome	76	18	8	2.0
Pyruvate + cytochrome	76	27	13	1.0
Acetate + cytochrome	76	70	70	3.6
Aconitate + cytochrome	76	50	18	2.4
Citrate + cytochrome	76	72	60	2.8
Succinate + cytochrome	76	61	29	3.8
Fumarate + cytochrome	76	75	73	1.8
Malate + cytochrome	76	28	9	1.5
No acid + cytochrome	76	55	38	12.3
Lactate + CoI	76	15	6	1.0
Pyruvate + CoI	76	36	9	1.0
Acetate + CoI	76	45	20	1.6
Aconitate + CoI	76	48	19	1.8
Citrate + CoI	76	54	23	1.7
Succinate + CoI	76	59	29	2.0
Fumarate + CoI	76	38	10	1.3
Malate + CoI	76	35	7	1.8
No acid + CoI	76	56	40	38.0
KCN (0.004M)	76	64	52	13.4
Azide (0.001M)	76	70	70	70.0
Fluoride (0.02M)	76	70	70	70.0
Malonate (0.02M)	76	52	43	11.0
Pyrophosphate (0.01M)	76	67	57	55.0
Iodoacetate* (0.0001M)	76	1	1	1.0
No substrate	76	70	54	27.3

* Discoloration was caused by the reaction of MB with iodoacetate, forming a brown precipitate.

Table 3 also shows the effect of cytochrome oxidase when added to the various acids in the dehydrogenase test systems. The reduction of MB with lactate and malate occurred faster when cytochrome oxidase was present in the system than when absent. The reason for the large inhibiting action of cytochrome oxidase in the presence of fumarate, acetate or citrate is not clear. It is possible that these acids formed some inhibitory complex with the cytochrome. (The preparation of cytochrome is not a pure system and may contain some substances reacting with the acids.) CoI did not produce this inhibition.

Enzyme inhibitors were used in the dehydrogenase tests without acid substrates. The results are shown in Table 3. The amount of inhibition was highest with iodoacetate. With 0.0001M-iodoacetate, whether or not homogenate was added to the system, the MB was irreversibly discoloured within 5 min. and formed a heavy precipitate. Azide, fluoride and pyrophosphate were very efficient inhibitors. The inhibition produced by potassium cyanide started to decrease after about 6 hr. Complete MB reduction occurred almost simultaneously with that of the control (after about 36 hr.). Malonate failed to produce inhibition.

Inhibitors were also used in the presence of acids in the dehydrogenase tests. Their effects were similar to those reported in the manometric experiments. For instance, the use of malonate together with succinate did not decrease the respiration rate. The reduction of MB occurred in about the same time as when no malonate was added.

DISCUSSION

The presence of CoI and cytochrome has been demonstrated in various plant materials. Euler & Nilsson (1931) showed that CoI was present in boiled extracts from jute seeds. Holmberg (1934) prepared a coenzyme from peas that had the same action as CoI in reducing MB in the presence of malate or lactate. Meeuse (1948) extracted a mixture from pea seedlings showing a high cytochrome oxidase activity. In the present investigation it was shown that CoI and cytochrome oxidase increased the rate of lactate and malate oxidation in *Chlorella*. This suggests that these enzymes may be involved in the oxidation of lactate and malate or they may participate in the enzyme system naturally occurring in the cells.

CoI and cytochrome did not affect the oxidation of pyruvate, and since the dehydrogenase tests showed that this acid was oxidized faster than any other, it would appear that the oxidation is more direct than that described in the tricarboxylic acid cycle. This points toward a mechanism of oxidation involving a pyruvic dehydrogenase and flavin-adenine dinucleotide instead of the CoI-cytochrome-lactic dehydrogenase system. Lipman (1940) found that in *Lactobacillus delbrückii* oxidation of pyruvate by pyruvic dehydrogenase could take place under both anaerobic and aerobic conditions.

Emerson (1927) found that 0.0001M-hydrogen cyanide inhibited about 60% of the respiration of a strain of healthy *Chlorella pyrenoidosa* cells which had been grown with glucose, but that the inhibition was small and temporary when lactic acid was used in the respiratory flasks with starved cells.

Warburg (1919) found a stimulation of respiration by cyanide in a strain of *C. pyrenoidosa*. If the

experiments of Emerson and Warburg were conducted with the same strain, it would appear that an alternative respiratory mechanism is available in that particular *Chlorella* strain. In the present experiments, 0.004M-potassium cyanide produced a decrease in the rate of oxygen uptake in all cases. This inhibition reached about 70 % with lactate and malate. It was possible to reverse the inhibition by adding cytochrome oxidase (Fig. 2).

It has been shown that potassium cyanide acts not only on cytochrome but also on several other oxidases (cf. Porter, 1946). Because of these other inhibitions, James (1946) stated, 'It cannot be assumed... that any respiration of a plant tissue inhibited by cyanide is necessarily due to the cytochrome system.' But since cytochrome oxidase produced an activation of the lactate oxidation, it is at least likely that this system exists in *Chlorella* cells.

Kohn (1935) noted that both respiration and photosynthesis of *C. pyrenoidosa* were inhibited by 0.001M-iodoacetate. The same concentration of iodoacetate produced a permanent inhibition with the *Chlorella* strain used in the present investigation. No specific effect could be studied with this inhibitor. Similarly other inhibitors, such as fluoride and pyrophosphate, produce a general toxicity and often give insignificant results (Bartlett & Guzman-Barron, 1947).

Though malonate characteristically inhibits succinic dehydrogenase it did not inhibit the oxygen uptake or the rate of MB reduction. This result does not fit in the scheme of the tricarboxylic acid cycle. Henderson & Stauffer (1944) reported that tomato roots also resist malonate poisoning. If succinic dehydrogenase is present, it is probably not the only path of succinate oxidation, as some alternate mechanism remains active in spite of malonate. This might fit with the theory advanced by Kalnitsky, Wood & Werkman (1943) on a succinate formation through the oxidative coupling of two acetates and the presence of a separate mechanism for the formation of C₃ and C₄ acids in *Chlorella*.

The utilization of high-energy phosphate bonds by cells has given an explanation for the reversal of the carbohydrate breakdown cycle in animal tissues (Kalckar, 1942). It was shown by Lepage & Umbreit (1943) that the energy release of sulphur bacteria is coupled with the formation of ATP from inorganic phosphorus and that the high-energy

phosphate bonds can be utilized to fix carbon dioxide in the dark for chemosynthesis.

The present experiments with ATP gave no evidence that phosphorylated compounds were utilized by *Chlorella* in the dark. Some indication was obtained that an enzyme capable of hydrolysing ATP is present.

The path of phosphorus in the strain of *Chlorella* used in the present work may be different from that in most animals and bacteria. Emerson, Stauffer & Umbreit (1944) were unable to identify the phosphorylated compounds present in other *Chlorella* cells with those normally present in most animal tissues and bacteria.

SUMMARY

1. The effect of several enzymes, inhibitors, and adenosinetriphosphate, together with organic acids, on the rate of oxygen uptake of *Chlorella* homogenate was studied by manometric experiments conducted in the dark. An investigation of the dehydrogenase system was also conducted by a slightly modified Thunberg technique.

2. Cytochrome oxidase and coenzyme I, in the presence of malate and lactate, produced an increase in the rate of oxygen uptake and also shortened the time of methylene blue reduction. Potassium cyanide inhibited this stimulation. These effects suggest that malate and lactate are oxidized in *Chlorella* in the same manner as in animal tissues.

3. Malonate did not produce an inhibition of respiration with or without succinate. This might indicate that succinic dehydrogenase is not the only possible path to fumarate formation and points toward a separate mechanism for the formation of C₃ and C₄ acids.

4. The comparatively rapid oxidation of pyruvate by *Chlorella* in the presence of methylene blue strongly suggests that pyruvate oxidation takes place through a pyruvic-dehydrogenase system.

5. The respiration rate of *Chlorella* was not affected by the addition of various amounts of adenosinetriphosphate to systems with or without organic acids. Some hydrolysis of adenosinetriphosphate was detected.

The author is indebted to Dr P. S. Krishnan of the Enzyme Laboratory at Cornell University for the purified potato apyrase.

REFERENCES

- Bartlett, G. R. & Guzman-Barron, E. S. (1947). *J. biol. Chem.* **170**, 67.
- DuBois, K. P., Albaum, H. G. & Potter, V. R. (1943). *J. biol. Chem.* **147**, 699.
- DuBois, K. P. & Potter, V. R. (1943). *J. biol. Chem.* **150**, 185.
- Emerson, R. (1927). *J. gen. Physiol.* **4**, 469.
- Emerson, R. L., Stauffer, J. F. & Umbreit, W. W. (1944). *Amer. J. Bot.* **31**, 107.
- Eny, D. M. (1950). *Plant Physiol.* **25**, 478.
- Eny, D. M. (1951). *Plant Physiol.* **26**, 268.
- Euler, H. von & Nilsson, R. (1931). *Hoppe-Seyl. Z.* **194**, 260.
- Henderson, J. H. M. & Stauffer, J. L. (1944). *Amer. J. Bot.* **31**, 525.
- Holmberg, C. G. (1934). *Skand. Arch. Physiol.* **68**, 1.
- James, W. O. (1946). *Ann. Rev. Biochem.* **15**, 427.
- Kalckar, H. M. (1942). *Biol. Rev.* **17**, 28.
- Kalnitsky, G., Wood, H. G. & Werkman, C. H. (1943). *Arch. Biochem.* **2**, 269.
- Keilin, D. & Hartree, E. F. (1938). *Proc. roy. Soc. B*, **125**, 171.
- Kohn, H. I. (1935). *J. gen. Physiol.* **19**, 23.
- Krishnan, P. S. (1949). *Arch. Biochem.* **20**, 261.
- Lepage, G. A. & Umbreit, W. W. (1943). *J. biol. Chem.* **147**, 263.
- Lipman, F. (1940). *Rep. Proc. 32nd Int. Congr. Microbiol.* (1939), 241.
- Mandels, G. R. (1943). *Plant Physiol.* **18**, 449.
- Meeuse, B. J. D. (1948). Program of the Amer. Soc. of Plant Physiol.
- Porter, J. R. (1946). *Bacterial Chemistry and Physiology*, p. 551. New York: John Wiley and Sons.
- Straub, F. B. (1940). *Biochem. J.* **34**, 483.
- Sumner, J. B. (1944). *Science*, **100**, 413.
- Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1945). *Manometric Techniques*, pp. 92, 8, 126. Minneapolis: Burgess Publishing Co.
- Wann, F. B. (1921). *Amer. J. Bot.* **8**, 1.
- Warburg, O. (1919). *Biochem. Z.* **100**, 230.
- Williamson, S. & Green, D. E. (1940). *J. biol. Chem.* **135**, 345.

The Reversible Dissociation of Insulin and its Minimum Molecular Weight

By H. GUTFREUND

Department of Colloid Science, University of Cambridge

(Received 5 June 1951)

In two previous papers (Gutfreund, 1948*a, b*) it has been shown that the molecular weight of insulin is dependent upon pH, temperature and protein concentration of the solution. In neutral solutions of moderate protein concentration insulin appeared to be homogeneous with respect to molecular weight, $M = 48,000$ (Gutfreund & Ogston, 1946; Gutfreund, 1948*a*). Under optimum conditions for dissociation (pH 2.6) a steep extrapolation to zero concentration of osmotic pressure against insulin concentration gave a value of 12,000 for the molecular weight. From this evidence, as well as from crystallographic and chemical information, it was concluded that insulin in solution consists of aggregates of loosely bound sub-units of molecular weight 12,000. It is not claimed that aggregation into molecules containing four sub-units is necessarily an upper limit.

More recently two papers gave an account of ultracentrifugal and diffusion studies on insulin solutions, and the authors drew different conclusions from their results. Pedersen (1950) believes that the molecular weight of insulin is 42,000 and that molecules of that size dissociate into two sub-units of molecular weight 21,000. His data would,

however, be quite consistent with the hypothesis that under varying conditions of solution the mean molecular weight can have any value from 12,000 to 48,000 or more. Fredericq & Neurath (1950) found that in dilute phosphate buffer of pH 2.65, insulin dissociates into sub-units of molecular weight 6000, this value being calculated from diffusion and sedimentation constants extrapolated to zero concentration. This extrapolation is, however, very steep, and since measurements of the diffusion constant in dilute solutions are likely to give values which are too high, the extrapolated value of the molecular weight is likely to be too low.

The present paper is confined to the determination of the molecular weight of the sub-unit (minimum molecular weight) into which insulin can be dissociated reversibly. It has already been emphasized by Gutfreund (1948*b*) that the study of the effect of pH, temperature and protein concentration upon the aggregation of the sub-unit of insulin should be complemented with an investigation of the influence of salt concentration on this phenomenon. The present paper describes this further investigation, and shows how it confirms and clarifies the author's previous findings.