I. INVESTIGATIONS IN ENZYMIC HISTOCHEMISTRY.

II. A MICRO-METHOD FOR THE DETERMINATION OF TRYPTIC ACTIVITY.

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(Received November 1st, 1935.)

THE development by Holter and Linderstrøm-Lang [1934] of methods and techniques for the micro-estimation of enzymes and other physiological substances in tissue sections may be said to mark the beginning of quantitative histological chemistry. In view of the applicability of these methods to biochemical problems, it becomes desirable to work out the technical details for the estimation of as many substances of physiological importance as possible.

The present paper describes a micro-method for the estimation of trypsin¹ using the apparatus developed by the above authors. The method is an adaptation of Sørensen's [1908] macro-method of formaldehyde titration of the carboxyl groups of proteins and their decomposition products. In preliminary studies it was found that the method of Foreman [1920] and Willstätter and Waldschmidt-Leitz [1921], namely, titration in alcoholic solution of the carboxyl groups liberated by tryptic action, is inapplicable because of precipitation of the protein substrate by the alcohol. A similar difficulty is encountered in the Linderstrøm-Lang acetone titration [1927]. Nephelometric measurement of tryptic activity, as described by Krijgsman [1934], is likewise unsuited to the micro-method because the instability of the turbidity may lead to considerable errors during a large series of experiments.

The formaldehyde titration method, on the other hand, was found to be very well suited to the present purpose. The addition of formaldehyde to the reaction mixture produces no precipitate, and at once prevents further enzyme action. The formaldehyde blocks the amino-groups liberated by the action of the trypsin, thus permitting the titration of an equivalent amount of carboxyl groups with alkali. As substrate, 4 % caseinogen solution at p_H 8.4 is used. Phenolphthalein is used as indicator, although thymol blue was also found to be satisfactory. Thymolphthalein cannot be used because of its insolubility in the aqueous formaldehyde solution. A standard for colour comparison was established by titration to a marked red colour, the latter being preserved by placing a soda-lime tube over the vessel.

The p_H optimum of the tryptic activity was found to extend over quite a wide range, in agreement with similar observations by Willstätter and Persiel [1925] and by Northrop [1922]. p_H 8.4 was selected as the optimum. In order that the formaldehyde titrations may be accurate, substances possessing dissociation constants within the titration range of p_H 6.8-9.5 must be avoided: phosphate buffer is, therefore, unsuitable for use in this titration. Borate buffer

¹ As is known, trypsin is a mixture of several proteolytic enzymes. The method described measures the tryptic activity of the whole complex.

reacts with the organic hydroxyl groups, and this may have a secondary influence on the enzyme. The veronal buffer at p_H 8.4, described by Michaelis [1930] is, therefore, to be preferred. This buffer, besides having a strong buffering power at this p_H , has the advantage of showing no reagent blank and, further, has no inhibiting effect upon tryptic activity.

Disturbances due to atmospheric CO_2 can be avoided during the reaction period by placing a soda-lime tube on the reaction vessel. Similar disturbances, which would affect the alkali titration, are prevented by employing the method developed by Linderstrøm-Lang *et al.* [1935], that is, the vessel in which the titration is carried out is closed by a bead, previously dipped in paraffin oil, the capillary end of the micro-burette passing through the hole in the bead and dipping into the solution being titrated. During the titration the mechanical stirring method described by Linderstrøm-Lang and Holter [1934] is used. A 0.05 *N* aqueous solution of tetramethylammonium hydroxide is employed to titrate the liberated carboxyl groups since, when sodium hydroxide is used, the carbonate is formed and crystallises out on the wall of the micro-burette, rendering the titration more difficult.

The method described, as shown by the experimental part, is suitable for histo-enzymic studies. It is reproducible to within $\pm 0.1 \mu l.$ of 0.05 *N* alkali.

EXPERIMENTAL.

Effect of formaldehyde concentration on accuracy of the titration. Increasing concentrations of formaldehyde were added to 0.1 *M* leucylglycine solution. The formaldehyde solutions were prepared by taking 1, 2, 4 and 8 ml. respectively of 40% formaldehyde solution, adding 2 ml. of 0.1% alcoholic phenolphthalein solution, titrating the solutions so obtained with 0.1 *N* sodium hydroxide to the first pink colour given by the indicator and diluting to 25 ml. with water. By means of a micro-pipette, as described by Linderstrøm-Lang and Holter [1934] and Glick [1935], 100 $\mu l.$ of the formaldehyde solutions thus prepared, containing 4, 8, 16 and 32 $\mu l.$ respectively of 40% formaldehyde, were added to 7 $\mu l.$ of 0.1 *M* leucylglycine solution. It is advantageous to use freshly prepared aldehyde solutions. The titration of the carboxyl groups was performed by the method described by Linderstrøm-Lang *et al.* [1935] using 0.1 *N* aqueous solution of tetramethylammonium hydroxide. In order to carry all the titrations to the same end-point, as shown by the same indicator colour, the first determination was titrated to a marked red colour (above p_H 10), and this colour, preserved by placing a soda-lime tube on the reaction vessel, served as a standard. Using this method of protection, no change in the intensity of the standard colour was observed over a period of 5–6 hours. The results obtained, expressed in $\mu l.$ of 0.05 *N* tetramethylammonium hydroxide, are given in Table I. The value of the reagent blank (amounting to 0.2 $\mu l.$) has already been deducted from the titration results to obtain the figures given.

Table I. *Effect of formaldehyde concentration on accuracy of titration.*

Volume of 40% formaldehyde in 100 $\mu l.$ solution $\mu l.$	Observed titra- tion (0.05 <i>N</i> alkali) $\mu l.$	Theoretical titration $\mu l.$
4	13.75	14.00
8	13.95	14.00
16	13.98	14.00
32	13.92	14.00

The solution containing 16 μ l. of 40 % formaldehyde in 100 μ l. solution, prepared as described above, was selected for further experiments since, as seen from Table I, further increase in the formaldehyde concentration causes no increase in the titration value. For each determination 100 μ l. of this solution were employed.

Micro-titration of known substances in presence of formaldehyde. To check the accuracy of the method, various concentrations of glycine, alanine and leucylglycine were titrated. The purities of these substances were checked by Kjeldahl determinations of total nitrogen. Solutions 0.1, 0.05, 0.033 and 0.02 *M* in concentration were made for each of the substances investigated, 7 μ l. of solution being taken for each determination. The free carboxyl groups were determined as described above. The results obtained (from which the reagent blanks have already been deducted) are shown in Table II.

Table II. *Micro-titration of known substances in presence of formaldehyde.*

(μ l. 0.05 <i>N</i> alkali.)				
Substrate	0.1 <i>M</i>	0.05 <i>M</i>	0.033 <i>M</i>	0.02 <i>M</i>
Glycine	13.97	6.95	4.59	2.76
	13.97	6.92	4.61	2.82
	14.05	6.90	4.57	2.78
	13.97	6.99	4.62	2.78
	13.99	6.94	4.60	2.78
Alanine	13.96	6.93	4.65	2.80
	13.97	6.93	4.67	2.82
	14.03	6.99	4.60	2.82
	13.99	6.97	4.63	2.80
	13.98	6.95	4.63	2.81
Leucylglycine	13.96	6.92	4.60	2.74
	13.99	6.96	4.58	2.76
	13.90	6.96	4.54	2.76
	13.94	6.98	4.58	2.80
	13.95	6.95	4.57	2.76
Calculated values	14.00	7.00	4.66	2.90

p_H optimum of tryptic activity. The trypsin solution used was prepared by taking 1 g. of dry trypsin preparation (Degoma S, Rohm and Haas Co.), suspending it in 10 ml. of water and allowing it to stand 1 day. To prevent bacterial infection a few drops of toluene were added. After 1 day the suspension was filtered and 0.1 ml. of the filtrate diluted to 25 ml. with water containing 30 % by volume of glycerol. To 7 μ l. portions of this enzyme solution 7 μ l. of 0.1 *M* veronal-hydrochloric acid buffer of *p_H* 6.4, 7.0, 8.0, 8.4, 9.0 and 9.5 were added together with 7 μ l. of a 4 % caseinogen solution of the same *p_H* as the buffer taken. "Hammarsten casein" was used and was adjusted to the required *p_H* by the addition of the necessary amount of *N* sodium hydroxide, the *p_H* being controlled by the hydrogen electrode.

The buffer and the enzyme solution were pipetted into the reaction vessel and the substrate carefully placed, as a drop, on the wall of the vessel and mixed with the reagents immediately before the experiments commenced. Simultaneously with each experiment a blank determination was done, identical with the determinations themselves except that the substrate was not mixed with the enzyme, but the reaction vessel was allowed to stand in a horizontal position (to avoid mixing of the solutions) under identical conditions. To avoid the effect of the CO₂ of the air on the experiments soda-lime tubes were placed on each

reaction vessel. After incubation for 30 min. at 37° the increase in the carboxyl groups was determined by the method of Linderstrøm-Lang *et al.* [1935].

The results are given in Table III, and are expressed in μ l. of 0.05 *N* tetramethylammonium hydroxide used. The corresponding blank determinations have already been subtracted.

Table III. p_H optimum of tryptic activity.

p_H	Increase in COOH groups (0.05 <i>N</i> alkali) μ l.
6.4	1.64
7.0	2.84
8.0	2.96
8.4	3.00
9.0	2.12
9.5	0.2

As the figures indicate, the p_H optimum of trypsin activity extends over a wide range, and for further experiments p_H 8.4 was selected.

Time course of tryptic activity as followed by the micro-method. 0.2 ml. of the enzyme extract (1 : 10), described above, was diluted to 25 ml. with water containing 30 % by volume of glycerol. To 7 μ l. of this enzyme solution 7 μ l. of 0.1 *M* veronal-hydrochloric acid buffer of p_H 8.4 and 7 μ l. of a 4 % caseinogen solution, also at p_H 8.4, were added. The increase in carboxyl groups was titrated, as previously described, using incubation times from 0 to 150 min. at 37°. The results are shown in Table IV, the figures given referring to μ l. of 0.05 *N* tetramethylammonium hydroxide required for the titration.

Table IV. *Time course of tryptic activity as followed by the micro-method.*

(μ l. 0.05 *N* alkali.)

Time min.	Control	Reaction mixture	Increase
0	2.80	2.84	0.0
7	2.76	4.46	1.70
15	2.76	6.07	3.31
22	2.84	7.44	4.60
30	2.80	8.55	5.75
45	2.78	9.85	7.07
60	2.80	10.79	7.99
90	2.84	11.41	8.57
120	2.80	11.95	9.15
150	2.82	12.36	9.54

As the figures show, the best condition for the reaction is that enzyme concentration, or that reaction time, which gives a carboxyl group increase requiring less than 7 μ l. of 0.05 *N* alkali.

Contact between enzyme and substrate. In histo-enzymic work using microtome tissue sections it is very important to show how far the enzymic activity is dependent upon the number of slices taken to obtain a given amount of tissue. It might be expected that by the use of several thin microtome slices, instead of one thick slice, to obtain a given amount of tissue, the contact between enzyme and substrate would be more complete. Fig. 1, taken from an unpublished study on tumour proteinase, shows that this factor, using the technique described, does not influence the results.

For this work a Walker No. 256 carcinoma was placed in a refrigerator for about 3 hours until frozen, after which a 2.5 mm. diameter pillar was prepared by

means of a cork-borer. The tumour pillar so obtained was placed on the freezing microtome and, from certain selected parts of it, two 25μ sections and four 12.5μ sections were taken immediately after each other for parallel trypsin determinations. Examinations of tumour tissues have shown that sections taken in this manner can be considered histologically identical.

Fig. 1. Contact between enzyme and substrate.

○—○ Tryptic activity of two 25μ sections.
 ×—× Tryptic activity of four 12.5μ sections.

For the activation of the trypsin $7\mu\text{l.}$ of enterokinase¹ [Waldschmidt-Leitz, 1924] containing 30 volume % of glycerol were taken. The microtome sections were placed by means of a very fine glass rod in the drop of activator and allowed to stand for 1 hour at room temperature. After this period of activation, $7\mu\text{l.}$ of $0.1\text{ }M$ veronal-hydrochloric acid buffer of p_H 8.4 were added together with $7\mu\text{l.}$ of a 4 % caseinogen solution also at p_H 8.4.

After 20 hours' incubation at 37° , using soda-lime tubes on the reaction vessels to prevent the diffusion of CO_2 from the atmosphere, the increase in the carboxyl groups was titrated as previously described.

As the curve demonstrates, no essential difference was observed between the results obtained by using two 25μ sections or four 12.5μ sections, thus showing that the present method is suitable for histo-enzymic studies.

The influence of extraction time upon the tryptic activity in microtome slices.

As Fig. 1 shows, the tryptic activity in the tumour tissue is independent of the thickness of the microtome slice over the range 12.5 – 25μ thickness. The second important question is whether, with increasing extraction time, a corresponding increase in the tryptic activity occurs. To study this problem slices from a carcinoma (Walker No. 256) were used, which were prepared from a frozen carcinoma pillar as described for Fig. 1.

Four 25μ sections were obtained from a selected portion of the tumour pillar, two of them being placed in $7\mu\text{l.}$ of 30 % (volume) aqueous glycerol solution and two, to give full activity, being placed in $7\mu\text{l.}$ enterokinase solution containing 30 % by volume of glycerol. These solutions were allowed to stand for 1 hour. Exact duplicates were made, using four 25μ slices from the adjacent portion of the tumour pillar, and these were allowed to stand for 6 hours. After addition of

¹ The enterokinase solution alone did not affect either the caseinogen or the leucylglycine solutions during the reaction period.

7 μ l. of 0.1 *M* veronal-hydrochloric acid buffer and 7 μ l. of 4% caseinogen solution, the solutions were allowed to stand 20 hours at 37°, and the increase of carboxyl groups was measured as described above.

As Fig. 2 shows, practically no difference was observed in the tryptic activity whether the extraction time was 1 or 6 hours. Preliminary experiments indicate

Fig. 2. The influence of extraction time upon the tryptic activity in microtome slices.

- Full activity after 1 hour's extraction time.
- ×—× Full activity after 6 hours' extraction time.
- Initial activity after 1 hour's extraction time.
- ×---× Initial activity after 6 hours' extraction time.

that only a certain part of the tryptic activity is thus extracted, the remainder of the activity being connected with the tissue. This phenomenon may be compared with the lyo- and desmo-enzymes studies by Willstätter and Rohdewald [1932] and with a similar observation on pepsin by Holter and Linderstrøm-Lang [1934].

SUMMARY.

The present paper describes a micro-method for the determination of tryptic activity in tissue sections. The reproducibility of the method is ± 0.00007 mg. amino-nitrogen. The method is suitable for histo-enzymic studies.

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