

PEPTIDASE ACTIVITY IN LEUCOCYTES, ERYTHROCYTES AND PLASMA OF YOUNG ADULT AND SENILE SUBJECTS^{1, 2}

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INTRODUCTION AND REVIEW OF THE LITERATURE

In the present work an attempt was made to find out whether the peptidase activity of the human blood changes with age. Preliminary to this a study of the characteristics of peptide hydrolysis was undertaken, and the degree of enzymatic activity in the various blood constituents was investigated.

Grassmann and Heyde (1) demonstrated the ability of serum to hydrolyse di- and polypeptides. Various authors have found that the peptidase activity of serum is increased in certain pathological conditions, such as during inflammatory processes, after bone fracture, and after burns (1-4). Concerning the possible source of the serum peptidases Zamecnik, Stephenson, and Cope (4) suggest that they may derive from muscle, skin, subcutaneous tissue and erythrocytes. Fruton and co-workers (5, 6) believe that peptidases may have a common origin, most probably the lymphoid cells present in the various tissues, and that the peptidase activity of serum might be due to the disintegration of lymphoid tissue.

Peptidase activity in erythrocytes has been demonstrated by a number of investigators (4, 7-10). Johansen and Thygesen (8) showed that per unit volume the rate of hydrolysis of alanylglutylglycine (AGG) by human erythrocytes is 40-50 times higher than by serum. Merten and Winschuh (11) report an increase in di-peptidase activity of human erythrocytes in various pathological conditions.

It has long been known that leucocytes are able to break down peptides [compare the literature review of Husfeldt (10); Merten and Winschuh (11)]. Husfeldt (10) demonstrated di-, tri-, and tetra-peptide hydrolysis by leucocytes

from a normal subject and a patient with myeloid leukemia. Hydrolysis of alanylglutylglycine by leucocytes from patients with lymphatic leukemia has been observed by Oelkers (12).

Little is known about the relation of the age of the organism to the proteolytic activity of blood. Goldenberg and Kondrachina (13) reported that the rate of autolysis of rabbit serum at pH 1 decreases with the age of the animal. Maver and co-workers (14), on the other hand, noted that serum from old normal rats apparently hydrolysed dl-leucylglycine at a faster rate than did the serum from younger rats.

SUBJECTS

Two groups of subjects were studied. The first group included 15 members of the staff, nine male and six female, ranging in age from 19 to 32 years. The second group of 12 comprised a number of senile patients, five male and seven female, in a mental institute. The members of this group were not bedridden, and none of them suffered from any known organic or infectious disease. Their ages ranged from 67 to 90 years.

METHOD

Twenty ml. of non-fasting blood was obtained from the cubital vein in the morning, and was poured immediately into flasks containing 40 mg. of potassium oxalate. A white blood count and differential count were made immediately. The white blood cells were separated by centrifugation of whole blood in waisted tubes (15). Plasma was removed by means of a capillary pipette. The buffy layer which contained white blood cells and platelets, plus a few red blood cells, was transferred with a second capillary pipette to a centrifuge tube, suspended in about 2 ml. of physiological saline solution and centrifuged at low speed for approximately two minutes. (Longer centrifugation caused the cells to adhere together in small clumps which could not be broken up on shaking.) After centrifugation the clear supernatant was sucked off with a pipette and the cells resuspended in from 2 to 4 ml. of saline and stored overnight in the refrigerator. The red blood cells were pipetted with a long capillary pipette from the bulb of the centrifuge tube and washed in the same manner as the white cells. The concentration of cells in these saline suspensions was determined by means of the standard

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² An abstract of this paper appeared in Proceedings of the Canadian Physiological Society, October, 1950.

clinical procedures, using Turck's and Hayem's dilution fluids. All counts were made in quadruplicate.

The substrate used was glycylglycylglycine (GGG). Hydrolysis was measured by the micro-titration technique of Grassmann and Heyde. The reaction mixture was set up in triplicate in glass-stoppered 2 ml. volumetric flasks. It was composed of 1 ml. of 0.1 M substrate, 0.2 ml. of 0.01 M cobalt sulfate, 0.01 ml. toluene and 0.8 ml. of plasma, or 0.6 ml. of distilled water and 0.2 ml. of cell suspension containing approximately 10^6 leucocytes or 10^6 erythrocytes.³ The reaction was carried out in a water bath at a temperature of 39°C and at a pH of 7.0, maintained within less than 0.1 unit by the buffering effect of the substrate which had been brought to pH 7.0 with 0.5 N NaOH. Hydrolysis during a two to four hour period was measured at frequent intervals by titrating 0.2 ml. samples with 0.01 N KOH (final concentration 90% alcohol), using thymolphthalein as indicator. At the dilution of red blood cells used no serious difficulty with the endpoint was encountered.

RESULTS

Preliminary experiments regarding some of the characteristics of the hydrolysis of GGG will be described first. The rate of hydrolysis by plasma,

³ In order to facilitate cytolysis the cell suspensions were incubated with the distilled water and the cobalt sulfate solution for 15 minutes before the addition of substrate, and the tubes vigorously agitated. Cell counts immediately after substrate addition (zero experimental time) and during the course of hydrolysis showed that the red cells were completely hemolyzed but only about 40% of the white cells from both young adult and senile subjects were cytolized at zero time and about 50% after two hours incubation. In all experiments the peptidase activity is based on the cell count before cytolysis occurred.

TABLE I

The effect of dilution of blood components on the rate of hydrolysis of GGG

Substrate 0.05 M GGG, pH 7.0

| Enzyme source | Concentration per ml. | Per cent hydrolysis per hour | |
|---------------|-----------------------|------------------------------|----------------------------|
| | | Observed | Calculated for 0.2 ml./ml. |
| Plasma* | 0.10 ml. | 7.0 | 14.0 |
| | 0.15 ml. | 9.5 | 12.7 |
| | 0.20 ml. | 12.0 | 12.0 |
| | 0.26 ml. | 17.0 | 12.6 |
| | 0.40 ml. | 24.4 | 12.2 |
| R.B.C. | 96 million cells | 45 | 0.48 |
| | 72 million cells | 34 | 0.47 |
| | 48 million cells | 23 | 0.48 |
| W.B.C. | 1.34 million cells | 16.0 | 11.2† |
| | 0.96 million cells | 12.7 | 12.3† |

* The plasma was slightly hemolysed which accounts for the high rate of hydrolysis.

† Values corrected by subtraction of the rate of hydrolysis due to the presence of erythrocytes.

white cells, or red cells, is constant until about 70% of the substrate is split, assuming complete splitting of one peptide linkage (see Figure 1). Beyond 100% theoretical splitting of a single bond of the tripeptide, hydrolysis continues but at a slower rate. To what extent the breakdown of the di-peptide contributes to the earlier stages of GGG hydrolysis is not known. The rate of hydrolysis by plasma, erythrocytes, and leucocytes was found

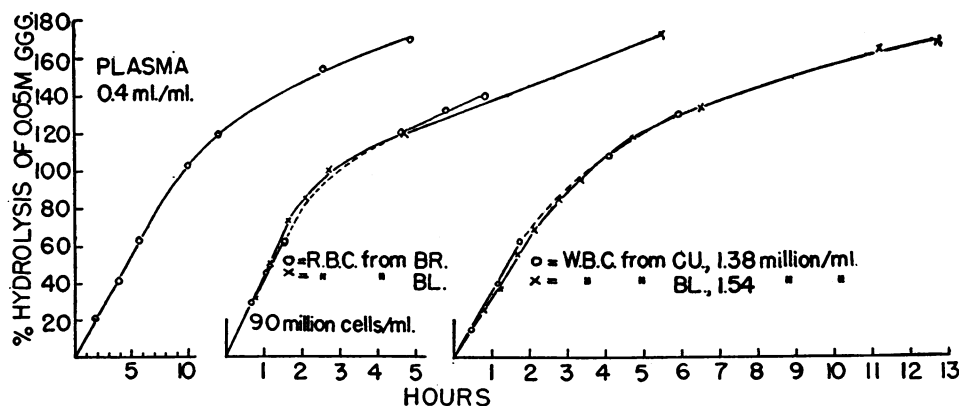


FIG. 1. THE COURSE OF HYDROLYSIS OF GGG BY PLASMA, LEUCOCYTES, AND ERYTHROCYTES

The reaction follows a similar pattern with all three enzyme sources, i.e., the hydrolysis rate is constant until about 70% of the substrate is split; after 100% theoretical splitting of one peptide bond of the tripeptide further hydrolysis occurs but at a slower rate.

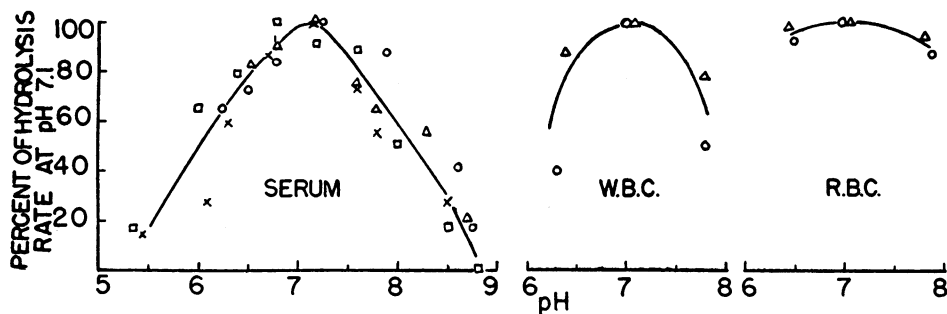


FIG. 2. EFFECT OF pH ON THE HYDROLYSIS RATE OF GGG BY SERUM, LEUCOCYTES, AND ERYTHROCYTES

The substrate was adjusted to the desired pH by adding the required amounts of HCl or NaOH. The drift in pH during the course of hydrolysis was negligible. Similarly marked points in each curve were obtained on samples of the same blood.

to be directly proportional to the concentration of these constituents in the reaction mixture (see Table I). The effect of metal ions on the hydrolysis rate is shown in Table II. Cobalt activates, magnesium and manganese inhibit the hydrolysis by plasma, red cells and white cells. It may be noted that a similar effect of these metal ions has been observed in this laboratory with the hydrolysis of l-leucylglycylglycine (LGG) by human serum (2). The effect of pH on the hydrolysis of GGG by various blood constituents is shown in Figure 2. Optimal hydrolysis occurs around pH 7.1.

The similar characteristics of the hydrolysis of GGG makes it likely that the same enzyme mechanism is involved in plasma, leucocytes, and erythrocytes.

The effect of temperature was studied using serum as the source of the enzyme. The rate of hydrolysis increases with increasing temperature

between 0° and 48°C, but at higher temperatures inactivation sets in (see Figure 3).

Regarding the relative activity of the blood components, it may be seen from Table III that in normal young people the average rate of hydrolysis per cell is about 40 times higher in white blood cells⁴ than in erythrocytes. In view of the high concentration of erythrocytes in blood, however, the activity of a sample of whole blood is mainly due to the hydrolysis by red blood cells. Using the average rates of hydrolysis of GGG in young adults and average normal values for red and white cell counts and plasma volume (16) it may be estimated that in a unit of whole blood the activity of the white blood cells is about six times greater, that of the red blood cells about 100 times greater than that of plasma. If the activity

⁴ The hydrolysis of GGG by white blood cells has been corrected by subtraction of the rate of hydrolysis due to the presence of erythrocytes in the reaction mixture. In most cases this value was less than 10% of the total rate.

TABLE II

Effect of metal ions on the hydrolysis of GGG by plasma, leucocytes, and erythrocytes

Per cent hydrolysis per hour of 0.05 M GGG of pH 7.0, calculated for a concentration of 0.2 ml. plasma, or 10⁶ cells per ml. reaction mixture.

| | Plasma | R. B. C. | W. B. C. | |
|--------------|--------|----------|----------------|----------|
| | | | Age of subject | |
| | | | 28 Years | 67 Years |
| No activator | 4.0 | 0.25 | 15 | 21 |
| Co (0.001 M) | 5.6 | 0.43 | 19 | 31 |
| Mn (0.001 M) | 3.1 | 0.16 | 10 | 16 |
| Mg (0.01 M) | 3.5 | 0.14 | 9 | 13 |

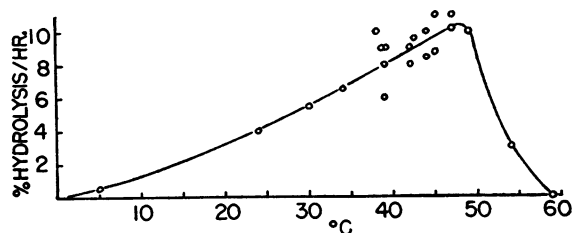


FIG. 3. EFFECT OF TEMPERATURE ON THE HYDROLYSIS RATE OF GGG BY SERUM

Values represent per cent hydrolysis by 0.2 ml. of serum per ml. reaction mixture. Serum was obtained at various times from one subject.

of one volume of plasma is compared with that of a volume of packed white and packed red cells, ratios of 1 to 480 to 125 are obtained. A similar proportion of enzyme activity among blood components was observed in experiments in which dl-leucylglycylglycine was used as substrate. The relative rates for plasma and red blood cells are somewhat higher, but of the same order of magnitude as the comparative values for AGG hydrolysis by human serum and erythrocytes estimated by Johansen and Thygesen (9). No clear cut data in the literature on the relative activity of white blood cells are known to us.

From Table III it may be seen that the rate of hydrolysis of GGG by white blood cells is considerably higher in the old age group than in the young adult group. The average values of hydrolysis of 0.05 M GGG per hour for one million cells per ml. of reaction mixture are 26 and 15% respectively. The difference is statistically significant ($P < 0.001$). (The averages for male and female subjects correspond closely in both groups.) When the values for plasma or red blood cells are compared no significant difference between the two groups is seen. No correlation between activity of white blood cells and the percentage of

TABLE III
Hydrolysis of GGG by leucocytes, erythrocytes, and plasma of young and old people

| Name | Age | Sex | Total W.B.C. | % Poly. | % Lym. | % Stab. | Hydrolysis % per hour * | | | Remarks | | |
|---------|-----|-----|-----------------|------------|-----------|------------|--|--|--|---|------|-----|
| | | | | | | | 10 ⁶ WBC/ml. reaction mixture | 10 ⁶ RBC/ml. reaction mixture | 0.2 ml. plasma/ml. reaction mixture | | | |
| VE | 27 | M | 6,900 | 17 | 47 | 26 | 18 | 0.35 | 5.5 | Repeat 2 weeks later 1st day menstruation 2nd day menstruation Jan. 6, 1950 April 4, 1950 May 15, 1950 | | |
| WO | 22 | M | | 26 | 67.5 | 0.5 | 17 | 0.27 | | | | |
| CH | 25 | M | | 56 | 42 | 0 | 14 | 0.27 | | | | |
| | | | | 59 | 35 | 4 | 15 | 0.41 | | | | |
| WI | 21 | F | 9,700 | 69 | 25 | 0 | 14 | 0.32 | 7.0 | | | |
| CA | 23 | M | | 61 | 33 | 0 | 18 | 0.25 | | | | |
| BR | 23 | M | | 34.5 | 39 | 19 | 13 | 0.26 | | | | |
| | | | | 50 | 41 | 6 | 12 | 0.34 | | | | |
| CU | 28 | F | 8,250 | 52 | 41 | 0 | 11 | 0.41 | 6.2 | | | |
| | | | 11,675 | 51 | 39 | 4 | 20 | 0.36 | | | | |
| | | | 21,800 | 54 | 33 | 8 | 15 | 0.46 | | | | |
| | | | 6,950 | 51 | 40 | 1 | 18 | 0.38 | | | | |
| DY | 22 | F | 7,500 | 57 | 34 | 5 | 16 | 0.39 | 6.5 | | | |
| PU | 22 | F | | | | | 9 | 0.44 | | | | |
| KO | 26 | F | | | | | 16 | 0.41 | | | | |
| DE | 28 | F | | | | | 19 | 0.43 | | | | |
| | | | 7,100 | 65 | 27 | 6.5 | 21 | 0.46 | 6.8 | | | |
| HO | 26 | M | | | | | 14 | 0.46 | | | | |
| MA | 32 | M | | | | | 20 | 0.42 | | | | |
| KA | 19 | M | | 11,300 | 56 | 38 | 0 | 10 | | | 0.40 | |
| EL | 26 | M | 4,800 | | | | 16 | 0.47 | | | | |
| Average | | | | | | | 15 | 0.38 | 6.4 | | | |
| DA | 76 | F | 13,450 | 70 | 14 | 13 | 28 | 0.33 | 7.5 | Repeat 13 days later Repeat 2 months later | | |
| SM | 68 | F | | 81 | 7 | 7.5 | 22 | 0.34 | | | | |
| MI | 82 | M | | 47 | 48 | 0 | 18 | 0.31 | | | | |
| | | | | | | | 26 | 0.45 | | | | |
| HA | 90 | M | 6,900 | 53 | 40 | 4 | 28 | 0.41 | 7.8 | | | |
| | | | 9,000 | 41 | 53 | 4 | 26 | 0.35 | | | 7.0 | |
| | | | 6,550 | | | 26 | 0.31 | 7.2 | | | | |
| | | | | | | 26 | 0.31 | | | | | 5.5 |
| KY | 94 | F | 11,050 | 60 | 32 | 4 | 30 | | 0.48 | | | |
| CA | 86 | M | 9,750 | 61 | 32 | 4 | 32 | | 0.43 | | 7.8 | |
| NO | 84 | M | 10,200 | 40 | 57 | 1 | 20 | 0.50 | 6.2 | | | |
| McG | 87 | F | 10,775 | 58 | 34 | 2 | 30 | 0.47 | | | | 6.2 |
| HE | 89 | F | 10,525 | 59 | 38 | 2 | 33 | 0.38 | | | | |
| ME | 86 | F | 8,250 | | | | 26 | 0.24 | | | 6.2 | |
| BA | 67 | F | | | | | 25 | 0.40 | 6.2 | | | |
| BR | 71 | M | | | | | 23 | 0.35 | | | | 6.2 |
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* Per cent per hour of the hydrolysis expected on the complete splitting of one peptide linkage.

polymorphonuclear leucocytes and lymphocytes in the differential count was apparent, and the slight difference in the distribution of the cell types among the two groups could not account for the difference in enzymatic activity. The response to metal ion activation was compared in two subjects (Table II) and no significant difference was observed. The possibility that the difference in rate among the two groups is due to an increased state of metal activation inherent in the white blood cells of the old age group is therefore unlikely. Although no pH optimum for hydrolysis of white blood cells from old people was determined, it is evident that the differences in activity cannot be due to a different response to pH since all studies were carried out in the optimum pH range for the activity of white blood cells in young people.

DISCUSSION

The increase in peptidase activity of white blood cells from aged subjects is noteworthy from a clinical standpoint in view of the role of leucocytes in processes of infection and tissue repair. It is possible, however, that the rise in peptidase activity might be a manifestation of increased protein metabolism in general, associated with old age. It must be pointed out that the old age group was composed largely of subjects who were institutionalized because of senile mental changes, and whose food intake was probably lower than that of the control group. The study will be continued on mentally normal old people. It is of interest that insulin, a polypeptide, has been found to lose its blood sugar reducing power when it is incubated with whole blood, and that blood from old people inactivates the hormone at a significantly higher rate than blood from young people (17). There was an indication that the leucocytes were responsible for this effect. This would be in line with the present observations, provided that a hydrolytic mechanism is involved in the inactivation of insulin.

The rise in peptidase activity of the white blood cells in the senile group was not accompanied by a significant rise in peptidase activity of plasma. This does not necessarily exclude the possibility that white blood cells are a source of plasma peptidase since the rate of dissolution of leucocytes rather than the actual activity of living cells may determine the level of enzymes in plasma. It has

been observed that the leucocytes circulating in the blood stream of old people are older cells than those in the blood from young persons (18). This indicates that the rate of turnover is lower in old age. It should, however, be kept in mind that per unit of whole blood by far the greatest enzyme activity is found in the red blood cells. Whether erythrocytes, leucocytes, platelets, or other tissue cells are the main source of the enzyme in plasma can only be determined if the rate of breakdown and turnover, as well as the relative enzyme activity of these constituents, is known.

SUMMARY

1. The hydrolysis of glycylglycylglycine (GGG) by the components of blood from young adult and from senile human subjects, and the effects of metal ions, pH, and temperature on the hydrolysis were studied.

2. Hydrolysis by plasma, erythrocytes and leucocytes was activated by cobalt, inhibited by magnesium and manganese and optimal at pH 7.1. In view of the similar characteristics of hydrolysis by the blood components it was concluded that the same enzyme mechanism must be involved in all cases.

3. The enzyme activity in the formed elements by far exceeded that of serum or plasma. In normal young people a volume of packed red cells was estimated to be over 100 times as active as a volume of plasma, and packed white blood cells nearly 500 times as active as plasma. Per unit cell the activity of leucocytes from young people was found to be about 40 times greater than that of the erythrocytes.

4. The rate of hydrolysis of GGG by leucocytes was found to be significantly higher in senile people than in young people, the average increase in rate being about 70%. On the other hand, no significant difference in rate of hydrolysis by plasma or erythrocytes of the two groups was observed.

5. The significance of these findings is discussed.

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REFERENCES

1. Grassmann, W., and Heyde, W., Alkalimetrische Mikrobestimmung der Aminosäuren und Peptide. *Ztschr. f. physiol. Chem.*, 1929. **183**, 32.

2. Barber, V. T., Stern, K., Askonas, B. A., and Cullen, A. M., Peptidases in the human serum. *Proc. Soc. Exper. Biol. & Med.*, 1948, **67**, 421.
3. Stern, K., Cullen, A. M., and Barber, V. T., Peptidase activity in human serum following bone fracture. *J. Clin. Invest.*, 1949, **28**, 419.
4. Zamecnik, P. C., Stephenson, M. L., and Cope, O., Peptidase activity of lymph and serum after burns. *J. Biol. Chem.*, 1945, **158**, 135.
5. Fruton, J. S., On the proteolytic enzymes of animal tissues. V. Peptidases of skin, lung and serum. *J. Biol. Chem.*, 1946, **166**, 721.
6. Holman, H. R., White, A., and Fruton, J. S., Relation of adrenal cortex to serum peptidase activity. *Proc. Soc. Exper. Biol. & Med.*, 1947, **65**, 196.
7. Willstätter, R., Bamann, E., and Rohdewald, M., Zur Kenntnis der proteolytischen Wirkungen farbloser Blutkörperchen, über Enzyme der Leukocyten. *Ztschr. f. physiol. Chem.*, 1929, **185**, 267.
8. Johansen, A., and Thygesen, J. E., Direct dilatometry of peptidase activity of normal human serum (and hemolysate). *Compt. rend. trav. lab. Carlsberg, Sér. Chim.*, 1948, **26**, 369.
9. Schwartz, T. B., and Engel, F. L., The adrenal cortex and serum peptidase activity. *J. Biol. Chem.*, 1949, **180**, 1047.
10. Husfeldt, E., Proteolytische Enzyme in den Leukocyten des Menschen. *Ztschr. f. physiol. Chem.*, 1931, **194**, 137.
11. Merten, R., and Wünsch, M., 1- und d-Dipeptidasen in den Formelementen des menschlichen Blutes. *Ztschr. f. Vitamin-Hormon-u-Fermentforsch.*, 1947, **1**, 35.
12. Oelkers, H. A., Untersuchungen über Fermente der Lymphocyten. *Arch. f. Exper. Path. u. Pharmacol.*, 1931, **161**, 344.
13. Goldenberg, E., and Kondrachina, M., Rate of proteolysis in serum in relation to age of experimental animal. *Bull. eksper. n. biol. i medits., U. R. S. S.*, 1940, **9**, 226.
14. Maver, M. E., Johnson, J. M., and Thompson, J. W., The d-peptidase activity of serum as an alleged diagnostic test for cancer. *J. Nat. Cancer Inst.*, 1941, **1**, 835.
15. Butler, A. M., Cushman, M., and MacLachlan, E. A., The determination of ascorbic acid in whole blood and its constituents by means of methylene blue, macro- and micro-methods. *J. Biol. Chem.*, 1943, **150**, 453.
16. Wintrobe, M. M., *Clinical Hematology*. Lea & Febiger, Philadelphia, 1936, pp. 72, 73, and 159.
17. Kohl, H., Selbach, H., and Janning, A., Über kristallinisches Insulin; der zeitliche Ablauf der Insulin-inaktivierung durch Normalblut. *Arch. f. exper. Path. u. Pharmacol.*, 1937, **185**, 212.
18. Olbrich, O., Blood changes in the aged. *Edinburgh M. J.*, 1947, **54**, 306.