CCL. THE RELATIVE CHOLINE-ESTERASE ACTIVITIES OF SERUM AND CORPUSCLES FROM THE BLOOD OF CERTAIN SPECIES.

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(*Received July 16th, 1935.*)

FOLLOWING the discovery by Stedman *et al.* [1932] that the destruction of acetylcholine by blood serum was brought about by a specific enzyme, which they termed choline-esterase, Stedman *et al.* [1933] examined the blood sera from a number of species for the presence of this enzyme and compared the choline-esterase activities of these sera with their activities towards two other types of esters, namely, methyl butyrate and tributyrin. The results offered ample confirmation, if such were needed, of the specific nature of choline-esterase. It was further demonstrated that sera from different species differed widely with respect to their content of the enzyme in question, those from certain species being, in fact, so deficient in it that none was detectable by the method of estimation which was employed. It was, however, emphasised that the virtual absence of the enzyme from serum did not necessarily imply its absence from the corpuscles, the choline-esterase activities of which could not at the time be determined since the method of estimation used with serum was not applicable to whole blood or to corpuscles. In order to examine this point the present authors have therefore developed a method for the estimation of choline-esterase in corpuscles, defibrinated blood or tissue and have used it to determine the distribution of the enzyme between corpuscles and serum in the blood of a number of species. This work is described in the present communication. Since this investigation, which was reported to the Meeting of the Biochemical Society in Glasgow in June 1934, was completed papers have appeared on the same subject by Ammon [1934] and Ammon and Voss [1935] in which a method for the determination of choline-esterase is described which is identical in principle with that employed in the present work. Inasmuch, however, as Ammon employs a Warburg apparatus whereas we utilise the Barcroft differential apparatus our experimental procedure differs from that of the German workers. The material which we have examined is, moreover, by no means completely identical with that investigated by Ammon.

Estimation of choline-esterase.

The method is an adaptation to choline-esterase of Rona and Lasnitzky's [1924] gasometric method for the determination of esterases, using, however, the Barcroft differential apparatus in place of Warburg's apparatus. The manometer is fitted with two flasks of the type described by Rona and Nicolai [1926] but of about 40 ml. capacity. In the actual estimation there are placed in each flask 5 ml. of bicarbonate Ringer's solution, prepared by mixing 100 ml. of 0·9% sodium chloride with 2 ml. of 1·2% potassium chloride, 2 ml. of 1·76% calcium chloride (calculated on the weight of the hydrated salt) and 20 ml. of 1·26% sodium bicarbonate, and 1 ml. of the liquid, or a weighed quantity of the solid, under investigation. Into the side-tube of one flask are then pipetted 0·5 ml. of the

bicarbonate Ringer's solution and 0·5 ml. of a solution of acetylcholine bromide of a concentration discussed below, the second flask being treated similarly except that water is used in place of the solution of acetylcholine. The apparatus is then assembled and partly immersed in a thermostat at 30°, leaving the stoppers of the side-tubes above the surface of the water. By removing the stoppers and attaching to the manometer, the stopcocks of which have been previously suitably adjusted, a reservoir containing a gaseous mixture composed of 95 % nitrogen and 5 % carbon dioxide, the flasks are swept out with this mixture, the stoppers are replaced, the stopcocks readjusted and the flasks immersed completely in the thermostat. Shaking is then commenced and continued until equilibrium is reached; the contents of the two compartments of the flasks are then mixed and readings of the manometer taken at definite intervals of time. Under the conditions chosen the rate of CO_2 production is linear with time. It should be clear from this description that the determination depends upon the liberation of a molecule of carbon dioxide from the bicarbonate by each molecule of acetic acid produced by hydrolysis of acetylcholine. The gas mixture, containing 5 % of carbon dioxide, serves in conjunction with the bicarbonate as a buffer to maintain the p_H of the reaction mixture at 7·4. Determination of the constant of the apparatus, which represents the volume of carbon dioxide liberated under the atmospheric pressure obtaining at the time of the experiment but at a temperature of 0° for each unit difference, on an arbitrary scale, between the meniscus levels in the two limbs of the manometer, can be conveniently effected by means of a standard solution of acetic acid.

That the above method yields consistent results is shown by Table I, in which are recorded the results obtained, using four different apparatus, with one and the same purified solution of choline-esterase. The maximum deviation from

Table I. *Comparison of results using different apparatus.*

Duration of experiment: 40 min.

Apparatus number	Constant	Manometer reading	Bar mm.	Carbon dioxide at N.T.P. ml.
1	0·0477	4·18	740	0·194
2	0·0292	6·98	740	0·198
3	0·0470	4·34	740	0·199
4	0·0354	5·58	740	0·192

the mean value is only 1·5 %. But perhaps the best demonstration of the accuracy of the method is the comparison of the results which it yields with those obtained under similar conditions of temperature, p_H and substrate concentration by the titration method described in our earlier communication. Thus, using 1 ml. of an 80-fold dilution of a purified solution of choline-esterase, the following results were obtained by the manometric method in two experiments of 50 min. duration: App. 1a; const., 0·0374; reading, 3·65; bar., 744·5; CO_2, 0·134 ml. App. 2; const., 0·0292; reading, 4·88; CO_2, 0·140 ml. The mean volume of CO_2 produced is thus 0·137 ml. In a titration experiment, using 20 times the amount of enzyme and of substrate in a solution of approximately 20 times the volume, 2·40 ml. of 0·02 N alkali were required to maintain the p_H of the solution at 7·4 during a period of 20 min. This would correspond with the liberation in 50 min. of 0·134 ml. of CO_2 by the amount of enzyme used in the manometric experiments, which is in excellent agreement with the value actually found.

Influence of substrate concentration on activity of enzyme.

For the purpose of ascertaining the best substrate concentration to employ in the determination of choline-esterase by the above method, five solutions of different dilutions of acetylcholine were prepared as follows: solution no. 1, 5 g. acetylcholine bromide in 10 ml. of water; no. 2, solution no. 1 diluted with an equal volume of water; no. 3, solution no. 2 diluted with an equal volume of water; no. 4, 2 volumes of no. 2 diluted to 5 volumes; no. 5, 2 volumes of no. 2 diluted to 8 volumes. The non-enzymic hydrolysis of acetylcholine in these solutions, under the conditions defined above for the estimation of choline-esterase, was first determined, with the results shown in Table II. A comparison was then

Table II. *Non-enzymic hydrolysis of acetylcholine.*

Duration of experiment: 30 min.

Solution of acetylcholine no.	CO_2
1	0·051
2	0·027
3	0·008
4	0·011
5	0·004

Table III. *Influence of substrate concentration on activity of choline-esterase.*

Duration of experiment: 30 min.

Solution of acetylcholine no.	CO_2	CO_2 (corr.)
1	0·390	0·339
2	0·346	0·319
3	0·324	0·316
4	0·321	0·310
5	0·248	0·244

made of the carbon dioxide liberated according to the above procedure when 1 ml. of a purified solution of choline-esterase was employed as enzyme with 0·5 ml. of each of the above solutions as substrate. The results are recorded in Table III, the figures in the last column being corrected for non-enzymic hydrolysis. It is evident that the optimum substrate concentration has not been reached in these experiments. Nevertheless, at concentrations higher than that of solution no. 4 the rate of increase of enzymic activity with increase in substrate concentration is small. A solution of this concentration has therefore been adopted for use with the method of estimation here described. Apart from economising material, the employment of this solution in preference to one of higher concentration possesses the advantage that the non-enzymic hydrolysis is so small as to be almost negligible.

Distribution of choline-esterase in normal human blood.

The method described above has been used for measuring the relative choline esterase activities of human blood from a number of normal individuals. The results obtained are given in Table IV, the figures representing ml. of carbon dioxide evolved in 50 min. by the action of the choline-esterase present in 0·05

Table IV. *Distribution of choline-esterase in normal human blood.*

Subject number	Defibrinated blood	Serum	Corpuscles
1	0·377	0·353	0·198
2	0·268	0·148	0·179
3	0·256	0·126	0·195
4	0·244	0·133	0·184
5	0·243	0·138	0·165
6	0·232	0·144	0·156
7	0·213	0·111	0·146
8	0·199	0·115	0·126
9	0·198	0·084	0·141

ml. of blood or serum or in the corpuscles originally contained in 0·05 ml. of blood. Defibrination of the blood was effected by shaking with glass beads. The serum was obtained by centrifuging the defibrinated blood, whilst the corpuscles were washed twice with saline and then suspended in sufficient of this fluid to bring them to a volume equal to that of the blood in which they were originally contained. In each case 1 ml. of the material (defibrinated blood, serum or suspension of washed corpuscles) so obtained was dissolved in water and diluted to 20 ml., 1 ml. of the dilute solution being used in each estimation; the corpuscles were, of course, laked in this process. It should, perhaps, be mentioned here that if the dilution of the material to be examined is made with water, this should be done immediately prior to the actual estimation. Otherwise the choline-esterase activity of the material may diminish owing to the smaller stability of the enzyme in dilute solution.

Distribution of choline-esterase in blood from other species.

The choline-esterase contents of blood from a number of other species have also been examined. The relevant data are recorded in Table V. The technique of the estimations was identical with that used with human blood except that,

Table V. *Distribution of choline-esterase in blood from various species.*

Species	Defibrinated blood	Laked defibrinated blood	Serum	Corpuscles	Laked corpuscles
Horse	0·108	0·111	0·129	0·044	—
Guinea-pig	0·071	0·068	0·069	0·034	0·033
Ox	0·071	0·079	0·005	0·071	—
Pig	0·065	0·061	0·024	0·052	—
Sheep	0·037	0·039	0·004	0·036	—
Cat	0·023	0·022	0·035	0·0	—
Fowl	0·023	0·022	0·035	0·0	—
Duck	0·022	—	0·038	0·0	—
Rabbit	0·022	0·023	0·024	0·012	—
Goat	0·020	—	0·006	0·015	—

in most cases, a larger amount of material, frequently 1 ml., was used. This was necessitated by the smaller content of choline-esterase in the blood of these species. In order, however, to make the figures comparable with one another and also with those of Table IV they have been recalculated to correspond with the volume of carbon dioxide which would be evolved in 50 min. by the action of the choline-esterase present in 0·05 ml. of the material in question. It will be noted that, in some instances, estimations have been carried out both on laked and unlaked blood or corpuscles. Laking, in these cases, was effected either by dilution with water or by repeated freezing and thawing. It is quite evident from the figures that the choline-esterase activity of blood is in no way dependent on the presence of the intact corpuscles.

Examination of cerebrospinal fluid and brain tissue.

The application of the technique described above has so far been mainly confined to blood and has not yet been extended to a systematic investigation of various tissues for the presence of choline-esterase. An examination has, however, been made of cerebrospinal fluid and of brain tissue. In the former case no trace of the enzyme could be detected. Cerebrospinal fluid from two species, cat and man, has been examined both with the titration and gasometric methods. The results were entirely negative even when as much as 1 ml. of the material was

employed in the latter method. The brain, on the other hand, contains relatively large quantities of choline-esterase, the concentration in the basal ganglia being, in the cat, approximately twice that in the cortex. Thus, the basal ganglia and cortex from a cat's brain, which had been perfused with saline until completely free from blood,[1] were separately minced and examined by the gasometric method using, in each case, 0·25 g. of the tissue. Duplicate estimations were carried out, the following volumes of carbon dioxide being produced in a period of 15 min.: basal ganglia, 0·463, 0·448; cortex, 0·216, 0·204 ml.

Summary and Discussion.

A method for the estimation of choline-esterase applicable to whole blood, corpuscles or tissue has been described and the results of its application to blood, cerebrospinal fluid and brain tissue recorded. The values obtained for the choline-esterase contents of blood sera from various species are in general agreement with those previously found by Stedman *et al.* [1933] using the titration method. Only in one case, namely, that of the cat, does any marked divergence appear between the results yielded by the two methods. This is no doubt due to the fact, exemplified by the results obtained with human blood, that considerable variations occur amongst individuals of a given species. In conformity with previous results, the blood sera from certain species (ox, sheep, goat) have been found to be, within the limits of experimental error, entirely deficient in choline-esterase. The corpuscles from these animals, however, are now shown by estimations both on defibrinated blood and on washed corpuscles to contain appreciable amounts of the enzyme in question. On the other hand the corpuscles from certain species (cat, fowl, duck) have proved to be devoid of any choline-esterase activity.

Choline-esterase is entirely absent from cerebrospinal fluid. Considerable quantities occur, however, in the brain, the concentration in the basal ganglia being approximately twice that in the cortex. This conforms with the findings of Dikshit [1934] that higher concentrations of acetylcholine or of an analogous substance are present in the former than in the latter material.

The expenses of this investigation have been partly met by grants from the Earl of Moray Research Fund of Edinburgh University.

[1] The authors desire to thank Dr B. B. Dikshit for preparing this material for them.

REFERENCES.

Ammon (1934). *Pflüger's Arch.* **233**, 486.
—— and Voss (1935). *Pflüger's Arch.* **235**, 393.
Dikshit (1934). *J. Physiol.* **80**, 409.
Rona and Lasnitzky (1924). *Biochem. Z.* **152**, 504.
—— and Nicolai (1926). *Biochem. Z.* **172**, 82.
Stedman, Stedman and Easson (1932). *Biochem. J.* **26**, 2056.
—— —— and White (1933). *Biochem. J.* **27**, 1055.