In early postnatal life the peaks of the loops are still covered by this type of epithelium, while in the second year the histological appearance is similar to that of the adults.

Further studies are needed to prove the validity of this observation and to further elucidate its mechanism.

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Chromodacryorrhea, a New Criterion for Biological Assay of Acetylcholine.

SHIRO TASHIRO, CARL C. SMITH,* ELIZABETH BADGER AND EDWARD KEZUR.

From the Department of Biochemistry, College of Medicine, The University of Cincinnati.

The peculiar phenomenon of the shedding of bloody tears by rats was reported in connection with the studies of dacryorrhetin, a compound prepared from muscle.[1, 2] This phenomenon is so unique and easily distinguishable from ordinary lacrimation that the term, chromodacryorrhea, is proposed for it. It has served as a very convenient criterion for the biological assay of dacryorrhetin.

When Selye[3] published a paper in which he quotes Freud's observation[4] that acetylcholine causes rats to shed tears tinged red by blood,† one of us (T) examined chromodacryorrhetic and other properties of acetylcholine to see if dacryorrhetin could be in reality acetylcholine.[5] The results of these investigations showed that these two compounds are not identical and at the same time suggested a possibility of using chromodacryorrhea as a new criterion for a biological assay of acetylcholine. We have thus determined how small amounts of acetylcholine can be detected accurately by this criterion under different conditions.

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* Charles Arnold Iglauer Fellow in Biochemistry.

1 Tashiro, Shiro, and Stix, Helen, *Biol. Bull.*, 1935, **64**, 327.

2 Tashiro, Shiro, *Proc. Am. Soc. Biochem.*, 1937, **8**, xcviii.

3 Selye, Hans, *Canadian Med. Assn. J.*, 1937, **36**, 200.

4 Freud, J., *Acta Brevia Neerl.*, 1933, **3**, 159.

† Although no casual observer would question the presence of blood in tears, and it gives a positive benzidine reaction and its bands are much like those of oxyhemoglobin when examined with a hand spectroscope, yet Tashiro and Badger have evidence that the red pigment in the bloody tears is not oxyhemoglobin.

5 Tashiro, Shiro, Kongressbericht. II. des XVI Internat. Physiologenkongress 1938, 46.

Experimental. The Criterion. The phenomenon of bloody tear flow in rats occurs within 2-5 minutes after an intraperitoneal injection, usually following salivation and clear tears; and it is almost instantaneous with an intravenous injection of effective doses. If salivation and clear tears are absent within 2-5 minutes after injection, usually bloody tears never flow. In the borderline cases, the naked eye may fail to recognize bloodiness in the tear, but with filter paper a slight tinge of red may be detected. In such a case, chromodacryorrhea is considered to be ±.

Animals. For intraperitoneal injections, rats of any size, sex and age are used; but for intravenous injections, young rats weighing 100-200 g are chosen. For intravenous injection, the tail is carefully washed, immersed in warm water and immediately the solution is injected into the caudal vein by means of a 27 gauge needle with a tuberculin syringe.‡ The same rats can be used repeatedly if they are injected only once a day.

Eserine Treatment. Preliminary experiments on optimum conditions for eserinization of rats show that an intraperitoneal injection of 50 γ eserine sulfate per 100 g body weight is best for both intraperitoneal and intravenous injections of acetylcholine. In either case, an acetylcholine solution is injected after the eserine effect becomes obvious (5-10 minutes). A convenient solution for eserinization is prepared by dissolving 10 mg of eserine sulfate in 20 cc H_2O, 0.1 cc of which contains 50 γ, the exact quantity necessary for each 100 g body weight.

The Minimum Chromodacryorrhetic Doses. The chromodacryorrhetic response was determined for 4 different cases, intraperitoneal injection with and without eserine and intravenous injection with and without eserine. The minimum effective doses chosen are those amounts with which a majority of rats shed bloody tears from both eyes detectable with the naked eyes without the aid of filter paper. They are expressed as the weights of acetylcholine iodide on the basis of 100 g body weight of the rats. The results of these experiments are given in Table I, in which the value for intraperitoneal dosage without eserine represents an average, as one naturally expects a wider variation under this condition. Freud states the normal effective dose (intraperitoneal) in rats of 100-120 g to be ±6 mg, which we presume to be of the iodide and to mean that the minimal dosages are around 6 mg.

The fact that the rat is more sensitive to intravenous injection

‡ Thanks are due to Dr. A. R. Sabin for his kind suggestions as to technic of intravenous injection in rats.

TABLE I.
Minimal Chromodacryorrhetic Dosages of Acetylcholine Iodide, Calculated on the Basis of 100 g Body Weight of Rats.

Mode of injection	Without eserine γ	With eserinized rats γ
Intraperitoneal	2,000	50.0
Intravenous	10-15	0.2

without eserine than to the intraperitoneal with eserine, and the fact that the eserinization increases sensitivity only about 50 times (10-15 γ to 0.2 γ) in the case of intravenous injection are rather interesting in view of the fact that one of us (S)[6] found an exceedingly small amount of the esterase in the serum of rats as compared to human serum.

The Quantitative Assay. These minimum effective dosages can be used for the basis of the quantitative assay of acetylcholine, if less than 1 cc of the solution contains more than 0.2 γ. Without eserinization, the intravenous injection gives fairly uniform results, but the results with intraperitoneal injection without eserine should be considered preliminary and approximate. The latter will be found useful when the available sample is large or highly concentrated such as in the study of acetylcholine synthesis and it will be exceedingly accurate for the estimation of other choline derivatives in which chromodacryorrhea is not enhanced by eserinization.

In either event, the quantitative assay of an unknown is done by determining the minimum positive and the maximum negative doses, accuracy depending upon the range between these two doses. For the actual analysis, a number of rats are weighed and about five rats are eserinized at the same time. Usually 0.1 cc of the solution per 100 g body weight is injected intraperitoneally. If the reaction is positive, a smaller amount or a diluted solution is similarly injected. If negative, the maximum injectable amount of the original solution should be injected intravenously to see if a measurable amount of acetylcholine is present. If positive a smaller amount or a diluted solution is injected. The cc per 100 g body weight with which the majority of rats give the reaction contain the minimum effective amount. Thus the concentration per cc of the original unknown solution will be: for intraperitoneal injection, 50 γ ÷ cc per 100 g body weight containing the minimum effective dose; and for intravenous injection, 0.2 γ ÷ cc per 100 g body weight. If the original solution is diluted, the value, of course, should be multiplied by the factor.

[6] Smith, Carl C., unpublished data.

Individual Variation of Rats. One should remember that occasionally he will find a rat which does not respond to dosages several times as large as the minimal effective dose. These variations must be due to the abnormal condition of Harder's glands, as one occasionally finds one or both glands lacking in some rats. There will be also a slight variation around the minimal dosage. Thus, if a large number of eserinized rats are injected intravenously with 0.2 γ acetylcholine iodide, there will be some which fail to shed bloody tears, although usually all will shed them with 0.3 γ dose. This variation is not due to a difference in sex or age. It is not probably due to variation in the esterase activity since a similar variation does occur when examined with the minimal dose of another choline derivative which is not destroyed by esterase. It is most likely due to a physiological condition of the glands, in which a slight change might be sufficient to produce a different response to the minimal dose. In any event, by selecting the minimum dosages in which the majority of rats shed bloody tears, this variation can be ignored.

Specificity of This Test. Chromodacryorrhea is not a specific test for acetylcholine any more than other methods of biological assay of acetylcholine. According to Freud, 0.5-1 mg of pilocarpine gives the reaction, but 20-80 mg of choline have no effect. We also find that choline and eserine do not produce chromodacryorrhea, although we noticed a few cases in which the animals shed the bloody tears during their death struggle following injection of very high toxic dosages of these compounds. Such rare cases will not interfere with the assay, for a mere dilution will eliminate the reaction. A powerful chromodacryorrhetic action of dacryorrhetin, although prepared from muscle, should not interfere with this test in a tissue analysis for acetylcholine, as this substance exists in the body as prodacryorrhetin which has no chromodacryorrhetic action. If necessary, one can easily distinguish dacryorrhetin from acetylcholine by comparing the effective doses with and without eserine, as the action of the former will not be appreciably enhanced by eserine.

Summary. 1. By using chromodacryorrhea, the phenomenon of the shedding of bloody tears by rats, as a criterion, acetylcholine can be detected in as small amount as 0.2 γ. 2. Since there are 4 ranges of minimal dosages detectable with this phenomenon, from 2 mg to 0.2 γ, depending on the mode of injection and treatment, this same criterion can be used for a wide range of concentration of acetylcholine with accuracy.