

HENLE'S REACTION OF THE CHROMAFFIN CELLS IN THE ADRENALS, AND THE MICROSCOPIC TEST FOR ADRENALIN.

BY TOMOSABURO OGATA AND AKIRA OGATA.

(From the Pathological Institute, and the Chemical Laboratory of the Pharmaceutical Institute of the Imperial University of Tokyo, Tokyo, Japan.)

PLATE 67.

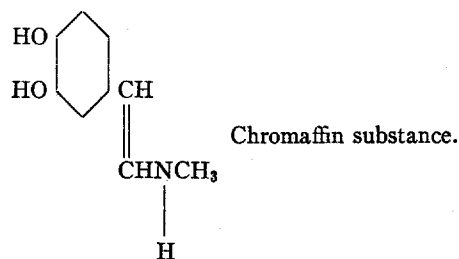
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HISTORICAL.

In 1865 Henle (1) discovered a brown coloration of the medullary cells of the adrenals when remaining in a solution of potassium dichromate, which at that time was generally used in histology for the hardening of tissues. Henle's reaction has since been confirmed by many investigators, and was also found to occur in other parts of the body; *e.g.*, in the carotid gland, in the sympathetic ganglia, in Zuckerkandl's gland, etc. The cells with a positive reaction are always found in groups, mixed with sympathetic ganglion cells; and numerous embryological investigations have made it clear that both are derived from the same ectodermal mother cells. On the other hand, comparative anatomical studies have shown that the cortex (interrenal system) and the medulla (suprarenal system) of the adrenals are genetically foreign to each other, and in lower vertebrates exist as two independent organic systems. On the basis of these results it can scarcely be doubted that the medullary portion of the adrenals represents, with all tissues giving a positive chrome reaction, a system which stands in close relation to the sympathetic nervous system (Kohn (2), Biedl (3), Wiesel (4, 5)). This characteristic behavior towards chromates was taken into consideration in naming these cells; *e.g.*, chromaffin (Kohn (2)), chromophil (Stilling (6)), chrome brown, or pheochrome (Poll (7)). The term chromaffin is the most commonly used. This term, as Kohn has stated, expresses only the ability of the cells, tissue, or system to be colored brown by dichromate or chromic acid, when in a fresh state, while with the chromaffin substance of Borberg and Ciaccio (17, 18) the term chromaffin indicates chemical affinity. While the chrome reaction was thus established as a characteristic of the chromaffin cells, numerous chemico-physiological investigations convinced us, on the other hand, that these cells produce adrenalin and supply it to the vascular system. This substance is considered an im-

portant hormone for the regulation of the vascular tonus. Langlois and Vincent were the first to show that adrenal extract causes an increase of blood pressure. Later Takamine isolated from adrenal extract a chemical compound which increased blood pressure, and which he named adrenalin. According to some authors (Biedl, Wiesel, etc.), the extract of extracapsular chromaffin tissues, *e.g.*, those of Zuckerkandl's gland, shows the same property of raising blood pressure as that of the adrenals. It is thus clear that adrenalin is a constant chemical constituent of chromaffin tissues. Biedl proposed the terms adrenal cells, adrenal tissue, adrenal bodies, adrenal organs, and adrenal system. He did not, however, always use the term adrenal cells, but substituted for it that of chromaffin cells (Kohn). In France the terms "*cellules adrénalogènes*" (Bonnamour (8)) and "*cellules adrénalinogènes*" have since been in use.

As has been explained above, two constant characteristics, affinity for chromic acid and chromates (Henle's reaction) as a histological, and the production of adrenalin as a physiological characteristic, were established in certain kinds of cells. However, it has not yet been made entirely clear whether these two peculiarities exist accidentally or whether there is a distinct relation between them. While the experiments of Kahn (10), Schur and Wiesel (11), etc., showed parallelism between the degree of the reaction and the adrenalin content, contrary results were published by Ingier and Schmorl (12), Popielski (13), and others. Biedl states that Henle's chrome reaction can serve only as a preliminary orientation as to the quantity of adrenalin contained. Though the presence of a definite relation between the two phenomena is assumed, proof of it is lacking. On the other hand, some investigators, as Mulon (14), Elliott and Tuckett (16), Ciaccio (17), Borberg (18), and others, believe that the chrome reaction is a purely chemical process, which occurs between chromates (chromic acid) and adrenalin or its derivatives. Borberg described this process in detail. He stated that adrenalin solution after the addition of a solution of potassium dichromate gradually becomes more and more reddish brown until in the course of a few hours a slowly increasing dark brown precipitate appears. According to him, the precipitate showed, besides its similarity in color, in its behavior towards various solvents complete agreement with the chrome reaction in the adrenal medulla. He therefore identified both reactions in the test-tube and on the slide. The reaction in the test-tube he explained thus: that potassium dichromate or the chromic acid resulting from the interaction of this salt and the acetic acid in the solution forms an insoluble brownish chemical compound. But he believed that the reaction on the slide was caused not by adrenalin itself but by substances in the preliminary stage of adrenalin formation (adrenalinogen, pre-adrenalin), which would be oxidized into adrenalin derivatives combined with chromates. This so called chromaffin substance may have, according to his hypothesis, the following chemical structure.



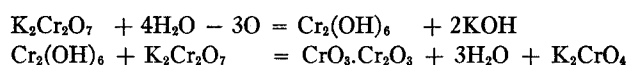
The relation between the chrome reaction and the presence of adrenalin is, however, not yet proved. This is why Biedl maintains that the chrome reaction could only serve as a preliminary orientation as to the quantity of adrenalin contained.

EXPERIMENTAL.

We believe that we have succeeded in solving this question. Our materials were mostly the fresh adrenals of healthy domestic animals, as cattle, horses, pigs, sheep, dogs, rabbits, guinea pigs, and rats. Human adrenals were also used, but were found not to be so well suited for our purpose as in them the reaction was much weaker than in the fresh adrenals of healthy animals. We also investigated in the test-tube the chemical process between adrenalin and potassium chromate, for the reaction can here be easily studied. It was only later we learned that Borberg had also investigated the reaction in this manner. But his results were different from ours. We too observed that an adrenalin solution turns brown on the addition of a solution of potassium chromate, to be followed by a dark brown precipitate which slowly increases. At first we believed with him that the precipitate was a chemical compound of chromic acid with some derivative of adrenalin, which arises by oxidation from adrenalin and dissolves in water, showing red coloration, for we perceived, in mixing both solutions, first the change of color into red and then the brownish precipitate. By closer examination of the properties of the brownish precipitate we learned that the latter was not a complicated organic compound but a simple inorganic one, namely chromium dioxide (CrO_2).

There are various methods for producing chromium dioxide; for instance, by introducing nitric oxide into a solution of potassium chro-

mate (Schweitzer (19)), by adding a solution of potassium chromate to a solution of chromium sulfate (Maus (20)), by digesting chromic acid solution and chromium hydroxide (Beusch (21)), by adding a solution of sodium thiosulfate to one of potassium chromate (Popp (22)), or reducing potassium dichromate by sunlight in the presence of alcohol (Vogel (23)). The process may be explained thus: that chromates are first reduced to chromium dioxide and the latter combines with the existing chromic acid into chromium dioxide according to the following equation.



It is conceivable that adrenalin, which is easily oxidized and therefore can exert a reducing effect upon other compounds, *e.g.*, the reduction compounds mentioned above, extracts part of the oxygen from the potassium dichromate, thus forming chromium dioxide.

If in the experiment mentioned above the precipitate in the test-tube is examined, it is found to agree in appearance and other properties with chromium dioxide, which has been extracted according to one of the methods described. The precipitate is a brownish powder and appears under the microscope in the form of yellow-brown amorphous granules, is insoluble in various solvents, such as alcohol, ether, chloroform, xylol, benzene, etc., but is easily soluble in caustic potash and mineral acids. In acetic acid and formic acid it is not easily soluble, but if these organic acids are present in the original mixture its elimination is interfered with. When dried at 100°C. it becomes dark brown; when brought to a glow it gives off no carbon dioxide, proving the absence of organic compounds. Quick heating causes it to flare up, as Schweitzer has observed with chromium dioxide.

If this precipitate, which we believe to be chromium dioxide, is washed, the water appears yellow on account of the admixture of a surplus of potassium dichromate, and further washings make the color disappear to a yellowish trace which cannot be got rid of. The same fact has been observed with chromium dioxide. Schiff (24) washed 1 gm. of chromium dioxide for 10 hours daily for 6 weeks, but chromic acid was still seen to dissolve. Popp also mentions that if chromium dioxide is washed until color no longer appears, it changes

to a new compound, $2\text{Cr}_2\text{O}_3 \cdot \text{CrO}_3$. For explanation of this chromium dioxide might be regarded as a loose compound of chromium oxide and chromic acid, so that in washing, a part of the chromic acid is continually dissolved. When the brownish precipitate was allowed to remain for several days on the water bath, a greenish shade of color gradually appeared; and if dried at a gradually increasing temperature it became black and gradually decreased in weight. This agrees with the fact observed with chromium dioxide, that its hydroxide does not easily give off water, and only under high temperature changes into green chromium oxide, setting oxygen free.

In order to convince ourselves further that the precipitate is chromium dioxide, we made it glow and examined the residue (chromium oxide). The method was as follows. To a 1 per cent solution of adrenalin hydrochloride a solution of potassium dichromate was added in excess; it was left for 3 days at 37°C ., centrifuged, and poured off. To this, water was added and the same operation was repeated several times, until the upper layer retained the same yellowish color. Absolute alcohol was then added to the precipitate, the mixture was shaken for 30 minutes, again centrifuged, and treated with ether, after which a brown powder was obtained, which was dried at 170°C . in a platinum crucible until the weight remained constant. This powder was gradually heated to red heat and the green residue, chromium dioxide, was weighed.

0.2026 gm. substance gave 0.1648 gm. chromium oxide.

0.1648 gm. of chromium oxide gave 0.1822 gm. of 2CrO_2 or 0.2017 gm. of $2\text{CrO}_2 + \text{H}_2\text{O}$.

From this it follows that the substance must have the molecular formula $2\text{CrO}_2 + \text{H}_2\text{O}$.

The following formulas have been given for the hydrate of chromium dioxide, dried at various temperatures.

Observer.	Formula.	Temperature.
		$^\circ\text{C}$.
Popp.	$2\text{Cr}_2\text{O}_3 \cdot \text{CrO}_3 + 9\text{H}_2\text{O}$	Not given.
Kopp.	$\text{CrO}_2 + 2\text{H}_2\text{O}$	Room.
Vogel.	$\text{CrO}_2 + \text{H}_2\text{O}$	100
Hinz.	$\text{CrO}_2 + \frac{1}{2}\text{H}_2\text{O}$	200
"	CrO_2	250

As the substance was dried at 170°C ., the formula $2\text{CrO}_2 + \text{H}_2\text{O}$ ($\text{CrO}_2 + \frac{1}{2}\text{H}_2\text{O}$) stands almost exactly between the third and fourth, and also agrees in this respect with the supposed hydrate of chromium dioxide.

As chromium dioxide is an amorphous substance, only a relative purity can be reached by washing. If the latter is continued too long, the chromium dioxide molecule loses part of its chromic acid; if it is not continued long enough, the precipitate may still contain some potassium chromate. It is therefore necessary to follow exactly the method of others in order to compare the results.

By the above investigation we believe we have established that the precipitate obtained by mixing solutions of adrenalin and dichromate in the test-tube is chromium dioxide.

We then prepared frozen sections of the adrenals, in which we had effected a chrome reaction. We left them in the solutions for a long time to test the conditions of solubility. In these sections we were able to ascertain the same conditions as those in the test-tubes. The shade of color in the sections was exactly that of chromium dioxide. By heating chromated adrenals to red heat we obtained a relatively large amount of chromium oxide. Though we were unable to analyze directly the brownish substance in the tissues, we may assume, according to the above investigation, that Henle's reaction is simply a reducing phenomenon of adrenalin upon dichromate of chromic acid. If the amount of adrenalin is great, a correspondingly large amount of chromium dioxide will be formed by reduction, so that the estimation of the content of adrenalin from the degree of the chrome reaction has thus been proved to be also theoretically correct. As the chrome reaction in the test-tube has shown, it is most distinct in a neutral medium; an acid or alkali medium disturbs the reaction. This explains why the chrome reaction in dead bodies is not proportional to the content of adrenalin, on account of postmortem changes which also affect the chemical reaction of tissues.

The above considerations explain why the chrome reaction in sections is most distinct in simple solutions of potassium dichromate. It can also be obtained with Mueller's fluid, Orth's fluid, dichromate-formalin solution, etc., because the other admixtures to these solutions do not disturb the reaction of the dichromate. In Zenker's

fluid, however, the chrome reaction becomes indistinct, not only because its constituent sublimate disturbs the reaction, but also because the large addition of acetic acid prevents the elimination of chromium dioxide.

As Henle has noted, the reaction can also be produced by chromic acid (Merck), but according to our observation, in the test-tube as well as in the sections, chromic acid must in this case be used only in a greatly diluted solution; 0.3 to 0.5 per cent is best. The reason is that the extracted chromium dioxide dissolves again in the abundant chromic acid. But microscopically the reaction caused by chromic acid is somewhat different on the slide. With chromic acid the atrabiliary part of the adrenals is of a diffuse brownish color, while with dichromate brownish granules appear in the protoplasm of the chromaffin cells. This may possibly be explained by the fact that the eliminated chromium dioxide is dissolved by the abundance of chromic acid, causing the diffuse coloration.

We have tried the reaction also with a solution of ammonium dichromate. The result was the same as with potassium dichromate.

The chrome reaction may also be effected by chromates as well as by dichromate and chromic acid. With chromates (we used potassium chromate), however, a large amount of adrenalin seems to be necessary for the reduction, for with the 1 per cent commercial adrenalin solution we could not produce the reaction in the test-tube, or in human materials, but it appeared distinctly in the fresh adrenals of healthy animals (rat, dog, pig). This reaction is similar to that of chromic acid,—microscopically it shows diffuse coloration.

We found that the chrome reaction appears best in the sections with 5 per cent solution of potassium dichromate, without any admixtures. But with this there is not sufficient hardening of the tissue, so we put the tissue first into 5 per cent potassium chromate solution for 6 to 12 hours, then added a tenth part of formalin, which gave the necessary hardening.

We also tried to combine the chrome reaction with subsequent coloring which some investigators have recommended for making the reaction more distinct. Such methods are Dewitzky's (25) (cresyl purple coloring) and Wiesel's (water blue safranin or toluidine blue safranin double coloring), but the simple toluidine blue coloring

recommended by Borberg (18) gave better results (discoloring in alcohol). We found that the grade of reaction can be estimated best without subsequent staining.

Since we have thus established that Henle's reaction is a simple reduction phenomenon of adrenalin upon chromic acid, especially dichromate, it follows that this phenomenon should not only occur with chromium compounds but also with other inorganic compounds. We have looked for such compounds, which should be easily reducible and insoluble in water. We first tried whether the reaction occurs in the test-tube, and if we obtained a precipitate we employed the medium also on tissues. But every reaction which succeeded in the test-tube was not always applicable to tissues. For instance, in a solution of potassium permanganate, manganese is at once precipitated by the addition of adrenalin, but because in tissues other organic substances besides adrenalin will reduce potassium permanganate, the adrenalin reaction is not recognizable by itself.

Fehling's solution, too, is reduced in the test-tube by adrenalin, but gave negative results in tissues. Fehling's solution contains a comparatively large amount of caustic soda, in consequence of which the adrenalin passes from the tissue into the solution and no copper oxide precipitates in the tissue.

We could easily reduce osmic acid by adrenalin in the test-tube. By using it on the adrenals the medulla becomes black but after this the lipoids of the cortex are also more markedly black. Afterwards we found that Mulon (15) in 1905 had observed the same phenomenon and also explained it as a reaction by adrenalin. He investigated the lipoid of the adrenals and came upon this fact by accident.

We made experiments with nitrate of silver. A solution of it is easily reduced by adrenalin in the test-tube and silver is precipitated; in the tissue of the adrenal medulla also precipitation takes place, but some precipitation occurs in all parts of the tissue, not only in the medulla; moreover, the simple solution does not penetrate deep into the tissue. We have, therefore, made various experiments and shall describe our method.

After completing this investigation we found that a former investigator had already observed this silver reaction of the adrenals and likewise explained it as a reduction by adrenalin. Laignel-Lavastine (26, 27), in the course of extensive

histological investigations of the sympathetic nerves, also examined the nerve filaments of the adrenal medulla. He applied Kajal's silver impregnation on the adrenals of a rabbit and accidentally found a silver precipitate in the medullary cells. He believed that adrenalin was the cause of this phenomenon.

We also tried Kajal's method, but as we had found already with the simple nitrate of silver solution, silver is precipitated throughout the whole tissue and the method is therefore not applicable for the microscopic reaction of adrenalin.

In our own method we employed ammoniacal silver solution as follows: (1) Cut the adrenals (chromaffin tissue) with a sharp knife into sections as thin as possible; (2) place the sections without previous fixing in 1 per cent ammonium solution for 2 hours; (3) transfer to 5 per cent ammoniacal silver solution for 3 to 5 hours; (4) place again in 1 per cent ammonium solution, repeating this several times, for 30 minutes; (5) transfer to 3 per cent sodium thiosulfate solution for 1 hour (these operations, Nos. 3 to 5, must be made in the dark room, protected from daylight); (6) wash in running water for 1 hour; (7) fix in 10 per cent formalin solution; (8) prepare sections by any method.

If the reaction is positive the medullary cells are filled with black and silver granules. Silver granules were also found in blood vessels, which also occurs with the chrome reaction and indicates the presence of adrenalin in the blood vessels.

Da Costa (28) reported his application of Wilshowsky's silver method in the adrenals. According to an abstract, he obtained results similar to ours, but as we have not seen the original paper we do not know his method of procedure. Wilshowsky's method refers only to tissues fixed in formalin. A reduction by adrenalin cannot take place. We believe, therefore, that our silver method is different from Da Costa's.

SUMMARY.

We have established the fact that the chrome reaction as well as the silver and osmium reactions are merely reductions by adrenalin. In our opinion the naming of the cells giving a positive reaction should not be based upon the reaction (*i.e.*, chromaffin cells), but on the presence of adrenalin itself. Biedl's terms, adrenal cell, adrenal organ,

adrenal body, adrenal system, and also Bonnamour's term, adrenalin-producing cells, are appropriate in this respect. We propose the names adrenalin cell, adrenalin tissue, adrenalin system, thereby indicating the presence of adrenalin.

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EXPLANATION OF PLATE 67.

Silver impregnation according to Ogata's method.

FIG. 1. Adrenal of pig.

FIG. 2. Human adrenal (beri-beri).

The chromaffin cells in the medulla of the adrenals are strongly impregnated.

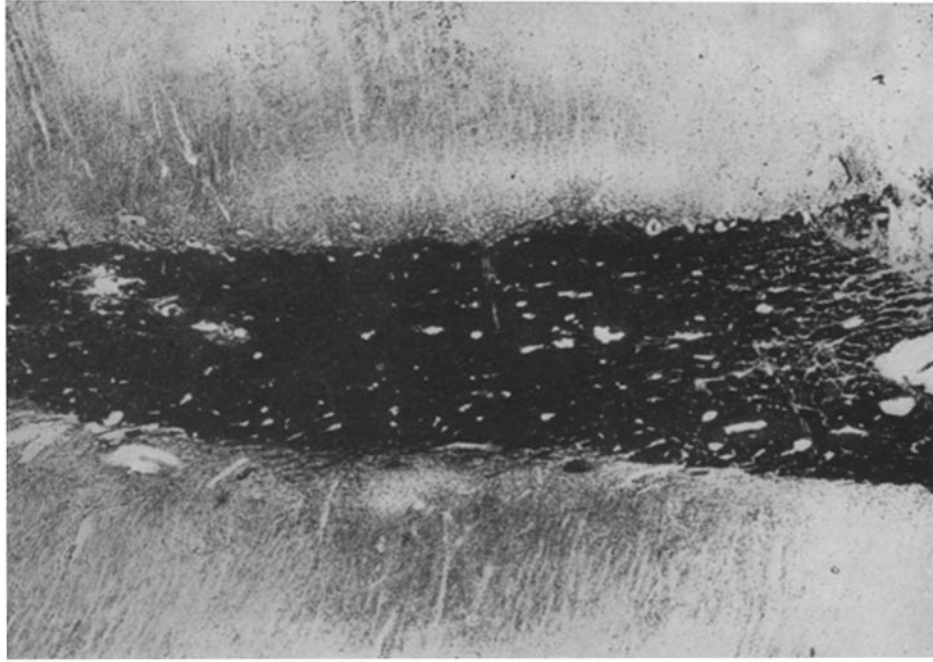


FIG. 1.

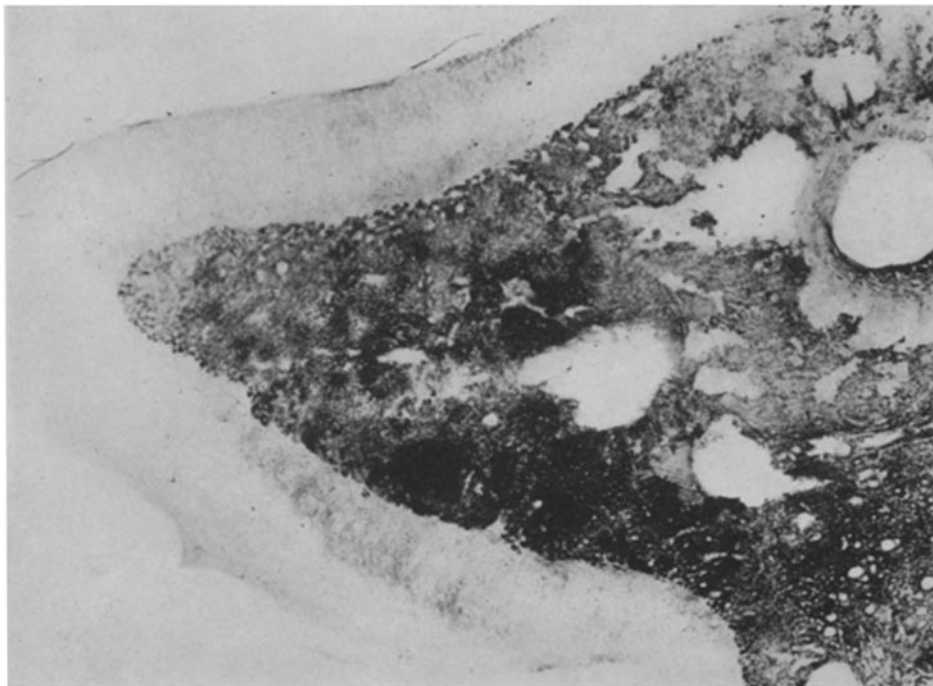


FIG. 2.

(Ogata and Ogata: Henle's Reaction of Chromaffin Cells.)