SCIENTIFIC SECTION

SALIVA TESTS. I. MORPHINE.*

BY JAMES C. MUNCH.[1]

The formation and secretion of saliva by the parotid, submaxillary and sublingual glands have been shown to be controlled by the nucleus salivoratus in the upper pons (21, 34, 59). The secretion is readily augmented by sensory stimuli, readily depressed by narcotics. Since the volume of saliva excreted daily by man normally amounts to 1000–1500 cc. and may be markedly increased, this pathway for excretion of foreign substances appears to deserve study.

It is reported that toad and cobra venoms are excreted in the saliva, as well as the virus of rabies and of mumps (5, 6, 7).

Rosenthal (5, 6) in 1893 appears to have been the first to demonstrate that morphine is eliminated in the saliva. Therapeutic doses were given to a number of hospital patients. The saliva was collected at half-hour intervals, and tested chemically with iodic acid, Husemann's and Froehde's reagents. Saliva from a number of normal and pathological individuals not receiving morphine or opium alkaloids gave uniformly negative results. When 19 mg. were administered daily, saliva tests were negative on the first and second days, then positive until one or two days after the morphine was discontinued. The saliva was estimated to contain between 0.05 and 0.2 mg. of morphine.

A detailed series of experiments on the excretion of medicinal substances by the salivary glands were conducted by Howe (23). Gelatin capsules containing test products were given by mouth and the saliva collected every five minutes for several hours. Many products appeared in the saliva within twenty minutes, and their presence was continually detected over a period of nine hours, but not after twenty-four hours. Attempts have been made to correlate the salivary excretion of NaCNS and KCNS.

The following products have been reported to be excreted in the saliva: Aconitine, amino-acids, ammonium compounds, arecoline, atropine, bismuth salts, bromides, brucine, chlorides, chlorates, creatinine, formic acid, glucose, guaiacol cinnamate, histamine, indican, iodides, iron salts, lead salts, menthol, mercury salts, methenamine, morphine, oil of peppermint, ouabain, potassium salts, quinine, salol, sodium benzoate, sodium salicylate, sodium sulphate, strophanthin, strychnine, thiocyanates, tyramine, urea and uric acid. The following are reported as not excreted in the saliva: Atropine, physostigmine, sodium ferrocyanide and sodium lactate (2, 3, 5, 6, 7, 9, 10, 11, 13, 16, 17, 19, 23, 24, 33, 37, 39, 43, 44, 45, 48, 53, 55, 57, 61, 62, 63, 66, 67, 70, 72, 74).

Saliva tests were developed to detect the "doping" of race-horses. Stimulants have been given to race-horses to obtain better (or poorer) performances. Varron

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(69), in 1533, reports that anise seed, honey and sandarach were given to race-horses, as stimulants. An English regulation dated June 14, 1666, prohibited the use of exciting substances and methods in races run at Worksop (50). The prohibition of doping has been the subject of various circulars and regulations by racing authorities in Argentine, Austria-Hungary, Belgium, Brazil, Chile, Czechoslovakia, Denmark, England, France, Germany, Italy, Poland, Spain and United States (12, 65). Published information (8, 12, 18, 27, 28, 29, 35) indicates that horses may be stimulated with alcohol, atropine, brucine, caffeine, cocaine, digitalis, heroin, kola nut, nitroglycerin, nux vomica, quinine, scopolamine, strychnine and veratrine. Thoroughbred horses are more sensitive to the action of drugs than ordinary work horses (15). Strychnine and cocaine have been used to dope hares for sport in England (32).

The parotid gland of a horse weighs about 400 Gm., the submaxillary 86 Gm. and sublingual 23 Gm. By cannulating Stensen's duct, 100 to 700 Gm. of saliva have been collected from a horse in 15 minutes (54).

Professor S. Fraenkel, of Vienna, apparently made the first scientific study of the detection of doping race-horses. Theobromine was apparently suggested as the "dope." Studies were made of excretion in the sweat, feces and urine (35, 73) but it appeared that the saliva was the most feasible medium for examination (14). Professor Kaufmann developed a specific chemical technique for testing saliva under race-track conditions (3, 4, 27, 28, 29, 71). Based upon his tests, the winners of several large races were disqualified and barred from tracks. Neuter (47) states that doping was very common in Belgium. Shortly before the World War, chickens of the guardian of the race-track of Stokel invariably died after pecking at the dung of competitors of the previous day.

A technique for collecting saliva was developed in France (12). A veterinarian, a representative of the Racing Commission and an assistant lead the horse into a box-stall. The veterinarian washes his hands in soap and water, then 95 per cent alcohol and dons sterile gloves. A wad of sterile gauze moistened in distilled water is introduced into the mouth of the horse and squeezed over the surface of tongue and lips, the escaping fluid being caught in the collecting basin. The tongue, lips and cheeks are then wiped with another piece of gauze. An attempt is made to express any saliva from the Wharton canals. The gauze and gloves are then placed in a jar, sometimes covered with alcohol, and sealed to prevent tampering with the sample.

Chemical methods of detection have been employed with only partial success. The method of Fraenkel (14) slightly modified by Lander (36) appears to be an improvement over the standard toxicological processes (1, 49). The saliva is extracted several times with 90 per cent alcohol, plus dilute acetic acid; the filtrate evaporated and extracted with ether. This is evaporated to a tacky consistency and exhausted with small amounts of warm absolute alcohol. The alcohol is removed and the residue dissolved in hydrochloric acid. This solution is made alkaline with sodium bicarbonate and extracted with chloroform and benzene. With iodine and Mayer's reagents the limit of sensitivity by morphine was 0.01 mg.; with phosphomolybdic acid and gold chloride, 0.001 mg., and with tannic acid 0.02 mg.

Chemical methods of procedure may fail in instances where threshold amounts of stimulants have been administered. Because tests upon animals are more sensi-

tive, a series of investigations were undertaken to determine their sensitivity for various stimulants which may be used on race-horses. Only the results obtained with morphine are reported in this communication.

Consideration of the bioassays for morphine (46) suggested the possibility of using the "mouse-tail reaction" (Mäuseschwanzphänomens) developed by Straub (64). Mice weighing 15 to 20 Gm. are injected with 0.5 cc. of test solution under the skin of the back or abdomen. If morphine is present the tail soon curves over the back in a characteristic S-curve. On stimulation, the mouse becomes somewhat restless, paresis of the posterior extremities becomes more noticeable, the back is humped and the fur stands out in a disheveled, shaggy manner. The mechanism of this reaction is still unsettled (20, 22, 25, 26, 30, 31, 38, 40, 41, 42, 51, 56, 58, 60, 64, 68). Other alkaloids may give a somewhat similar response, but different symptoms are produced. Positive reactions may be obtained with doses of 0.01 mg. of morphine and heroin (46, 56) to 0.02 mg. (42) amounts which are far below the sensitivity of chemical procedures on an extract of an organ or on the saliva.

Van Rijn (56) reported in 1914 on twenty-five cases of morphine poisoning. A corpse was examined and extracts of viscera prepared 59 days after death and again 38 days later. The extracted morphine injected subcutaneously under the skin of the back of white mice caused their tails to become stiffly erect in S-shape curves in two to twenty minutes.

Maier (42) injected 10 mice at each of a series of increasing doses, from 0.01 mg. per 20-Gm. mouse to 0.50 mg. per 20 Gm. (0.5 to 25 mg. per Kg.) (Table I). The animals showing a questionable reaction were divided arbitrarily, half being considered positive and half negative. A definite relationship was found between the dose administered and the duration of the reaction, but this relationship was not as close as the dose:percentage response relationship. Using the factors of duration and percentage, he tested a series of samples of unknown potency, obtaining results which, in general, were ten per cent less than theory. He concluded that this method was suitable for legal and toxicological assays, and had a sensitivity of 0.02 mg. of morphine, an amount much less than could be determined by chemical methods. Keil and Kluge (31) confirmed the nature of the dose percentage and dose:time relationships, using 100 mice at each dose from 1/100 mg. of morphine hydrochloride per 10-Gm. body weight, down to 1/40 mg. (1 to 2.5 mg. per Kilo) (Table I). Solutions containing amounts of morphine unknown to the investigator at the time assays were made, were tested with an average error of 6 per cent by the dose:percentage reaction, and 2 per cent by the dose:time reaction, or an average of 4 per cent. For the determination of morphine, 0.0125 mg. can be determined with an accuracy of about 5 per cent.

A review of the literature showed that about 0.01 mg. of morphine may be detected with an accuracy of five per cent by means of the tail reaction of white mice.

EXPERIMENTS.

In my investigational work preliminary experiments showed that the presence of morphine dissolving in saliva could be detected by the mouse-tail method with the same precision as when morphine had been dissolved in distilled water. A large number of tests with differing doses of morphine led to the development of a

specific technique for this test. It did not alter experimental results to inject solutions under the skin of the abdomen rather than under the skin of the back, as recommended by previous workers. The volume of injected solution did not make much difference in the nature of morphine response produced, but for uniformity it was decided to inject approximately 25 cc. of solution per kilo, which corresponds to 0.5 cc. for a 20-Gm. mouse. In those instances where weak solutions were tested and larger volumes injected up to 4 cc. per 20-Gm. mouse, dependable results were still obtainable, although a somewhat longer time period was usually necessary for the development of the characteristic phenomena.

After injection the mice were placed in individual cages in a quiet place and observed every minute or so for a period of half an hour. It was arbitrarily decided to fix a time period for reading the results of the injection as fifteen to twenty minutes after injection. Those animals which showed positive reactions in five minutes, for example, still showed a positive reaction in fifteen to twenty minutes. On the other hand, those animals which showed uncertain responses in fifteen to twenty minutes and gave questionably positive reactions in half an hour when injected with a given dose usually failed to show a consistent response in subsequent trials with the same quantity given to the same animal.

The degree of response varied with the dose of morphine injected: (1) Doses below the accepted threshold gave evidence of somewhat increased irritability and alterations in respiratory rate; the tail did not leave the ground voluntarily or after stimulation of the back. These responses were noted, but considered negative. (2) Threshold doses producing the satisfactory response caused definite alterations in respiratory rate. The mice tended to stand with the back arched and head depressed. When they were gently stimulated by a current of air or stroking the back with a lead pencil, the back became more arched. The hair tended to stand out in a shaggy manner. The posterior limbs showed rapid fibrillary twitchings tending toward paresis and the tail was lifted from the floor of the cage to or toward a definite S-curve. After the stimulus was discontinued, the tail reaction and the appearance of the posterior extremities extended for several seconds to several minutes. (3) When a supramaximal dose was administered, a very rapid development of the characteristic symptoms was followed by marked apnea, the fibrillations tending to extend to the anterior portions of the injected animals and the tail response developing normally without external stimulus and persisted for some time. In conformity with the literature findings, the duration of the effect was roughly proportional to the dose administered. One mouse in Fig. 1 shows the supramaximal effect, another shows the threshold.

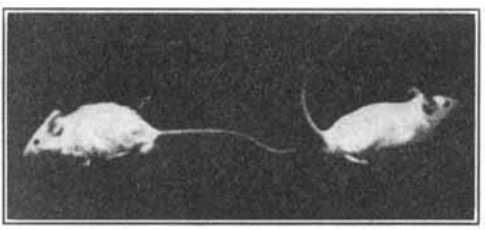

Fig. 1.—Tail response of mice after injection of morphine derivatives.

The procedure, based on my experiments, was outlined: Weigh a series of mice of either sex with an accuracy of one gram; inject morphine solution in a dose of approximately 25 cc. per kilo subcutaneously under the skin of the abdomen; place injected animals in a quiet place. Between fifteen and twenty minutes after

injection of the threshold dose gentle stimulation produces arching of the back, posterior partial paresis and elevation of the tail to or toward an S-curve. A number of animals (at least ten) should be injected, after preliminary tests have indicated an approximate threshold concentration. Animals should show a positive response within 15–20 minutes in determining the threshold dose.

In applying this procedure to the determination in the saliva of horses, a series of trials have been made. Collections of about 2 cc. of saliva from each of fifty horses not injected with morphine were made; 1 cc. and, in many instances, 2 cc. of each saliva were injected by the method outlined, and no response obtained. This appears to justify the conclusion that the normal saliva of a horse not injected with morphine does not give symptoms suggesting a tail reaction. A series of doses of morphine, ranging from 100 mg. to 1 Gm. per horse were then injected; in general, the horses weighed about one thousand pounds. Typical results of this test are given in Table II.

No chemical tests were made upon the samples of saliva. In each instance a control sample of saliva was collected immediately before the injection, then saliva samples taken fifteen minutes and thirty minutes after injection. In some instances samples of saliva were collected at periods greater than thirty minutes after injection, but in general, the results obtained with them were in agreement with the results obtained at the thirty-minute interval. Injection of saliva in the pre-

TABLE I.—MOUSE-TAIL RESPONSES AFTER INJECTION OF MORPHINE; LITERATURE DATA REARRANGED.

Dose Mg./Kg.	Per Cent of Mice Showing Positive Reactions. Maier.	Keil & Kluge.	Munch.
0.5	16	..	...
1.0	53	..	0
1.11	...	8	...
1.25	...	20	1
1.43	...	39	...
1.67	...	60	5
2.0	79	85	10
2.5	91	..	20
3.0	100	..	31
4.0	...	..	40
5.0	100	..	65
6.0	...	..	90
7.0	...	..	100

TABLE II.—TYPICAL PROTOCOL: MOUSE-TAIL TEST ON HORSE SALIVA.

Horse No.	Amount Morphine. Mg. per Horse.	Mg./Kg.	Results of Saliva Collected Various Periods after Injection. 0 Min.	15 Min.	30 Min.	45 Min.	60 Min.
141	100	0.22	Neg.	Neg.	Neg.	Pos.	..
9325	200	0.45	Neg.	Pos.	Pos.	Pos.	Pos.
9971*	200	0.45	Neg.	Pos.	Pos.	Pos.	Pos.
9949	250	0.55	Neg.	Neg.	Pos.	..	..
9112*	300	0.66	Neg.	Neg.	Pos.	Neg.	Pos.
9326	400	0.88	Neg.	Pos.	Pos.	Pos.	Pos.
9975	500	1.10	Neg.	Neg.	Pos.	..	..
9321*	800	1.75	Neg.	Pos.	Pos.	Pos.	Pos.
X	1000	2.20	Neg.	Pos.	Pos.	Pos.	Pos.

* 1/4 grain arecoline.

liminary tests was made in doses of 0.25, 0.5 and 1 cc. into each of three mice. Based upon these results, further samples were injected in proper dilutions, as indicated.

No effort was made to follow through a complete collection of saliva at various intervals after injection in order to obtain the absolute amount of morphine quantitatively eliminated in the saliva. This rather complicated problem is being considered and will be reported subsequently. No difficulty was encountered in detecting morphine in the saliva of horses that had received subcutaneous injections of 100 mg. of morphine sulphate per 1000 pounds of horse (approximately 0.2 mg. per kilo body weight of horse, or $1^1/_2$ grains of morphine sulphate per horse). The therapeutic dose of morphine for a horse (15) is listed as 0.3 to 0.6 Gm. It would, therefore, appear that after the administration of large therapeutic doses of morphine its presence might be detected in the saliva over a period of half an hour. Doses of morphine suggested for "doping" race-horses correspond to 3 to 5 grains of morphine sulphate per horse, amounts which may be detected by this procedure, even without attempting to concentrate the saliva. If the saliva is concentrated by chemical procedure, the sensitivity may be greatly increased. The administration of $^1/_4$ grain of arecoline per horse increased salivation, but did not appear to affect the elimination of morphine.

CONCLUSIONS.

1. Morphine may be quantitatively determined by the mouse-tail reaction.
2. The amounts used in "doping" race-horses can be readily detected in the saliva fifteen and thirty minutes after administration.
3. A standardized technique has been developed for this test.

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ABSTRACT OF DISCUSSION.

W. B. D. Penniman said he would discuss this report by Dr. Munch, in view of the fact that we are collaborating on this race-track work, to a certain extent: "It must be remembered that the collection of saliva from a race-horse is not a quantitative procedure, although a specific technique has been developed for this purpose. The race-horse is most emphatically *not* a laboratory animal. After the sample is obtained the toxicological difficulties develop because of the presence of iron from the bit, traces of blood and other substances which may interfere with our tests. By a chemical method I have succeeded in detecting or identifying fractions of a mg. of the morphine alkaloids, caffeine, strychnine and cocaine. These are only four of the substances which we have to seek. Everybody who has worked on this subject to date seems disposed to keep it a secret. If any chemist or any pharmacologist is interested in this matter, I know I am speaking for Dr. Munch as well as my own organization, in saying we will be glad to confer with them and get, as well as give any possible assistance. In making saliva tests at this time, the chemist is in a little better shape than the pharmacologist. Tests upon the saliva by chemical and pharmacological methods may be depended upon to show whether horses have been 'doped.'"

F. A. Upsher Smith said that this matter is of tremendous importance because when you

find harmful poisons in a horse's mouth after a race, you are questioning the honor of the men who own and train the horses: "The work Dr. Munch is doing is a credit to him and to the country, because all decent racing men will appreciate the fact that it is only the 'rotters' who are doping their horses and the good men don't want it. I would like to suggest that the perspiration from under the saddle would be a more handy substance to use than the gooey saliva. The favorite mixture for doping horses is nitroglycerin, strychnine, digitalis and heroin. Is it possible to get an accurate result between the time the horse comes back from weighing in and the time he returns to the stable?"

The author stated that the chemical methods developed by Dr. Penniman are much more sensitive than those published in the literature, and he deserves to be commended for his studies on this subject: "We are uncertain whether the chemical or biological methods are the most delicate, as well as the most specific, for testing the horse's saliva. With the animal test you get definite symptoms more rapidly than with the chemical: with the chemical method you can often isolate the specific alkaloids and identify them. The combination of both methods appears highly desirable. I have had no experience with perspiration from under the saddle, but believe that it might be contaminated with too much dirt. It is hoped that the application of these methods of chemical and biological testing will stop the doping of race-horses."

THE STABILIZATION OF SYRUP OF FERROUS IODIDE, U. S. P. X.

BY WILLIAM J. HUSA AND LYELL J. KLOTZ.

(*Concluded from page 683, July Journal.*)

As indicated in Table II, the hydrolysis of aqueous solutions of ferrous iodide corresponds predominantly to the equation:

$$FeI_2 + HOH \rightleftharpoons Fe(OH)I + HI$$

and apparently, two equilibria exist since reasonably constant values of K_1 were obtained for solutions having either a p_H of 3.2 or 4.1. In the former case, the degree of hydrolysis is approximately 0.30% at the concentration of the U. S. P. Syrup; in the latter instance, it is approximately 0.027%. Solutions of ferrous iodide of p_H 4.1 are hereafter designated as solutions at primary equilibrium; those at p_H 3.2 are considered to be at a condition of secondary equilibrium.

The Mechanism of Iodine Formation.—The decomposition of aqueous solutions of ferrous iodide consists in the formation of free iodine and ferric hydroxide. If sucrose, or other peptizing agent is present, however, ferric hydroxide does not precipitate and the appearance of iodine is dependent upon the rate at which the peptizing agent reacts with free iodine as well as upon the rate of auto-oxidation. The presence of soluble ferric ion is prohibited by the presence of iodide ion which reduces it to the ferrous state.

Several equations to account for the decomposition of ferrous iodide preparations have been advanced. Salzer (9) and Sadtler and Coblentz (10) formulated the equation

$$\text{(I)} \qquad FeI_2 + 2HOH + {}^1/_2O_2 \rightleftharpoons Fe(OH)_3 + HI + I$$

Mylius (11) suggested

$$\text{(II)} \qquad 2FeI_2 + 3O_2 + 3HOH \rightleftharpoons 2Fe(OH)_3 + I_2$$

and Bentley and Driver (12) account for the decomposition by two reactions: