Red urine has not been described as a symptom of pellagra, either endemic or alcoholic. The amounts of urinary porphyrin reported by Beckh, Ellinger and Spies were in many instances so large that the urine might have been expected to be red, since the amounts were as large as are often encountered in the wine red or dark red urines of idiopathic porphyria. This discrepancy is probably explained by the fact that the simple procedure they used is not specific for porphyrins and is not suitable for quantitative determination of the porphyrins. In the 3 cases discussed here, in which fluorimetric determinations were made, the per diem amount of coproporphyrin in no instance exceeded 0.6 mg (600γ), while Beckh, Ellinger and Spies reported 4 cases in which the amount was *over* 100 mg per liter, 3 in which the amount was between 10-100 mg, and 3 in which it was between 1-10 mg. In all of the present cases red pigments have been encountered differing from porphyrins or known hemoglobin derivatives. To what extent these pigments are related is not known. Considerable similarity in spectroscopic absorption was observed. The possibility must be considered that a common parent substance is altered variously in different urines and under different conditions, possibly undergoing esterification or conjugation. A change of the latter type was suggested particularly in the first of the present 4 cases, in which 25% HCl extracted a red pigment from the ether extract of the fresh urine, but later failed to remove it.

Prof. J. F. McClendon, Department of Physiological Chemistry, University of Minnesota, suggested that these red pigments might be indigo derivatives. Indirubin (indigo red, indipurpurin) exhibits similar absorption in xylene at 561 and 522 mμ (max.) and crystallizes in crimson needles which sublime without melting. The possibility is being investigated that this is the red substance which has been encountered in pellagra urines.

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A Rapid and Sensitive Method for Bioassay of the Adrenal Cortical Hormone.

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In view of the ever growing interest in the physiology of the cortical hormone, it seemed of importance to develop a method

which would allow the detection of minute amounts of this principle in tissues and body fluids. It is well known that animals, deficient in a certain hormone, are especially sensitive test objects for it. Use has been made of this observation for instance in methods for the bioassay of the pituitary hormones which can be detected most readily in hypophysectomized animals. As previous experiments have shown, there is a particularly acute need for cortical hormone during the alarm reaction, in fact, it appears that one of the most important, if not the most important function of the adrenal cortex is to make adaptation possible to such acute damaging agents as are capable of eliciting this reaction.[1,2] It seemed advisable, therefore, to assay the cortical hormone in adrenalectomized rats during exposure to an alarming stimulus. Exposure to cold proved to be a stimulus which elicits an uncomplicated type of an alarm reaction, that is, the characteristic symptoms of damage as such (gastro-intestinal ulcers, hemoconcentration, hypoglycemia, etc.) appear without being significantly altered by changes due specifically to cold. We, therefore, decided to use adrenalectomized rats exposed to low temperature for the bioassay of the cortical hormone and our preliminary experiments soon showed that our supposition was correct and that under these conditions, minute doses of cortical hormone exert a marked effect and may readily be detected.

For such an assay we use nine male or female "hooded" rats weighing between 35 and 50 g, the optimum being 45 g, for each level of hormone concentration. We make it a rule to use 9 untreated adrenalectomized controls of the same size for each assay. Twelve to 24 hours after adrenalectomy, food and water are removed and the rats are placed in a refrigerator at a temperature of $+2^\circ$ - $+5^\circ$C. At the same time, the 9 treated receive a subcutaneous injection of the hormone preparation dissolved in 0.2 cc of distilled water. Two more injections of the same type are given at 3-hour intervals, care being taken to make injections as rapidly as possible so that the animals are not kept outside the refrigerator too long. The total amount of the hormone preparation is thus administered in 3 doses within 6 hours. If the material which is to be assayed is toxic, one might administer it orally. The controls begin to show signs of hypoglycemia (convulsions and coma) within 4 to 5 hours, and most of them die between the sixth and the tenth hour, although occasionally some of the larger rats survive as long as 12 hours. The treated rats, on the other hand, survive much longer and show

1 Selye, Hans, *Arch. internat. Pharm. e. Ther.*, 1937, **55**, 431.

2 Selye, Hans, PROC. SOC. EXP. BIOL. AND MED., 1938, **38**, 728.

no obvious signs of damage. We found it advisable to base our unit of the hormone on the difference between the survival rate of the controls and the treated animals, rather than on the absolute survival time of the treated animals alone, because in this case, variations in the temperature of the refrigerator are less likely to affect the result. *We regard as one unit the minimum amount of the cortical hormone which suffices to maintain at least 6 out of 9 experimental animals alive at the time when 6 of the 9 controls are dead.* Of course if a sufficient amount of material is available, statistically more significant results will be obtained if a larger number of rats is used, but even in this case, we would advise making the reading when two-thirds of the controls are dead and to consider as one unit the minimum amount which maintains alive at least two-thirds of the treated animals at this time. This comparative method of assaying has the further advantage of eliminating the factor of varying sensitivity to cold of rats from colonies normally kept at different temperatures and the influence of seasonal variations on the length of survival of adrenalectomized rats. The following examples of assays on Wilson's* preparation will illustrate the manner in which the bioassay is performed.

Twenty-seven female rats weighing 35-50 g were adrenalectomized between 8 and 10 o'clock in the evening. Next morning at 8 o'clock, 9 of them received 0.3 cc of the cortical extract subcutaneously, 9 others, 0.1 cc and the remaining 9 were not treated. All animals were placed in a refrigerator having a temperature of +2° - +5°C without food or water. The treated rats received 2 more injections of the same magnitude as the first after 3 and 6 hours respectively. The first control died after 6 hours and all controls were dead after 10 hours at which time, none of the injected rats had succumbed. This experiment showed that even as small a total dose, 0.3 cc is above the unit.

In a second experiment conducted in exactly the same manner, we had 4 groups, each consisting of 9 rats of the same size. The first group received a total of 0.3 cc, the second 0.1 cc and the third 0.03 cc in 3 divided doses. In order to make these injections accurate, we diluted the extract so that the above mentioned amounts were always contained in 0.2 cc of fluid. The first 2 controls died after 8 hours. After 10 hours, 6 out of the 9 controls were dead, while in each of the 0.1 and 0.03 cc groups only one animal was dead at this time. In the 0.01 cc group, 2 animals were dead out of

* We are greatly indebted to the Wilson Laboratories who, through the courtesy of Dr. David Klein, have generously supplied us with their adrenal cortical preparation.

the total of 9 which according to our definition would indicate that even this amount is above the unit, but since 2 others were dying at this time, we believe that as far as one can tell from an experiment performed on a relatively small number of rats, this amount of the Wilson preparation corresponds approximately to one unit according to the above definition.

From these experiments, it appears that a total amount of 0.03 cc of Wilson's preparation per rat is still demonstrable with this method.

Since not every laboratory would always have a sufficiently large number of "hooded" rats of this size, we assayed the total doses of 0.6, 0.3, and 0.1 cc given in 3 injections on hooded rats weighing 63-80 g and found them all to be above the unit. As we assumed that these larger rats would be less sensitive, we unfortunately did not assay as low a dose as 0.03 cc, but the experiment indicates that the minimum amount detectable in the 63-80 g rat does not differ significantly from that detectable in the 35-50 g animal if the reading is made when two-thirds of the controls are dead. It should be noted, however, that all animals survived several hours longer in this series.

The advantages of our method may be summarized as follows: The test is *specific* because it is based on the preservation of life in adrenalectomized animals exposed to an alarming stimulus during fasting. While salt treatment may also increase resistance in adrenalectomized animals which receive food, such treatment has been found to be entirely ineffective in the case of the fasting animal.[3] Fasting has the further advantage of making the results obtained in different laboratories more comparable as differences in the diet have been proven to exert a marked effect on the length of survival.

The test is *sensitive* for the amount just detectable with our method is 0.03 cc of Wilson's preparation per rat, while in other survival tests on fed rats such as that of D'Amour and Funk,[4] 0.5 cc of the same preparation has to be administered daily over a period of 10 days to obtain a positive test, that is, the total amount necessary per rat is 5 cc or 167 times as much as can be detected with our method.

The test is *rapid,* as it gives positive results within 8 hours at the most—and within no more than 24 hours, even if we count the time from adrenalectomy—while ordinary survival tests take 10 to 20 days.

The test is *simple* as it involves no complicated surgical technic,

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3 Selye, Hans, *Brit. J. Exp. Path.*, 1936, **17**, 234.

4 D'Amour, Fred E., and Funk, Dorothy, *J. Pharm.*, 1938, **62**, 307.

since adrenalectomy in the rat is much easier to perform than in other animals, and no chemical determination is necessary.

The fact that any animal is used only for one test eliminates the danger of acquired resistance against the cortical hormone, such as has been described by Hartman, *et al.*,[5] and Toby and Lewis.[6] Since the assay is performed almost immediately after adrenalectomy, accessory cortical tissue, if present, has no time to undergo compensatory hypertrophy, so that in our experience no untreated control survived longer than 12 hours.

Several investigators advised the use of the body temperature of adrenalectomized animals as an index for cortical hormone assays either following exposure to cold[7, 8] or during the hypothermia induced by ether.[9] The reason why these testes never enjoyed great popularity is that the relationship between the dose of the cortical hormone and its temperature-maintaining effect is not as close as that between the dose of the hormone and its life-maintaining effect.

Summary. A method for the bioassay of the adrenal cortical hormone is described which allows of its detection in minute quantities. It is based on the maintenance of life of adrenalectomized rats weighing 35-50 g exposed to a temperature of +2° - +5°C. The hormone is administered subcutaneously in 3 doses with periods of 3 hours between injections. The unit is defined as the minimum amount necessary to maintain the life of two-thirds of the treated rats at a time when two-thirds of the untreated controls succumb. Because of the simplicity, sensitivity, and rapidity of this test, it appears to be particularly suitable for clinical and physiological studies necessitating the detection of the adrenal cortical hormone in tissues and body fluids in which only traces are present.

5 Hartman, Frank A., Lewis, L. A., and Toby, C. G., *Endocrinol.*, 1938, **22**, 207.

6 Toby, C. G., and Lewis, L. A., PROC. SOC. EXP. BIOL. AND MED., 1937, **37**, 352.

7 Hartman, Frank A., Brownell, Katharine A., and Crosby, Alford A., *Am. J. Physiol.*, 1931, **98**, 674.

8 Widström, G., *Acta Med. Scandinavica*, 1935, **87**, 1.

9 Martin, Steven J., and Maresh, Frank, *Am. J. Physiol.*, 1933, **105**, 273.