References and Notes

1. C. Heidelberger, *J. Cellular Comp. Physiol.* **64** (suppl. 1), 129 (1964).
2. W. H. Kirsten, Ed., "Malignant transformation by viruses," in *Recent Results in Cancer Research* (Springer-Verlag, New York, 1966).
3. K. K. Sanford, *Intern. Rev. Cytol.* **18**, 249 (1965).
4. Y. Berwald and L. Sachs, *Nature* **200**, 1182 (1963); *J. Nat. Cancer Inst.* **35**, 641 (1965).
5. C. Borek and L. Sachs, *Nature* **210**, 276 (1966).
6. E. Borenfreund *et al., Proc. Nat. Acad. Sci. U.S.* **56**, 627 (1966).
7. K. K. Sanford, reported at Decennial Conf. Cell, Tissue, and Organ Culture, Bedford, Pa., 15 Sept. 1966.
8. I. Lasnitzki, in "Biology of the prostate and related tissues," *Nat. Cancer Inst. Monograph 12* (1963), p. 381.
9. M. R. Roeller and C. Heidelberger, in preparation.
10. H. Eagle, *Science* **130**, 432 (1959).
11. J. M. Vasiliev and V. L. Guelstein, *J. Nat. Cancer Inst.* **31**, 1123 (1963); R. T. Prehn, *ibid.* **32**, 1 (1964).
12. L. Diamond, *J. Cellular Comp. Physiol.* **66**, 183 (1965).
13. G. Gomori, *Stain Technol.* **25**, 81 (1950).
14. H. J. Tagnon and A. Steens-Lievens, in "Biology of the prostate and related tissues," *Nat. Cancer Inst. Monograph 12* (1963), p. 297.
15. L. Hayflick and P. S. Moorhead, *Exp. Cell Res.* **25**, 585 (1961).
16. V. J. Evans and W. F. Andresen, *J. Nat. Cancer Inst.* **37**, 247 (1966).

17. Aided by NIH grant CA-07175. C.H. is an American Cancer Society professor of oncology; P.T.I. is a fellow of the Damon Runyon Memorial Fund. We thank Katherine Cato for technical assistance and Manuela Roeller and Howard Temin for discussions. The histological slides were read by H. C. Pitot and S. Inhorn.

25 November 1966

Tryptophan Hydroxylation: Measurement in Pineal Gland, Brainstem, and Carcinoid Tumor

Abstract. *Development of a rapid and sensitive radioassay has permitted study of the conversion of tryptophan to 5-hydroxytryptophan in mammalian tissues. Of normal tissues examined, beef and rat pineal gland contained the highest activity. This is the first direct demonstration of tryptophan hydroxylase in this hydroxyindole-rich tissue. Rat and rabbit brainstem and human carcinoid tumor also had quantities of enzyme that could be measured easily. The reaction requires a reduced pteridine and oxygen and is inhibited by para-chlorophenylalanine.*

It is generally accepted that hydroxylation of tryptophan at the five position is the first and presumably rate-limiting step in 5-hydroxytryptamine (serotonin) biosynthesis. Although there have been reports (*1–4*) on the detection of tryptophan hydroxylase activity in mammalian tissues in vitro, study of this enzyme has been elusive, owing to low activity and the lack of a sensitive and specific assay. We reported (*5*) that a malignant mast cell tumor of the mouse is rich in tryptophan hydroxylating activity, and this enzyme source has been used to develop a radioassay. The assay, together with the finding that enzyme activity is enhanced in the presence of the reducing agent, 2-mercaptoethanol (*6*), has permitted detection of tryptophan hydroxylation in several mammalian tissues and assay of enzyme activity in certain serotonin-rich tissues including pineal gland, brainstem, and carcinoid tumor.

The major steps and principles of the assay are as follows. (i) L-Tryptophan-2-^{14}C is incubated with enzyme, a monoamine oxidase inhibitor, and necessary components of the reaction mixture in the presence of a known amount of 5-hydroxytryptophan (5HTP). The latter serves as an inhibitor of tryptophan decarboxylation (*7*) as well as a trap for the newly formed 5HTP. (ii) After the reaction has proceeded, a portion of the 5HTP is enzymatically decarboxylated with partially purified aromatic L-amino acid decarboxylase (*8*). (iii) The serotonin formed is isolated and its specific radioactivity determined. The amount of tryptophan converted to 5HTP can then be calculated.

Rat, rabbit, and guinea pig tissues were removed immediately after decapitation; beef pineal glands were obtained at a slaughter house and removed within 10 minutes of exsanguination; carcinoid tumor was a liver metastasis obtained at surgery. All tissues were iced immediately and homogenized in two volumes of ice-cold 0.05*M* tris-acetate buffer, *p*H 7.4.

A typical assay is done as follows (*9*): 0.05 to 0.2 ml of a 33 percent tissue homogenate is incubated in the presence of 0.25*M* tris-acetate buffer (*p*H 7.5), 1.8 m*M* DL-5HTP, 0.4 m*M* pargyline hydrochloride, 1 m*M* 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine ($DMPH_4$), 0.05*M* 2-mercaptoethanol, 0.1 m*M* ferrous ammonium sulfate, and 0.12 m*M* L-tryptophan-2-^{14}C (16.7 μc/μmole) in a total volume of 0.25 to 0.50 ml. After the desired period of incubation in air at 37°C, 0.5 ml of 1*M* tris-acetate buffer, *p*H 9.0, is added to stop the hydroxylation reaction. The mixture is diluted to 2 ml, and 250 units of the decarboxylase enzyme (*10*) and an additional 1.2 μmole of DL-5HTP are added. This mixture is incubated for an additional 20 minutes at 37°C. At the conclusion of the second incubation the sample is diluted to 20 ml and applied to a 0.5 by 3 cm Permutit column (*10*). The column is washed with 20 ml of boiling H_2O. Tryptophan and 5HTP do not adhere to the column and are discarded in the wash. Serotonin is eluted from the column with 4 ml of 2*N* NH_4OH. Two milliliters are placed in a counting vial; 0.3 ml of glacial acetic acid is immediately added to the remaining 2.0 ml of eluate to stabilize the serotonin. A 0.4-ml portion of this latter fraction is diluted to 2.0 ml with H_2O, 0.6 ml of concentrated HCl is added, and the serotonin content is determined by fluorescence assay (*11*). To the portion of the Permutit eluate in the counting vial, 10 ml of fluorophore solution (*12*) are added, and radioactivity is determined in a Packard Tricarb liquid scintillation counter.

The validity of the assay was first established with the use of the mouse mast cell tryptophan-hydroxylating enzyme which is normally assayed by a colorimetric procedure (*5*). Comparison of the results of radioassay and

Table 1. Comparison of tryptophan hydroxylation as measured by radioassay and colorimetric assay. Enzyme preparations used in these experiments were ammonium sulfate fractions of the mouse mast cell tumor (*5*). The enzyme solution contained 20.0, 8.1, and 5.5 mg/ml of protein for experiments 1, 2, and 3, respectively. One milliliter of enzyme solution was used for the colorimetric assay (*5*) and 0.1 ml for the radioassay. Incubations were for 1 hour, as described in the text. Results for radioassay in this and subsequent experiments were calculated with the following formula: Tryptophan hydroxylated = (specific activity of serotonin × total L-5HTP added)/specific activity of substrate. Tryptophan and L-5HTP are expressed in micromoles; specific activity of serotonin and the substrate in counts per minute per micromole.

Exp. No.	Activity (mμmole/mg protein per hour)	
	Radioassay	Colorimetric assay
1	14.3	17.5
2	16.3	17.3
3	12.3	14.9

Table 2. Tryptophan hydroxylase activity in various tissues. Incubation volume for pineal tissue was 0.25 ml and for other tissues 0.50 ml. In the case of pineal tissue, 10 rat glands and 3 or 4 beef glands were pooled for homogenization. Each value for enzyme activity represents the average of at least two separate incubations done with the same homogenate. Net radioactivity in the counting vials ranged from 5000 to 50,000 count/min for the values shown. Results in other tissues studied to date were: approximately 0.01 mμmole per milligram of protein per hour for rat liver and kidney and for guinea pig intestinal mucosa, and no detectable activity in rat heart and spleen.

Tissue	Enzyme activity (mμmole/mg protein per hour)
Beef pineal	0.50, .53, .54, .44
Rat pineal	.53
Rat brainstem	.062, .057
Rabbit brainstem	.061, .055
Carcinoid tumor	.23

colorimetric assay of mast cell enzyme activity is shown in Table 1; the findings with the two techniques are similar. Isolation of the reaction product by Permutit chromatography followed by high-voltage paper electrophoresis in 6.8 percent formic acid showed a single radioactive spot with the same mobility as serotonin. No radioactivity was detected in areas corresponding to the locations of tryptamine and tryptophan.

A variety of other tissues were then examined (Table 2). Pineal glands had the highest activity, but easily measured activity was also found in rabbit and rat brainstem and in carcinoid tumor. In addition, tryptophan hydroxylase could be detected in rat liver and kidney and in guinea pig intestinal mucosa. Beef pineal was used to confirm the validity of the radioassay technique. The hydroxylation reaction in pineal homogenates is proportional both to time (up to 60 minutes) and amount of enzyme (up to 5 mg), and the final product of incubation was identified as serotonin by paper chromatography in a mixture of butanol, acetic acid, and water, 12 : 3 : 5.

The properties of tryptophan hydroxylation in tissues where activity was easily measured were quite similar. As shown in Table 3, all require $DMPH_4$, oxygen, and 2-mercaptoethanol. The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) failed to stimulate the pineal and mast cell enzymes. The only differences observed so far are that the mast cell enzyme is dependent upon exogenous iron and its *p*H optimum is 6.8 while the other enzymes operate optimally at about *p*H 7.5. Although exogenous iron is not required for tissues such as pineal homogenates, the role of iron in the reaction can be demonstrated by the fact that $6.4 \times 10^{-3}M$ α,α-dipyridyl, an iron chelator, causes complete inhibition. Para-chlorophenylalanine (PCP), a powerful depletor of tissue serotonin (*13*) in vivo, appears to be a potent inhibitor of tryptophan hydroxylase (Table 3). It seems likely that the mechanism of serotonin depletion in vivo by PCP is through inhibition of synthesis.

The requirements of mercaptoethanol, ferrous ion, and a reduced pteridine for the tryptophan hydroxylase measured in the current system are very similar to those reported for other aromatic ring hydroxylases, particularly tyrosine hydroxylase (*14*). Other workers (*1–4*) did not observe these requirements and in general have found the reaction not to be dependent upon the reduced pteridine cofactor. While it is not possible to reconcile these differences directly, the variations in incubation and assay conditions have been so great that they certainly account in part for differences. Further, we estimate that in other studies (*1–4*) the activity of tryptophan hydroxylase in rat brainstem has been in the order of 0.01 mμmole per milligram of protein per hour, which is only about one-fifth of the activity reported here. This latter difference is probably due to the use of 2-mercaptoethanol, since it was found (Table 3) that brainstem activity is only about 0.01 mμmole per milligram per hour in the absence of a high concentration of this reducing agent. The enzyme requirements in our work are most similar to those recently reported by Gal *et al.* (*3*). The latter investigators, using various brainstem enzymes, observed some stimulation by NADPH and biopterin and inhibition by α,α-dipyridyl, although they did not include 2-mercaptoethanol in their system.

Our assay system offers several advantages over existing methods. The procedure can be done rapidly, requiring only about 3 hours to complete. It is possible to measure accurately the hydroxylation, irrespective of the recovery of final product, since calculations are based only on specific radioactivity of the serotonin. Finally, the presence at all times of a large excess of 5HTP eliminates a serious and probable error in some studies, namely, decarboxylation of the ^{14}C-tryptophan to ^{14}C-tryptamine which can be mistaken for ^{14}C-serotonin.

With the finding of tryptophan hydroxylase in pineal gland, all of the enzymes involved in the conversion of tryptophan to melatonin have now been measured. The question may be raised as to which enzymatic step is rate-limiting in melatonin biosynthesis. Based on the observations on the concurrent diurnal variation in the pineal levels of melatonin and hydroxyindole-*O*-methyltransferase, Axelrod *et al.* (*15*) suggested that the latter enzyme is the controlling step. In our work the specific activity of tryptophan hydroxylase appears to be considerably higher than that reported for hydroxyindole-*O*-methyltransferase. This would tend to support the conclusions of Axelrod *et al.* (*15*), although experiments on the diurnal variation of tryptophan hydroxylase in pineal tissue would be of considerable interest.

There have been numerous hypotheses regarding the possible roles of serotonin in physiologic and pathologic states and in the responses to drugs. The availability of a simple means of estimating the rate of endogenous synthesis of the amine would offer a valuable new approach. This assay procedure for tryptophan hydroxylase, the

Table 3. Properties of tryptophan hydroxylation in four tissues. Incubations were carried out for 1 hour as described in the text.

Addition (+) or omission (−)	Activity (mμmole/mg protein per hour)			
	Beef pineal	Carcinoid tumor	Rabbit brainstem	Mouse mast cell
None (complete system)	0.44	0.23	0.050	16.5
−$DMPH_4$	.04	0	.002	0.1
−Fe^{++}	.54	.22	.062	1.2
−Oxygen	.04			0
−2-mercaptoethanol	.01	.06	.009	0.6
+NADPH ($10^{-3}M$)	.47			13.0
+PCP ($10^{-4}M$)	.16	.11		7.2

rate-limiting synthetic enzyme, should prove useful in this respect. The discovery of inhibitors, such as *p*-chlorophenylalanine, may also lead to therapeutic agents which would be effective in control of the exaggerated hydroxyindole pathway of tryptophan metabolism in patients with the carcinoid syndrome.

WALTER LOVENBERG
ERIC JEQUIER
ALBERT SJOERDSMA
Experimental Therapeutics Branch, National Heart Institute, Bethesda, Maryland

References and Notes

1. D. G. Graham-Smith, *Biochem. Biophys. Res. Comm.* **16**, 586 (1964).
2. H. Green and S. L. Sawyer, *Anal. Biochem.* **15**, 53 (1966).
3. E. M. Gal, J. C. Armstrong, B. Gimberg, *J. Neurochem.* **13**, 643 (1966).
4. A. Nakamura, A. Ichiyama, O. Hayaishi, *Federation Proc.* **24**, 604 (1965).
5. W. Lovenberg, R. J. Levine, A. Sjoerdsma, *Biochem. Pharmacol.* **14**, 887 (1965).
6. S. Hosada and D. Glick, *J. Biol. Chem.* **241**, 192 (1966).
7. W. Lovenberg, dissertation George Washington University, Washington, D.C. (1962).
8. C. T. Clark, H. Weissbach, S. Udenfriend, *J. Biol. Chem.* **210**, 139 (1954).
9. 2-Amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine was obtained from Calbiochem Inc.; *p*-chlorophenylalanine was a gift of Chas. Pfizer & Co.; L-tryptophan-2-C^{14} was purchased from New England Nuclear Corp.
10. W. Lovenberg, H. Weissbach, S. Udenfriend, *J. Biol. Chem.* **237**, 89 (1962).
11. D. F. Bogdanski, A. Pletscher, B. B. Brodie, S. Udenfriend, *J. Pharmacol. Exp. Therap.* **117**, 82 (1956).
12. G. A. Bray, *Anal. Biochem.* **1**, 279 (1960).
13. B. K. Koe, and A. Wiessman, *J. Pharmacol. Exp. Therap.*, in press.
14. T. Nagatsu, M. Levitt, S. Udenfriend, *J. Biol. Chem.* **239**, 2910 (1964).
15. J. Axelrod, R. J. Wurtman, S. H. Snyder, *ibid.* **240**, 949 (1965).

13 October 1966

Association of Illness with Prior Ingestion of Novel Foods

Abstract. *Rats were permitted to ingest a novel food and a familiar food. One hour later they were x-irradiated. When they were subsequently allowed to choose between these foods, their preference for the novel food was less than that exhibited by appropriate controls.*

If animals eat a particular food a few hours before they are x-irradiated, they will subsequently avoid that particular food because they associate the early symptoms of radiation sickness with it (*1*). Furthermore, injection of apomorphine, which like x-irradiation produces a gastrointestinal syndrome, also results in aversions to previously eaten foods (*2*). This ability to form associations between ingestion and subsequent illness (*3*) is probably responsible, in part, for the avoidance by rats of slow acting poisons (*2*). However, another factor must also be involved. Since rats are likely to eat a number of foods over a long period of time, there must be a mechanism by which they can associate the poisoned food with the effects of the poison. A poisoned food is bound to be novel; otherwise the rat would probably already be dead. Our hypothesis is that rats associate radiation sickness with novel food rather than familiar food if they have eaten both types before they become sick. Conventional conditioning procedures suggest that association of a stimulus with a consequence is likely to be inhibited if there has been a number of earlier presentations of the stimulus in the absence of that consequence (*4*). Furthermore, neophobia, the hesitancy with which rats approach novel foods (*5*), seems to indicate the existence of an investigatory reflex (*6*) to novel foods which may predispose rats to associate novel illnesses with them.

Forty-eight male Sprague-Dawley rats were housed in individual cages (Hoeltge HB-11A) throughout the experiment and were given continual access to water and to ground rat chow for 1 hour each day. During days 1 through 8, half the rats were made familiar with milk (equal parts of condensed milk and water, by weight) by being allowed to drink it for 5 minutes per day, 17 hours after they had eaten their meal of chow; the remainder of the rats were allowed to drink sucrose solution (19.7 percent by weight) under the same conditions. The rats ate these foods in test cages similar to the home cages, except that the test cages had provisions for recording the number of times each rat licked the spout.

The conditioning trial was administered on day 9. Half the rats under each familiarization condition were allowed 100 licks of the familiar food in one test cage; they were then immediately transferred to a second test cage, where they were permitted 100 licks of the other (novel) food (termed the f→n procedure). The remaining rats received the novel food prior to the familiar food (n→f). After they had eaten both foods the rats were returned to their home cages, from which the water bottles had been removed. After 60 to 70 minutes, half the rats exposed to each of the four previous combinations of treatments were x-irradiated and half were not, so that there were eight groups with six rats in each group. Radiation consisted of 50 roentgens received in the course of 21.3 seconds from a GE Maxitron 250 KVP unit at 30 ma, 250 kv, filtered through 1 mm of aluminum and 0.5 mm of copper. The control rats were placed under the unenergized x-ray unit. The rats were given water at their regular feeding time, about 4½ hours after they were irradiated, and it was available for the remainder of the experiment.

Conditioning was tested on day 12. Each rat was deprived of food for 15 to 18 hours and then was placed in the test cage for 30 minutes with free access to both the novel and the familiar food. Preference for the novel food was defined as the number of licks to the novel food divided by the total number of licks.

The irradiated rats showed a lower preference for the novel food than did the controls regardless of which earlier experimental procedures were used (Table 1). However this effect was not statistically reliable if only the rats for which milk was novel were considered. A probable reason is that there was a strong overall preference for sucrose, so that when milk was novel, the preference for it among the control rats was too small to permit a reliably smaller preference among the irradiated rats. Since rats prefer milk to grape juice (*7*), the procedure was repeated

Table 1. Mean preference for novel food: choice between sucrose and milk. An F test (novel food by conditioning sequence by irradiation) shows the following factors had reliable effects; novel food ($p < .001$), irradiation ($p < .01$), and irradiation by novel food interaction ($p < .05$). All other effects had $p > .25$. Because of the significant interaction, separate F tests were used to analyze the irradiation effect for each novel food; the irradiation effect was reliable when sucrose was novel ($p < .01$), but not when milk was novel ($p = .18$). Preference expressed as ratio between number of licks to the novel food and total number of licks.

Conditioning procedure	Preferences	
	Irradiates	Controls
	Novel food: sucrose	
f→n	0.534	0.948
n→f	.673	.904
	Novel food: milk	
f→n	.004	.036
n→f	.005	.015