R. Paysinger and Dr. C. S. Simons for their assistance during these experiments.

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Received November 13, 1953. P.S.E.B.M., 1954, v85.

Inhibition of Brain Respiration *in vitro* by Bilirubin. Reversal of Inhibition By Various Means.*† (20849)

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Kernicterus is the yellow staining of areas of gray matter found at autopsy in infants dying of erythroblastosis fetalis(1). The prevention of kernicterus and its concomitant nerve cell damage by replacement transfusion therapy provides the chief justification for that procedure(2). Other conditions than hemolytic anemia affecting the newborn, and associated with jaundice, may also be complicated by kernicterus(3,4). Although the pigment as it is found within nerve cells has been identified as mesobilirubin, the toxic agent or agents remain unknown(5). The present research was undertaken to see if bilirubin itself could be a contributing factor to the death of nerve cells. Although clinical experience indicates that jaundice is well tolerated by adults, high serum levels of indirect bilirubin in neonatal life are associated with a high death rate stemming apparently from kernicterus(6), and follow-up studies of infants recovered from erythroblastosis show an association between severe jaundice and a later impairment of intelligence(7).

Material and methods. Rats weighing from 25 to 75 g were decapitated, their cerebral hemispheres removed and at once chilled and chopped with scissors into pieces less than approximately 2 mm in their largest diameter. From 200 to 300 mg of chopped brain were placed in each of 4 to 6 Warburg flasks, the air replaced with oxygen, and oxygen uptake at 24.6°C recorded for the first 40 to 50 minutes after temperature stabilization. The fluid bathing the brain consisted of an electrolyte solution either as such for controls or with bilirubin and other test substances dissolved in it. The composition of the fluid, before the addition of bilirubin and adjustment of pH was as follows: NaCl .126, KCl .0025, Na_2CO_3 .082, Buffer .008 molar (Tris-(hydroxymethyl)Aminomethane). After the test substances were dissolved, the control and

* Appreciation is expressed for valuable suggestions and help received from Drs. Louis K. Diamond, Lytt Gardner and John Taggart.

† This research was supported by a grant from the Public Health Service.

TABLE I.
Oxygen Uptake of Chopped Rat Brain in Various Media Expressed in μl/mg/hr (Wet Wt).

Additions to medium	Observations	Mean ± stand. dev.	Significance of difference from 1) Control	2) Bilirubin
1) None—control	22	.663 ± .065		
2) Bilirubin	31	.490 ± .066	$p < .001$	
3) Biliverdin	9	.623 ± .035	$p > .05$ (not sig.)	$p < .01$
4) Bilirubin + cytochrome C	8	.689 ± .094	$p = .5$ (not sig.)	
5) Cytochrome C	8	.733 ± .048	$p < .05$	$p < .01$
6) Bilirubin + methylene blue	6	.680 ± .106		$p < .01$

experimental solutions were adjusted with HCl to a pH similar to each other within 0.05. Osmolarity, estimated by determining the freezing point, was adjusted with distilled water to within 4 milliosmoles. From day to day, larger variations were permitted, pH varying from 7.9 to 8.3 and milliosmolarity from 265 to 291. Variations of this size did not affect the results appreciably. The substances tested consisted of bilirubin, oxidized bilirubin ("biliverdin"), cytochrome C and methylene blue. The bilirubin was obtained from the Amend Drug and Chemical Co., N. Y. City, lot No. C79142. This bilirubin is indirect. The cytochrome C was obtained from the Treemond Co., N. Y. City, lot No. 3719. Oxidized bilirubin, presumed to be biliverdin because of its green color, was prepared by bubbling oxygen through the bilirubin solution in a bath of boiling water for 3 hours. Neither oxidation with oxygen, nor the addition of cytochrome C in a 10^{-4} M concentration resulted in any visible precipitation. Methylene blue, however, when mixed with bilirubin causes a fine granular precipitation.

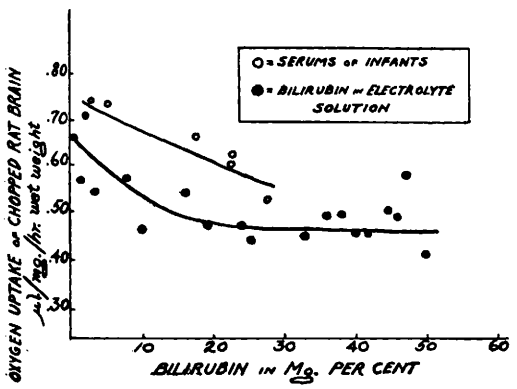

FIG. 1.

Results. The results appear in the Table and in the Figure. The Figure suggests that above about 25 mg % of bilirubin, the degree of respiratory inhibition becomes constant. Accordingly, for statistical validation of the depression in Q_{O_2} the comparison is made between the oxygen uptake by brain in the control solution and that in bilirubin solutions varying in concentration from 20 to 55 mg %. Twenty-two control flasks yielded a Q_{O_2} of 0.663 ± 0.065 (mean ± S.D. of the series) μl per mg per hour. Thirty-one flasks containing bilirubin solutions yielded a mean value of 0.490 ± 0.066 μl/mg/hr. The difference between these 2 means is 9.6 times its standard error, so that p is less than 0.01. The difference is highly significant.

Nine experiments using "biliverdin" yielded a mean of 0.623 ± 0.035 μl/mg/hr, a value slightly less than that for the controls, but not quite significantly so. Whether the slight apparent depression of Q_{O_2} by biliverdin is the result of experimental error, of contamination of the sample with bilirubin or depends upon a slight action by biliverdin itself is not apparent from these experiments. The difference in action between bilirubin and biliverdin, however, is highly significant, being 8 times its standard error ($p < 0.01$).

Cytochrome C added to the bilirubin solution in a concentration of 10^{-4} M increased oxygen consumption to a level slightly above that of the controls. Cytochrome C, when brain tissue is present, converts bilirubin to biliverdin. This oxidation presumably involves cytochrome oxidase from broken cells,

since cytochrome C alone does not accelerate the conversion of bilirubin to biliverdin when the solution stands at room temperature. However, even when brain tissue is present, the conversion to biliverdin is slow, a moderate amount of green color being present after 2 hours. In contrast with this slow action, the effect of cytochrome C on the bilirubin inhibition of oxygen uptake is noticed in the first period after the introduction of the cytochrome. Inspection of the flasks showed no visible green at this time, and even after 1 hour there was very little green to be seen. Cytochrome C alone raised the respiration of brain to 10% above the control rate, whereas the bilirubin solutions to which cytochrome C had been added supported respiration of the brain at a rate 43% greater than when the bilirubin was not accompanied by cytochrome C. A possible interpretation is that cytochrome C overcomes the inhibition by bilirubin of part of the oxidative process.

Methylene blue in equimolar concentration with that of the bilirubin reverses the inhibition, but the action is not immediate. In fact, the methylene blue must be mixed several hours prior to an experiment. During this period, there is a slow conversion of bilirubin to biliverdin, and, in addition, a precipitate forms consisting of fine blue-green granules. When added to the control flasks, methylene blue induced no change in Q_{O_2} in 4 observations.

In addition to the above, observations have been made with rat brain bathed in serums from newborn infants with varying degrees of indirect bilirubinemia. The results are indicated by the open circles in the Figure. Each point is the average of from 2 to 4 flasks. There were 9 flasks with serum bilirubin concentrations of 7 mg % or less and 9 with concentrations of from 17 to 27 mg %. Comparison of these two sets of flasks discloses that the probability of the difference between the two being the result of chance is 0.05. Further work with heavily jaundiced serums from infants is needed, but because of the use of replacement transfusion, such serums are scarce in this clinic.

Three observations were made with commercial bilirubin purified by crystallization, and 2 observations with bilirubin purified by extraction with chloroform and washing with water. These preparations gave results identical to those with the product as obtained from the supply house.

Two observations using liver slices showed a depression of Q_{O_2} by bilirubin similar to that found with brain. It is presumed that the liver slices could be cut with less trauma to the cells than might be expected by the process of chopping the brain. Additional evidence that bilirubin can exert its action on intact cells is obtained from the fact that no greater inhibition was obtained in two observations in which a brain homogenate was used. A final answer to the question of whether intact cells can be affected by bilirubin, however, awaits *in vivo* observations.

Comment. These observations indicate that bilirubin depresses the respiration of brain *in vitro*. It seems likely, though not certain, that this action can be exerted upon intact cells. It is of interest to note that Najjar(8) has postulated that bilirubin might have a toxic action upon intact cells.

It remains for future work to show whether the phenomena described in this report also take place in the living animal. One does not know what the margin of oxidative safety for a neuron is. One might postulate the participation in the infant of other factors leading to tissue anoxia, such as anemia, incomplete expansion of lungs, sludging of red blood cells, and depression of enzyme systems by excessive use of therapeutic oxygen. Were the respiratory center depressed by the action of these factors combined with the inhibitory effect of bilirubin, a vicious cycle would be set up. In support of such a theory is the observation in this clinic that respiratory rates are very slow for many hours prior to death from kernicterus.

The confinement of kernicterus to the newborn period is another fact which is not explained by the theory that bilirubin is the toxic agent. The blood-brain barrier is known to be less resistant to trypan blue(9) and to bilirubin(10,11) in young animals than in older ones. The rapid maturation of cellular enzyme systems shown by Potter and coworkers(12) might also have some bearing

on the age incidence of kernicterus. These subjects need more attention than they have been so far accorded.

Summary and conclusions. Bilirubin added to the ambient fluid in concentrations above 20 to 25 mg % depresses the respiration of chopped rat brain by approximately 25%. This inhibition can be counteracted by oxidation of the bilirubin and by the action of methylene blue on the bilirubin. Cytochrome C also reverses the inhibition. The mechanisms of these reversals appear to be different.

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Received January 4, 1954. P.S.E.B.M., 1954, v85.

Urinary Estrogens and Related Compounds in Postmenopausal Women With Mammary Cancer: Effect of Cortisone Treatment. (20850)

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This paper presents data on the quantification of estrogens and related compounds in the urines of 15 postmenopausal women. Eight were normal and 7 had cancer of the breast. The effect of cortisone therapy upon urinary estrogens was investigated at various times in 4 of the 7 cancer patients. Observations were also made concerning the influence of advancing disease and of the surgical removal of X-rayed ovaries.

Methods. From the 8 normal women 72- to 96-hour specimens were analyzed while only 24- to 48-hour collections were available on the cancer patients. The accuracy of each collection was checked by creatinine determination and the volume corrected when necessary. One aliquot of each specimen was subjected to the usual HCl hydrolysis(1) and a second to Zn-HCl hydrolysis(2) prior to continuous benzene extraction(1) for bioassay. The total estrogenic activity released by these 2 procedures is designated T_o and T_{zn} respectively. The residual urines after benzene extraction of the HCl hydrolyzed aliquots were further studied by methods previously described(3) in order to determine how much of the difference between T_o and T_{zn} values might be accountable to more complete hydrolysis of conjugated estrogens. The results of these latter studies are not included in this report since in no instance could the difference observed between T_o and T_{zn} values be explained on this basis. It would appear to be safe to assume that the very high T_{zn} values found in many of the cancer urines represent unknown constituents, possibly estrogen oxidation products or precursors(3), related to the estrogens but exhibiting estrogenic activity only after Zn-HCl treatment. T_o values, on the other hand, represent estrogens excreted as such.

Bio-assay was performed by a standardized procedure(3) on spayed mature female rats. Unless more than 50 I.U. of T_o or more than