Some Effects of Nonoptimally High Incubation Temperatures on Chicken Blood Chemistry[1]

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Introduction

Chicks hatched from eggs incubated for varying numbers of days initially or terminally at 41° (all temperatures are reported as centigrade) have shown significant reduction in body weight when compared with control animals hatched from eggs incubated under normal temperatures of 37.5° for the entire incubation period (8). Marked behavioral aberrations have also been noted in the same birds (7, 9, 10, 11).

In an effort to assess at physiological levels the extent and nature of damage to the nervous system caused by the heat treatment, pilot biochemical investigations have been initiated. The first type of tissue studied was blood, since observations to date have indicated its involvement in a number of ways: the blanching of combs and wattles, the inability or apparent inability of temperature-stressed chicks to regulate body heat effectively (unpublished data), and the tendency of experimental birds to huddle (11). Consequently, this report is based upon results obtained through tests of hemoglobin, blood sugar, blood lactic acid, and hematocrit.

Materials and Methods

Group I chicks

Three hundred twenty-two White Leghorn (DeKalb) eggs were incubated for the first 3 days at 41° and were then placed in a normal temperature incubator at 37.5° for the remainder of the incubation period. Sixty-three chicks hatched under these conditions. (All such temperature-stressed chicks are referred to as experimental animals.) Of 96 eggs incubated at the normal temperature of 37.5° for the entire incubation period, 60 hatched. (All chicks incubated at optimal temperature are referred to as control animals.)

When the birds were 4 weeks old, 15 experimental and 15 control animals were selected at random for hemoglobin determination. The direct photometric method for hemoglobin, as described by Hawk, et al. (12), was used. The addition of a small amount of hydrochloric acid (2, 4, 14, 16) was found necessary in order to decrease the turbidity of the solution.

For determination of blood sugar, 5 control and 5 experimental animals were selected at random from the chicks not used in hemoglobin tests. These chicks were 5 weeks old when tested. The micromethod of Folin and Malmros for blood glucose, as described in Hawk, et al. (12), was used. Prior to the tests the birds were deprived of food but not water for 6 hours.

1. Supported by research grant B-2128, U. S. Public Health Service, Council on Neurological Diseases and Blindness.

Group II chicks

These chicks were hatched from a different lot of White Leghorn (DeKalb) eggs under the same conditions as those in Group I. Of the 276 eggs exposed to 41° for the first three days of incubation, only 13 hatched. Forty-nine control birds were obtained by incubating 84 eggs at normal temperatures. Eleven control and 11 experimental chicks were selected at random for all of the blood tests. Hemoglobin and sugar tests were run on the blood of these chicks, as described above for Group I. Blood lactic acid was determined by the micromethod of Natelson (15). The hematocrit values were ascertained by the Guest-Siler (6) technique, as modified by Johnston (13). Tests were run when the chicks were 4, 6, and 13 weeks of age. Prior to the tests the birds were deprived of food but not water for 6 hours.

Group III chicks

This group of 60 chicks (30 experimentals and 30 controls) was selected at random from a third lot of White Leghorns (DeKalb). Incubation conditions of the eggs from which these chicks were hatched were the same as those described for Groups I and II. A total of 68 chicks was obtained from 492 eggs incubated for the first 3 days at 41°, and 65 chicks were obtained from 108 eggs incubated at normal temperatures for the entire incubation period.

When these chicks were 2 weeks old, hemoglobin, sugar, lactic acid, and hematocrit values were determined by the methods described for Group II chicks. Prior to the tests the birds were deprived of food but not water for 6 hours.

For all groups, venous blood was obtained from the right atrium or ventricle and was ejected from the syringe into a heparinized-oxalated test tube. The blood used for each specific test was pipetted from the test tube by appropriate micro-pipets. For the hematocrit, blood from the same source was obtained in heparinized capillary tubes. All tests were begun immediately after the blood was withdrawn from the heart. Time was a particularly critical factor in the case of lactic acid tests, so that glycolysis, which would result in the conversion of blood sugar to lactic acid, would not occur and thus give false values. However, it was inevitable that attempting to run the tests simultaneously would result in some time lag.

Since comparative results rather than absolute values were desired, the blood pipets were not recalibrated, although care was taken to insure that the experimental groups were tested with the same pipets as those used on the control groups. In other words, pipets were randomized within groups, but the same pipets were used among groups. A further precaution observed was that of using the most accurate pipets obtainable, either class "A" or those certified by the Committee on Microchemical Apparatus, Division of the American Chemical Society.

Results

The results of blood glucose and hemoglobin tests of Group I chicks are shown in Table 1. The mean values for the control chicks are higher in the cases of both types of tests. Student's "t-test" elicited

TABLE 1. Ranges and means of blood tests of Group I chicks and results of Student's "t-test."

Blood constituent		Controls	Experimentals
Glucose (mg/100 ml blood)	Range	137.0-186.0	101.0-134.0
	Means[2]	155.8	115.2
	Number of chicks tested, all 5 weeks old	5	5
Hemoglobin (g/100 ml blood)	Range	4.2-10.0	1.4-9.8
	Means[3]	8.9	5.0
	Number of chicks tested, all 4 weeks old	15	15

2. Highly significant difference: df = 8; t = 3.671; $P < .01$
3. Highly significant difference: df = 28; t = 5.097; $P < .001$

values of t which were highly significant for these mean differences ($P<.01$ for sugar; $P<.001$ for hemoglobin). Error variances were tested by the "F-max" procedure and were found to be homogeneous.

The chicks in Group II were tested for all 4 blood constituents: sugar, hemoglobin, hematocrit, and lactic acid. Table 2 summarizes the results of these tests. It is seen that the mean values for hemoglobin, hematocrit, and lactic acid are higher for the control than for the experimental animals. However, experimental birds have higher mean blood sugar values than do control birds. Analyses of variance failed to yield significant F-values for hemoglobin, hematocrit, and blood sugar. Since the error variances for the lactic acid data were non-homogeneous by the "F-max" test, the mean difference for this blood component was tested by means of "the t-test for samples with non-homogeneous error variances" as described by Edwards (3), which elicited a significant value of t ($P<.05$).

The birds in Group III consisted of 30 controls and 30 experimentals; it is emphasized again that all tests for this Group were run simultaneously. Table 3 is a summary of the test results for Group III. While the means for glucose and for lactic acid are higher for the controls, the means for hematocrit and hemoglobin are higher for the experimental animals. Analyses of variance failed to yield significant values of F for glucose and hematocrit, but the F-values for hemoglobin and lactic acid are significant ($P < .05$ and $P < .01$, respectively), with hemoglobin content higher for experimentals and lactic acid higher for controls. For these data, error variances are homogeneous by the "F-max" test.

TABLE 2. Ranges and means of blood tests of Group II chicks and results of "t-test" and analyses of variance.

Blood constituent		Controls	Experimentals
Lactic acid (mg/100 ml blood)	Range	3.7-8.4	3.5-4.7
	Means*	5.6	4.2
	Posthatching age of chicks	4 weeks	4 weeks
Glucose (mg/100 ml blood)	Range	54.2-208.5	54.2-188.5
	Means $(NS)_1$	125.1	147.2
	Posthatching age of chicks	6 weeks	6 weeks
Hemoglobin (g/100 ml blood)	Range	7.3-9.9	6.6-11.3
	Means $(NS)_2$	8.8	8.2
	Posthatching age of chicks	13 weeks	13 weeks
Hematocrit (% volume packed red blood cells)	Range	29.0-33.8	28.4-33.5
	Means $(NS)_3$	31.9	30.7
	Posthatching age of chicks	6 weeks	6 weeks

*Significant difference: $t = 2.604$; $P < .05$ ("t-test" df = 20)
$(NS)_1$ Not significant: $F = 1.216$; $P > .05$ Analyses of variance df:
$(NS)_2$ Not significant: $F = 1.450$; $P > .05$ Between Groups = 1
$(NS)_3$ Not significant: $F = 3.239$; $P > .05$ Within Groups = 20

Discussion

The reliability of the methods used in these determinations might be questioned on the basis that other techniques exist which would give more accurate results. A comparison of the means and ranges of the data for controls with "standards" (1) indicates that the hematocrit and hemoglobin values reported herein compare favorably with results obtained by other and similar methods (2, 4, 13, 16). The blood sugar values differ markedly in mean and minimal values, but maximal glucose content compares favorably with "standards" (1). The authors were unable to find a "standard" value for chicken blood lactic acid in the

TABLE 3. Ranges and means of blood tests of Group III chicks and results of analyses of variance (all chicks 2 weeks old).

Blood constituent		Controls	Experimentals
Lactic acid (mg/100 ml blood)	Range	0.4-3.6	0.05-3.1
	Means**	2.3	1.0
Glucose (mg/100 ml blood)	Range	108.7-193.1	113.0-186.2
	Means $(NS)_1$	151.8	146.3
Hemoglobin (g/100 ml blood)	Range	5.1-11.4	6.2-11.4
	Means*	8.2	9.0
Hematocrit (% volume packed red blood cells)	Range	22.6-39.3	23.7-36.5
	Means $(NS)_2$	28.1	29.9

*Significant difference: $F = 4.400$; $P < .05$
**Highly significant difference: $F = 10.013$; $P < .01$
$(NS)_1$ Not significant: $F = 1.021$; $P > .05$
$(NS)_2$ Not significant: $F = 1.126$; $P > .05$
df Between Groups = 1; df Within Groups = 58

literature. Perhaps it is unnecessary to point out again that absolute values are not of particular interest in this research, since the comparison between the values for control and experimental animals is of prime concern. In fact, the optical density values alone, as read from the spectrophotometer, could have supplied equally valid information from the comparative viewpoint. The reproducibility of these values must await further research. As far as studies conducted to date are concerned, it appears that the results can be reproduced within the limits of biological variability. Further investigation is currently in progress in an attempt to verify this.

When the glucose values for the chicks of Group I (Table 1) are compared with those for the chicks in Groups II and III (Tables 2 and 3, respectively), there appears to be some inconsistency. While the differences in glucose between controls and experimentals of Group I reflect a highly significant reduction in blood sugar in the experimentals, the difference for the Group II animals reveals an opposite effect—an increase in sugar content of the blood of experimentals. This increase, however, is not significant. The experimentals in Group III show a lower blood sugar content, but the difference again is not significant. The appropriate interpretation of these findings appears obscure at the present time. The smaller amount of glucose in Group I animals could be a reflection of inherent organismic variability which may have resulted in statistical significance because of the small number of animals employed (5 in each lot). Possible procedural errors might also account for the conflicting results. On the other hand, it is entirely possible that the differences obtained are reliable and that they appear

only at certain times. Each lot of chicks might manifest a different order of appearance and magnitude of these defects. The different groups of chicks were not all tested at the same ages, and thus age may also be a factor in the onset of these differences. Further tests must be conducted in order to make a final decision. Speculation on the implication of significant differences in sugar content would be premature prior to resolution of the apparent inconsistencies in the data.

The accurate determination of hemoglobin in chicks is particularly difficult because of the turbidity of the solutions which is caused by the nucleated red blood cells. However, this undesirable feature is eliminated by the method used, and satisfactory results can be readily achieved and rapidly reproduced. Total hemoglobin is measured, and the values obtained probably do not accurately reflect the absolute hemoglobin content.

The highly significant reduction in hemoglobin content of the experimental chicks in Group I is contradicted by a significant increase in hemoglobin in the experimental chicks in Group III. While the mean is lower for Group II experimental birds as compared with controls, the difference is not significant. What makes this puzzling and certainly unique from a statstical viewpoint is that the number of chicks from all groups is ample for statistical sampling. It is unfortunate that the hematocrit was not taken on the blood of the Group I birds, for in the Group III birds the ratio of packed red blood cells to total serum volume was greater (but not statistically significant) in the experimentals than in the controls. One could almost predict this in view of the significant increase of hemoglobin in the experimentals. Any consistent difference in hemoglobin would have to be investigated further with respect to the red blood cells. Are the latter different in number, size, or development? Has their oxygen-carrying capacity been impaired or enhanced in the case of the experimentals? The hematocrit can give information relative to number and size of red blood cells, but it cannot distinguish between them. In passing, it can be noted that the means for hematocrit obtained of chicks in Groups II and III are remarkably similar, and they appear to be positively correlated with the means for hemoglobin.

The test for blood lactic acid gives the most consistent results. Although this test has been given to only two different hatches of chicks, the total number of chicks tested (82 in the two groups) is quite impressive from the standpoint of biological numbers and lends credence to the reliability of the results of statistical analyses. In both Groups II and III the experimental birds have mean values for blood lactic acid which are significantly lower than those for the control birds. To be sure, the means and ranges are quite dissimilar in the two groups, but so are their ages. In view of what one is tempted to call the "retarded mentality" of the experimental chicks, their lowered blood lactic acid appears to be highly significant. However, what precise effect (if any) this has on the brain or behavior of the hatched chicks remains obscure.

The brain absorbs lactic acid as well as glucose from the circulating cerebral blood. Lactic and pyruvic acids, the principal end products

of anaerobic glycolysis, are resynthesized to glycogen during the aerobic phase. Presumably, this reconversion of lactic and pyruvic acids to glycogen occurs in brain cells as well as in muscle. However, the brain stores little, if any, glycogen. Consequently, the brain must rely almost entirely on free blood glucose for anaerobic glycolysis. This implies that there must be a readily available supply of free blood sugar to the brain at all times. (Perhaps the glucose-lactic acid route is not used to any appreciable extent by brain cells, or is not the only one traveled in brain metabolism.)

The sensitivity of the brain to lack of sugar is well known. Lactic acid effects are not so well documented, however. Blood lactic acid increases considerably during violent exercise but decreases rapidly during the resting phase. Pathologic increases in blood lactic acid are noted whenever a deficiency of oxygen occurs (hypoxia). Recently Grabowski (5) has shown that lactic acid accumulation induced by subjection to hypoxia is responsible for abnormal development in chick embryos. He further indicated that direct application of lactic acid to the embryo produced results similar to those induced by hypoxia. His hypoxia- and lactic acid-induced malformations produced embryos with severe edema followed by formation of cutaneous blisters and hematomas. In work previously reported by Gunther and Jones (8) it was found that chick embryos subjected to abnormally high temperatures for the first 5 days of incubation were significantly heavier and more advanced in development than controls. Some of these embryos resembled those described by Grabowski (5), particularly with respect to the general edema and to the formation of subcutaneous blisters. As a purely speculative interpretation, it is quite possible that this condition in our embryos resulted from lactic acid accumulation, although it is difficult to conceive of a lack of oxygen in our experimental set-up.

If there is a lactic acid accumulation in the heat-stressed embryos, and if the heat treatment somehow causes a lowering of the oxygen supply (perhaps through loss of hemoglobin), there would appear to be a remarkable transformation of embryonic biochemical processes to just the opposite effect in heat-stressed hatched chicks. As indicated in Tables 2 and 3, there is a decrease in blood lactic acid of Groups II and III experimental chicks from that of the controls, and an increase in hemoglobin of Group III experimental chicks over that of the controls. The increased hemoglobin content of the blood of the Group III experimentals over that of the control chicks agrees in theory with the decreased blood lactic acid in the experimentals from that of the controls. That is, increased hemoglobin content of the red blood cells could make available more oxygen for reoxidation of reduced diphosphopyridine nucleotide (DPNH) through the riboflavincytochrome hydrogen transfer system (DPNH→DPN). This might increase lactic acid dehydrogenase activity so that the equilibrium between lactic and pyruvic acids is disturbed. The net result would be a lowering of lactic and an increasing of pyruvic acid content in the blood. We found a consistent and significant decrease in blood lactic acid of the experimentals from the controls, but did not run tests for pyruvic acid. Our current research activity focuses also on this problem. The simultaneous

determination of blood pyruvic and lactic acids in hatched chicks may shed some light. Also, determination of lactic and pyruvic acids in the blood of embryos during the first five days of incubation at the nonoptimally high temperature (41°) may yield information as to whether the edema and subcutaneous blisters are caused by lactic acid accumulations. At any rate, it seems reasonable to assume that the short-term nonoptimally high incubation temperatures cause alterations either in metabolic pathways or activity.

Summary

Chickens were permitted to hatch from eggs which had been placed in a nonoptimally high temperature of 41°C. during the first 3 days of the incubation period, and from eggs which had been incubated at the same time at normal temperature (37.5°C.) for the entire period.

The hatched chicks were tested at different ages for hemoglobin, blood sugar, blood lactic acid, and the hematocrit.

In two groups of chicks hatched from eggs at different times, hemoglobin values differed significantly between the control and experimental birds. In one group the blood of heat-stressed chicks contained less hemoglobin than the blood of control chicks. In the second group of heat-stressed chicks the hemoglobin content was higher than in the controls.

Blood glucose levels were significantly lower in one group of heat-stressed animals than in controls. Differences among other groups were present but the mean differences failed to attain significance.

The hematocrit was similar for all groups investigated, and means did not differ significantly.

Blood lactic acid measured significantly lower in heat-stressed birds than in controls in all groups studied. One experimental group with a significantly lower blood lactic acid content had a significantly higher hemoglobin content than did control chicks.

These data are compared with available "standards." Discussion of the significance of these differences relative to aberrant behavior and to retarded mentality centered around the possible biochemical mechanisms involved.

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