Significance of the Level of Serum Aldolase in Tumor-bearing Animals*

JOHN A. SIBLEY, GERARD A. FLEISHER, AND GEORGE M. HIGGINS

WARBURG AND CHRISTIAN (9) REPORTED THAT FIVE OF THE ENZYMES INVOLVED IN GLYCOLYSIS ARE NORMALLY PRESENT IN THE BLOOD SERUM OF RATS AND THAT THE LEVELS OF TWO OF THESE, ALDOLASE AND TRIOSE ISOMERASE, ARE CONSISTENTLY ELEVATED IN THE SERUM OF RATS BEARING LARGE TUMORS. THEY SHOwed THAT THE INCREASE IN SERUM ALDOLASE IS PROPORTIONAL TO THE SIZE OF THE TUMOR BUT CONCLUDED THAT THE TUMOR ITSELF WAS NOT THE SOURCE OF THE EXCESS ALDOLASE. WARBURG POSTULATED THAT THE GLYCOLYTIC ENZYMES OF TUMORS MIGHT HAVE A COMMON ORIGIN IN MUSCLE AND THAT THE TUMOR MIGHT THEN ACT AS A BIOLOGIC PARASITE.

EMPLOYING A NEW METHOD FOR THE DETERMINATION OF ALDOLASE, SIBLEY AND LEHNINGER (6, 7) CONFIRMED THE BASIC OBSERVATIONS OF WARBURG AND CHRISTIAN. WITH THIS NEW ANALYTICAL METHOD THEY DEMONSTRATED MUCH HIGHER ALDOLASE VALUES FOR NORMAL TISSUES, SUCH AS LIVER AND KIDNEY, THAN HAD BEEN REPORTED PREVIOUSLY (9), AND THEY SUGGESTED, THEREFORE, THAT MUSCLE IS NOT NECESSARILY THE ONLY SOURCE OF THE ENZYME IN THE SERUM.

KUN AND CO-WORKERS (2) REPORTED A HIGH ALDOLASE ACTIVITY IN THE ASCITIC FLUID OF MICE BEARING THE EHRLICH ASCITES TUMOR. WARBURG AND HIPPELER (10) SHOWED THAT, WHEN ASCITIC FLUID CONTAINING THE CELLS OF THE EHRLICH MOUSE ASCITES TUMOR WAS INCUBATED ANAEROBICALLY, A STEADY INCREASE IN ITS ALDOLASE CONTENT OCCURRED BUT THAT, WHEN IT WAS INCUBATED AEROBICALLY, THERE WAS LITTLE OR NO INCREASE IN THE ENZYME CONTENT. IN CONTRAST TO WARBURG'S EARLIER THEORY, THEY (10) SUGGESTED THAT THE INCREASE IN SERUM ALDOLASE IN ANIMALS BEARING SOLID TUMORS MIGHT BE THE RESULT OF ANAEROBIC CONDITIONS IN PART OF THE TUMOR. SCHADE (8) REPORTED AN INCREASE OF ALDOLASE IN THE BLOOD SERUM AND ASCITIC FLUID OF MICE BEARING THE MOUSE ASCITES THYMOMA OR THE EHRLICH ASCITES CARCINOMA. HE ALSO NOTED THAT ANAEROBIC INCUBATION OF THE EHRLICH CARCINOMA INCREASED THE ALDOLASE CONTENT OF THE ASCITIC FLUID MUCH MORE THAN DID AEROBIC INCUBATION.

OUR PURPOSE IN THE PRESENT INVESTIGATION WAS TO STUDY IN MORE DETAIL THE FACTORS THAT GOVERN THE LEVEL OF SERUM ALDOLASE IN BOTH NORMAL AND TUMOR-BEARING RATS AND TO DETERMINE, IF POSSIBLE, THE SIGNIFICANCE OF THE SERUM-ENZYME LEVEL AND ITS RELATIONSHIP TO MALIGNANCY.

METHODS

ALDOLASE ACTIVITY WAS DETERMINED BY THE METHOD OF SIBLEY AND LEHNINGER (7). THE MATERIAL FOR ASSAY WAS INCUBATED FOR 30 MINUTES AT 38°C. WITH FRUCTOSE DIPHOSPHATE IN THE PRESENCE OF HYDRAZINE, WHICH SERVED TO BIND THE TRIOSES FORMED. THE BUFFER WAS TRIS (HYDROXYMETHYL)AMINOMETHANE AT pH 8.7. THE REACTION WAS STOPPED WITH TRICHLOROACETIC ACID, AND THE TRIOSES WERE DETERMINED THROUGH THE FORMATION OF A 2,4-DINITROPHENYLHYDRAZINE DERIVATIVE WHICH PRODUCED A CHARACTERISTIC COLOR IN ALKALINE SOLUTION. THE COLOR DENSITY MEASURED AT 540 MU WAS DIRECTLY PROPORTIONAL TO THE AMOUNT OF TRIOSES FORMED AND, HENCE, TO THE ACTIVITY OF THE ENZYME. IN THIS PAPER, THE ALDOLASE CONTENT IS EXPRESSED AS THE NUMBER OF ML. OF FRUCTOSE DIPHOSPHATE SPLIT IN 1 HOUR AT 38°C. AND pH 8.7 PER STATED VOLUME OF SERUM OR WEIGHT OF TISSUE.

IN ACCORDANCE WITH THE SUGGESTION OF DOUNCE AND CO-WORKERS (1), WE FOUND THAT THE ENZYME WAS INACTIVATED AT 38°C. WHEN IT WAS PRESENT IN MUSCLE EXTRACTS OR OTHER DILUTE SOLUTIONS, BUT NOT WHEN IT WAS PRESENT IN SERUM. A PROTECTIVE ACTION OF THE PROTEINS APPEARED TO BE THE EXPLANATION. INSTEAD OF RUNNING THE DETERMINATIONS AT A LOWER TEMPERATURE, WE ADDED 0.5 ML. OF A 5 PER CENT SOLUTION OF EGG ALBUMIN TO THE INCUBATION MIXTURE IN ALL ASSAYS OF TISSUE EXTRACTS. WITH THE ADDITION OF THIS PROTEIN, THE ENZYME WAS NOT INACTIVATED AT 38°C. DURING THE 30 MINUTES REQUIRED FOR THE TEST.

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Rats of the Sprague-Dawley strain and the Walker carcinosarcoma 256 were used in these experiments. Pieces of the tumor were ground in normal saline solution with added penicillin, and 0.5 ml. of the suspension was injected through a large-gauge needle into the subcutaneous tissues of the back. The transplantation was successful in almost all cases. The surgical procedures, including extirpation of the tumor, adrenalectomy, splenectomy, nephrectomy, ureteral ligation, and bile-duct ligation, were performed on the rats under ether anesthesia.

For measurements of serum levels, blood was obtained from the heart while the rats were lightly anesthetized with ether. Great care was exercised in withdrawing the samples of blood to avoid hemolysis and trauma to the muscle, since the higher enzyme content of erythrocytes and of muscle tissue would then lead to falsely high results. For the aldolase assays of various tissues, the animals were killed and exsanguinated, and the specimens were rapidly removed and placed on ice. Portions were weighed, ground with an all-glass homogenizer in cold water, and diluted to an appropriate volume with cold water.

For the experiments with tissue slices, the organs were rapidly removed and placed on ice. As soon as possible, thin slices of the organs were made by hand with a chilled razor blade. Slices were washed 3 times in cold normal saline solution and then placed in vessels containing Krebs-Ringer bicarbonate buffer at pH 7.4 in a water bath kept at 38° C. Glucose was added to some of the vessels, and an atmosphere of either oxygen and carbon dioxide or nitrogen and carbon dioxide was maintained. Aliquots of the medium were removed at intervals for determination of aldolase. At the end of the experiment, the slices were removed, dried in an oven at 110° C., and weighed.

RESULTS

Serum aldolase in normal and in tumor-bearing rats.—The levels of serum aldolase of a large number of normal rats ranged from 30 to 100 units per milliliter, the majority being between 60 and 80 units. The age, size, or sex of the animal did not influence the enzyme level. These values are in full agreement with those previously reported (6).

All rats bearing large tumors displayed significant increases of serum aldolase. In contrast to previous reports (6, 9) our data show that the level of serum aldolase did not rise steadily in proportion to the increase of tumor volume. Rather, the enzyme level remained within normal limits for about 2 weeks of tumor growth and then rose rapidly to a maximum, where it usually remained in spite of continued increase in the size of the tumor (Chart 1). The maximal level tended to vary between 200 and 400 units per milliliter without apparent relationship to the appearance or size of the tumor.

Histologic study of the Walker carcinosarcoma 256 at different stages of its growth revealed that many microscopic foci of necrosis soon developed throughout the tumor. In subcutaneous implants which had grown to tumors about 2 cm. in diameter, the central parts were already very necrotic; these necrotic foci became extensive with the overall enlargement of the tumor. The microscopic foci of necrosis were noted not only in small solid tumors but also in the shell of apparently viable tissue of the very large growths.

Effect of removal of tumor.—Following the total surgical removal of large subcutaneous tumors, the elevated value for serum aldolase decreased rapidly and reached normal within 12 hours (Chart 2). This rate of decrease after excision of the tumor was found to parallel the rate of decrease that followed the intravenous injection of crystalline aldolase. If, from a rat bearing two tumors of different size, the larger one was removed, thus eliminating most but not all of the malignant tissue, the value for aldolase decreased to normal just as it did after total removal of the tumor from a rat that had only a single large tumor; but if the smaller tumor was removed, thus leaving a large volume of tumor tissue, the high value for aldolase was not affected.

Arteriovenous differences in aldolase levels.—The serum aldolase content of blood drawn from a vein leaving the tumor was compared with that of heart blood obtained at approximately the same
time. The value for serum aldolase in the heart blood was considered equivalent to that in the arterial blood supplying the tumor. In all instances, the value obtained from venous blood leaving the tumor was higher than that obtained from heart blood (Chart 3). The differences between the two were greatest in those cases in which the values obtained from heart blood were particularly high.

Specificity of increase in serum aldolase.—Cer-

Splenectomy did not affect the level of serum aldolase in normal rats, and it did not prevent a rise in the enzyme level in tumor-bearing rats. Pronounced enlargement of the spleen was therefore not considered of importance in producing the marked increase of serum aldolase in such animals. Likewise, bilateral adrenalectomy did not alter the enzyme level in normal rats, nor did it influence the elevation in animals with massive tumor growth.

Effect of x-rays and other inhibitors of tumor growth.—Exposure of the entire body of a normal rat to 350 r of radiation did not influence the level of serum aldolase, although it caused severe leukopenia. The effect of a single exposure of a rat bearing a large tumor, and hence showing a high level of serum enzyme, to 350 r was variable. When the tumor alone or the body alone was exposed to such ionizing radiation, the other part being shielded, there was usually little change in serum aldolase. However, when the entire animal including the tumor was exposed to the same amount of radiation, the serum aldolase decreased to normal (Chart 6). With higher doses, ranging from 450 to 550 r, serum aldolase decreased similarly, although often not entirely to normal, after exposure of either the tumor alone or the body alone.

The results of experiments involving exposure of tumor-bearing rats to ionizing radiation prompt-

[Diagrams and charts are shown in the original text, but not transcribed here.]

Anemia was produced by the repeated withdrawal of relatively large amounts of blood from the heart. The concentration of hemoglobin and of serum protein decreased as a result of the fast (Chart 4).

By withholding food for a 12-day period, animals lost about a third of their body weight, a decrease comparable to that sustained in animals bearing large tumors; but during this period significant changes in the level of serum aldolase did not occur. The concentration of blood hemoglobin and of serum protein decreased as a result of the fast (Chart 4).

Anemia was produced by the repeated withdrawal of relatively large amounts of blood from the heart. The concentration of hemoglobin thereby decreased to less than 25 per cent of its normal value, yet there were no changes in the value for serum aldolase (Chart 5). These experiments demonstrate that the decrease in serum aldolase after excision of the tumor was not dependent upon loss of blood with resulting hemodilution incident to surgical removal.

The possibility that these factors in themselves might influence the level of serum aldolase in these animals was considered and tested.

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Chart 4.—Effect of starvation on the body weight, the level of serum aldolase, the level of total serum protein, and the concentration of blood hemoglobin in the rat. Determinations on single animal representative of series.

Chart 5.—Effect of excessive loss of blood on the level of serum aldolase, the level of total serum protein, and the concentration of blood hemoglobin in the rat. Determinations on single animal representative of series.
of animals, the single intraperitoneal injection of 200 μg of nitrogen mustard (methylbis[β-chloroethyl]amine hydrochloride)/100 gm of body weight also tended to lower the high levels of serum aldolase.

Elimination of aldolase from the body.—After bilateral nephrectomy in normal animals the concentration of serum aldolase increased steadily, reaching about 2.00 units/ml in 48 hours, the usual duration of life following this procedure (Chart 7). A similar rise occurred after bilateral ligation of the ureters, suggesting that this increase of enzyme following nephrectomy was caused by urinary retention rather than by failure of a nonexcretory function of the kidney. Unilateral nephrectomy or unilateral ureteral ligation, however, did not alter the level of serum aldolase. Aldolase activity was not detected in voided urine; inactivation of the enzyme might occur, however, during the process of excretion. In vitro studies showed that incubation of urine with crystalline aldolase was not followed by any loss of activity.

Following the intravenous injection of varying amounts of crystalline aldolase into normal animals there were immediate marked increases in serum aldolase. The increases were proportional to the amount of the enzyme injected, and blood volume could be estimated from data thus obtained. The concentration of aldolase decreased promptly and reached normal in about 12 hours in those cases in which postinjection values of 450 units or less per milliliter were produced; proportionately longer intervals were required for the return to normal when the initial rise was greater (Chart 8).

In rats subjected to bilateral nephrectomy shortly before the intravenous injection of crystalline aldolase, the enzyme level decreased but at a much slower rate than in normal rats. The values for serum aldolase 24 hours after injection plus nephrectomy were always higher than those obtained 24 hours after bilateral nephrectomy alone. It appeared, therefore, that the loss of renal function retarded, but did not entirely abolish, the elimination of excess aldolase from the circulation. Other means of elimination were thus indicated.

TABLE 1

<table>
<thead>
<tr>
<th>TISSUE</th>
<th>NORMAL RATS</th>
<th>TUMOR-BEARING RATS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Aldolase content</td>
<td>Aldolase content</td>
</tr>
<tr>
<td></td>
<td>Av. Range</td>
<td>No. Av. Range</td>
</tr>
<tr>
<td>Muscle</td>
<td>180.0</td>
<td>106.0–140.0</td>
</tr>
<tr>
<td>Brain</td>
<td>20.4</td>
<td>17.0–24.7</td>
</tr>
<tr>
<td>Liver</td>
<td>17.4</td>
<td>11.1–21.0</td>
</tr>
<tr>
<td>Adrenal</td>
<td>16.4</td>
<td>10.8–18.0</td>
</tr>
<tr>
<td>Kidney</td>
<td>14.2</td>
<td>10.0–17.7</td>
</tr>
<tr>
<td>Spleen</td>
<td>7.2</td>
<td>6.3–8.1</td>
</tr>
<tr>
<td>Walker tumor</td>
<td>14.9</td>
<td>11.8–18.2</td>
</tr>
</tbody>
</table>

Units per milligram of tissue (wet weight).
The attempted blocking of the reticuloendothelial system by the intravenous injection of India ink did not reduce the normal value for serum aldolase; nor did such attempts produce any change in the rate of disappearance of crystalline aldolase injected intravenously.

Total hepatectomy caused a variable increase in the serum aldolase during the 12–24 hours such animals usually live. The multiple and extensive physiologic abnormalities to which these animals are subjected made interpretation of these data most uncertain. Complete experimental obstruction of the bile duct caused either no change in the level of serum aldolase or a slight elevation which soon returned to normal.

**Liberation of aldolase from tissue slices.**—When a slice of tumor tissue was incubated in a physiologic medium at 38° C. with added glucose and under an atmosphere of oxygen and carbon dioxide, there was only a slight increase in the aldolase content of the medium during a period of more than 8 hours (Chart 9). In a similar preparation with added glucose but under nitrogen and carbon dioxide, there was a similar slight increase in enzyme content during incubation. However, when glucose was omitted from the medium, there was a moderate liberation of the enzyme under aerobic conditions and a very marked liberation under anaerobic conditions (Chart 9). When similar experiments were performed with slices of normal rat liver, there was a relatively slow increase in the aldolase content of the medium when incubated aerobically either with or without added glucose, but a rapid liberation under anaerobic conditions, irrespective of the addition of glucose. These
data thus confirm the observations of Warburg and Hiepler for the Walker tumor 256 and of Schade for the Ehrlich mouse ascites tumor, namely, that anaerobic conditions cause a more rapid liberation of aldolase than do aerobic conditions. It is interesting that with tumor slices the liberation of the enzyme, certainly indicative of injury to the cells, was caused chiefly by the lack of glucose, while with slices of liver the liberation of the enzyme was increased more by the lack of oxygen.

**DISCUSSION**

In confirmation of previous reports, we found that rats bearing large Walker tumors showed a pronounced elevation in serum aldolase. The remarkably constant level of this enzyme in normal animals and the prompt return to normal of an elevated level when the cause of such elevation was eliminated suggest that the content of aldolase in the serum is the resultant of a dynamic balance between its continuous liberation from some source in the body and its constant removal from the circulation. Since it is probable that all tissues of the body manufacture sufficient aldolase for their own use, transport of the enzyme from one tissue or organ to another would not be essential to the over-all body economy. An elevated serum level would thus indicate a massive production exceeding the rate at which the enzyme can be eliminated.

In the case of tumor-bearing rats, it is proposed that the tumor itself is the source of the excessively high content of serum aldolase. This appears to be true for the following reasons: (a) After excision of the tumor, the elevated level of aldolase in the serum immediately begins to fall and within 12 hours returns to normal. The rate of disappearance of the enzyme in animals from which the tumor has been removed closely parallels the rate of disappearance of crystalline aldolase injected intravenously. This indicates that complete cessation of the excessive elaboration of the enzyme occurs at the time of removal of the tumor. (b) A significantly higher aldolase content was found in the blood from a vein leaving the tumor than in heart blood obtained at the same time. (c) The experimental reproduction in normal animals of marked anemia or extreme cachexia, comparable to those seen in tumor-bearing rats, did not influence the level of serum aldolase. Similarly it was shown that the spleen or the adrenal glands, organs which undergo pronounced hyperplasia during tumor growth, did not influence the serum enzyme level. (d) The similar aldolase content of the tissues of normal and tumor-bearing rats did not suggest that some tissue other than the tumor might be
the source of excess aldolase. (e) The level of serum aldolase was not elevated until the tumor had reached a large size, and partial excision of the tumor often resulted in a fall to normal levels. This would seem to demonstrate that an elevated serum level was indicative of a tumor of greater than a certain volume, rather than indicative of malignancy per se.

The increased liberation of aldolase within the tumor may be attributable to the many small foci of necrosis, which were invariably found on microscopic examination at all stages of tumor growth. Whereas the cellular material in the massive foci of central necrosis is largely isolated from the circulation, the normally intracellular substances in the small foci have ready access to the circulation when they are released through cellular injury. The experiments with tissue slices seemed to confirm this hypothesis. In vitro studies with slices of both tumor and normal liver demonstrated that environmental conditions that were detrimental to the life of the cell caused release of aldolase. Inadequacy of the supply of glucose and oxygen must certainly exist in the parts of the tumor that have outgrown their blood supply and are seen as foci of necrosis. The observation that viability of the malignant cells was particularly dependent upon an adequate supply of glucose, while oxygen was more important for the survival of liver cells, is very interesting. This might be related to the known relatively greater importance of glycolytic than respiratory mechanisms in the metabolism of malignant cells.

Since high levels of serum aldolase may be the result of excessive tissue destruction, normal serum levels may be the result of the constant physiologic breakdown of tissue cells. The great amount of aldolase normally present in the tissues, particularly in skeletal muscle, compared with the relatively small amount in the serum, would make such a source quantitatively feasible. For example, the aldolase in about 5 mg. of muscle would equal that in the entire circulation of a large rat.

In other studies (3) we have shown that focal necrosis produced in the liver of rats by the inhalation of carbon tetrachloride vapors is accompanied by a considerable rise in the level of serum aldolase. An elevation in enzyme level is therefore not specific for malignancy, but is apparently related to acute cellular destruction from any cause. Similarly, in a survey of patients with a wide variety of diseases, the elevated levels of serum aldolase were encountered in conditions characterized by extensive injury to a tissue rich in aldolase (4).

Ionizing radiation or drugs known to have a cancerocidal action, when given in amounts sufficient to produce definite histologic effects on the tumor, lowered the increased values for serum aldolase. The mechanism of this action is uncertain, but it is possible that the rapid destruction of a high percentage of the tumor cells causes sudden liberation of their aldolase, which is then promptly eliminated (a rise in enzyme level soon after irradiation was often seen). The volume of remaining viable tumor cells was then insufficient to maintain a continuous high level of aldolase.

While it is apparent that the rat can rapidly dispose of excess aldolase and maintain a more or less constant level in the circulation, the mechanism by which it is eliminated from the body was not determined in our study. Renal excretion of the enzyme is certainly indicated by our data, but it is probably not the only method utilized to maintain the delicate balance. Although the enzyme was not excreted in the bile, its destruction in the liver may possibly occur.

SUMMARY

In this investigation of the significance of elevated levels of serum aldolase in rats bearing the Walker carcinosarcoma 256 it was found that the enzyme content in the blood leaving the tumor was higher than in heart blood. Surgical removal of the tumor caused the level of serum aldolase to fall promptly to the normal range, the rate of fall paralleling the rate of disappearance of crystalline aldolase injected intravenously. Anemia, cachexia, and the presence of the spleen or the adrenal glands were unrelated to the level of the serum enzyme. X-ray treatment of the tumor, or the administration of urethan, aminopterin, or nitrogen mustard, usually caused a fall in the elevated level of the enzyme.

In experiments with tissue slices, aldolase was released from tumor tissue into the medium under anaerobic conditions, particularly when glucose was not added to the medium. In the presence of oxygen and glucose, aldolase was retained in the cells.

Bilateral nephrectomy or bilateral ligation of the ureters caused an increase of serum aldolase in normal rats; these procedures delayed but did not prevent the disappearance of intravenously injected crystalline aldolase. Ligation of the bile duct did not produce an increased amount of aldolase in the blood serum.

The evidence provided by the data assembled indicates that the elevated levels of serum aldolase result from its increased liberation from the tumor, at a rate exceeding its elimination from
the circulation. It is suggested that this increased liberation derives from foci of necrosis in the tumor and that it is not a characteristic of malignant tissue per se.

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