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# THE EFFECT OF TOTAL BODY X RADIATION ON THE ACTIVITY OF SEVERAL ENZYMATIC SYSTEMS OF RAT SPLEEN

C.E. CARTER

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C. E. Carter

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# THE EFFECT OF TOTAL BODY X RADIATION ON THE ACTIVITY OF SEVERAL ENZYMATIC SYSTEMS OF RAT SPLEEN

C. E. Carter

Although several investigations have established the inactivation of purified and crystalline enzymes in dilute solution by X radiation (1, 2, 3), the phenomena of radiation injury to living tissue is, in general, remarkable for the lack of effect on enzyme activity following exposure to high doses of ionizing radiation. Because of the sensitivity of mammalian hematopoietic tissue to damage from ionizing radiation (4, 5), rat spleen has been assayed for enzymatic activity catalyzing the degradation of benzoyl-L-arginineamide, glycyl-L-leucine, leucylglycylglycine, and glycyldehydroalanine, substrates for intracellular proteolytic enzymes; the phosphate activated desamidation of glutamine, and the degradation of ribonucleic and desoxyribonucleic acid in spleens from normal animals and from animals exposed to X radiation. Although such a study is not exhaustive, the results have indicated that of the systems so far investigated only ribonuclease activity shows striking deviations from normal following X irradiation.

Materials and Methods. Male and female rats weighing approximately 175 g were exposed to X radiation delivered from a 250 KV Maximar machine at a rate of 50-75 r per minute. Spleens were removed at various intervals following exposure, weighed on a torsion balance, homogenized in all-glass homogenizers with 5 volumes of water and centrifuged lightly to clarify the solution. All assays for enzymatic activity were based on wet weights of normal and irradiated spleens.

Ribonucleic (yeast) acid used in these experiments was obtained from Schwartz Laboratories. Solutions of ribonucleic acid for enzymatic assay were prepared by dissolving 4 g of sodium ribonucleate in 50 ml of water, dialyzing overnight against distilled water in the cold, and diluting to 100 ml with

distilled water. This solution was then adjusted to pH 7.7 with 1 N NaOH and stored in the cold. Assayed spectrophotometrically the solution contained 17.75 mg ribonucleic acid per ml. Ribonuclease activity was determined by potentiometric titrations. This assay depends on the liberation of secondary phosphoric acid dissociations from the ribonucleic acid molecule by the nuclease and subsequent increments in titratable acidity. The method is essentially the same as the manometric determination of ribonuclease activity described by Zittle (6) where the increase in titratable acidity liberates carbon dioxide from a bicarbonate buffer. The equipment required for the potentiometric titration determination is simple and the method rapid and susceptible of great accuracy. A pH meter with external leads is connected with a glass electrode assembly mounted on a magnetic stirrer. Assays were conducted in 10 ml beakers at the glass electrode in a constant temperature room by the following procedure: 1 ml of tissue extract was adjusted to pH 7.7 with 0.02 N NaOH and 2 cc of ribonucleic acid solution (pH 7.7) containing 17.75 mg ribonucleic acid per ml added with rapid stirring. A horizontal microburette described by Schwert et al. (7) containing 0.02 N NaOH was introduced into the beaker just above the digest solution and touching the wall. Increments of base were added at intervals to maintain the pH of the mixture at 7.7 and the appearance of titratable acidity then plotted against time.

Peptidase determinations were conducted by a potentiometric formol titration described by D. G. Doherty (8). This method for following the liberation of free amino acids from peptide linkage is based on the increment of titratable acidity arising from the reaction of formaldehyde with free amino groups. Titrations were done at a glass electrode using 0.02 N NaOH in a horizontal microburette. The procedure consisted of adding an 0.2 ml aliquot of an enzyme digest containing 0.028 molar peptide to 1 ml of neutralized formol (37%), adding 1 ml of water and titrating to pH 8.1. Mixing during titration was accomplished

with a magnetic stirrer. Titration values for complete hydrolysis of the peptide were derived from data on aliquots of mixtures of composite amino acids equimolar with the peptide.

The desamidation of benzoyl-L-arginineamide by spleen extracts was followed in digests adjusted to pH 6.0 with veronal buffer by aeration of the ammonia which is enzymatically hydrolyzed from the molecule into .05 N $H_2SO_4$ and directly nesslerizing the solution for nitrogen determination.

The phosphate activated glutaminase of rat spleen was determined as previously described by Carter and Greenstein (9).

Desoxyribonuclease degrades desoxyribonucleic acid to dialyzable components and this is the basis for the assay described by Carter and Greenstein (10) used in this investigation. Desoxyribonucleic acid, prepared by a modification of the Bang-Hammarsten procedure, was dialyzed against distilled water overnight and adjusted to a concentration of 1 mg per ml. Digests consisted of 0.5 ml of spleen extract, 1 ml DNA solutions and 1 ml of water inside a small cellophane bag which was suspended in 20 cc of 0.05 M NaCl. The dialysate, compared against a tissue blank, was examined in the spectrophotometer at 2600 A for increments of dialyzable nucleic acid components. Although this method is susceptible to kinetic analysis with purified desoxyribonuclease, the complexity of the degradation processes in tissue extracts makes difficult any quantitative statement regarding tissue desoxyribonuclease activity using the procedure. Because the loss of viscosity of DNA solutions in tissue extracts has a large nonenzymatic component, this method of assay for desoxyribonuclease activity is also open to criticism. It was found that tissue extracts did not liberate significant titratable acidity from added desoxyribonucleic acid to use this phenomena as a basis of desoxyribonuclease assay.

Dehydropeptidase activity was determined spectrophotometrically employing glycyldehydroalanine as a substrate according to the procedure described by

Carter and Greenstein (11). This substrate was synthesized by the method of Price and Greenstein (12).

Benzoyl-L-arginineamide was generously supplied by Dr. D. G. Doherty. Glycylleucine and leucylglycylglycine were synthesized according to procedures described by Fischer (13) and melting points of these compounds were in agreement with published values. Crystalline ribonuclease was purchased from Armour and Company.

Results. 1. Ribonuclease activity. The data illustrated in Fig. 1 indicates, that, in the range of spleen extract concentrations assayed, the rate of reaction is limited by enzyme concentration and that the time course of the reaction deviates from zero and first order kinetics, while the reaction with crystalline ribonuclease approximates first order kinetics. In Fig. 2, ribonuclease activity of spleens of normal and irradiated rats are compared. There is no immediate effect of X radiation on the ribonuclease activity of spleen. Twenty-four to 48 hours following exposure, marked decreases are apparent in the ribonuclease activity and, in the group exposed to 500 r, there was no recovery up to 12 days following radiation. Doses as low as 100 r produce significant depressions of ribonuclease activity in spleen and, in animals assayed 7 days following this exposure, ribonuclease activity remained lower than normal.

The tissues of kidney are generally considered to be comparatively insensitive to damage from ionizing radiation[4]. However, a depression of ribonuclease activity in kidney extracts 48 hours following exposure to 500 r X radiation was found. It was also found that kidney ribonuclease approximates zero order reaction kinetics in contrast to spleen ribonuclease. It is therefore impossible to compare ribonuclease activity in several different tissue extracts on the basis of a rate constant dependent on the order of reaction. The inverse time relationship is independent of the apparent order of the reaction and may be used to assign quantitative values to enzyme activity (14). Using the time

Figure 1

The degradation of ribonucleic acid with crystalline ribonuclease and normal spleen extract. Digests consisted of 2 cc of dialyzed RNA pH 7.7 plus 0.2 cc of crystalline ribonuclease solution containing 1.5 mg enzyme per cc and, in the case of enzyme determination in spleen, 1.0 ml, 0.5 ml, and 0.25 ml of aqueous spleen extract (5 vols. of water) made to a total volume of 3.0 cc with water. Initial pH was 7.7 and titrations were performed at 5, 10, 20 and 30 minutes after addition of enzyme by adding 0.02 N NaOH from a microburette to pH 7.7.

A log plot of the data is shown to illustrate the different kinetics of the crystalline ribonuclease reaction as compared with degradation of ribonucleic acid by normal spleen extract. Previous investigations have shown that ribonuclease liberates $7 \times 10^{-7}$ equivalents $H^+$ per mg ribonucleic acid[10] and, assuming this as the value for 100% hydrolysis, in the system described this would correspond to 1.4 cc of 0.02 N NaOH.

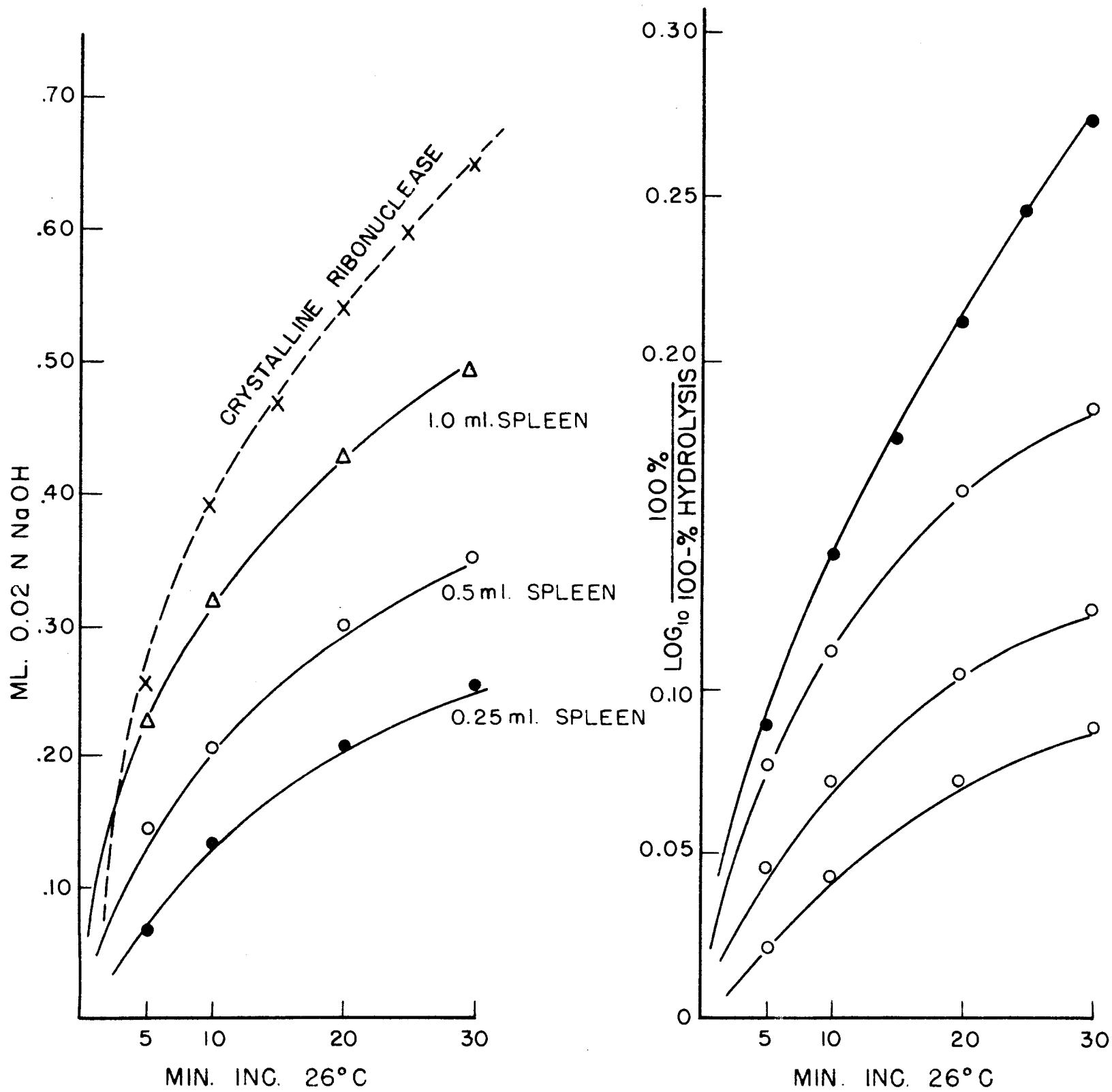

ML. 0.02 N NaOH
.70
.60
.50
.40
.30
.20
.10
CRYSTALLINE RIBONUCLEASE
1.0 ml. SPLEEN
0.5 ml. SPLEEN
0.25 ml. SPLEEN
5
10
20
30
MIN. INC. 26° C
$LOG_{10} \frac{100}{100-\%\ HYDROLYSIS}$
0.30
0.25
0.20
0.10
0.05
0
5
10
20
30
MIN. INC. 26° C

FIG. 1

Figure 2

Ribonuclease activity of spleen extracts from normal and X-irradiated rats. Values for normal and irradiated spleen derived from data on 4 animals in each group. Assays conducted on 1 ml spleen extract as described in text.

RIBONUCLEASE

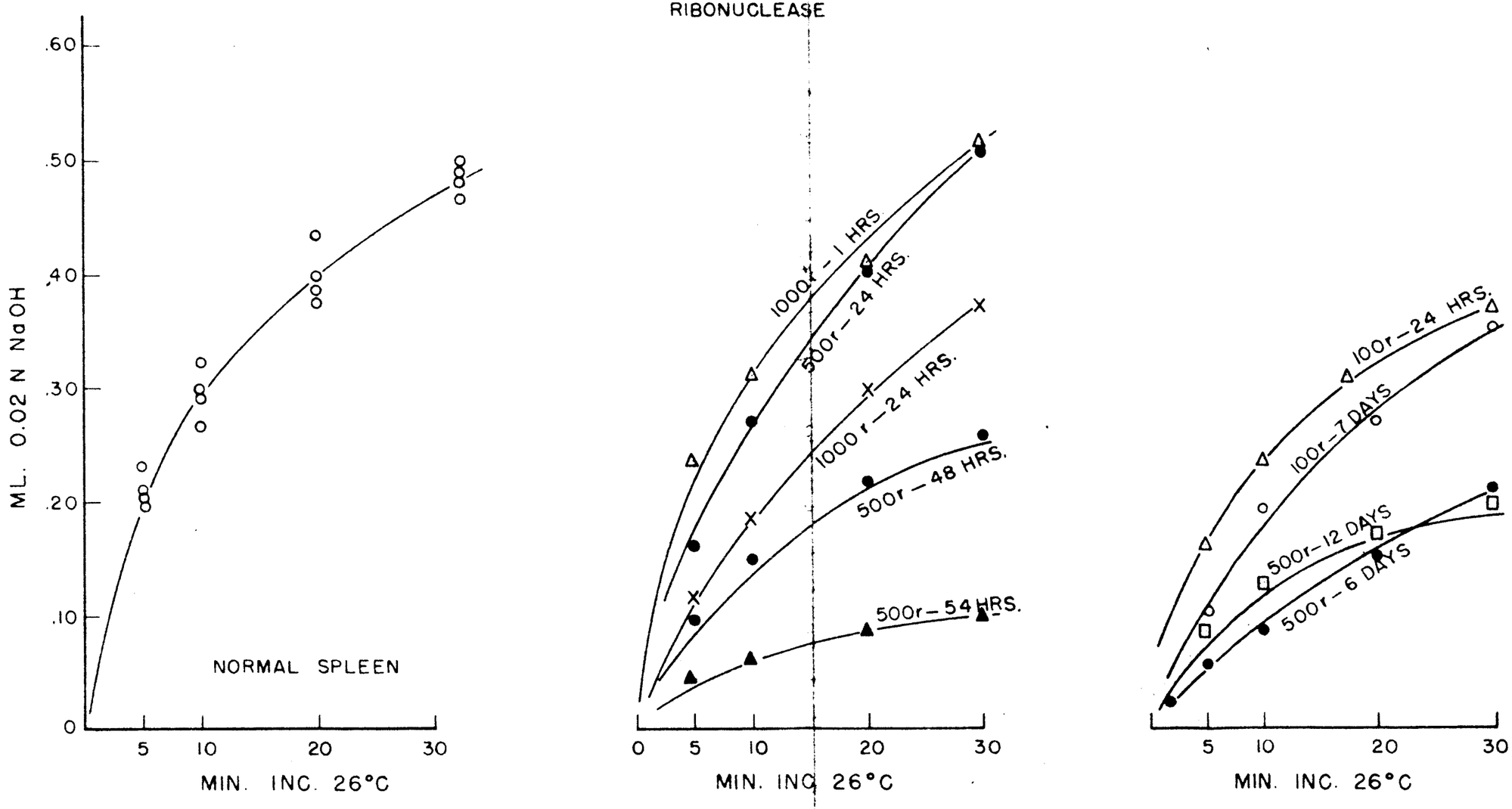



FIG. 2

Drawing # 6304

required for 18% (see legend to Fig. 1) hydrolysis of ribonucleic acid in spleen and kidney extracts assayed by the techniques described, the activity values for normal and irradiated animals are shown in Table I. Time values are interpolated from hydrolysis curves. The data in Table I also include activity values for spleen extracts obtained with two different preparations of nucleic acid adjusted to the same pH and concentration. The variability of molecular size (polymerization) of different ribonucleic acid preparations is well known and probably accounts for the differences of ribonuclease activity values using the two preparations. The relative depression of ribonuclease activity in spleen extracts following irradiation is, however, comparable for the two preparations. Ribonuclease activities of normal and irradiated spleens were also compared on the basis of total phosphorous of the spleen extract and the results were of the same order as when wet weight of the tissue was employed as a reference for extract concentration.

2. Proteolytic enzymes. Benzoyl-L-arginineamide was found by Fruton and Bergman (15) to be a synthetic substrate for an intracellular proteolytic enzyme of spleen which they designated as Cathepsin II. This enzyme system, which is distributed in several mammalian tissues, hydrolyzes the amide group from benzoyl-L-arginineamide. In Fig. 3 the time course of the hydrolysis of benzoyl-L-arginineamide in extracts of normal spleen and in extracts of spleens from animals exposed to X radiation is shown. Twenty-four hours following exposure to 500 r X radiation the hydrolysis curve demonstrates a considerable augmentation of activity in spleen extract in the 3 animals assayed. The activity approaches that of the normal in the 48-hour and 72-hour groups.

Leucylglycylglycine and glycyl-L-leucine have been widely used as substrates for intracellular peptidases (16). Assays for peptidase activity in spleen extracts of normal and irradiated rats employing these substrates are illustrated in Fig. 4. The spleen extract from the irradiated animal exhibited less than 0.1

TABLE I

Ribonuclease activity of normal and irradiated rat spleen and kidney

Activity expressed as $\frac{1}{\text{time 18\% hydrolysis}} \times 100$

| | Activity | |
|---|---|---|
| | Nucleic Acid # 1 | Nucleic Acid # 2 |
| Spleen** | | |
| Normal | 15-18 | 5 |
| Irradiated | | |
| 100 r - 24 hrs. | 12 | |
| 48 hrs. | 8 | |
| 168 hrs. | 6.8 | |
| 250 r - 24 hrs. | 11 | |
| 500 r - 48 hrs. | 3.4 | 2 |
| 144 hrs. | 2 | 1.0 |
| 12 days | <1 | |
| 1000 r - 24 hrs. | 5.9 | |
| Kidney** | | |
| Normal | 4 | |
| Irradiated | | |
| 500 r - 48 hrs. | 2.2 | |

** Activity values are averages of determinations on four animals in each group.

Figure 3

Hydrolysis of benzoyl-L-arginineamide in extracts of normal and irradiated spleens. Digests consisted of 0.5 cc of spleen extract (5 vols. of water), 1.0 cc of veronal buffer to adjust pH to 6.0, 1.0 cc of a solution of benzoyl-L-arginineamide containing 6 mg per cc. Under these conditions the substrate was present in excess and the concentration of enzyme was the rate limiting factor. At intervals of 15 minutes during the incubation period duplicates were removed from the series, alkali added and ammonia aerated into 0.05 N $H_2SO_4$ where it was determined by direct nesslerization.

Normal values are based on experiments with 4 animals and, in the irradiated group, 3 animals for each 24-hour period following radiation.

FIG. 3

BENZOYL-L-ARGININE AMIDASE

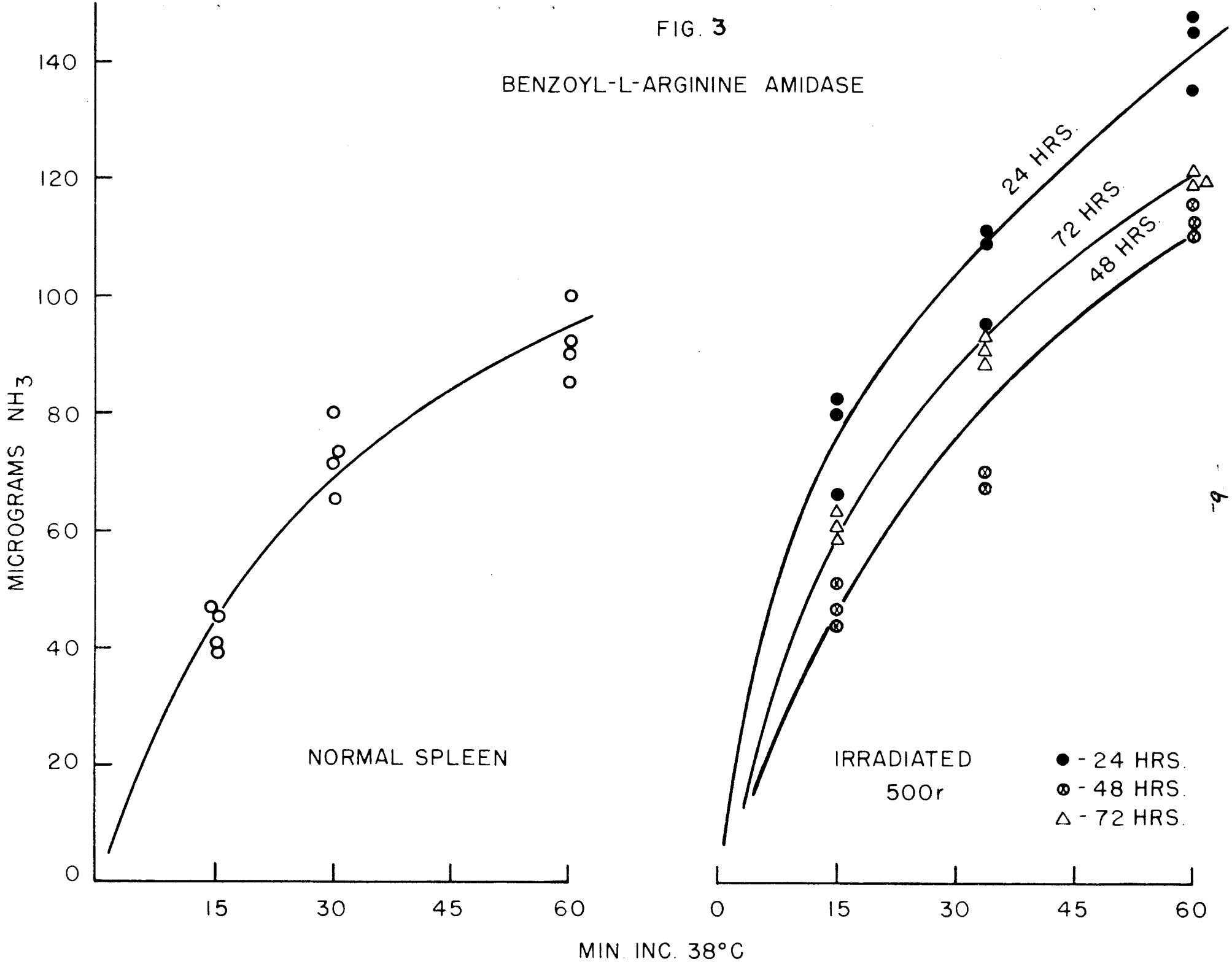


Figure 4

Peptidase activity of spleen extracts from normal and irradiated rats. Hydrolysis of the peptide bond was followed by potentiometric formol titrations in aliquots of digest consisting of 0.25 ml spleen extract, 0.75 ml of .06 M phosphate buffer pH 7.8 and 1.0 cc of 0.056 M leucylglycylglycine or glycyl-L-leucine adjusted to pH 7.8 in .06 M phosphate buffer. Under these conditions the concentration of enzyme in spleen extract is the rate limiting factor. Approximately 0.3 ml .02 N NaOH represents 100% hydrolysis in the case of all 3 reactions.

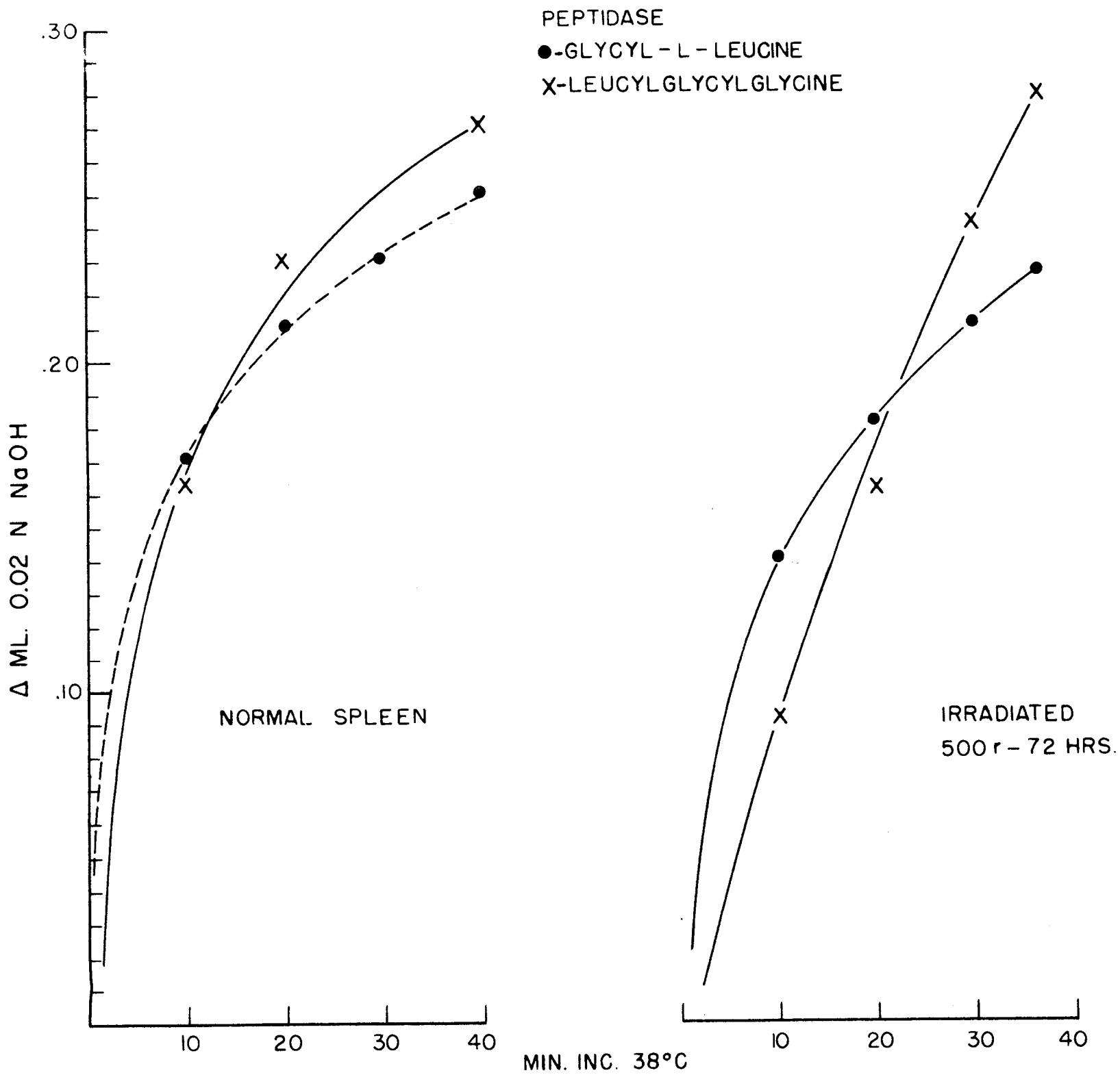

PEPTIDASE
●-GLYCYL - L - LEUCINE
X-LEUCYLGLYCYLGLYCINE
.30
.20
.10
Δ ML. 0.02 N NaOH
NORMAL SPLEEN
IRRADIATED
500 r - 72 HRS.
10
20
30
40
10
20
30
40
MIN. INC. 38°C

FIG. 4

the ribonuclease activity of normal spleen, this is in contrast to the peptidase activity of the same extract which approximated normal.

Dehydropeptides are rapidly hydrolyzed by enzymes which are widely distributed in plant and animal tissues (17). These unsaturated peptides may arise from the condensation of amino acid amides with keto acids and thus may represent important pathways of protein metabolism. The effect of X radiation on the activity of this enzyme in spleen extracts from animals exposed to 500 r, 72 hours before assay is shown in Fig. 5 where a slight increase of activity of the irradiated spleen as compared with the normal is demonstrated. The contrast with the ribonuclease activity of the spleen from the irradiated animals is apparent.

3. Glutaminase activity. The amino acid amide, glutamine, is found in relatively high concentration in several mammalian tissues, and is considered to be an important intermediate in nitrogen metabolism (18). The enzymatic hydrolysis of the amide group of glutamine is greatly accelerated by the presence of inorganic phosphate in several tissues including spleen[9]. As shown in Fig. 6, the phosphate activated glutaminase of spleen extract from irradiated animals is significantly depressed 24-48 hours following exposure to 500 r. Here again, however, the magnitude of the effect is much less than noted for the ribonuclease system.

4. Desoxyribonuclease. The formation of dialyzable components from high molecular weight desoxyribonucleic acid results from incubation of this compound with purified desoxyribonuclease and extracts of several mammalian tissues[10]. Spleen is high in the enzymatic activity which catalyzes the degradation of desoxyribonucleic acid to dialyzable components; however, the complexity of the degradation process in crude tissue extracts and the fact that the nature of the dialyzable product is not defined make quantitative statements of desoxyribonuclease activity in normal and irradiated spleen extracts tentative. In Table II it is

Figure 5

Dehydropeptidase activity of spleen extracts from normal and irradiated rats. Enzymatic hydrolysis of glycyldehydroalanine followed spectrophotometrically in digests consisting of 0.1 ml spleen extract (5 vols. of water), 0.1 ml of glycyldehydroalanine solution containing 3.7 mg per cc, and 10 cc of borate buffer pH 8.0. Digests were incubated in spectrophotometer curvettes at 25° C and optical density determined at intervals against a tissue blank at 2500 A. Assays were conducted on 3 animals from the normal and irradiated group.

FIG. 5

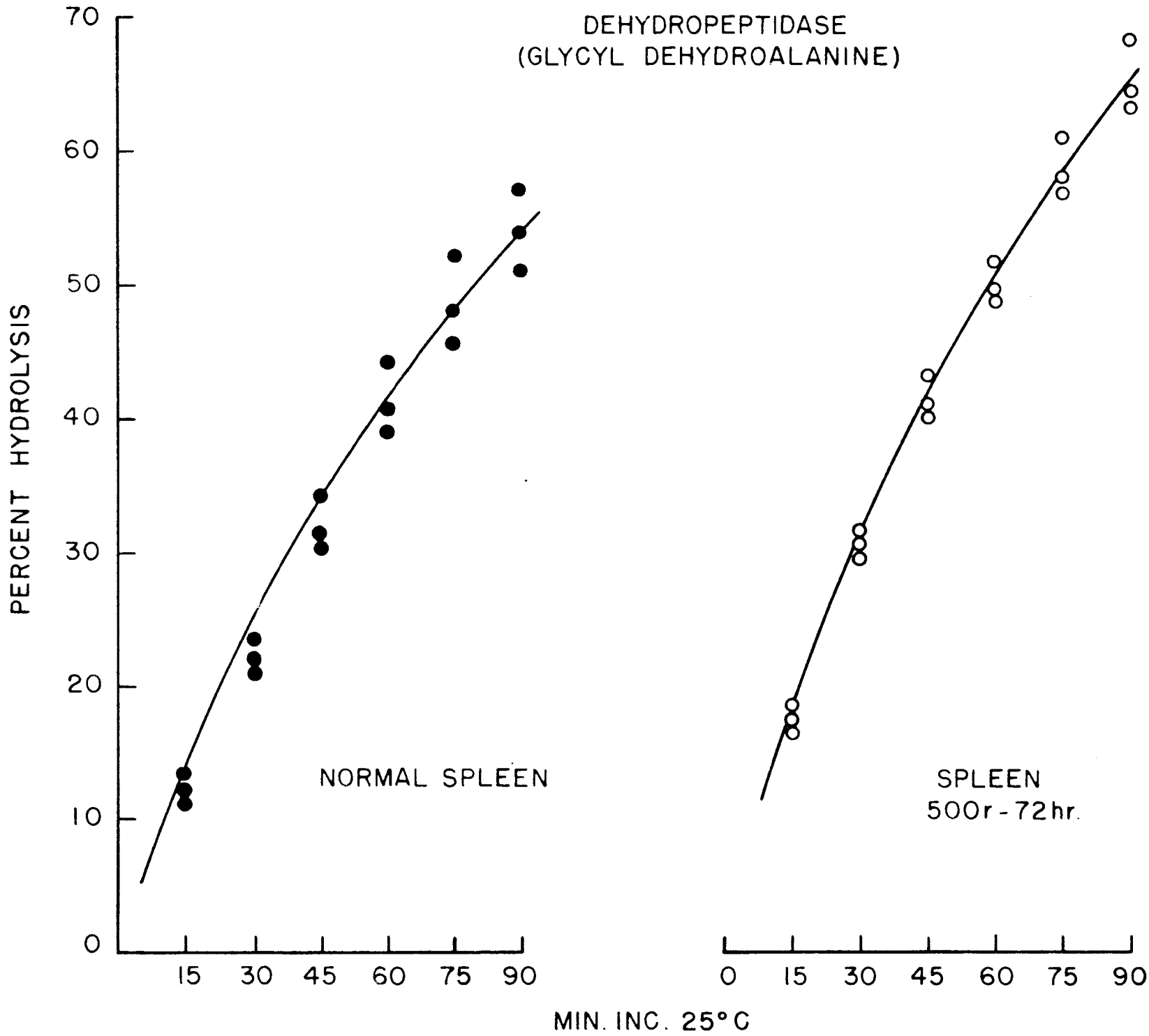
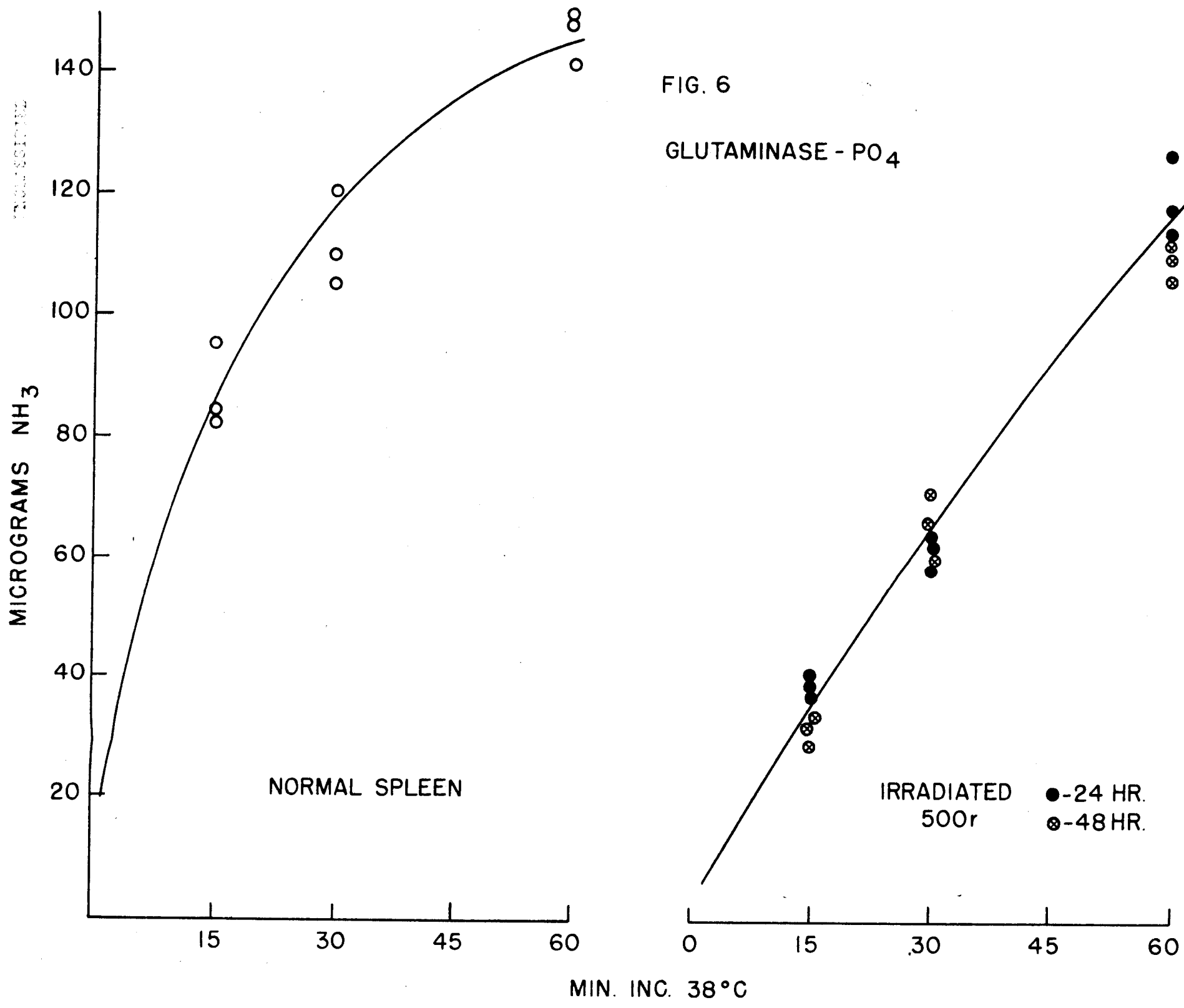

DEHYDROPEPTIDASE
(GLYCYL DEHYDROALANINE)
PERCENT HYDROLYSIS
70
60
50
40
30
20
10
0
NORMAL SPLEEN
SPLEEN
500r - 72hr.
15 30 45 60 75 90
0 15 30 45 60 75 90
MIN. INC. 25° C

Figure 6

Phosphate activated glutaminase of spleen extracts from normal and irradiated rats. Enzymatic hydrolysis of the amide group followed by determining ammonia formed in digests at pH 8.0 consisting of 0.5 cc spleen extract (5 vols. of water), 1.0 cc phosphate buffer and 1.0 cc of glutamine solution containing 2 mg per cc.




Drawing # 6868

TABLE II

Enzymatic degradation of desoxyribonucleic acid to dialyzable components in spleen extracts from normal and irradiated rats***

| Spleen extract (5 vols. $H_2O$) | Per cent dialyzable components formed in 4 hours incubation at 38° C |
|---|---|
| Normal | 53 - 58 |
| Irradiated | |
| 100 r - 48 hours | 42, 47 |
| 250 r - 72 hours | 61, 56 |
| 500 r - 48 hours | 42, 47 |
| 500 r - 72 hours | 40, 38 |

*** Spleens from two animals in each group were assayed at stated periods following exposure to X radiation.

shown that, following exposure to doses of X radiation which produce depression of ribonuclease activity in spleen, the enzymatic degradation of desoxyribonucleic acid to dialyzable components shows no significant deviation from normal.

Discussion. The results of enzymatic studies using several substrates associated with protein and nucleic acid metabolism in extracts of spleens from normal rats and from rats exposed to X radiation emphasize the sensitivity of ribonuclease activity to the late effects of radiation injury. This may be a consequence of the damage and subsequent disappearance of one or more sensitive cellular systems of spleen rather than a consequence of the progressive denaturation of the enzyme protein caused by the ionizing radiation. Enzyme chemistry at the histochemical level is necessary to elucidate this problem.

Radiation injury is a process characterized by a generalized destruction of tissue elements and the data presented herein are not intended to support the contention that depression of a single enzyme system is responsible for the late effects of radiation injury in spleen. However, if the data of Caspersson et al. (19) describing the integration of protein and nucleic acid metabolism are considered, the depression of an enzymatic mechanism concerned with the synthesis of ribonucleic acid may be a critical factor in the survival of a tissue following exposure to ionizing radiation.

Further investigation of the effects of radiation on the activity of several enzymes concerned with the metabolism of nucleic acids and their component molecules is in progress in this laboratory.

Summary. The late effects of whole body X radiation on several enzyme systems of rat spleen are described. Of the enzymes studied, ribonuclease shows the most striking alteration of activity following radiation, depressions of activity being found 24 hours following exposure to doses as low as 100 r. In rats exposed to 500 r, ribonuclease activity in spleen is severely depressed and no evidence of recovery was found in periods up to 12 days following radiation.

REFERENCES

1. Barron, E. S. G., Atomic Energy Commission, 1946, Declassified Document No. MDDC-484.

2. Forssberg, A., Nature, 1947, 159, 308.

3. Dale, H. S., Nature, 1943, 151, 280.

4. Bloom, W., Atomic Energy Commission, Declassified Document No. MDDC-488.

5. Dunlap, C. E., Archives of Pathology, 1942, 34, 562.

6. Zittle, C. A., and Reading, E. H., J. Biol. Chem., 1945, 160, 519.

7. Schwert, G. W., Neurath, H., Kaufmann, S., and Snoke, J. E., J. Biol. Chem., 1948, 172, 221.

8. Doherty, D. G., Unpublished work.

9. Carter, C. E., and Greenstein, J. P., J. Nat. Canc. Inst., 1947, 7, 433.

10. Carter, C. E., and Greenstein, J. P., J. Nat. Canc. Inst., 1946, 7, 29.

11. Carter, C. E., and Greenstein, J. P., J. Nat. Canc. Inst., 1946, 7, 51.

12. Price, V. E., and Greenstein, J. P., J. Biol. Chem., 1947, 171, 477.

13. Fischer, E., Untersuchungen uber Aminosauren, Polypeptide und Proteine, Julius Springer, Berlin, 1906.

14. VanSlyke, D. D., Advances in Enzymology, Vol. 2, Interscience, New York.

15. Fruton, J. S., Bergman, M., J. Biol. Chem., 1939, 130, 19.

16. Johnson, M. J., and Berger, J., Advances in Enzymology, Vol. 2, Interscience, New York.

17. Greenstein, J. P., Advances in Enzymology, Vol. 8, Interscience, New York.

18. Archibald, R. M., Chemical Reviews, 1945, 37, 161

19. Caspersson, T. O., Society for Experimental Biology, Vol. 1, Cambridge University Press, 1947.