6. Johnson, P. C., Circulation Res. 7, 992 (1959).
7. Johnson, P. C., Circulation Res. 15, (Suppl. 1), 225 (1964).
8. Johnson, P. C., Gastroenterology 52, 435 (1967).

Received Jan. 23, 1969. P.S.E.B.M., 1969, Vol. 131.

# Effects of Restraint-Stress on Enzymes Involved in DNA Synthesis* (33882)

ANTHONY R. IMONDI, M. EARL BALIS, AND MARTIN LIPKIN

*Department of Medicine, Cornell University Medical College, The Sloan-Kettering Division, Cornell University Graduate School of Medical Sciences, and the Sloan-Kettering Institute for Cancer Research, New York, N. Y. 10021*

Recent investigations have shown that restraint-stress in rodents results in decreased cellular proliferation in the stomach (1, 2) and an inhibition of DNA[1] synthesis along the entire gastrointestinal tract (3). The synthesis of DNA is the result of several enzymatic steps which begins with the synthesis of DNA precursors and ends with their polymerization. The absence or inhibition of any of the enzymes involved in DNA synthesis would result in diminished synthesis. Certain of these enzymes are thought to play a more important role in the regulation of DNA synthesis because their activities have been shown to parallel closely the rate of cell proliferation in some tissues. Among these are TdR kinase (4) and TMP kinase (5) required for the synthesis of TTP, DNA nucleotidyltransferase (4) responsible for the polymerization of the deoxynucleoside triphosphates, and ATCase (6) which catalyzes the first step in the *de novo* synthesis of pyrimidines.

A reduction in the levels of any of these enzymes after restraint-stress but prior to the inhibition of DNA synthesis would indicate a possible site of action and lead to a better understanding of the factors involved. In the present study, the activities of these enzymes were measured in gastrointestinal tissues of restrained mice shortly before the observed inhibition of DNA synthesis. The results indicate that changes in the levels of these enzymes are not responsible for the decreased synthesis of DNA resulting from restraint-stress.

*Materials and Methods.* Male CFW mice weighing 25 g were restrained in a wire mesh screen (7) for 4 hr, injected intraperitoneally with 20 $\mu$Ci of TdR-methyl-$^3$H (New England Nuclear, Boston; sp act 16.5 Ci/mmole) and killed 1 hr later. Control animals were kept individually in wire bottomed cages without food or water for the treatment period. The glandular stomach and jejunum were removed, homogenized in cold 0.01 *M* Tris–HCl (pH 7.5), washed free of acid-soluble material and assayed for DNA by the diphenylamine method. An aliquot of the DNA hydrolysate was used to measure the amount of acid-insoluble tritium by liquid scintillation counting. Details of this procedure have been reported (3).

Enzyme assays were performed on the 15,000*g* supernatant fraction of whole tissue homogenates, and the activities were expressed on a per unit DNA basis. The TdR kinase was assayed using the reaction mixture of Behki and Morgan (8) containing TdR-methyl-$^3$H with the addition of 1 $\mu$mole of NaF. After a 30-min incubation at 37°, the reaction tubes were immersed in boiling

* Supported by National Institutes of Health Grants and Awards CA-08921; CA-08748; K3-AM-4468; and 5-F2-CA-37, 131-01.

[1] Abbreviations: DNA, deoxyribonucleic acid; TdR, thymidine; TMP, thymidine-5′-monophosphate; TTP, thymidine-5′-triphosphates; ATCase, aspartate carbamoyltransferase; Tris, tris(hydroxymethyl) aminomethane; DEAE, diethylaminoethyl cellulose; ATP, adenosine-5′-triphosphate; dCMP, deoxycytidine-5′-monophosphate.

TABLE I. Incorporation of Thymidine-methyl-$^3$H into DNA of Stomach and Jejunum of Mice Restrained for 4 hr.

| Tissue | Control | Restrained |
|---|---|---|
| Stomach | 182[a] | 112[b] |
| Jejunum | 492 | 178[c] |

[a] (cpm/$\mu$g of DNA) mean of 3 mice.
[b] Different from control ($p < 0.05$).
[c] Different from control ($p < 0.01$).

water for 3 min. An aliquot was spotted on DEAE cellulose discs which were then washed with $10^{-3}M$ ammonium formate, water, and ethanol (9). The radioactivity retained by the paper was measured in a liquid scintillation spectrometer. The TMP kinase reaction mixture was identical to that for TdR kinase except that TMP-2-$^{14}$C replaced TdR-$^3$H. After stopping the reaction with heat the incubation mixture was reacted with 5′-nucleotidase (Sigma Chemical) to convert unreacted TMP to TdR, and the total amount of TdR di- and triphosphates was determined using DEAE papers as described above.

The ATCase activity was assayed by the method of Bresnick (6) and the amount of carbamylaspartate-$^{14}$C formed was determined after passing the reaction mixture through Dowex 50 $H^+$ to remove unreacted asparate-$^{14}$C.

The DNA nucleotidyltransferase activity (4) was measured by reacting the deoxynucleoside triphosphates of guanine, adenine, cytosine, and thymine-methyl-$^3$H with the enzyme source in the presence of ATP, Mg, mercaptoethanol, and heat denatured DNA primer for 80 min at 37°. The reaction was terminated by cooling to 0° and acidifying with cold perchloric acid. The precipitate was then washed three times with cold 0.25 *N* perchloric acid and hydrolyzed in 0.5 *N* perchloric acid. The amount of TdR-$^3$H in the DNA hydrolysate was measured in a liquid scintillation spectrometer.

*Results and Discussion.* There was a significant decrease in the incorporation of TdR-methyl-$^3$H into DNA of stomach and jejunum of mice restrained for 4 hr (Table I). It was assumed, therefore, that if the levels of enzymes related to DNA synthesis were in some way involved in the inhibition of DNA synthesis, then changes in the activities of these enzymes should have been detectable in less than 4 hr of restraint. However, there were only a few instances of decreased enzyme activities after 3 hr of restraint (Tables II and III). These differences were not statistically significant and could not be held responsible for the marked reduction in DNA synthesis. The unaltered level of TdR kinase is especially significant because it serves to validate this and previous studies which measured the uptake of TdR-$^3$H into DNA based on the assumption that the phosphorylation of TdR is not limiting.

It has been suggested that the phosphorylation of TdR and TMP by their respective kinases may be involved in the regulation of DNA synthesis because these enzymes are known to increase in regenerating liver (4) and in certain tumors (10). This view was strengthened by the reports that TTP inhibits TdR kinase (9), dCMP deaminase (11)

TABLE II. Activities of Thymidine Kinase and Thymidylate Kinase in Tissues of Mice Restrained for 3 hr.

| | Thymidine kinase[a] | | Thymidylate kinase[a] | |
|---|---|---|---|---|
| Tissue | Control | Restrained | Control | Restrained |
| Liver | 8.1 ± 1.3 | 9.0 ± 0.5 | 274 ± 66 | 295 ± 36 |
| Stomach | 11.6 ± 2.1 | 8.7 ± 1.9 | 169 ± 43 | 145 ± 29 |
| Duodenum | 0.5 ± 0.2 | 0.4 ± 0.1 | 67 ± 15 | 46 ± 6 |
| Jejunum | 16.6 ± 4.6 | 10.4 ± 2.1 | 93 ± 19 | 68 ± 16 |
| Ileum | 10.6 ± 3.2 | 8.6 ± 1.7 | 35 ± 6 | 35 ± 11 |
| Colon | 6.1 ± 0.7 | 6.2 ± 0.7 | 38 ± 10 | 50 ± 8 |

[a] Substrate phosphorylated ($\mu$moles/hr/mg of DNA) mean of 4 mice ± SE.

TABLE III. Activities of Aspartate Carbamoyltransferase and DNA Nucleotidyltransferase in Tissues of Mice Restrained for 3 hr.

| Tissue | ATCase[a] | | DNA nucleotidyltransferase[b] | |
|---|---|---|---|---|
| | Control | Restrained | Control | Restrained |
| Liver | 1.4 ± 0.3 | 1.4 ± 0.2 | 56.2 ± 7.5 | 58.2 ± 8.0 |
| Stomach | 0.8 ± 0.1 | 0.8 ± 0.2 | 8.2 ± 0.7 | 8.0 ± 2.4 |
| Duodenum | 0.4 ± 0.1 | 0.2 ± 0.1 | Trace | Trace |
| Jejunum | 0.8 ± 0.2 | 0.8 ± 0.2 | 97.8 ±19.1 | 79.2 ±18.0 |
| Ileum | 0.5 ± 0.1 | 0.6 ± 0.1 | 12.0 ± 3.1 | 11.8 ± 2.8 |
| Colon | 0.6 ± 0.2 | 0.4 ± 0.1 | 23.5 ± 6.0 | 14.2 ± 5.6 |

[a] Carbamoylaspartate formed (μmoles/hr/mg of DNA) mean of 4 mice ± SE.
[b] Acid-insoluble $^3H$ (cpm/80 min/μg of DNA) mean of 3 mice ± SE.

and pyrimidine ribonucleotide reductase (12). However, there has been some doubt concerning this hypothesis due to the failure to detect high amounts of TdR kinase in such rapidly proliferating tissues as chick embryo (13) and mouse duodenal epithelium (14). The results of the present study demonstrate that the level of TdR kinase is not necessarily indicative of the amount of DNA synthesis occurring within a tissue.

Another enzyme implicated in the regulation of DNA synthesis is ATCase because of its prevalence, too, in those tissues characterized by high rates of cell proliferation (15) and its inhibition by cytosine nucleotides (16). Yet, as shown in the present study, the level of this enzyme did not change at a time when DNA synthesis decreased. It is, therefore, suggested that the level of ATCase and the potential for synthesing pyrimidine nucleotides is not involved in the stress-induced depression of DNA synthesis.

The role of DNA nucleotidyltransferase in the regulation of DNA synthesis is not clear. The assay for this enzyme may be subject to error due to the presence of DNAse in unpurified preparations (17). However, the existence of two forms of the nucleotidyltransferase suggests that it may, indeed, be a regulatory enzyme (18). The present study shows only that this enzyme is present in the tissues of the restrained mice and that the observed block in DNA synthesis is not a result of an inability to polymerize deoxynucleoside triphosphates.

The evidence presented herein has not established how restraint-stress inhibits DNA synthesis, but does show that certain enzymes thought to have regulatory roles in DNA synthesis and cell proliferation are not involved. It is clear that the levels of these enzymes, in themselves, are not indicative of the DNA synthesizing activity of the tissues. Although these studies in no way invalidate the hypothesis that in some tissues the regulation of DNA synthesis is effected by variations in the activities of these enzymes they show that there are at least some exceptions.

*Summary.* The uptake of thymidine-methyl-$^3H$ into DNA of stomach and intestine was significantly decreased within 4 hr after subjecting mice to restraint-stress. The failure to detect any appreciable change in thymidine kinase, thymidylate kinase, asparatate carbamoyltransferase, or DNA nucleotidyltransferase after 3 hr of restraint indicated that these enzymes were not directly involved in the stress-induced inhibition of DNA synthesis.

1. Lahtiharju, A. and Rytomaa, T., Exptl. Cell Res. **46**, 593 (1967).
2. Kim, Y. S., Kerr, R., and Lipkin, M., Nature **215**, 1181 (1967).
3. Imondi, A. R., Balis, M. E., and Lipkin, M., Exptl. Mol. Pathol. **9**, 339 (1968).
4. Bollum, F. J. and Potter, Y. R., Cancer Res. **19**, 561 (1959).
5. Cannellakis, E. S. and Mantsavinos, R., Biochim. Biophys. Acta **27**, 643 (1958).
6. Bresnick, E., J. Biol. Chem. **240**, 2550 (1965).
7. Brodie, D. A. and Hanson, H. M., Gastroenterology **38**, 353 (1960).
8. Behki, R. M. and Morgan, W. S., Arch. Biochem. Biophys. **107**, 427 (1964).
9. Breitman, T. R., Biochim. Biophys. Acta **67**,

153 (1963).

10. Bukovsky, J. and Roth, J. S., Cancer Res. **25**, 358 (1965).

11. Maley, G. F. and Maley, F., J. Biol. Chem. **239**, 1168 (1964).

12. Moore, E. C. and Hurlbert, R. B., J. Biol. Chem. **241**, 4802 (1966).

13. Roth, J. S. and Askew, J., Biochim. Biophys. Acta **130**, 28 (1966).

14. Imondi, A. R., Lipkin, M., and Balis, M. E., Federation Proc. **27**, 787 (1968).

15. Young, J. E., Prager, M. D., and Atkins, I. C., Proc. Soc. Exptl. Biol. Med. **125**, 860 (1967).

16. Gerhart, J. C. and Pardee, A. B., J. Biol. Chem. **237**, 891 (1962).

17. Burdon, M. G., Smellie, R. M. S., and Davidson, J. N., Biochim. Biophys. Acta **91**, 46 (1964).

18. Keir, H. M., *in* "Progress in Nucleic Acid Research and Molecular Biology" (J. N. Davidson, and W. E. Cohn, eds.), Vol. 4, p. 81. Academic Press, New York (1965).

Received Dec. 2, 1968. P.S.E.B.M., 1969, Vol. 131.

# The Cardiac Lymphatics in Experimental Chronic Congestive Heart Failure* (33883)

HERMAN N. UHLEY, SANFORD E. LEEDS, JOHN J. SAMPSON, AND MEYER FRIEDMAN
(With the technical assistance of Shantilal Patel and Vaida Hoffman)
*Mount Zion Hospital and Medical Center, San Francisco, California 94115*

It has been shown that the right duct lymph system plays an important role in removing fluid from the lung in experimental chronic congestive heart failure (6). While there has been recent interest in the cardiac lymphatics in various pathologic states (3), the role of the cardiac lymphatics in chronic congestive heart failure has not been established, principally because it is difficult to cannulate the small lymphatics and to produce chronic congestive heart failure in the experimental animal. Both of the technical obstacles are surmountable and the present paper reports the effect of experimentally induced chronic congestive failure on canine cardiac lymph flow.

*Methods.* Six dogs weighing between 15 and 30 kg were anesthesized with sodium pentobarbital (29 mg/kg). Respirations were maintained with an intermittent positive pressure respirator. A left lateral thoracotomy was performed to provide adequate exposure for visualization of the heart and mediastinum. The pericardium was opened and 1 ml of T 1824 dye (Evans blue 0.5% aqueous solution) was injected into the subepicardial layers of the pulmonary conus. After a few minutes the dye filled the lymphatics in the region of the cardiac lymph node lying between the superior vena cava and innominate artery at the base of the heart. Using a Zeiss dissecting microscope a cardiac lymphatic was cannulated with a polyethylene tube (size 10, 20, or 50) and the lymph samples were collected in 15-min aliquots for 3 hr.

Chronic congestive heart failure was produced in a second group of six dogs weighing between 17 and 21 kg by the creation of an aorticocaval anastomosis below the renal arteries and the administration of 25 mg of deoxycorticosterone trimethylacetate twice a week and a 6 g salt diet (6). The animals were edematous and in marked congestive failure after 19–23 days. They were anesthesized with sodium pentobarbital (23 mg/kg) and placed on a positive pressure respirator. A left lateral thoracotomy was performed and the cardiac lymphatics were visualized and cannulated. Lymph was collected as described above in the control group. In both groups femoral artery blood was drawn after 2 hr of lymph flow. The RBC, WBC, SGOT, LDH, total protein, sodium, potassium, and chloride concentrations were determined. Similar studies were done on pooled lymph flow samples after periods of 1, 2, and 3 hr.

*Results.* The effluent cardiac lymphatics

* Aided by Grants from the National Heart Institute, National Institutes of Health, U.S. Public Health Service (H-3180).