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# INCORPORATION OF RADIOACTIVITY FROM $^{14}$C-NITROGLYCERIN INTO RAT LIVER GLYCOGEN, LIPID, PROTEIN, RIBONUCLEIC ACID AND DEOXYRIBONUCLEIC ACID

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**Abstract**—Rats were given a single oral dose of $^{14}$C-nitroglycerin and their livers were excised after 1 hr. Fractionation of the livers showed the lipid, glycogen, protein, RNA and DNA to be radioactive. In order to be confident that the nucleic acids were actually labeled, pure RNA was isolated and hydrolyzed to its component nucleotides. The nucleotides, resolved chromatographically, were found to be labeled, indicating that radioactivity from $^{14}$C-nitroglycerin was indeed incorporated into RNA (and therefore, presumably, into DNA as well). The single treatment with nitroglycerin did not affect the glycogen, protein, lipid, RNA or DNA content of the liver.

ADMINISTERING $^{14}$C-nitroglycerin to rats by gavage has been shown in this laboratory to lead to the rapid elimination of respiratory $^{14}CO_2$ and urinary $^{14}$C-glycerol.[1] Additionally, a considerable quantity of the label was found in the tissues and some of this radioactivity did not yield to solvent extraction. This latter observation suggested that $^{14}$C from nitroglycerin was incorporated into normal tissue components. The objective of the present work was to study some of these tissue components and to determine whether the administration of nitroglycerin affected the quantity of these substances normally present. The investigation was limited to the liver. The tissue components studied were protein, glycogen, lipid, RNA and DNA, and these were assayed 1 hr after $^{14}$C-nitroglycerin administration because our previous study[1] showed a maximal ratio of nonextractable to extractable radioactivity at this time.

## MATERIALS AND METHODS

*$^{14}$C-nitroglycerin.* Labeled nitroglycerin was synthesized by the Lawrie procedure[2] from (1,3-$^{14}$C)-glycerol. After purification, as described by Dunstan *et al.*,[3] the product was 99·9 per cent radiochemically pure. The explosion hazard was minimized by mixing the nitroglycerin with 19 parts by weight of c.p. lactose. The specific activity of the mixture was 0·183 mc/g.

*Treatment of animals.* Normally fed female Wistar rats (KG Farms) weighing 180–200 g were used in pairs. One member of each pair was untreated and the other was dosed by gavage with 10 mg $^{14}$C-nitroglycerin per kg body weight. One hr later, both animals were sacrificed and their livers were immediately excised, weighed and frozen. Nine pairs of rats were processed in this manner.

*Fractionation of liver.* The quantity of glycogen, lipid, protein, RNA and DNA in each liver was determined by the fractionation procedure of Shibko *et al.*[4]

*Radioactivity counting.* After the entire liver of each rat was homogenized,[4] 0·5-m aliquots containing 22–27 mg of fresh tissue were digested for 24 hr at 37° with 1·5 ml of hyamine 10X (1 M in methanol) and counted for radioactivity by scintillation spectrometry. The scintillation solution consisted of 7·0 g of PPO (2,5-diphenyloxazole), 0·3 g of dimethyl POPOP [1,4-*bis*-2-(4-methyl-5-phenyloxazolyl)benzene] and 100·0 g naphthalene in 1·0 l. of redistilled dioxane. Similarly, aliquots of the isolated fractions were digested with hyamine and counted.

*RNA extraction with trichloroacetic acid.*[5] The starting material was frozen liver homogenate[4] prepared from one $^{14}$C-nitroglycerin-treated rat. The assay data[4] on this liver were: lipid, 131; glycogen, 36; protein, 49; DNA, 802; and RNA, 827, all values being expressed as dpm per mg of liver.

To 5 ml of liver homogenate was added 5 ml of 20% trichloroacetic acid (TCA). After 15 min at 0°, the precipitate was washed twice with 5-ml portions of cold 10% TCA. To remove any residual TCA as well as some lipid, the pellets were extracted with 5 ml of 95% ethanol saturated with sodium acetate. Further lipid extractions were performed with 5 ml of ethanol:ether (3:1) and 5 ml of anhydrous ether. To the pellet was added 40 ml of 0·3 N KOH and the samples were incubated at 37° in a water bath for 60 min. After this hydrolysis, the separation of DNA and protein from RNA was effected by adding an equal volume of 5% TCA and 0·2 vol. of 6 N HCl. After centrifugation, the supernatant was collected and concentrated by flash evaporation.

*RNA isolation with phenol–cresol mixture.*[6] As above, two rats were dosed with $^{14}$C-nitroglycerin and sacrificed. The livers were excised and immediately placed on solid carbon dioxide. The frozen livers were transferred to a cooled Waring blendor containing cold 6% sodium 4-aminosalicylate and cold phenol–cresol mixture. The quantity of each solution was 15 ml/g of liver. The phenol–cresol mixture was prepared from phenol, detached crystals, 500 g; *m*-cresol, 70 ml; water, 55 ml; and 8-hydroxyquinoline, 0·5 g.

After homogenzing for 3 20-sec periods at top speed, the contents were stirred for 20 min at 20°, and then the mixture was centrifuged at 7000 *g* for 30 min at 5°. The clear aqueous phase was removed by means of a syringe, care being taken to avoid aspirating any of the interphase material which contains denatured protein and DNA. Any emulsion was removed and recentrifuged. To each 100 ml of the clear top layer was added 3 g sodium chloride. This layer was again extracted with 0·5 vol. of phenol–cresol mixture for 10–15 min at 20° and then centrifuged for 10 min at 8000 *g* at 0°. The aqueous phase was carefully removed and mixed with 2 vol. of anhydrous ethanol:*m*-cresol (9:1) at freezer temperature. The mixture was stored overnight in a freezer and the precipitate was collected by centrifigation at 8000 *g* for 20 min. The precipitate was extracted twice with 25-ml portions of cold sodium acetate solution (3M, pH 6·0) to remove glycogen, DNA and soluble RNA. The residual RNA was then washed once with a cold 1% solution of sodium chloride in 75% ethanol, once with 75% ethanol and twice with absolute ethanol. The yield was about 4 mg/g of liver.

*Hydrolysis of isolated RNA.* RNA prepared by the phenol–cresol procedure[6] was hydrolyzed by the method of Davidson and Smellie.[7] The hydrolysis involved treatment of the RNA with 5 ml of 0·3 N KOH for 18 hr at 37°. Potassium ions were removed as $KClO_4$ after neutralization with 70% perchloric acid. The solution of

nucleotides was taken to dryness by flash evaporation and the residue was dissolved in a minimum volume (*ca.* 0·5 ml) of 0·01 M Tris (hydroxymethyl) aminomethane hydrochloride buffer at pH 7·4.

*Spectrophotometry.* Ultraviolet absorption spectra were taken on the nucleotide mixture produced by the method of Shibko *et al.*[4] and on the nucleotide mixture obtained by hydrolyzing RNA isolated with the phenol–cresol method.[6] The usual solvents were employed for these preparations, namely, 0·3 M NaOH and 0·01 M Tris (hydroxymethyl) aminomethane hydrochloride at pH 7·4, respectively.

*Paper chromatography.* Aliquots of 30–100 $\mu$l of the nucleotide solutions produced by the TCA and phenol–cresol methods were applied to Whatman No. 1 paper Descending chromatography was run for 18 hr with Wyatt's solvent[8] consisting of isopropanol:water:conc. HCl (680:156:164). $R_f$ determinations were made by viewing the chromatograms under ultraviolet light. The ultraviolet-absorbing areas and appropriate blanks were cut out, transferred to scintillation vials, covered with scintillation fluid and counted for radioactivity. $R_f$ values in the same solvent system were obtained for authentic samples of adenylic, guanylic, cytidylic and uridylic acids.

## RESULTS

In a previous study,[1] 6·6 per cent of the radioactivity administered as $^{14}$C-nitroglycerin was found in the liver after 1 hr. The present experiment was confirmatory; it showed 6·4 per cent of the $^{14}$C to be present after the same interval. The five components assayed accounted for 18·6 per cent of the liver weight (Table 1) and 14·6 per cent of its radioactivity (Table 2).

TABLE 1. YIELDS OF LIVER COMPONENTS FROM CONTROL AND NITROGLYCERIN-TREATED RATS

| Liver fraction | Amount of fresh liver (mg/g ± S.E.) Control | Treated |
|---|---|---|
| Lipid | 30·2 ± 1·2 | 32·6 ± 1·1 |
| Glycogen | 12·1 ± 2·9 | 11·3 ± 2·6 |
| Protein | 133·3 ± 5·8 | 134·8 ± 6·6 |
| RNA | 5·1 ± 0·7 | 5·5 ± 0·9 |
| DNA | 1·6 ± 0·2 | 1·4 ± 0·2 |

TABLE 2. INCORPORATION OF RADIOACTIVITY INTO LIVER FRACTIONS OF RATS TREATED WITH $^{14}$C-NITROGLYCERIN

| Sample | dpm/mg of sample (±S.E.) |
|---|---|
| Liver homogenate | 96 |
| Lipid | 137 (±13·1) |
| Glycogen | 48 (±6·4) |
| Protein | 72 (±10·3) |
| RNA | —* |
| DNA | —† |

* 1226 (±605) dpm/mg on the product obtained by the method of Shibko *et al.*[4]

† 2144 (±395) dpm/mg on the product obtained by the method of Shibko *et al.*[4]

The administration of nitroglycerin to rats did not affect their liver levels of lipid, glycogen, protein, RNA or DNA (Table 1). The values for these components agreed reasonably well with the earlier assays for lipid,[4,9] protein[4,9,10] and DNA,[4,9] but were much lower than the data reported for glycogen[4,9,10] and RNA.[4,9]

All of the fractions, as produced by the method of Shibko *et al.*,[4] were radioactive. Three of the components showed specific activity of the same order of magnitude as the entire liver homogenate, but the nucleic acids were calculated to have incorporated far more $^{14}C$ (Table 2). Specifically, the dpm per mg values were 96 for liver, 137 for lipid, 72 for protein, 48 for glycogen and 1200–2100 for the nucleic acids. These high values obviously required re-evaluation before acceptance, especially because RNA and DNA were assayed spectrophotometrically not gravimetrically as were the other liver fractions. Thus, contamination of these minor components might have yielded essentially correct data for their contribution to the liver weight but incorrect data for their specific activity. This problem was discussed by Shibko *et al.*,[4] who indicated that 2–5 per cent of the liver protein contaminated their DNA and RNA fractions.

The results of re-evaluating the RNA labeling are shown in Table 3. Applying the Schmidt–Thannhauser[5] TCA method to samples of previously assayed liver showed a

TABLE 3. $^{14}C$-LABELING OF LIVER RNA BY $^{14}C$-NITROGLYCERIN ADMINISTRATION TO RATS

| Preparation | Method of isolation | dpm/mg RNA |
|---|---|---|
| Nucleotides | Shibko *et al.*[4] | 827* |
| RNA | Schmidt and Thannhauser[5] | 257* |
| RNA | Kirby[6] | 21† |

* From liver of $^{14}C$-nitroglycerin-treated rat No. 7.

† From livers of $^{14}C$-nitroglycerin-treated rats Nos. 10 and 11.

far lower level of $^{14}C$-labeling. The phenol–cresol procedure of Kirby,[6] generally accepted as the best method to isolate RNA, indicated that the RNA was indeed radioactive, but less so than was the lipid, glycogen or protein.

Figure 1 presents the ultraviolet absorption spectra of the nucleotide mixture produced by the assay method[4] and the RNA isolated by the Kirby procedure.[6] The $A_{260m\mu}/A_{230m\mu}$ ratios are markedly different namely, 1·1 for the product of the assay method and 2·1 for the isolated RNA. Absorbance at 230 m$\mu$ indicates contamination, often by polypeptides.

Paper chromatography of reference compounds yielded $R_f$ values of 0·23 for guanylic acid, 0·42 for adenylic acid, 0·54 for cytidylic acid and 0·74 for uridylic acid. Except for guanylic acid, which was resolved well in our hands, the data agree with those reported earlier.[8] Corresponding $R_f$ values were obtained from the labeled RNA hydrolysates and each nucleotide was found to be radioactive (Table 4). The blank areas on the chromatograms showed no trace of radioactivity and, taking into account the low $^{14}C$ levels present, the recovery of radioactivity from the chromatograms was very satisfactory (73 per cent). Except for adenylic acid, the amount of $^{14}C$ found in the resolved nucleotides was quite uniform. This exception may well be due to the very low solubility of adenylic acid rather than to low incorporation of radioactivity into this nucleotide.

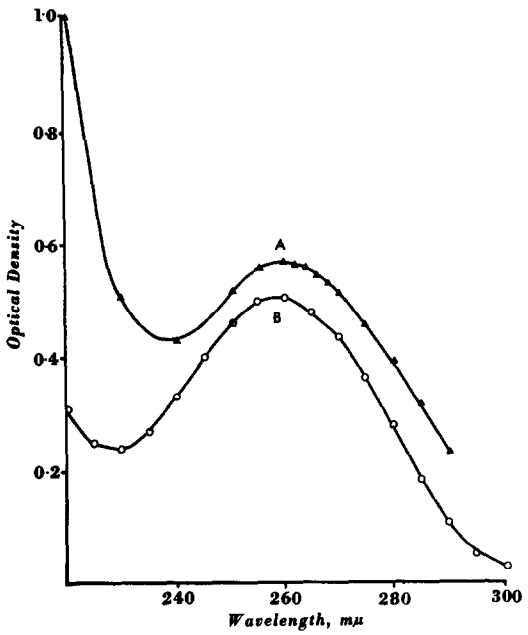


FIG. 1. Ultraviolet absorption spectra of (A) nucleotides obtained from rat liver by the method of Shibko *et al.*[4] and (B) RNA isolated from rat liver by the Kirby method.[6]

TABLE 4. RADIOACTIVITY OF NUCLEOTIDES ISOLATED FROM LIVER RNA OF RATS TREATED WITH $^{14}$C-NITROGLYCERIN

| Nucleotide | Total counts accumulated* | Net dpm/nucleotide† |
|---|---|---|
| Blanks | 1840 | 0 |
| Guanylic acid | 2950 | 15 |
| Adenylic acid | 2075 | 3 |
| Cytidylic acid | 3190 | 18 |
| Uridylic acid | 3250 | 19 |

* The total counting time was 100 min.

† The total quantity of $^{14}$C spotted on the duplicate chromatograms was 75 dpm. The total counted on the four areas was 55 dpm (73 per cent); no other area on the sheets was radioactive.

## DISCUSSION

The early literature on nitroglycerin metabolism concluded that the drug is degraded only slightly by the rat.[11,12] Further study, facilitated by the availability of $^{14}$C-nitroglycerin, contradicted the limited degradation thesis by showing the major urinary metabolite to be glycerol.[1] The present findings go far beyond this position and indicate that the glycerol derived from nitroglycerin enters into a variety of normal anabolic processes.

To our knowledge, the present data represent the first demonstration that some of the end products of glycerol metabolism are utilized in the synthesis of RNA. In addition, each purine and pyrimidine nucleotide is labeled. At this time, however,

the label cannot be assigned specifically to either the base or the ribose moiety. Also, there does not seem to be an earlier report of the conversion of glycerol into protein. It is well known, however, that glycerol serves as a precursor in the formation of glycogen[13–15] and various lipids.[14,15]

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