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EFFECT OF WHOLE-BODY GAMMA IRRADIATION ON SYNTHESIS AND EXCRETION OF GLUCURONIDES IN RATS

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EFFECT OF WHOLE-BODY GAMMA IRRADIATION ON SYNTHESIS AND EXCRETION OF GLUCURONIDES IN RATS

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ABSTRACT

Urinary excretion of glucuronides and free glucuronic acids in rats was found to be increased during the first 24 hr after irradiation with 1000 R when the rats had been starved for 3 days before irradiation.

Duodenal tissue from whole-body irradiated rats, tested in vitro, had a reduced ability to conjugate anthranilic acid, going down to 30% of the control level 48 hr after irradiation. At 192 hr after irradiation the conjugation again reached the normal level. Glucose added to the medium had no effect on control tissue but increased the conjugation of irradiated tissue. DPN increased the conjugation of tissue from normal animals but had no effect on tissue from irradiated animals. UDPG and UDPGA had no effect on normal or irradiated tissue.

In live animals given naphthalene intragastrically or anthranilic acid subcutaneously at different intervals after irradiation with 1000 R, excretion of total glucuronic acid (free plus conjugated) was the same as in the controls.

Levels of beta-glucuronidase excreted in urine by starved and irradiated rats were found to increase between 48 and 72 hr after irradiation. Urinary excretion of beta-glucuronidase inhibitor in irradiated rats was found to be the same as in the controls.

A machine is described for automatic step-gradient elution in column chromatography of soluble uridine nucleotides. Preliminary results are reported on the determination of their specific activity by using P^{32} in rat tissues.

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EFFECT OF WHOLE-BODY GAMMA IRRADIATION ON SYNTHESIS AND EXCRETION OF GLUCURONIDES IN RATS*

INTRODUCTION

Many methods of detoxification are available to higher animals when toxic substances are introduced or formed in the organism: oxidation, reduction, conjugation, deacetylation, hydroxylation, etc.

Many normal metabolites are regulated in their action by processes similar to those used for controlling toxic substances. One of the most important detoxification processes is conjugation with glucuronic acid. This metabolic conjugation is one of the most widespread reactions in drug metabolism and also has an important role in carbohydrate metabolism.

In mammals glucuronic acid in the free form is found only as beta-D-glucopyranuronic acid. The glucuronolactone is the gamma-lactone, beta-D-glucofuranurono-6,3-lactone. The

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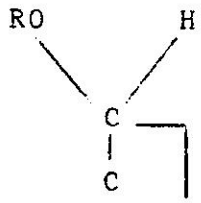
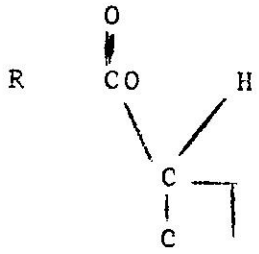
conjugated forms of glucusiduronic acids or glucuronides could be the following:

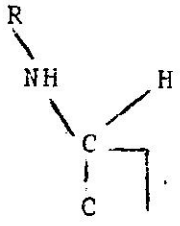
O-Glucusiduronic acid (a glucoside of the uronic acid, where R may be an alcoholic or phenolic hydroxyl radical),



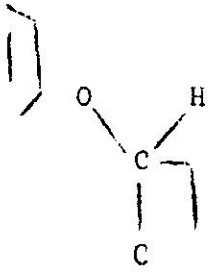
Acyl O-glucusiduronic acid,



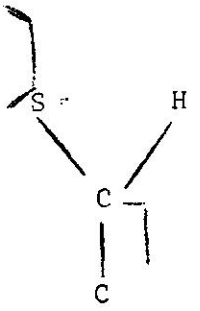
N-Glucusiduronic acid,



Enol-O-glucusiduronic acid



Or S-Glucurosiduronic acid



Until recently the synthesis of glucuronic acid was unknown, but the discovery of uridine disphosphate glucose (UDPG) by Leloir and his group[1] led to the finding that the glucose is oxidized while it is bound to the nucleotide.[2]

The sequence from glycogen to glucuronides is as follows:

glycogen ⟶ glucose-1-P (1)

glucose-1-P + UTP ⟶ UDPG + PP (2)

UDPG + 2DPN ⟶ UDPGA + 2 DPNH (3)

UDPGA + acceptor $\xrightarrow{\text{glucuronyl transferases}}$ glucusiduronic acid + UDP (4)

The mechanism of synthesis of glucusiduronic acid is a group transfer mechanism. Glucuronyl is transferred to an acceptor group by a group of enzymes of microsomal origin called UDP-glucuronyl transferases. Some recent reports suggest that the enzymes may be different when different kinds of glucusiduronic acid are synthesized.[3]

In studying the synthesis and excretion of glucuronides after whole-body irradiation of rats, three possibilities were taken into consideration.

1. The possibility of finding substances used by the organism to prevent impairment of homeostasis by radiation, which are eventually excreted as glucuronides. A process for repair after irradiation does exist at least in the mitotic apparatus, but there is no evidence for the existence of any physiological mechanism associated with this process in higher animals. It is well known that the conjugation of many substances, especially hormones, controls the homeostatic mechanism in many species.

2. The possibility of the formation of toxic substances at the moment of irradiation or after cell destruction, which could be treated as such and conjugated and excreted.

3. The possibility that radiation could impair the detoxification mechanism, especially the conjugation with glucuronic acid - this would aggravate the so-called radiation syndrome.

There are some indications that the detoxification by conjugation with glucuronic acid is not normal in irradiated animals. Hartiala et al. have reported the low conjugation of O-aminophenol by intestinal tract[4] and liver[5] irradiated *in situ* with x rays and tested *in vitro*. We have reported

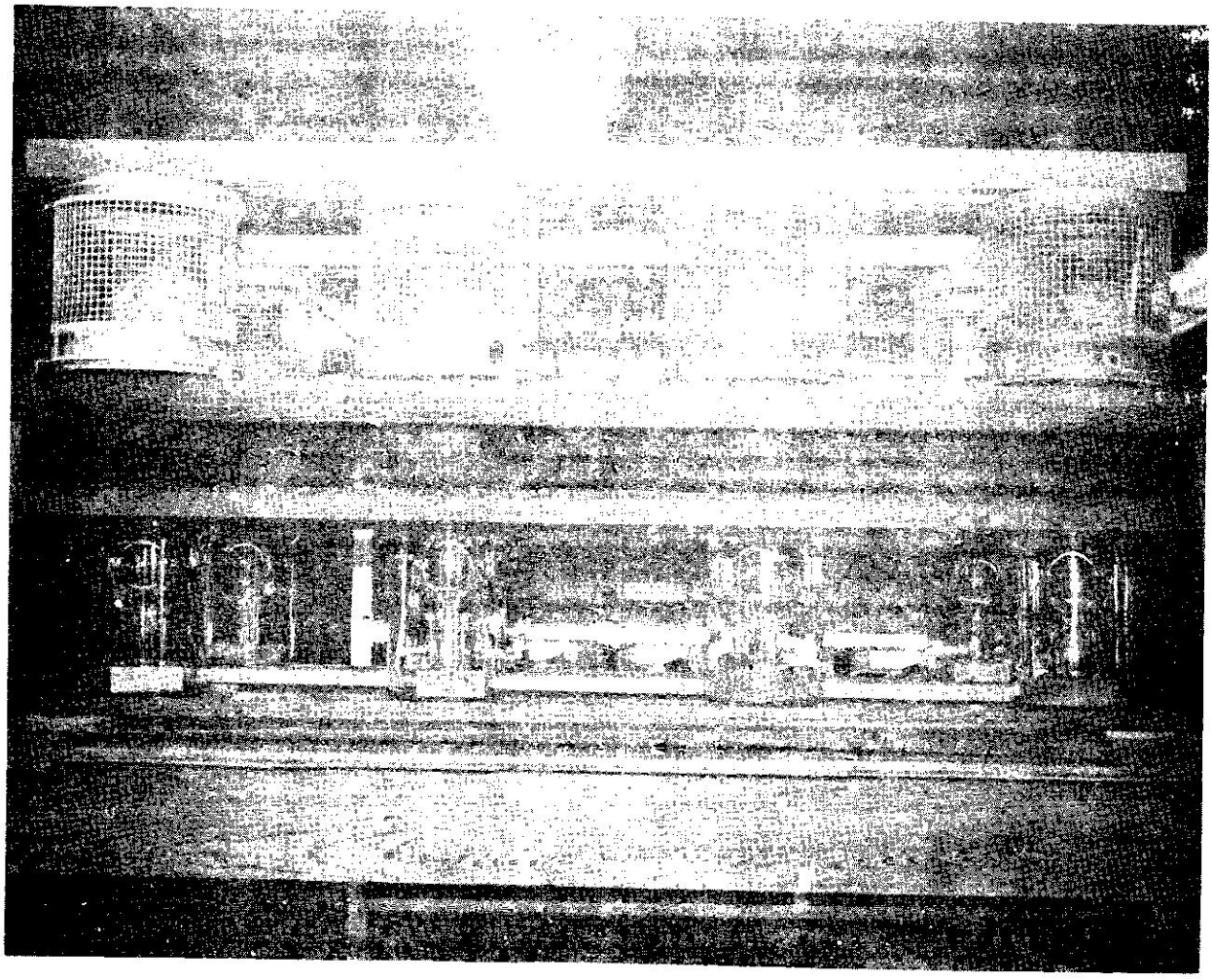


Figure 1

Metabolic cages used in this experiments to collect feces-free urine

that whole-body gamma irradiated rats are more sensitive to toxic doses of naphthalene administered orally.[6] Stevens has found low conjugation of some steroids by liver slices *in vitro* after whole-body irradiation of mice.[7]

These considerations induced us to perform experiments that could answer some of the questions raised above. However, it is necessary to keep in mind that glucuronic conjugation is only one of the many detoxification mechanisms in higher animals.

Experiments *in vitro* were checked against experiments *in vivo*. Also, experiments were done in which starvation was one of the parameters, since radiation produced low food intake in the rats almost immediately, and this introduced a confusing factor into the metabolic studies. Experiments *in vitro* showed reduced ability of irradiated tissue to conjugate glucuronic acid, but experiments *in vivo* did not show the same lack of conjugation judging by the excretion of urinary glucuronides.

EXPERIMENTAL PROCEDURE

Male Sprague-Dawley rats weighing from 250 to 300 g were used. They were maintained under standard conditions of temperature and humidity, with 12 hr of light per 24-hr cycle.

The rats were confined in Plexiglass cages during irradiation by a cobalt-60 source at a dose rate of 2.35 R/min measured in air. The irradiations were carried out in the

mornings. The urine, free of fecal contamination, was collected under toluene in metabolic cages (Figure 1).

The urine was removed from the collection device at 24-hr intervals and stored at -18°C until chemical analysis was made. Daily individual food intake was measured. The previously filled and weighed food containers were weighed each day at 9:00 a.m., and the amount of food consumed was found by difference.

Total glucuronic acid in urine were generally determined by the naphthoresorcinol method described by Meads et al.[8] using glucuronolactone as standard. Results are expressed as "Total glucuronides" in mg per 24 hr of glucuronic acid infering that no free glucuronic acid is excreted by the kidney; but when it was necessary to determine free and conjugated glucuronic acid separately, the method of Fishman et al.[9] was used, which gives consistently higher values for glucuronic acid.

When it was necessary to keep the animals in starvation, the food container was removed from the cage but the water supply was kept ad libitum.

The ability of duodenal tissue to conjugate anthranilic acid with glucuronic acid was tested in the following manner. The animals were anesthetized with ether and sacrified by decapitation. The duodenum was immediately removed and put

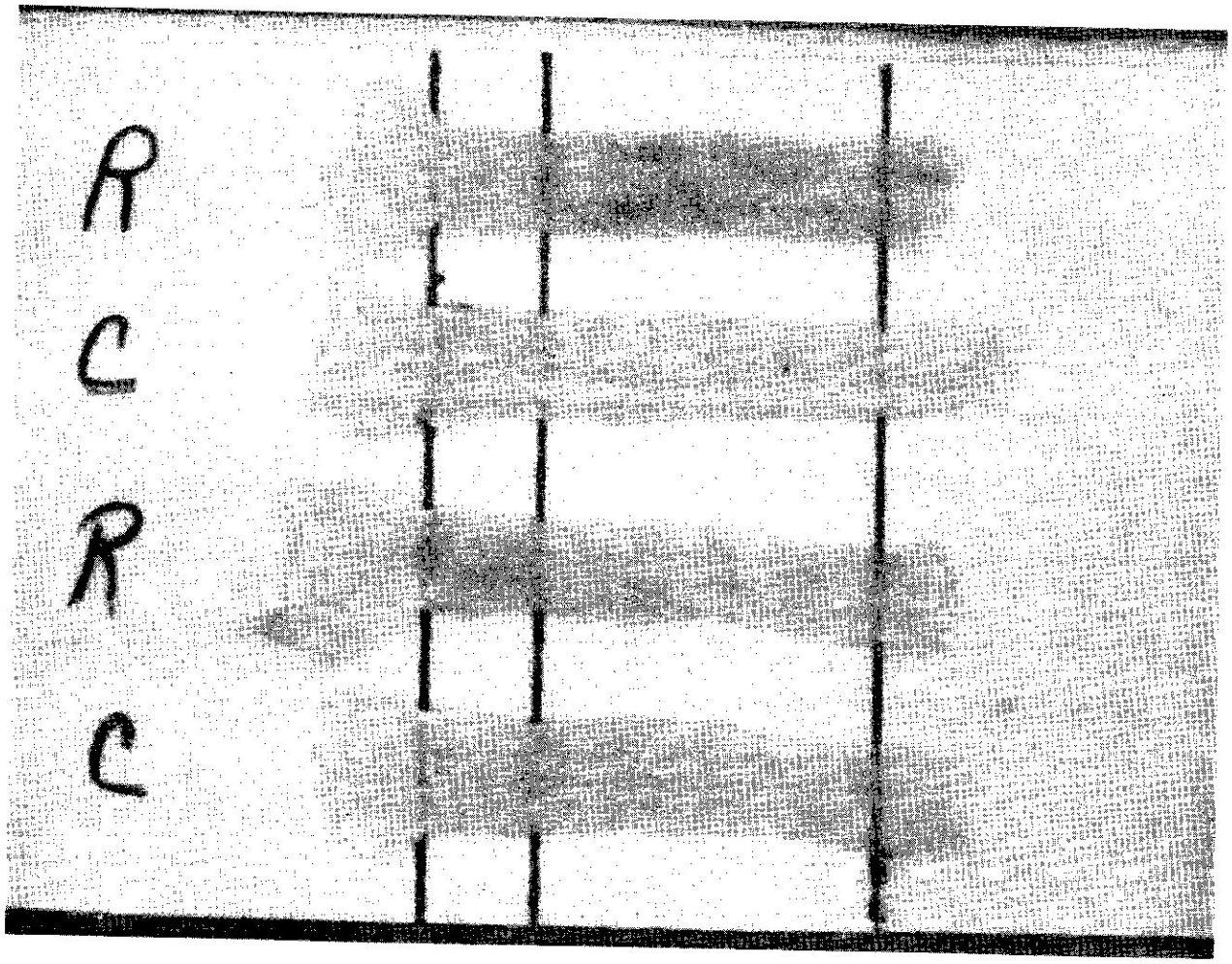


Figure 2

Actual handling of duodenal segment sample on pre-marked and frozen blotting paper

into chilled Krebs-Ringer-phosphate (KRP) medium (with the $MgSO_4$ replaced by $MgCl_2$) at pH 7.4. The duodenum was then opened, washed in chilled KRP, and placed on wet, frozen premarked blotter paper (Figure 2).

Three cm of duodenum, take 1 cm below the pylorus, was placed in a 50-ml Erlenmeyer flask containing 4 ml KRP solution and 52 µg anthranilic acid. The length of the duodenal slice was used as reference because in previous experiments it gave less variable results than weight and DNA concentration used as the basis for comparison. Glucose 20 mmoles, DPN 1.43 mmoles, UDPG 0.67 mmoles, and UDPGA 0.67 mmole, were then added to the incubating flask, if they were used. Samples were exposed for 1 min to O_2, then stoppered and shaken for 90 min at 37°C in the Dubonoff shaker.

Conjugated anthranilic acid formed by the duodenal slices was quantitatively separated by the method of Shirai and Ohkubo[10] with the modification that the pH of the sample was adjusted to 3.8 in a pH meter before the ether extraction. The Bratton-Marshall reaction was used to detect anthranilic acid.

In experiments with live animals two kinds of substances were used to challenge the formation of total and specific glucuronides, naphthalene administered intragastrically and anthranilic acid administered subcutaneously.

The naphthalene was a 4% solution freshly prepared in

Mazola oil, and 2 ml was given to each rat intragastrically with a cannula. In the experiments with napthalene-1-C^{14} (1.92 mCi/mmole) 1 mg was dissolved in 5 ml Mazola oil and 1 ml was given to each rat in the same way. The urine of the control and irradiated animals was collected for the next 24 hr and extracted quantitatively first with ether, which removes the oxidized derivatives, and then with n-butanol. This was followed by paper chromatography by the method of Terriere et al.(11) After autoradiography of the paper, the substances were identified by their Rf with use of Terriere data and quantitatively determined by densitometry and integration of the curve.

When anthranilic acid was used, a 0.3% solution in water was prepared and 15 mg was immediately injected subcutaneously in the rear flanks of the rats. Total anthranilic acid in urine was determined after alkaline hydrolysis according to the method of Ashima et al.(12) Anthranilic glucuronides in urine were determined according to the principles worked out by Shirai and Ohkubo.(10) In both cases the Branton-Marshall reaction was used for the development of color. Anthranilic glucuronides in urine were also measured after paper chromatographic separation with a butanol-acetic-water solvent (4:1:1). The anthranilic glucuronide spot was located with ultraviolet light, then quantitatively determined by using the paper chromatography

attachment of the Turner fluorometer. Both methods used were equally suitable, and the results agree very well.

Urinary beta-glucuronidase was determined by the method of Fishman et al.[13] with phenolphthalein glucuronide used as substrate. To detect beta-glucuronidase inhibitor in urine, an aliquot of the urine to be tested was added to the enzymatic system. For the controls the aliquot of urine was replaced with water. As an enzyme source a normal urine sample was used in each determination.

Dose rates, numbers of animals per treatment, and information about controls are given in the text, tables and Figures.

RESULTS AND DISCUSSION

A. EXCRETION OF TOTAL GLUCURONIDES AFTER WHOLE-BODY IRRADIATION IN RATS

In preliminary studies in which rats were irradiated with 1000 R, the excretion of total glucuronides was found to diminish and to reach the lowest values on the third day after irradiation.[6]

1. Irradiation Dose, Food Intake, and Total Glucuronide Excretion

In order to gain more information on this effect, experiments were done in which groups of 8 rats were irradiated with different doses: 1000, 400 and 100 R. Their daily food intake and daily excretion of total glucuronide were measured.

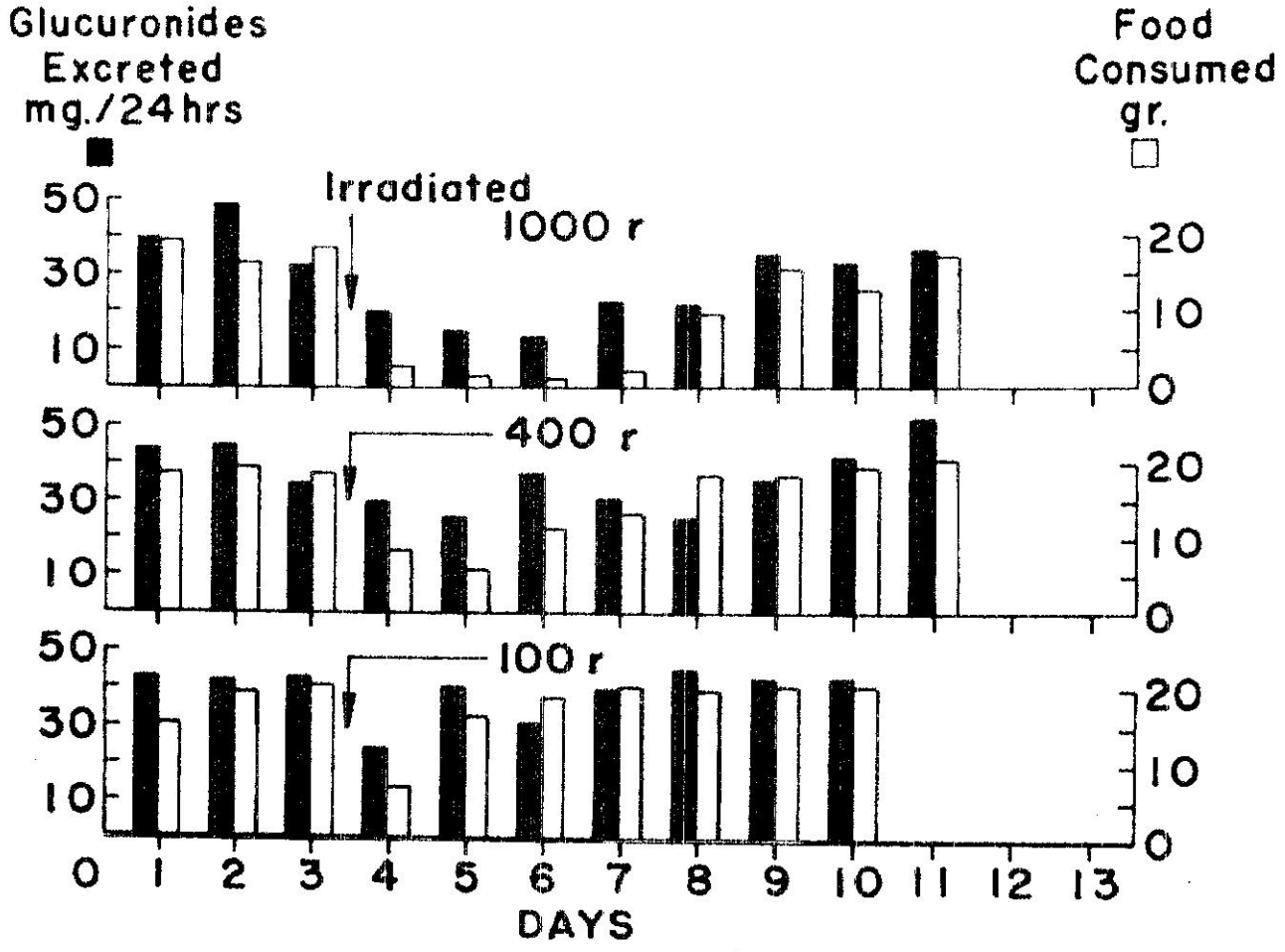


Fig. 3

Relation between radiation dose, food uptake and urinary excretion of total glucuronides.

The results (Figure 3) show the correlation of irradiation dose with food intake and with the amount of glucuronide excreted.

With 1000 R the food intake decreased severely for 4 days. With 400 R, there was a slight decrease during the first 3 days after irradiation. Therefore, the reduction of glucuronide excretion after irradiation must be correlated with the low food intake of the animals during this period.

2. Effect of Starvation on Total glucuronide Excretion

An experiment was conducted on 8 rats to test the effect of starvation on the excretion of total glucuronides. Total glucuronide excretion was measured for 3 days before food was withheld. After 7 days food was again supplied. With a standard Purina diet, normal excretion of total glucuronides averages 40.5 mg/day determined by Meads's method. After food withdrawal, the excretion decreases, reaching values < 10 mg/day. This level of excretion could be accounted for by endogenous sources (Figure 4).

The results indicated the following: (a) Four-fifths of the total glucuronides excreted by our normal rats are of exogeneous origin. These are healthy rats with a 50% rate of spontaneous tumorogenesis with aging. (b) The effect of gamma irradiation in producing low food intake in rats could have some influence on the rate at which endogenous or exogenous substances are conjugated. (c) The amount of total glucuronide excreted

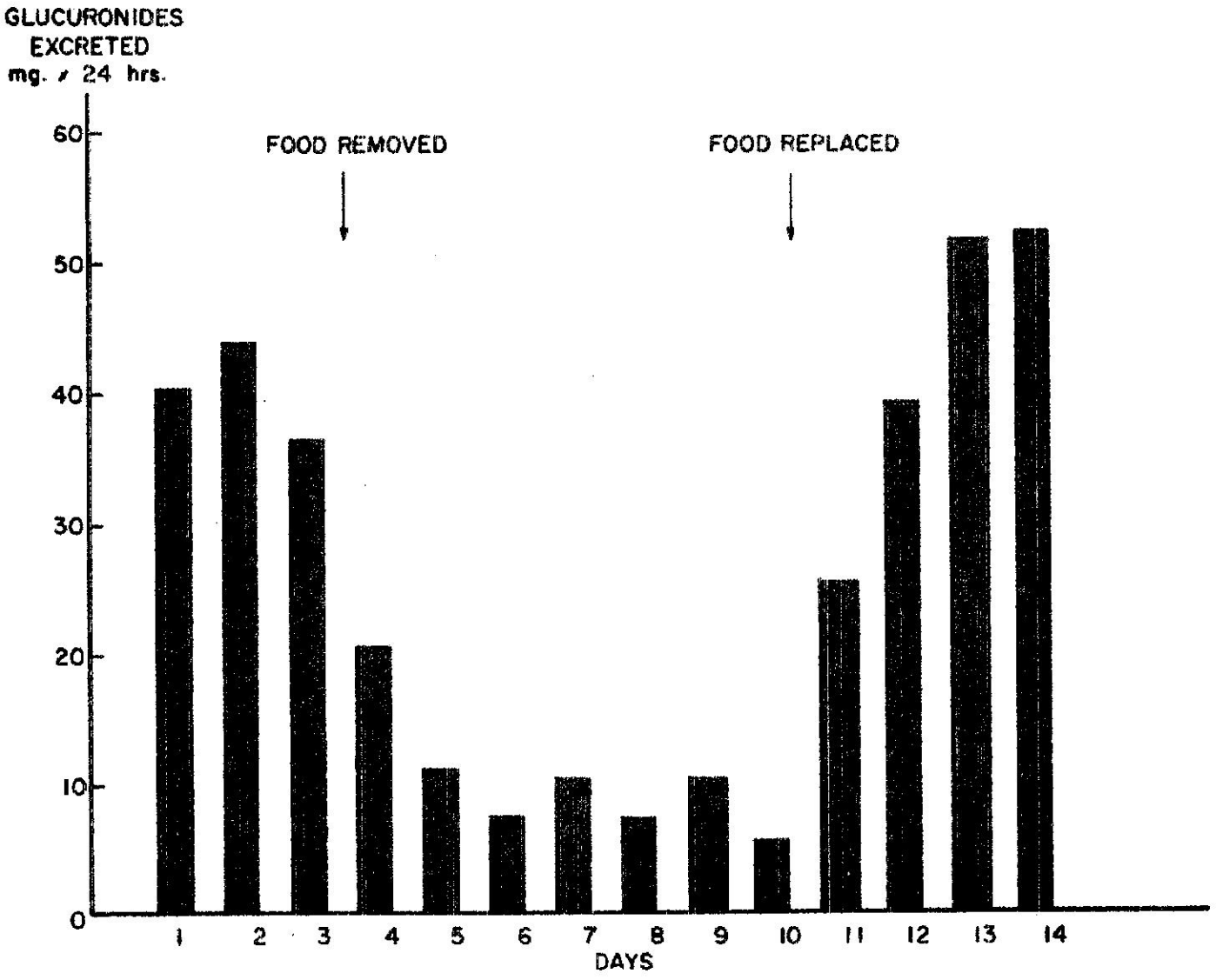


Fig. 4

Total urinary glucuronides excretion in normals rats under starvation.

per gram of food eaten was calculated, and the following values were obtained after substraction of the theoretical amount considered endogenous in irradiated rats. Before irradiation each gram of food produces an excretion of 1.8 mg, but the first day after 1000 R irradiation this increased immediately to 4.0 then to 4.6, 4.6, and 3.3 mg/g food, and it drops back to normal levels during the following days.

3. Excretion of Glucuronides of Endogenous Origin After Irradiation

The decrease in food intake of irradiated rats produces a corresponding decrease in the normal total glucuronide excretion that could mask the changes due to irradiation. In an experiment done to test this possibility, 8 rats were put under starvation for 3 days and then irradiated with 1000 R. The excretion of total glucuronides was followed each day.

The results (Table 1) clearly show the increased excretion during the first 24 hr after irradiation compared with that of the controls (8.82 $\pm$ 0.39 mg for control and 14.56 $\pm$ 0.56 for irradiated animals). A *t* test comparing the two groups indicates the high significance of these values.

It is possible to differentiate free glucoronic acid from glucusiduronic acid by the method of Fishman and Green.[9] This method was used in an experiment similar to the one just described. The results (Figure 5) show that the increased

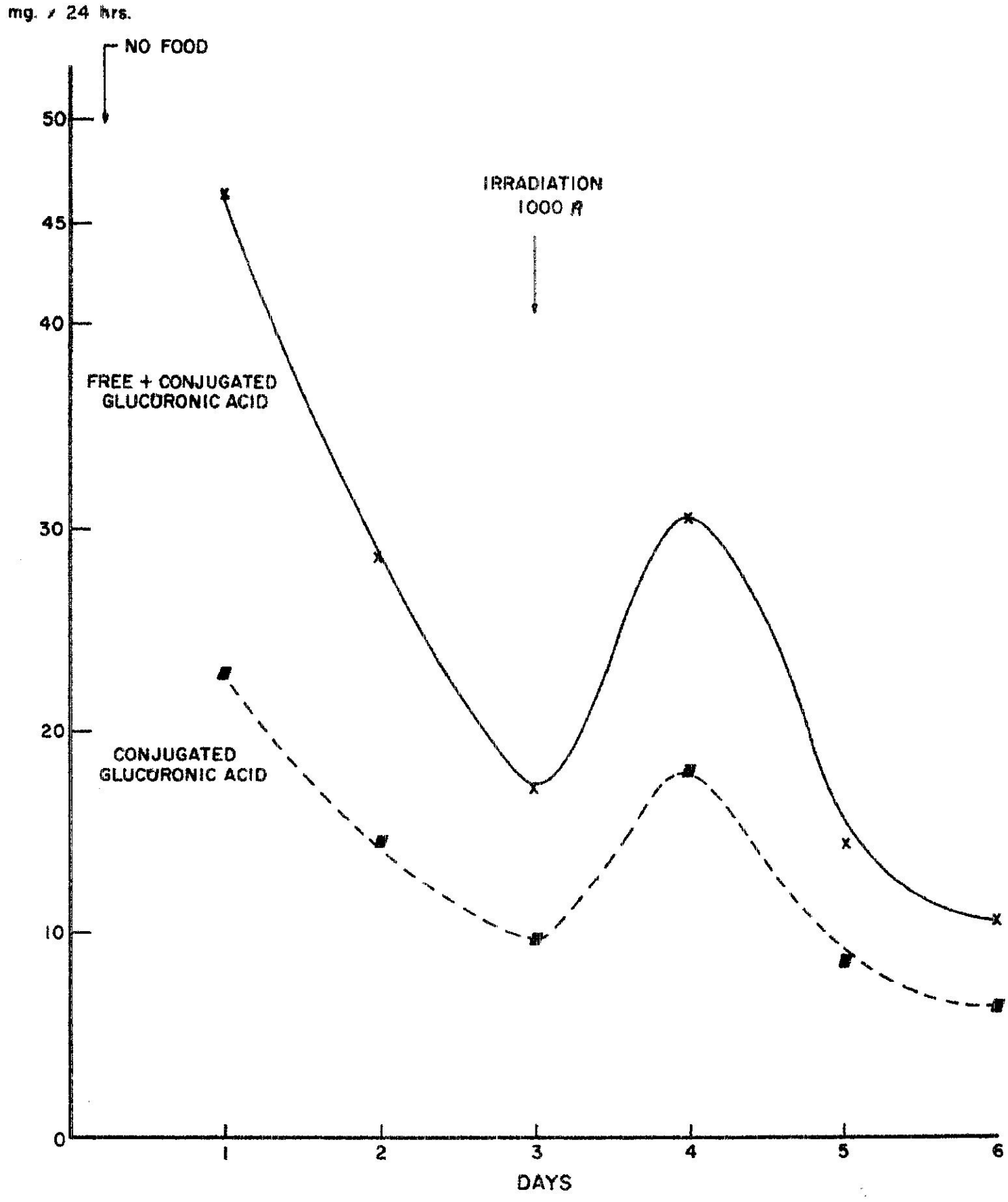


Fig. 5

Urinary excretion of free and conjugated glucuronic acid in starved rats after 1,000 R

Table 1

Total Urinary Glucuronide Excretion of Starved Rats (mg per 24 hr) After Whole-Body Irradiation

Day	Controls	Irradiated	Significance of Δ between control and irradiated group
1	19.59±0.55**	18.59±0.84	not significant
2	12.58±0.59	14.22±0.66	not significant
3	11.22±0.83	9.94±0.65	not significant
		1000 R administered	
4	8.82±0.39	14.56±0.86	$P < 0.005$
5	8.00±0.51	8.88±0.80	not significant
6	6.48±0.28	7.85±0.81	not significant

*Starved at day 0.

**Standard error of the mean.

excretion includes both the free and the conjugated acid forms.

The free acid found may be due to the action of beta-glucuronidase present in the urine. Many substances, principally hormones or degradation products, could be responsible for the increased glucuronide excretion in starved rats after irradiation. Elucidation of this point could contribute to the understanding of the physiological reaction of higher animals to radiation. Identification of these substances is the next step in this line of study.

B. DUODENAL SYNTHESIS OF GLUCURONIDES IN VITRO AFTER WHOLE-BODY IRRADIATION

We have reported that whole-body irradiated rats are more sensitive to naphthalene administered intragastrically.[6] The idea that this may be due to some impairment in the detoxification process seems to be supported by Hartiala's results[4,5] showing a decrease in glucuronic acid conjugation by tissues irradiated in situ. We decided to test this ability to conjugate using the same condition of irradiation as in our previous experiments. The conjugation of glucuronic acid with anthranilic acid in the gastrointestinal tract was tested because it was not possible to test its conjugation with naphthalene.

Immediately after 1000 R of whole-body irradiation the conjugation of anthranilic acid in duodenal slices from rats was slightly impaired (Table 2); in tissue from rats killed

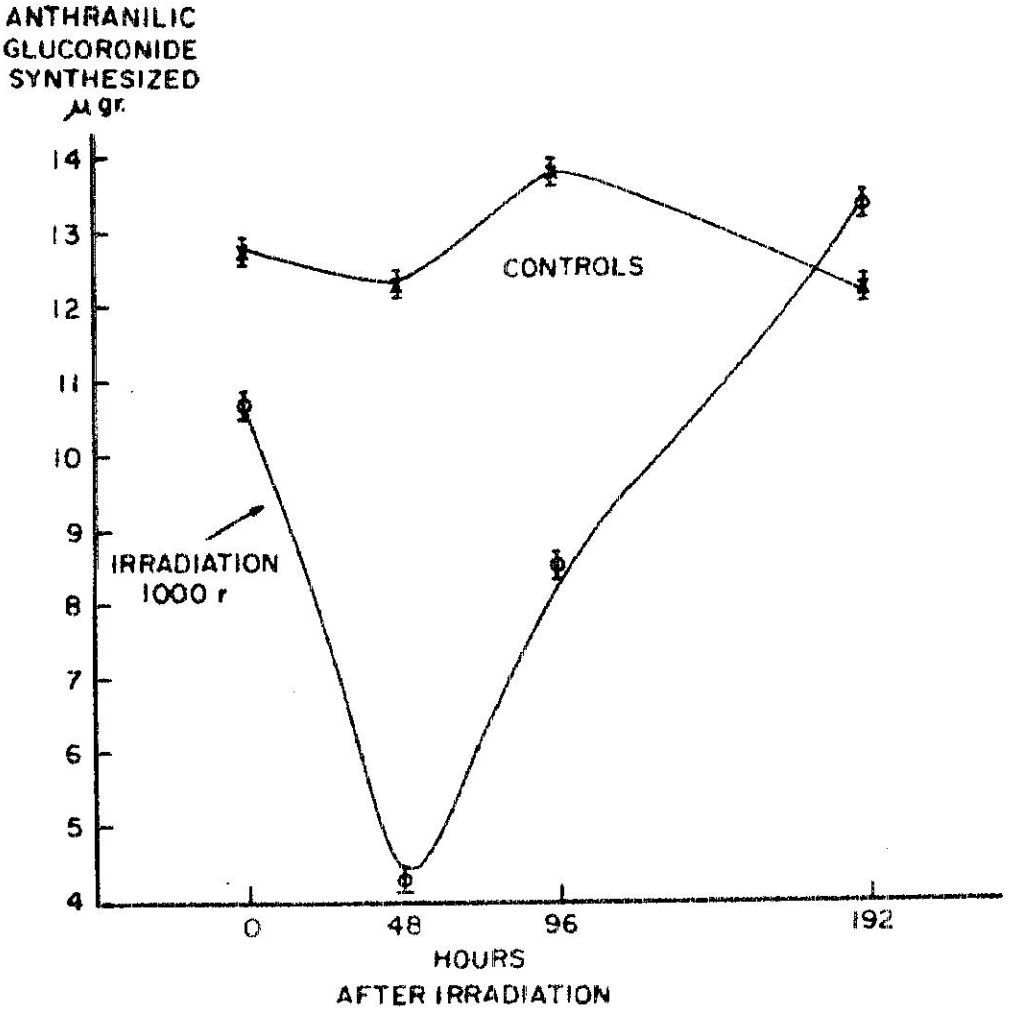


Fig. 6

In vitro conjugated anthranilic acid by duodenal tissue from rats which received whole body irradiation.

48 hr later the conjugation was only 34.5% of control values. After 96 hr some of the tissues reached normal values but others had very low values, giving an average of 53.4% of control. After 192 hr the duodenal slices conjugated anthranilic acid at a normal rate with some tendency to overcompensate (Figure 6).

In contrast to the results of studies in which glucose was found to depress the conjugation of anthranilic acid in liver slices,[14] the anthranilic acid conjugation in duodenal segments was not affected in normal rats (13.10 ± 0.76 without glucose in 14 determinations compared with 14.3 ± 0.68 with glucose in the same number of animals). But the conjugation was somewhat higher with glucose added in segments from starved or irradiated animals (Table 2).

DPN increased, slightly but significantly, the *in vitro* duodenal conjugation with glucuronic acid in normal animals. (Without DPN, 20 animals had an average of 11.16 ± 0.34 and with DPN, 13.08 ± 0.53; - this indicates a significant difference according to the student *t* test with *P* 0.02.) DPN had no effect on the conjugation of segments from irradiated or starved animals. UDPG or UDPGA added to the incubation medium had no effect on the conjugation of any group, possibly because of the membrane barriers or because the nucleotides were destroyed before reaching the effective site.

Table 2

In Vitro Synthesis of Anthranilic Glucuronides (μg) by Duodenal Slices From Irradiated and Starved Rats

Time of sampling	Control	Irradiated (1000 R)	Irradiated +glucose
Immediately after irradiation	12.81±1.29[a] (4)[b]	10.65±0.97* (8)	14.52±0.54* (8)
48 hr after irradiation	12.39±1.18 (4)	4.22±0.59** (8)	6.90±0.57** (11)
96 hr after irradiation	13.98±0.86 (4)	8.59±2.27 (8)	-----
192 hr after irradiation	12.27±0.45 (4)	13.32±0.59 (8)	-----
96 hr after starvation	13.06±0.71 (11)	6.98±0.40* (12)	9.40±0.05* (6)

[a] Standard error of the mean.

[b] Number of animals.

*$P < 0.01$.

**$P < 0.02$.

The conjugation *in vitro* in a radiosensitive tissue could be the expression of cell death plus some metabolic disturbances in the cells remaining alive. The effect reported in liver[5,7] which is a radio-resistant tissue, must be exclusively metabolic and is probably related to UDPGA synthesis.

Some reduction of conjugation by duodenal slices was also produced by a non-specific stress due to handling or confining the animals for some hours.

The *in vitro* results reported above could partly explain the high sensitivity of irradiated rats to naphthalene administered intragastrically; however, other mechanisms could also be involved.

C. SYNTHESIS AND EXCRETION OF GLUCURONIDES *IN VIVO* AFTER INTRODUCTION OF TOXIC SUBSTANCES INTO IRRADIATED ANIMALS

The assumption that the detoxification mechanism, especially the conjugation of glucuronides, does not work properly after irradiation is supported by our own results, the results of Hartiala et al.[4,5] on testing conjugation *in vitro* by tissue irradiated in situ, the report by Stevens[7] mentioning reduced conjugation of steroids in mice irradiated with x rays, and the study by Gerber et al.[15] on the effect of irradiation on the rate of conjugation of bilirubin in perfused liver.

We decided to do a sistematic study on the response to toxic substances in irradiated and starved rats. The excretion

Table 3

Total Urinary Glucuronide Excretion (mg per 24 hr) After Administration of Naphthalene and Anthranilic Acid to Rats Starved for 96 hr Before Dose

	Naphthalene (4 rats)		Anthranilic acid (3 rats)	
Day	Control	Starved	Control	Starved
1	24.04±1.67*	7.14±0.45	24.30±1.37	7.95±0.10
Dosed				
2	41.29±1.45	27.55±0.74	35.05±0.45	17.59±0.59
3	27.55±3.70	13.43±0.40	24.4±1.73	5.66±1.00

*Standard error of the mean.

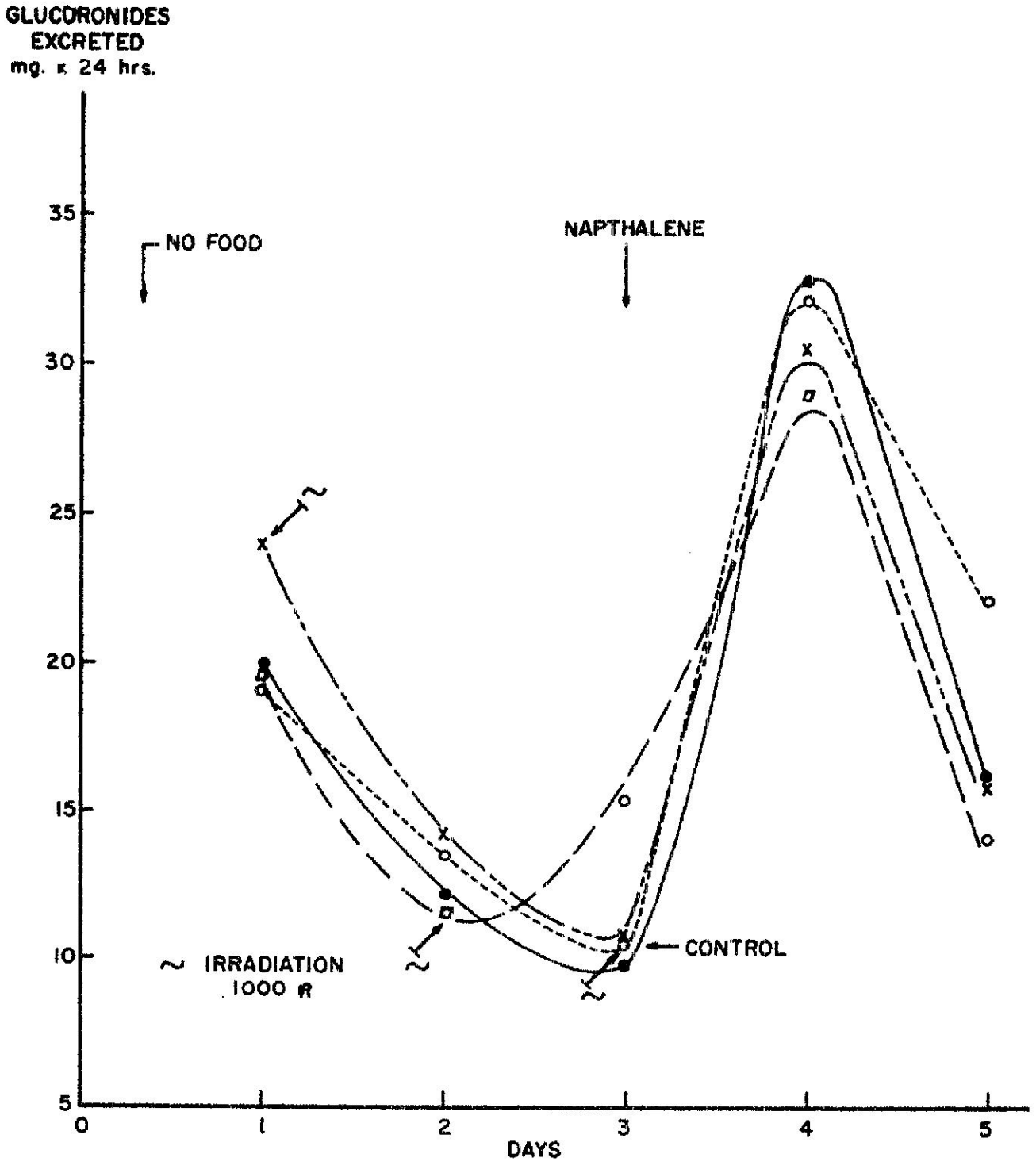


Fig. 7

Total glucuronides excreted in rat urine after starvation and administration of naphalene at different intervals after irradiation.

Table 4

Total Urinary Glucuronide Excretion (mg per 24 hr) of Irradiated Rats After Intragastric Administration of Naphthalene

Day(no food; water ad libitum)	Control	Dosed immediately after irradiation	Dosed 24 hr after irradiation	Dosed 72 hr after irradiation
1	20.18±0.55*	19.00±1.7	19.78±0.21	24.23±0.06**
2	12.57±0.63	13.32±0.96	11.56±0.28	14.28±1.41
3	9.99±0.23	10.00±0.85	15.02±0.48**	10.51±0.07
Dosed with naphthalene		**		
4	33.40±1.40	32.97±0.46	29.64±0.34	30.38±1.73
5	16.66±0.42	22.07±0.60	13.99±0.38	16.01±1.4

*Standard error of the mean.

**Day of irradiation with 1000 R.

of total and specific glucuronides in urine was used as a measure of the response, considering that glucuronides are substances that do not have a renal threshold.

Preliminary experiments, in which toxic substances were administered and the total glucuronides excreted by irradiated animals were measured, did not give clear results because the change in food intake after radiation masked the response. Therefore, it was considered necessary to keep the animals under starvation.

First an experiment was done which showed that starvation itself had no effect on the synthesis of the total glucuronides when the animals were challenged with toxic substances (see Table 3). Then an experiment to test the response of irradiated animals was done on sets of 32 rats (divided into 4 groups of 8 rats) maintained in metabolic cages of the type shown in Figure 1. Feeding was suspended, and 3 days later all the rats were given anthranilic acid subcutaneously or naphthalene intragastrically. Total glucuronides excreted in the urine were determined in both cases, and, in addition, anthranilic glucuronides and total anthranilic acid excreted were determined in the first case. For each drug one group of 8 rats was used as a control, and the other 3 groups had been irradiated with 1000 R, one group immediately before dosing, another 24 hr before, and the third 72 hr before. In this way the parameter

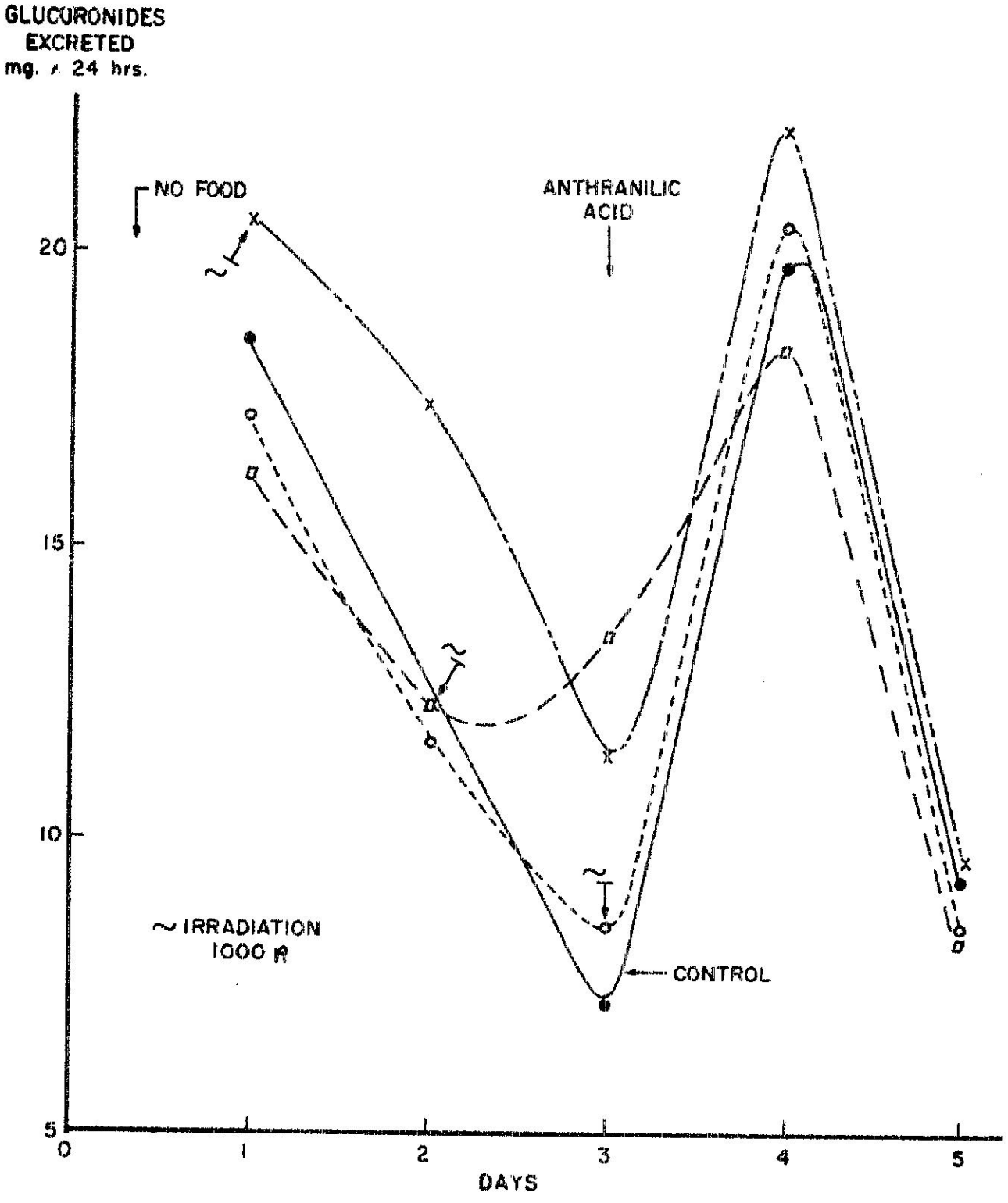


Fig. 8

Total glucuronides excreted in urine of rats under starvation and dosed with anthranilic acid at different intervals after irradiation.

Table 5

Total Urinary Glucuronide Excretion (mg per 24 hr) of Irradiated Rats After Subcutaneous Administration of Anthranilic Acid

Day (no food; water ad libitum	Control	Dosed immediately after irradiation	Dosed 24 hr after irradiation	Dosed 72 hr after irradiation
1	18.60±0.53*	17.29±0.92	16.36±0.33	20.52±0.55**
2	12.90±0.42	11.76±0.44	12.29±0.22	17.47±0.34
3	7.11±0.42	8.59±0.42	13.46±0.33**	11.22±0.35
Dosed with anthranilic acid		**		
4	19.07±0.89	20.50±0.62	18.45±0.55	22.05±0.50
5	9.25±0.42	8.43±0.32	8.17±0.31	9.58±0.33

*Standard error of the mean
**Day of irradiation with 1000 R.

of food consumption was maintained constant and only the time of irradiation was varied.

The results in Tables 4 and 5 show the excretion of total glucuronides determined by the naphthoresorcinol technique of Meads et al. There was practically no difference from the controls in the degree of response when the animals were challenged with the toxic substances immediately and 72 hr after irradiation.

When the animals were irradiated 24 hr before administration of naphthalene or anthranilic acid, in both cases apparently less total glucuronides were formed than in the controls. This result is related to the finding reported above that irradiated animals show increased excretion of glucuronides during the first 24 hr after irradiation when dosed with the drug in starved condition (Figures 7-8). The result of these experiments indicate that <u>in vivo</u> the mechanisms by which glucusiduronic acids are formed after irradiation are very stable.

Anthranilic glucuronides were measured by solvent extraction and also by paper chromatographic procedures, and the results were the same. In the case of anthranilic glucuronides no significant difference in excretion was found between controls and rats irradiated immediately and 24 hr before administration, but the excretion by rats irradiated 72 hr before administration was considerably less (Table 6). The fact that there was no

Table 6

Urinary Excretion (mg per 24 hr) of Total Anthranilic Acid and Anthranilic Glucuronide in Irradiated Rats After Administration of Anthranilic Acid

Group* and time after irradiation	Total anthranilic acid**	Anthranilic glucuronides**
Controls	10.38±0.52	4.19±1.10
Dosed immediately	11.00±0.25	4.47±0.36
Dosed 24 hr after	10.64±0.53	4.64±1.37
Dosed 72 hr after	12.16±0.36	1.36±0.50***

*Rats under starvation.

**Average for 8 animals.

***$P < 0.02$.

difference between test rats and controls in total anthranilic acid excretion led us to hypothesis that for unknown reasons some hydrolysis of the glucuronides occurred in the urine of this set of animals after excretion.

An increase in beta-glucuronidase excretion, or a decrease in the excretion of some inhibitor of this enzyme, was suspected and will be discussed below.

The excretion of specific glucuronides was tested also by administering napththalene-1-C^{14} intragastrically immediately after irradiation with 1000 R. Table 7 shows the results of quantitative analysis, after chromatography, by the method of Terriere et al.[11] of the two glucuronides excreted by the rats. Autoradiography of the paper chromatograph from the quantitative butanolextraction of the urine shows no systematic differences in the pattern of excretion (Figure 9). Ether extraction showed that the irradiated rats excreted higher amount of the oxidized metabolites 1-naphtol and 1,2-dihydro-1,2-dihydroxy-naphthalene; this point is under study.

The normal ability of the irradiated animals to conjugate glucuronic acid (judging by the urinary excretion of total and specific glucuronides) in contrast with the lowered ability of tissues tested in vitro could be explained by the fact that in the whole animal the conjugation takes place in a broad area and one organ can compensate for another. It is too early,

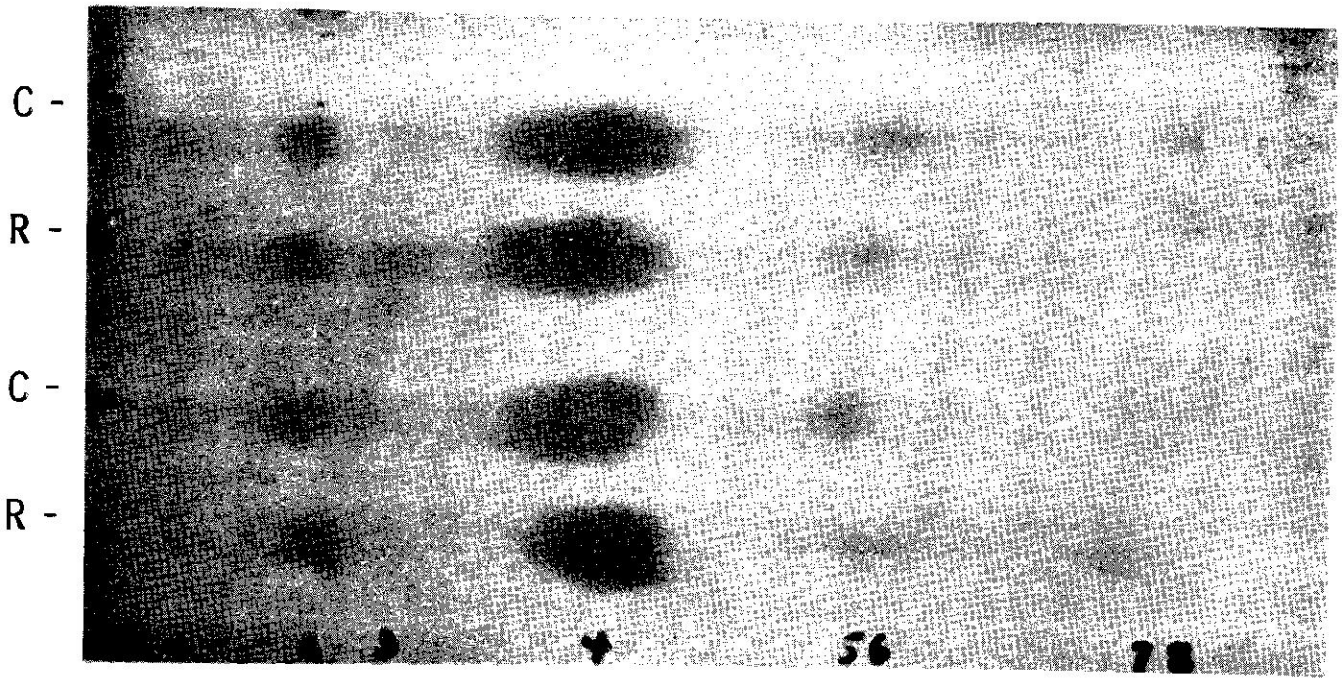


Figure 9

Autoradiography of a paper chromatograph of naphthalene products excreted in urine. R = radiation C = control. 2 and 3 glucuronides.

Table 7

Comparison of Excretion of Glucuronides of Naphthalene by Irradiated and Normal Rats After Administration of Naphthalene-1-C^{14}

	Irradiated[a]		Control	
	Rat 1	Rat 2	Rat 1	Rat 2
1,2-dihydro-2-hydroxy-1-naphthyl-glucosiduronic acid	0.0561[b]	0.0377	0.0446	0.0481
1,2-dihydro-1-naphthyl-glucosiduronic acid	0.0173	0.0241	0.0257	0.0288

[a] Intragastric administration immediately after 1000 R.

[b] Area (cm^2) under the densitometric curve from an autoradiography after paper chromatography.

to discard the idea that tissues tested in vitro need some circulating substances to compensate for the failure of their detoxification mechanism or the idea that some reaction inhibitor is present under the test conditions. What is interesting is the stability of the rate of glucuronic acid conjugation in intact animals after irradiation, considering that changes in glucuronic acid precursors due to radiation have been reported. Glycogen concentration in liver suffers considerable changes after irradiation,[16] and the rate of formation of some nucleotides changes also.[17] However, irradiated animals respond similarly to controls when are challenged with toxic substances. It is difficult to explain the normal response of irradiated animals, judged by total urinary glucuronide excretion, when they are challenged with toxic substances administered through the intestinal tract. It is well known that the gastrointestinal epithelium suffers histologic changes and changes in absorption properties when irradiated.[18]

D. EXCRETION OF beta-GLUCURONIDASE AND beta-GLUCURONIDASE INHIBITOR AFTER IRRADIATION AND STARVATION

As mentioned above, the excretion of anthranilic glucuronides was considerably lower in the group irradiated 72 hr before dosing than in the controls. However, the total amount of anthranilic acid excreted, conjugated and free, was more or less the same in both groups (Table 6). This finding led us to suspect

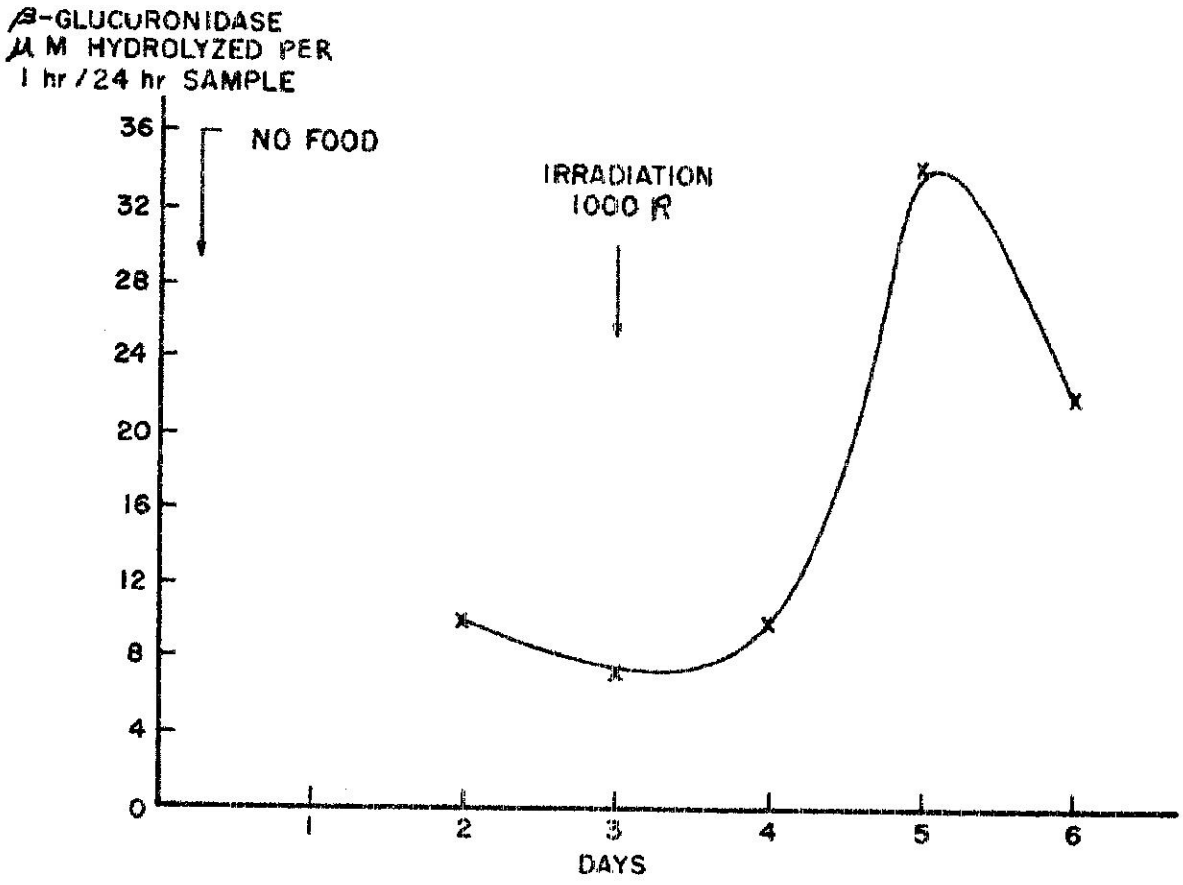


Fig. 10

Excretion of β-glucuronidase in urine of starved rats before and after whole body irradiation.

that there could be some change in the amount of beta-glucuronidase or its inhibitor in tissue or in the urinary excretion.

The data presented in Table 8 show the 24-hr excretion of anthranilic glucuronide and of beta-glucuronidase in a group of animals to which anthranilic acid was administered subcutaneously 72 hr after irradiation. The beta-glucuronidase concentration was higher in the irradiated group than in the control, 27.8 ± 9.6 mg versus 60.2 ± 8.6 mg hydrolyzed per hour per 24-hr sample. The mean anthranilic glucuronide excreted in 24 hr was 2.13 ± 0.62 mg for the control group and 0.69 ± 0.05 in the irradiated group. Thus it is very possible that hydrolysis of glucuronides occurs in the collection device after the urine has been excreted.

In order to learn more about the excretion of beta-glucuronidase in rats starved for 3 days and irradiated with 1000 R, an experiment was done in which excretion of the enzyme was measured before and after irradiation. The data in Figure 10 show the average excretion before irradiation and 24, 48, and 72 hr after. The increase at 48 hr was very significant compared with the values for excretion before irradiation (Table 9). No difference from controls was found in the excretion of beta-glucuronidase inhibitor in rats irradiated under the same conditions.

Table 8

Comparison of the Excretions of Anthranilic Glucuronides and of beta-Glucuronidase in Irradiated Rats after Administration of Anthranilic Acid

Rat No.*	Total anthranilic acid, mg per 24 hr	Anthranilic glucuronides, mg per 24 hr	beta-Glucuronidase, μmoles hydrolyzed per hr in 24-hr sample
Control			
1	7.00	2.75	5.1
2	7.50	0.85	24.9
3	9.00	0.40	33.6
4	9.00	0.58	79.5
5	8.95	4.95	5.1
6	9.62	3.70	8.1
7	9.25	3.20	10.8
8	8.50	0.58	55.5
Mean	8.60 ± 0.32**[a]	2.13 ± 0.62[b]	27.8 ± 9.6[c]
Dosed 72 hr after irradiation			
9	8.20	0.50	33.7
10	9.37	0.68	57.6
11	8.60	0.62	45.9
12	5.88	0.65	53.7
13	9.63	1.00	56.7
14	11.00	0.82	79.2
15	10.00	0.62	111.1
16	9.31	0.62	44.1
Mean	3.999 ± 0.537[a]	0.69 ± 0.05[b]	60.2 ± 8.6[c]

*All rats in starvation.

**Standard error of the mean.

[a] $0.40 < P < 0.60$.

[b] $0.02 < P < 0.05$.

[c] $0.02 < P < 0.05$.

Table 9

beta-Glucuronidase Excretion After Whole-Body Irradiation of Starved Rats

	μMoles substrate hydrolyzed per hr per 24-hr sample			
		After irradiation		
Rat No.	Before irradiation*	1st day	2nd day	3rd day
1	13.00	11.50	15.50	14.50
2	2.00	4.00	83.00	11.50
3	8.00	9.00	22.00	12.00
4	5.00	8.00	27.50	8.00
5	2.00	11.00	25.00	6.00
6	12.50	8.00	76.50	102.00
7	2.50	22.00	4.00	22.00
8	17.00	6.50	27.50	2.00
Av.	7.75 ± 2.05**	10.00 ± 1.91	35.12 ± 10.13	22.25 ± 11.58
Av. difference		2.25	27.37	14.50
Probability		< 0.5	$0.02 < P < 0.05$	< 0.2

*Rats were starved for 72 hr before irradiation with water ad libitum.

** Standard error.

The enzyme beta-glucuronidase is a lysozyme enzyme. Increase in the beta-glucuronidase content of organs after irradiation has been reported, and it is related to cell damage.[19] The lysozyme membrane has been described as very sensitive to radiation.[20] This could be the explanation for the increase in urinary beta-glucuronidase excretion in our animals. However, the physiological significance of this finding has not yet been evaluated in relation to the radiation syndrome.

E. CHROMATOGRAPHIC SEPARATION OF URINE NUCLEOTIDES*+

Our results and results reported by others have shown that tissue from irradiated rats has a reduced ability to conjugate toxic substances with glucuronic acids when tested in vitro. However, when the synthesis of glucuronides was tested in live animals after administration of toxic substance, no difference between control and irradiated animals was found, judging by the amount of glucuronide excreted in urine. It was surprising that even when the toxic substances were given intragastrically

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* The abbreviations are: UMP = Uridine Monophosphate, UDP = Uridine Diphosphate, UTP = Uridine Triphosphate, UDGP = Uridine Diphosphate Glucose, UDPGA = Uridine Disphosphate Glucuronic Acid, ADP = Adenosine Disphosphate, ATP = Adenosine Triphosphate and DPN = Diphospho Pyridine Nucleotide.

+ Collaborate in this part of the work Dr. Javier Barua, Fulbright Fellow from the University of San Marcos, Lima, Peru.

(the gastrointestinal epithelium is very sensitive to radiation) no difference was found in the urinary excretion of glucuronides. In order to discover the mechanism responsible for this finding, we decided to study the amount of uridine nucleotide and its turnover in different tissues, principally those in which its metabolism is directly concerned with the detoxification process. In order to separate the nucleotides and determine them quantitatively, a machine was built for automatic analysis with a sufficient degree of reproducibility and accuracy for this type of research. This part of the work was not completed because the author left Oak Ridge. Preliminary data are reported here.

1. Description of the Machine

An automatic analysis machine was assembled (Figure 11) to process the nucleotide mixture in a step-gradient fashion, in which it would also be possible to detect radioactivity in the eluent flow. The machine has the following parts:

1. An automatic stepwise eluent dispenser, partially described elsewhere. This was modified to make it possible to change from one buffer to another with a minimum probability of mixing them. An electronic device, constructed to take the place of the float, counterbalance, and switch, was installed in the machine, which consisted of a photocell activated by the buffer level in the main reservoir (Figure 12).

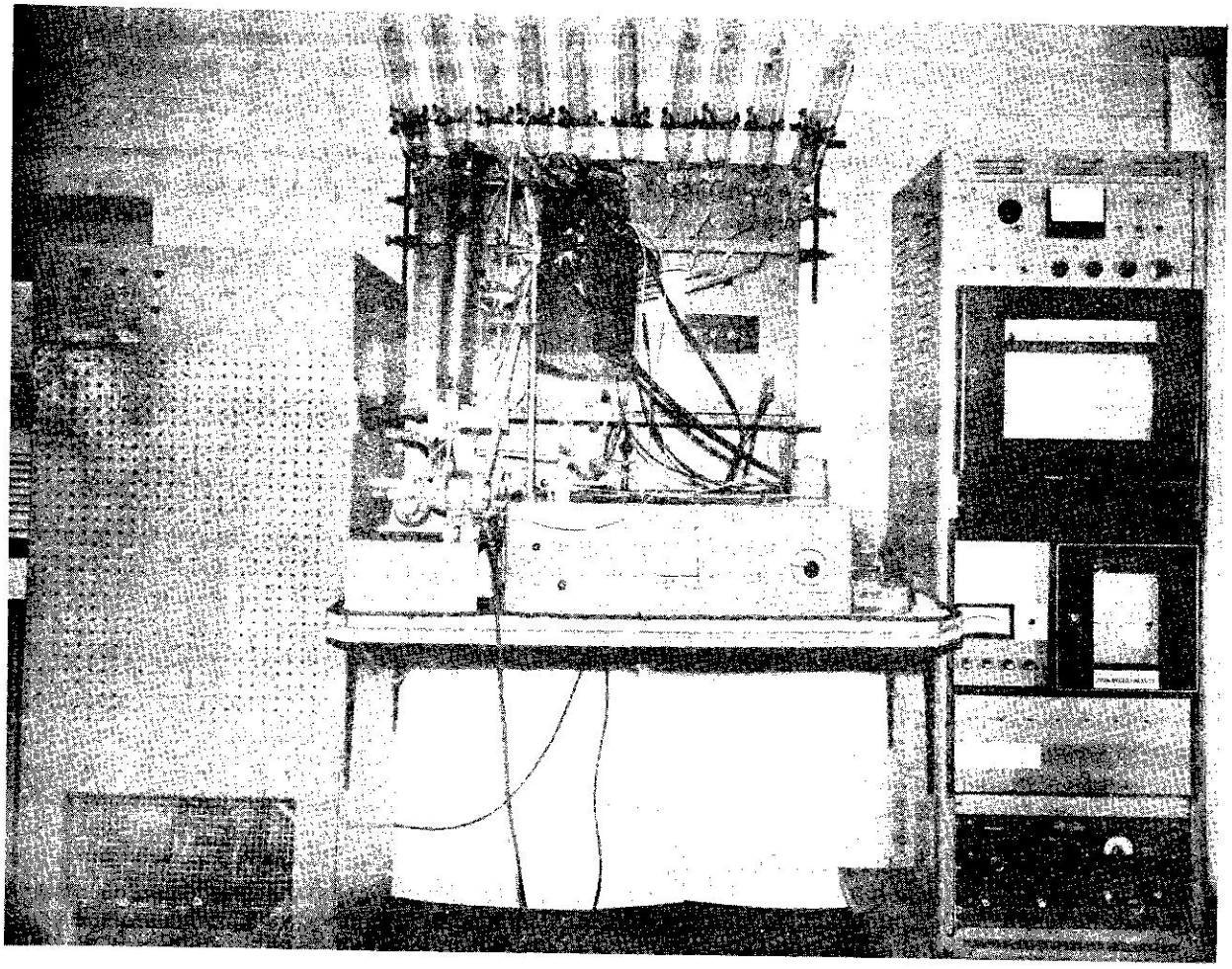

Figure 11

Automatic machine for step gradient chromatographic elution of nucleotides

The amplifier had no coupling capacitor between stages, and therefore only the inertia of the relay limited the speed of operation. In addition the device is positive in its action, being unaffected by normal power supply variation and parameter changes over a wide range (Figure 13).

2. A fraction collector.

3. A cooling unit that pumps cooling solution containing antifreeze through a copper tube inside the fraction collector to the jacket column, to permit changes in the temperature of the column.

4. A Roy millipump to pump the elution buffer across the column to the detectors.

5. A double-beam detector in the ultraviolet range, with a Vanguard instrument for selecting the wavelength and recording the elution pattern.

6. A detector for beta emission with a cartridge full of anthracene crystals. Light from the other face of the cartridge is sent back to the phototube by a plastic mirror at a 45° angle to increase the sensitivity of the detector (Figure 14). The signal from the amplifier is recorded in a Honeywell recorder.

To operate the machine, the resin column is charged with the mixture to be analyzed, and the dispenser containers are filled with the different buffers to be used. A button

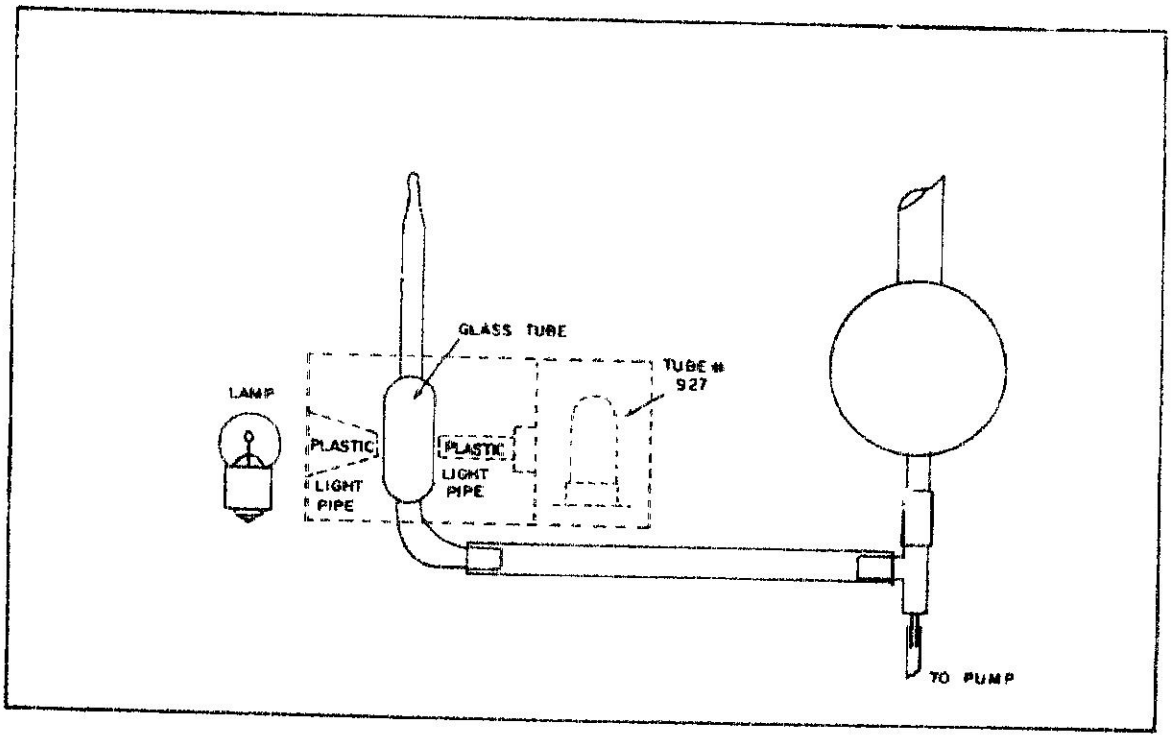
GLASS TUBE
TUBE # 927
LAMP
PLASTIC
LIGHT PIPE
PLASTIC
LIGHT PIPE
TO PUMP

Fig. 12

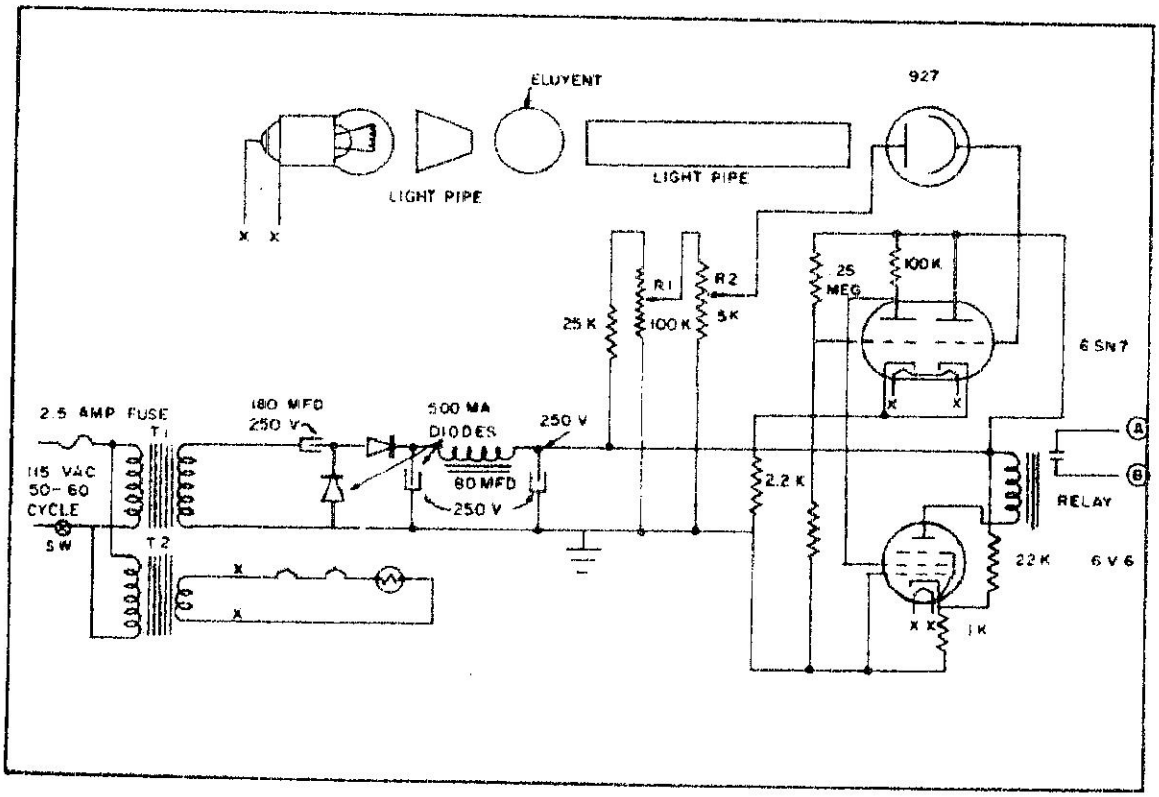
ELUYENT
927
LIGHT PIPE
LIGHT PIPE
X X
R1
R2
25 K
100 K
5K
25 MEG
100 K
6 SN7
2.5 AMP FUSE
T1
115 VAC 50-60 CYCLE
SW
T2
180 MFD 250 V
500 MA DIODES
250 V
80 MFD 250 V
2.2 K
22 K
6 V 6
1K
RELAY
A
B

Fig. 13

is push to start the machine. Operation continues until the last buffer is dispensed across the column. It is possible to locate sections on the recording chart because the machine makes a mark on the paper strip each time the fraction collector changes a tube.

2. Column Preparation

In the first experiments the method of Hurlbert et al.[22] was used, with a formic-formate system, but later the elution liquid was changed to acetate buffer at pH 4.4 according to the method of Anderson.[23] The resin used was Dowex 1 x 8 200-400 mesh purified by Bio-Rad and treated with normal HCl and KOH and washed with distilled water. After this pretreatment the resin was treated with 3 M acetate buffer, pH 4.4, and then equilibrated with the first buffer used, 0.15 M acetate. The column was 1 x 50 cm, and it was charged under 10-lb pressure from a nitrogen tank.

The column was first tested with pure Sigma compound alone and with a standard mixture of the same compound, and later the different mixtures were run that were obtained from rat tissue after the nucleotides had been labeled with P^{32} in the live animal.

3. Preparation of P^{32}-Labeled Acid-Soluble Nucleotide Mixture From Rat Tissue

The method used was adapted from Hurlbert et al.[22] and the tissue was processed in the following way. Rats were

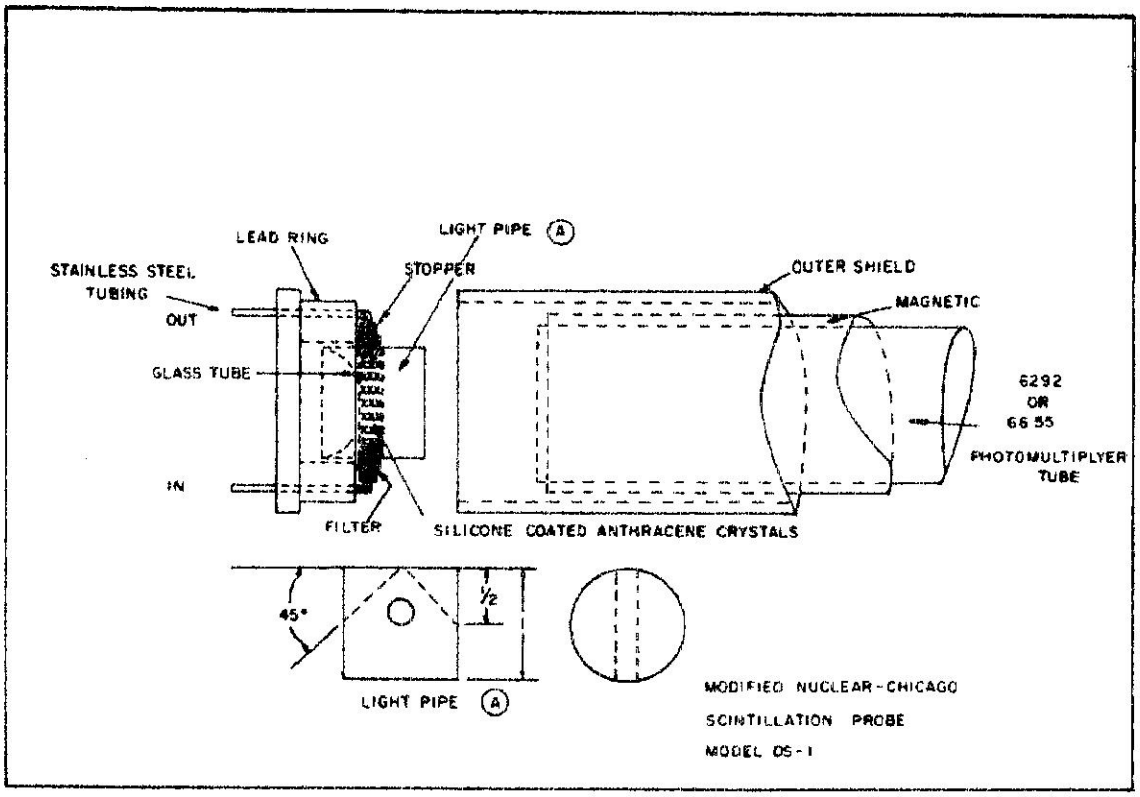
LEAD RING
LIGHT PIPE (A)
STAINLESS STEEL TUBING
STOPPER
OUTER SHIELD
MAGNETIC
OUT
GLASS TUBE
6292 OR 6655
PHOTOMULTIPLYER TUBE
IN
FILTER
SILICONE COATED ANTHRACENE CRYSTALS
45°
½
LIGHT PIPE (A)
MODIFIED NUCLEAR-CHICAGO
SCINTILLATION PROBE
MODEL DS-1

Fig. 14

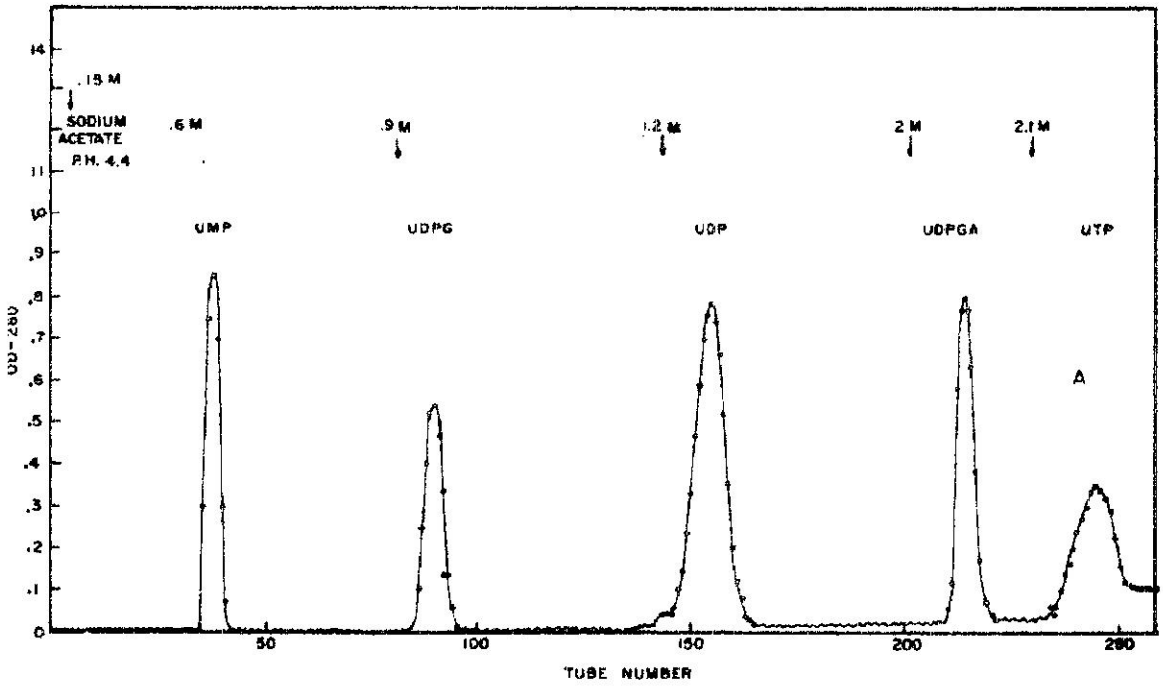
.15 M
SODIUM ACETATE
P.H. 4.4
.6 M
.9 M
1.2 M
2 M
2.1 M
UMP
UDPG
UDP
UDPGA
UTP
A
OD-280
TUBE NUMBER
50
100
150
200
250

Fig. 15

dosed intraperitoneally with 0.4 mCi P^{32} in 9% NaCl and killed 1 hr later. When liver was to be used, 10 g liver was frozen immediately by immersion in dry ice acetone mixture and covered with Saranwrap. It was later cut into pieces and homogenized in a Potter-Ehlvejem homogenizer at 2°C. When intestinal tissue was to be used, the whole small intestine was cleaned and frozen in the same way. It was then passed through a stainless steel press, and 6 g of it was homogenized. The homogenizate was put into 2 volumes of 0.6 N cool perchloric acid and centrifuged under refrigeration. The precipitate was washed with 2 volumes of 0.2 N perchloric acid and centrifuged again. The two supernatants were mixed and adjusted to pH 6-7 with phenol red used as indicator and then treated with potassium hydroxide. The potassium perchlorate was precipitated overnight at 0°C. After centrifuging the supernatant was ready to use in the column. Half of the sample was used in each run. The separation was made with different concentrations of the acetate buffer at pH 4.4.

4. Preliminary Results

Many different ways of separating nucleotides are described in the literature. Some use gradient systems, others step-gradient systems. Since we were interested in a group of only a few nucleotides, all of the uridine family, we tried to find the most useful elution pattern that would provide quick and

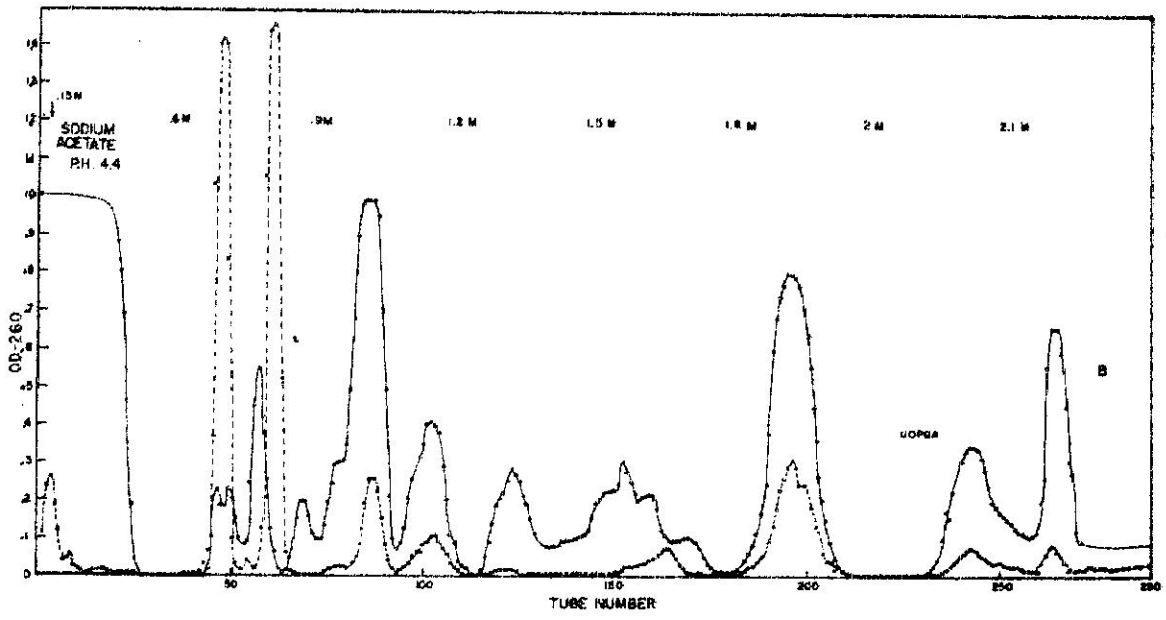

Fig. 16

Separation of acid soluble nucleotides from liver of rat after been dosed with P^{32}. Dotted line radioactivity in CPM x 10^4.

quantitative information about specific activity.

It was found that UDPGA is eluted at 2 M concentration of the acetate buffer at pH 4.4 with Dowex 1 used as described above. When a mixture of UDP, ADP, ATP, UDPG, and UDPGA was eluted with the same buffer at 1.8 M and 2 M, only UDPGA was eluted at 2 M.

By trial and error an elution pattern was found which could be considered useful in the separation of the nucleotides in tissue samples. When 3 mg of each of the uridines were mixed and applied to a column, they were separated by using 200 ml of different concentrations of acetate buffer at pH 4.4. The rate of flow was kept constant at 1.1 ml/min. Under these conditions UMP eluted at 0.6 M, UDPG at 0.9 M, UDP at 1.2 M, UDPGA at 2 M, and UTP at 2.1 M (Figure 15).

The separation of acid-soluble nucleotide mixtures prepared from rat tissue was accomplished with a similar pattern of elution. The conditions are stated in each figure showing the results. Only UDP and UDPGA have been identified up to now, with similar samples the reproducibility has been very good. Identification was done by paper chromatography, ultraviolet spectrum, and colorimetric analysis of the glucuronyl moeity in the case of UDPGA (Figure 16). The amount of UDPGA in liver was found higher than in small intestine with correction made for difference in sample weight.

Future research in this area could clarify some aspects of the detoxification process in general and the effect of

irradiation on it.

SUMMARY

1. Whole-body irradiation decreased the urinary excretion of glucuronides in relation to food intake in rats.

2. When rats were put on starvation and then irradiated, there was an increase in the excretion of glucuronides and free glucuronic acid during the first 24 hr.

3. Whole-body irradiation decreased the ability of duodenal tissue to conjugate anthranilic acid with glucuronic acid in vitro. Starvation also diminished this ability.

4. Glucose added to the incubation vessel had no effect on normal duodenal segments but increased anthranilic acid conjugation by irradiated and starved tissues.

5. DPN increased the conjugation of anthranilic acid by normal duodenal segments but had no effect on tissue from irradiated or starved rats.

UDPG and UDPGA had no effect on the conjugation by normal or irradiated or starved duodenal tissue.

6. Whole-body irradiated rats excreted in the urine the same amount of total glucuronides as the controls when challenged with toxic substances such as subcutaneously administered anthranilic acid and intragastrically administered naphthalene.

7. The amount of anthranilic glucuronide excreted by rats irradiated 72 hr before dosing was lower than by the controls. This was found to be related to an increased excretion of beta-glucuronidase in the urine.

8. In order to study the discrepancy between the conjugation of toxic substances by tissue *in vitro* and by the live animal, a machine was built for automatic determination of the quantity and turnover of uridine nucleotides with P^{32} in the live rat. Preliminary results are reported.

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