The Effect of Tannic Acid on the Synthesis of Protein and Nucleic Acid by Rat Liver

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1. As early as 1 hr. after the intraperitoneal administration of tannic acid to rats, it could be demonstrated in the liver. At 3 hr. the nuclear fraction contained the largest amount of tannic acid. 2. Nuclear RNA synthesis was inhibited *in vivo* 2 hr. after the administration of tannic acid. Induction by cortisol of tryptophan pyrrolase was 90% inhibited at 24 hr. 3. Incorporation of [^{14}C]leucine into protein by liver slices from treated rats was decreased by 50% after 24 hr. Its incorporation into postmitochondrial supernatant from treated animals was not inhibited. Incorporation into slices and postmitochondrial supernatants were inhibited *in vitro* by tannic acid. 4. The sequence of events: concentration of tannic acid in nuclei, inhibition of nuclear RNA synthesis, inhibition of protein synthesis and production of necrosis, is discussed.

Tannic acid causes hepatic necrosis when administered to man (Lucke, Hodge & Patt, 1963) and horse (Begović & Ožegović, 1959). Cameron, Milton & Allen (1943) detected a fall in serum albumin concentration in tannic acid-poisoned goats and suggested that this may result from a direct action on the liver since there was insufficient tannic acid in the serum to precipitate the albumin. Horváth, Sólyom & Korpássy (1960), by using histochemical methods, demonstrated a decrease in the protein and RNA content of liver cells in tannic acid-poisoned rats.

We examined the effect of tannic acid on protein and nucleic acid synthesis by rat liver in an attempt to throw light on the mechanism of tannic acid-induced liver damage.

MATERIALS AND METHODS

Animals. Male Wistar rats (170–400 g. body wt.) were used. They were fed on Oxoid 41B diet and water *ad libitum*.

Chemicals. Tannic acid (pentadigalloylglucose) from Hopkin and Williams Ltd., Chadwell Heath, Essex, was found to contain the acid equivalent of 26.4% gallic acid when a sample was titrated (0.1 M-NaOH) with Methyl Red as indicator. It was freed from gallic acid and *m*-digallic acid by the method of King & White (1961). Purity was assessed by paper chromatography by the method of Horler & Nursten (1961). For intraperitoneal injection, a 2% (w/v) solution in 0.9% NaCl was used (final pH 7.0). Crystalline bovine serum albumin was from Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex; ATP, phosphoenolpyruvate, pyruvate kinase and hydrocortisone 21-acetate were from the Sigma Chemical Co., St Louis,

Mo., U.S.A. Yeast RNA was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany, and a highly polymerized calf thymus DNA (sodium salt) was from British Drug Houses Ltd., Poole, Dorset. The rest of the chemicals from the latter firm were of AnalaR grade except for L-leucine and L-tryptophan.

For radioactivity experiments, [^{14}C]leucine and [^{14}C]orotic acid (specific radioactivities 34.2 mc/m-mole and 44.5 mc/m-mole respectively) were from The Radiochemical Centre, Amersham, Bucks. Toluene, 2,5-diphenyloxazole and 1,4-bis-(5-phenyloxazol-2-yl)benzene, all scintillation grade, were from Nuclear Enterprises (G.B.) Ltd., Edinburgh.

Histology. Rat liver sections were stained with haematoxylin-eosin or haematoxylin-Sudan IV.

Total lipids and dry matter in liver. Total lipids were determined by the method of Folch, Lees & Sloane-Stanley (1957). The residue left after extraction of fat was dried at 100° for 2 hr. and then dried at room temperature in a vacuum desiccator over CaCl_2 to constant weight. Total lipids were expressed as mg./g. of fat-free dry residue. The water content was obtained from the difference between the wet weight and the combined weights of fat and fat-free dry residue.

Chemical and enzymic determinations. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with crystalline bovine serum albumin as standard. A more stable copper solution was used as described by Hall & Cocking (1965). DNA was determined by the diphenylamine method (Dische, 1955, p. 287) and RNA was determined by the orcinol method (Dische, 1955, p. 301). Because DNA interfered with the RNA estimation, a standard curve was made for DNA by the orcinol method and a correction applied where necessary. Tannic acid was determined with FeCl_3 (Cameron *et al.* 1943). Oxygen uptake by liver slices was measured by the usual Warburg technique (Umbreit, Burris & Stauffer, 1957). Tryptophan

pyrrolase was assayed by the method of Feigelson & Greengard (1961). The μ moles of kynurenine produced/g. wet wt. of liver/hr. were plotted against incubation time and the slope of the line during the linear phase gave the enzyme activity. For induction experiments, hydrocortisone 21-acetate (2mg./100g. body wt., dissolved in 0.1 ml. of propylene glycol) was injected intraperitoneally into rats. Control animals received equal volumes of propylene glycol, which was found to exert no effect on the enzyme activity. All animals were killed 3 hr. after injection.

Preparation of liver slices and homogenates. The animals were killed by stunning and cervical dislocation and their livers removed within 30 sec. into the appropriate ice-cold medium. Slices were prepared by hand with a razor blade. Homogenates were prepared with a Teflon-glass Potter-Elvehjem homogenizer at 1100 rev./min. for 1 min. All operations were carried out at 0–4°.

Radioactivity measurements. Protein samples (dissolved in *m*-NaOH) and nucleic acid, nucleotide or amino acid samples (dissolved in 0.6 *M*-trichloroacetic acid) were counted in a well-type liquid-scintillation counter (approx. 48% efficiency) by the method of Hall & Cocking (1965). The sample (0.1 ml.) was added to 5 ml. of scintillator and 0.1 ml. of 23 *M*-formic acid. Under optimum conditions of sample volume (2 ml.) and voltage, radioactivity was measured until 10000–12000 counts had been obtained. The background radioactivity was very small (36–42 c.p.m.).

Incorporation of [14 C]leucine into proteins. Incorporation by rat liver slices was determined by the procedure of Smuckler (1966). Controls and tests were done in quadruplicate. [14 C]Leucine was added to give 1 μ C/flask (14.6 μ M). After incubation the supernatant fluid was decanted. Slices were removed and homogenized in 0.6 *M*-trichloroacetic acid containing 0.1 *M* non-radioactive leucine. The sedimented residue was washed twice by repeated centrifugation and decantation in 0.6 *M*-trichloroacetic acid. The radioactivity of the acid-soluble extract (amino acid pool) was counted and expressed as c.p.m./mg. wet wt. of liver. The protein in the sedimented residue was purified by the method of Siekevitz (1952). The air-dried protein was dissolved in 1 ml. of *m*-NaOH. Radioactivity was counted and protein estimated as described above. Specific radioactivity was expressed in c.p.m./mg. of protein as the mean value of four determinations.

[14 C]Leucine incorporation into proteins by the rat liver postmitochondrial supernatant (after 10 min. at 10000 *g*) was performed as described by Clifford & Rees (1967). Both controls and tests were performed in quadruplicate. After incubation the protein was isolated and dissolved in alkali, as above. Protein was determined and radioactivity counted and expressed as above.

Incorporation of [14 C]orotic acid into liver-cell nuclear RNA, total liver RNA and nucleotides. The incorporation *in vivo* was performed by the procedure of Clifford & Rees (1967). [14 C]Orotic acid (100 μ C/kg. body wt., dissolved in 0.9% NaCl) was injected into the tail vein. Then 15 min. later the rats were killed; their livers were removed and two 200 mg. portions extracted for total liver nucleotides. Nucleotide radioactivity was expressed as c.p.m./ E_{260} unit. The nuclei were isolated from the remainder of the liver by the method of Rees & Rowland (1961). RNA was extracted from the nuclei by heating them for 15 min. at 90° with 0.6 *M*-trichloroacetic acid. Radioactivity and RNA were measured as above. DNA was determined in this extract by

the diphenylamine method and a correction was applied to obtain the RNA content of the nuclear extract. Specific radioactivity was expressed as c.p.m./mg. of RNA.

The incorporation of [14 C]orotic acid into RNA by control and test liver slices was performed in quadruplicate. Each flask contained 200 mg. ($\pm 10\%$) wet wt. of liver slices in 3.0 ml. of ice-cold Krebs–Ringer phosphate medium (Umbreit *et al.* 1957) and 0.1 ml. of [14 C]orotic acid was added to give 1 μ C of radioactivity/flask. The flasks were incubated at 37° for 1 hr. under O_2 with shaking. The reaction was stopped by the addition of 1.9 ml. of 0.6 *M*-trichloroacetic acid and the contents of the flask were centrifuged. The supernatant was decanted and the slices were homogenized in 0.6 *M*-trichloroacetic acid and centrifuged. The sedimented residue was washed twice with the same reagent. The residue was then extracted for RNA by heating it with 2.0 ml. of 0.6 *M*-trichloroacetic acid at 90° for 15 min. The RNA content and radioactivity of the separated supernatant solution were determined. Specific radioactivity was expressed as the mean value of four determinations, in c.p.m./mg. of RNA.

Sedimentation of DNA and tannic acid. DNA (10 mg./ml.) and tannic acid (1 mg./ml.) were dissolved in 0.01 *M*-NaCl–0.01 *M*-tris–HCl buffer, pH 7.4. Portions of each solution were diluted with buffer and a mixture was prepared by mixing equal volumes of the original undiluted DNA and tannic acid solutions so that final concentrations were 5.39 mg. of DNA and 0.58 mg. of tannic acid/ml. Two tubes each of the mixture, the diluted DNA solution and the diluted tannic acid solution were centrifuged in a Spinco model L preparative ultracentrifuge (no. 40 rotor) for 10–14 hr. at 105000 *g*. DNA was determined in portions of both the supernatants from the diluted DNA solution and supernatants of the mixture. Tannic acid was determined in the diluted tannic acid and the mixture.

Detection of tannic acid in the liver and in subcellular fractions of rat liver after administration *in vivo* and *in vitro*. Tannic acid (68 mg./kg. body wt.) was injected intraperitoneally into rats. After the specified time-interval the animals were killed and their livers were extracted for tannic acid. In the experiment *in vitro* normal liver was homogenized in medium containing 1.7 mg. of tannic acid/ml. (a concentration found by trial to give about the same size of spots on the chromatogram as in the studies *in vivo*). Each 10 g. (wet wt.) of liver was homogenized in 20 ml. of 0.25 *M*-sucrose containing 5 *mM*- $CaCl_2$. The homogenate was filtered in the cold through metal sieves of 60 and 90 mesh/in. to remove unbroken cells and cell debris. The filtrate was centrifuged at 1000 *g* for 20 min. The upper nuclear layer of the sediment was removed and mixed by homogenization with the decanted supernatant. The erythrocyte layer was discarded. The homogenate obtained in this way was the total liver homogenate free from cell debris and erythrocytes. Each homogenate was divided into two equal parts by volume.

(a) Tannic acid from total liver homogenates. The first half of this homogenate was mixed with 3 vol. of acetone and the mixture was homogenized and centrifuged at 12000 *g* for 10 min. The clear yellow supernatant was treated with 0.2 ml. of *m*-HCl and shaken with an equal volume of ethyl acetate. After separation, the lower aqueous layer was discarded. The upper layer of ethyl acetate and acetone was filtered through Na_2SO_4 and the filtrate was evaporated to dryness under reduced pressure and at a temperature not

exceeding 40°. The resultant solid residue was dissolved in acetone (0.1 ml./g. wet wt. of liver).

(b) Tannic acid from liver subcellular fractions. The nuclear fraction was sedimented from the second half of the homogenate (the suspending medium was 0.25 M-sucrose containing 5 mM-CaCl₂) and purified from any remaining cell debris and erythrocytes by the method of Rees & Rowland (1961). The supernatant containing the mitochondrial, microsomal and cell-sap fractions was centrifuged at 12000g for 10 min. to give the mitochondrial fraction, then at 105000g for 50 min. to give the microsomal and cell-sap fractions. The nuclear, mitochondrial and microsomal pellets were homogenized in 75% (v/v) acetone and centrifuged at 12000g for 10 min. The supernatants were treated as for the total liver homogenate method. The cell-sap fraction was treated with 3 vol. of acetone, centrifuged at 12000g for 10 min. and the supernatant solution was extracted with ethyl acetate as above.

(c) Paper chromatography. Ascending paper chromatography in butan-1-ol-acetic acid-water (20:5:12, by vol.) was performed at 20 ± 1° with 0.1 ml. extracts from total liver homogenates and subcellular fractions. Authentic gallic acid and tannic acid and extracts from normal and treated livers were compared, after the dried paper had been sprayed with ammoniacal 10% (w/v) silver nitrate.

RESULTS

Histology. Fatty granulation was noticed at 2 hr., and extended through to the central zones 12 hr. after intraperitoneal administration of tannic acid (68 mg./kg. body wt.). There were very few damaged cells around the central vein and no haemorrhage, and the portal tracts showed no reaction. At 24 hr. after the injection there was a well-marked centrilobular cell necrosis. Liver cells in the affected zones were either completely destroyed or in advanced stages of cytolysis and nuclear destruction.

The liver total lipids (expressed as mg/g. of fat-free dry residue) were increased by 17, 48, 62 and 1% at 12, 24, 48 and 72 hr. respectively.

Effect of tannic acid on incorporation of [1-¹⁴C]-leucine into liver proteins. The incorporation of leucine into proteins was inhibited significantly

24 hr. after injection of as little as 50 mg. of tannic acid/kg. body wt. (Table 1). There was rapid recovery (Table 2). Treatment of slices with tannic acid *in vitro* also inhibited incorporation of leucine and this was little increased by preincubation. The mean rate of incorporation of [1-¹⁴C]leucine into proteins (c.p.m./mg. of protein) was decreased from a control rate of 14207 to 6878 by preincubation of liver slices with 0.5 mM-tannic acid for 1 hr. at 37° before the addition of [1-¹⁴C]leucine, and to 8096 by a similar preincubation with tannic acid followed by transfer to fresh medium without tannic acid before the addition of [1-¹⁴C]leucine. Thus the possibility that tannic acid acted by precipitating the [1-¹⁴C]leucine in the incubation medium was excluded. The method of incubation was as described in Table 1 and each group had four animals. In other experiments under the same conditions, exposure *in vitro* to tannic acid for 15 min. decreased incorporation by 9%, and exposure for 30 min. decreased it by 38%.

A tenfold excess of Mg²⁺ in the incubation medium did not alter the inhibition of liver slices by tannic acid added *in vitro*. When liver slices were preincubated for 1 hr. at 37° with or without tannic acid in a Ca²⁺- and Mg²⁺-free medium, transferred to fresh medium containing the usual concentrations of cations and were incubated for a further 1 hr. with labelled leucine, the inhibition of incorporation (46–51%) due to tannic acid was similar to that in the direct incubation experiment (52%). As inhibition could result from an increase in the intracellular amino acid pool, this was examined by measuring the uptake of labelled leucine. After 1 hr. soluble radioactivity in control slices was 267 c.p.m./mg. of liver (293, 182, 326) whereas tannic acid-treated slices retained 298 c.p.m./mg. of liver (321, 292, 282), a difference that was insignificant.

Tannic acid, injected intraperitoneally, did not significantly affect oxygen uptake by liver slices

Table 1. *Inhibition of incorporation of [1-¹⁴C]leucine into proteins of rat liver slices by intraperitoneal injection of tannic acid*

The animals were killed 24 hr. after the injection. Each flask contained 80 mg. wet wt. of liver slices, 1.7 ml. of Ringer phosphate-bicarbonate medium (Smuckler, 1966) and 0.2 ml. of 0.25 M-sucrose containing 1 μ C of [1-¹⁴C]-leucine. Incubation was at 37° for 1 hr.

Expt. no.	Dose of tannic acid (mg./kg. body wt.)	No. of animals	Incorporation of [1- ¹⁴ C]leucine (c.p.m./mg. of protein)	Inhibition (%)	Significance (P)
1	0	3	13226	—	—
	68	4	6466	51	<0.001
2	0	3	8142	—	—
	50	4	5867	28	<0.050
	84	4	3657	55	<0.010
	100	4	3435	58	<0.002

in vitro. At 12 and 24 hr. after administration of tannic acid, oxygen uptake rates were 5 and 11% less than control values respectively. Normal liver slices incubated in the presence of 0.25 mM- and 0.5 mM-tannic acid showed negligible inhibition of respiration.

Table 2. *Time-course of the effect of intraperitoneal injection of tannic acid on incorporation of [1-¹⁴C]-leucine into proteins by rat liver slices*

The method of incubation was as described in Table 1. Each group had four animals, except the control group at 24 hr., which had three. The dose of tannic acid was 68 mg./kg. body wt.

Time after injection (hr.)	Mean incorporation (c.p.m./mg. of protein)		Inhibition (%)	Significance (P)
	Control animals	Treated animals		
12	12312	11170	9	> 0.1
24	13226	6466	51	< 0.001
48	12191	10650	12	> 0.1
72	12191	14945	-22	> 0.1

Table 3. *Effect of tannic acid in vitro on incorporation of [1-¹⁴C]leucine into proteins by rat liver post-mitochondrial supernatant*

Each flask contained 1 μ C of labelled leucine, 2 μ moles of ATP, 10 μ moles of phosphoenolpyruvate, 100 μ g. of pyruvate kinase and 0.7 ml. of postmitochondrial supernatant. Incubation was at 37° for 20 min. Each measurement was made in quadruplicate.

Concn. of tannic acid (mM)	Mean incorporation (c.p.m./mg. of protein)	Inhibition (%)	Significance (P)
0	2104	—	—
0.05	1634	22	> 0.100
0.1	1372	35	< 0.050
0.5	1420	33	< 0.050

Tannic acid (added *in vitro*) inhibited incorporation of leucine into proteins by the postmitochondrial supernatant of liver (Table 3). Incorporation by the postmitochondrial supernatant of liver from rats that had received tannic acid *in vivo* was not inhibited. Incorporation (c.p.m./mg. of protein) was 3164 and 3397 at 12 and 24 hr. respectively after the administration of 68 mg. of tannic acid/kg. body wt., compared with 3125 in control flasks.

Effect of tannic acid on incorporation of [6-¹⁴C]-orotic acid into liver nuclear RNA, total liver RNA and nucleotides. At 14 hr. after administration of tannic acid, the incorporation *in vivo* of labelled orotic acid into nuclear RNA was very strongly inhibited (Table 4). Incorporation into nucleotides was not significantly altered by tannic acid. The inhibition of incorporation into total RNA was also studied by adding the orotic acid *in vitro* to liver slices (Table 5). Although significant at 2 and 6 hr., the inhibition was not large until 12 hr. When added *in vitro* 15 and 60 min. before the orotic acid, 0.5 mM-tannic acid inhibited the incorporation of labelled orotic acid into RNA in liver slices by 31 and 49% respectively. Induction of tryptophan pyrrolase by cortisol was almost completely inhibited by giving tannic acid (68 mg./kg.) 17 hr. earlier (Table 6).

In an attempt to demonstrate binding between tannic acid and DNA, preparations of tannic acid, DNA and a mixture of the two were centrifuged at 105000g. The amount of DNA that sedimented (45–46%) was not affected by the presence of tannic acid. Although tannic acid alone did not sediment, in the presence of DNA 59% of the tannic acid sedimented.

Detection of tannic acid in poisoned livers. At 3 hr. after the intraperitoneal injection of tannic acid (68 mg./kg. body wt.) the liver was homogenized and fractionated by differential centrifugation. The tannic acid in each fraction was determined by solvent extraction and paper chromatography. Most of the tannic acid in the liver was associated with the nuclear fraction. The microsomal fraction contained the second largest proportion of tannic

Table 4. *Effect of administration of tannic acid on the incorporation in vivo of [6-¹⁴C]orotic acid into rat liver nuclear RNA and total liver nucleotides*

At 14 hr. after administration of tannic acid (68 mg./kg.) labelled orotic acid (100 μ C/kg.) in 0.9% NaCl was injected into the tail vein. The animals were killed 15 min. later. There were two control and two treated rats.

	Incorporation into nuclear RNA (c.p.m./mg. of RNA)	Mean change (%)	Incorporation into nucleotides (c.p.m./E ₂₆₀ unit)	Mean change (%)
Control rats	33707 35000	—	1388 1927	—
Tannic acid-treated rats	3015 3263	-91	3070 1594	+41

Table 5. *Time-course of the effect of administration of tannic acid on incorporation of [6-¹⁴C]orotic acid into liver-slice RNA*

Tannic acid (68mg./kg.) was administered intraperitoneally. Each flask contained 200mg. wet wt. of liver slices, 3ml. of Krebs-Ringer phosphate medium (Umbreit *et al.* 1957) and 1 μ C of labelled orotic acid. Incubation was at 37° for 1 hr. Each group had two rats and duplicate flasks were set up for each animal.

Expt. no.	Time after tannic acid administration (hr.)	Mean incorporation (c.p.m./mg. of RNA)	Inhibition (%)	Significance (P)
1	0	18157	—	—
	2	15078	17	<0.002
	6	16361	10	<0.050
2	0	15240	—	—
	12	7663	50	<0.001
	24	8246	46	<0.001

Table 6. *Effect of administration of tannic acid on the induction of tryptophan pyrrolase by cortisol*

Tannic acid (68mg./kg. body wt.) was injected 20 hr. and cortisol 21-acetate (2mg./100g. body wt.) was injected 3 hr. before the rats were killed. The determinations were made in duplicate. Values are means $\pm$ S.E.M.

Treatment	No. of rats	Tryptophan pyrrolase (μ moles of kynurenine formed/g. wet wt. of liver/hr.)	Inhibition (%)
Nil	4	5.2 $\pm$ 0.25	—
Tannic acid	4	5.5 $\pm$ 0.32	—
Cortisol	6	13.7 $\pm$ 0.58	—
Tannic acid + cortisol	6	6.2 $\pm$ 0.36	92

acid. A trace of tannic acid was detectable in the mitochondrial and cell-sap fractions. To determine whether these proportions were due to redistribution after homogenization control experiments were set up. Tannic acid was added to the medium in which normal liver was homogenized. Much tannic acid now appeared in the mitochondria and nuclei, and some was in the microsomes. To investigate how quickly injected tannic acid reached the liver, animals were killed 1, 3, 5 and 13 hr. after administration. Tannic acid was present in the poisoned liver as early as 1 hr., reached a maximum at 3 hr., but fell at 5 hr. During the following 8 hr. the intensity of the stain on the chromatogram varied.

DISCUSSION

Accumulation of lipid and death of liver cells are the most common results of a number of liver poisons. Tannic acid is necrogenic rather than steatogenic, in contrast with carbon tetrachloride, which Schotz & Recknagel (1960) found to double the triglyceride content at 3 hr. although necrosis did not appear until much later.

Our studies (Table 7) showed that liver necrosis was a late phenomenon in tannic acid poisoning. It was absent at 12 hr., by which time other events were well established. Tannic acid appeared in the liver by 1 hr. and was concentrated in the nuclei at the earliest time we examined them (3 hr.). Our control experiments in which tannic acid was added to the homogenate *in vitro* showed a more general distribution of tannic acid, and suggested that the concentration in nuclei was not an artifact of the fractionation procedure.) The inhibition of RNA synthesis (most of which occurs in the nuclei) was already significant at 2 and 6 hr. (Table 5) and was 50% at 12 hr. By 14 hr. the inhibition of incorporation of orotic acid into liver nuclei was about 90% (Table 4). Since incorporation of nucleotide radioactivity into nuclear RNA takes place via DNA-dependent RNA polymerase we examined a specific system that requires this enzyme, cortisol-induced tryptophan pyrrolase. At 20 hr. after administration of tannic acid, pyrrolase induction was inhibited by 90% (Table 6). After a further 4 hr. the animals showed a well-marked centrilobular hepatic parenchymal-cell necrosis with, in particular, destruction of nuclei.

In contrast with the effects on RNA synthesis, protein synthesis was not affected at 12 hr., but inhibition was maximal (51%) at 24 hr. (Table 2). Recovery was almost complete at 48 hr. Taken together the evidence suggests that inhibition of nuclear RNA synthesis is an early biochemical effect, preceding the inhibition of protein synthesis.

A number of experiments were done to eliminate simple explanations of the inhibitory effects. The effects of tannic acid could be demonstrated when it was administered *in vitro* to liver slices. As preincubation did not enhance the effect on protein synthesis, which appeared in 1 hr., it was concluded that the effect was due to tannic acid itself rather than a more toxic product from it. The possibility that tannic acid precipitated the labelled leucine

Table 7. *Sequence of events after intraperitoneal administration of tannic acid to rats*

—, Not tested; +, minimal effect; ++, moderate effect; +++, marked effect; (+), slight positive effect.

	Time ...	2hr.	6hr.	12hr.	24hr.
Necrosis		Nil	Nil	Nil	+++
Accumulation of fat		+	+	++	++
Tannic acid in liver		+	++	+	—
Inhibition of protein synthesis (in liver slices)		—	—	(+)	+++
Inhibition of protein synthesis in postmitochondrial supernatant		—	—	Nil	(+)
Inhibition of RNA synthesis in nuclei		+	+	+++	+++
Inhibition of induction of tryptophan pyrrolase		—	—	—	+++

was tested by temporary exposure of the slices to tannic acid before addition of the labelled leucine. Tannic acid did not inhibit leucine uptake. Tannic acid forms complexes with Ca^{2+} and Mg^{2+} , but the possibility that it acts by removal of these cations, which are essential for RNA and protein synthesis, was eliminated by increasing the concentration of cations, or by temporary exposure of the slices to tannic acid for an interval before addition of the cation. The effect was also not dependent on a general depression of metabolism since exposure *in vitro* to tannic acid (0.5 mM), which inhibited incorporation of both leucine and orotic acid by 50%, did not alter the oxygen consumption.

Although most of the findings were consistent with the possibility that inhibition *in vivo* of protein synthesis was due to a block in RNA synthesis, one feature was inconsistent. Although protein synthesis was 51% inhibited in liver slices from animals poisoned 24 hr. previously (Table 2), the post-mitochondrial fraction of these livers had a normal rate of incorporation of leucine. This may be explained by the much lower rate of incorporation by postmitochondrial supernatant than by intact slices. This may be because short-lived messenger RNA may be more abundant in the slices, or possibly externally added energy sources may be limiting in the postmitochondrial supernatant. Any factor lowering the incorporation in the controls might hide the effect of tannic acid. We have not clarified this point.

Inhibition of protein synthesis by blocking the production of messenger RNA has been suggested as the mode of action of actinomycin D (Greengard, Smith & Acs, 1963) and aflatoxin B (Clifford & Rees, 1967). The latter authors found that adding aflatoxin B *in vitro* to postmitochondrial supernatant had no effect, unlike our experience with tannic acid (Table 3). Although tannic acid can produce this direct effect on protein synthesis when added *in vitro*, the absence of effect after intraperitoneal administration may arise from an almost complete localization in the nuclear fraction under these circumstances.

Both aflatoxin B (Clifford & Rees, 1966) and

actinomycin (Reich & Goldberg, 1964) interact with the DNA on which RNA polymerase depends. Our ultracentrifugation experiments in which DNA caused tannic acid to sediment suggested that tannic acid also combined with DNA. The fact that tannic acid appeared in the liver 1 hr. after administration and was concentrated in the nuclei as early as 3 hr. is consistent with the possibility that it acts by interference with production of messenger RNA. These findings are also compatible with the view that a failure of protein synthesis leads to necrosis. Our studies merely establish that inhibition of RNA synthesis precedes inhibition of protein synthesis and that necrosis occurs later than these two events.

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