ALKALINE RIBONUCLEASE ACTIVITY INCREASE IN RAT KIDNEY CORTEX AND LIVER AFTER TRYPAN BLUE AND OTHER AZO DYES ADMINISTRATION

M. RABINOVITCH, M.D., R. BRENTANI, S. FERREIRA, M.D., N. FAUSTO, M.D., and T. MAACK

From the Laboratory for Cell Physiology, Department of Histology and Embryology, Faculty of Medicine, University of São Paulo, Brazil

ABSTRACT

Acid azo dyes, most of them naphtholdisulfonic acid derivatives, were given intraperitoneally to rats and their effect on "alkaline" ribonuclease activity was studied in total homogenates of kidney cortex and liver. Acid treatment was used to release bound enzyme activity. Several of the dyes, including trypan blue, increased RNase activity in both organs 3 days after administration of single doses, while others, like Evans blue, were inactive. Activity was apparently bound to the sulfonic substitution in the 3, 6 positions in the naphthalene rings, substitutions in the benzidine rings being not critical. All of the active and most of the inactive compounds were taken up by tubule cells of kidney cortex and by reticular and parenchymal cells of liver. While the effect on both liver and kidney was obtained 1 day after trypan blue administration, RNase remained increased for only about 3 days in the first organ, and for at least a month in the second. However, repeated trypan blue doses increased liver enzyme activity for at least 9 days. Serum RNase activity was decreased after trypan blue administration. Ethionine administration together with trypan blue markedly blocked the effect of the dye on liver RNase activity; simultaneously given methionine partially reversed the action of the antimetabolite. This suggests that *de novo* synthesis of RNase is induced in liver by trypan blue. The action of ethionine on the kidney RNase response to trypan blue was less marked although significant; in view of the possible kidney uptake of the plasma enzyme, interpretation of this finding must be postponed. Results are discussed with reference to the mechanism of the structural specificity of the compounds used, cytological localization of the dyes and their mechanism of action on liver and kidney RNase.

Disclosure of the participation of ribonucleic acids in the mechanism of protein synthesis led to a renewal of interest in enzymes involved in RNA synthesis and degradation (1). Moreover, the findings of Brachet (2) and others of *in vivo* effects of pancreatic ribonuclease (RNase) on a variety of single-celled and multicellular organisms, pointed to the need of further knowledge on the biochemistry and physiology of inter- and intracellular hydrolytic enzymes of this group (*cf.* 3). Previous work from this laboratory has shown that in mammals plasma alkaline RNase is inactivated by the kidneys (4) and suggested that the kidney enzyme could be taken up from the plasma (5).

During the course of a study on the effect of administering several proteins on RNase activity of the renal cortex (6), azo dyes known to be bound to proteins were also assayed. It was found that trypan blue and some related dyes increase

RNase activity of kidney cortex and liver. The time course of this effect, structural requirements of the dye molecules, and experiments bearing on the mechanism of the phenomenon will be presently described.

MATERIALS AND METHODS

Albino rats of the Wistar strain, both males and females, weighing 100 to 200 gm. were used. The dyes were dissolved in saline and the solutions were administered intraperitoneally in single or repeated injections of 5 ml. per 100 gm. body weight. The rats received the dyes in doses of from 1.7 to 30 mg per 100 gm. body weight (*cf.* Table I). Control animals received only saline. Trypan blue was purified by ethanol extraction (7), and in some experiments the ethanol-soluble "red fraction" was recovered and assayed. In a few experiments animals were simultaneously treated with DL-ethionine (0.22 mmoles per 100 gm. body weight) with or without the administration of DL-methionine (0.25 or 1.25 mmoles per 100 gm. body weight).

Under ether anesthesia kidney cortex and liver samples (weighing 20 and 120 mg., respectively) were obtained, one before and the others at different time periods after experimental treatments were started. Tissues were stored at −10°C. until assayed. Homogenates were prepared in 0.05 M citric acid, neutralized with 0.25 M K_2HPO_4, and assayed at pH 7.3 as previously described (8). Assays were performed in triplicate on 0.17 mg. kidney cortex or 3.0 mg. liver. In some experiments RNase activity of blood serum was determined on 5 μl samples. It is known that acid treatment (0.25 N H_2SO_4) unmasks liver alkaline RNase activity (9). Preliminary experiments have shown that citric acid can be conveniently used for this purpose, giving similar activities as the H_2SO_4 treatment. Maximal activities were also obtained by making homogenates with 0.5×10^{-3} M *n*-ethyl maleimide (*cf.* 10). Results of RNase assays are expressed either as arbitrary optical densities or in percentages of the activity of initial biopsies.

For histological study of the dye uptake, formol-calcium-fixed pieces of kidney and liver were frozen sectioned, the sections lightly counterstained with hematoxylin and mounted in balsam.

Although, for descriptive purposes, means and standard errors are given, in the statistical analysis of the results non-parametric tests (11) were mainly used. The significance level was taken at 0.05.

RESULTS

Effects of Different Azo Dyes on RNase Activity of Liver and Kidney: Fig. 1 lists the dyes assayed together with their structural formulae. Table I gives the RNase activities of liver and kidney, in percentages of the initial biopsies 3 days after a single injection of the dyes in the doses indicated. Doses are not uniform because of marked toxicity or low solubility of some of the dyes. Each compound was assayed in at least two separate experiments.

It can be seen that the dyes used fell into two groups. The active ones (Fig. 1, left column) included symmetrical dyes of the trypan blue type, with sulfonic groups in positions 3 and 6 of the naphthalene rings (C.I. (color index) numbers 23850, 24400, 22610, 24340, and Dye A). Two other asymmetrical dyes were also active; in one of them (C.I. 22590) the sulfonic group in position 3 of one of the naphthalene rings was lacking and the amino group in the same ring shifted to position 2. The other active dye was a more complex trisazo compound (Dye B, Table I), in which positions 3 and 6 of the terminal naphthalene rings were also substituted with sulfonic groups.

In view of the above mentioned non-uniformity of dosage, and lack of information as to the actual dye content of the samples used, no stress is being placed on the differences in response towards the dyes of this group. It seems, however, that, except for the hydroxyl group in position 8 of the naphthalene rings, which was present in all of the dyes assayed, the other substitutions in these rings as well as in the benzidine rings were not critical.

To the inactive group of dyes (Fig. 1, right column) belonged the symmetrical compounds Evans blue and Niagara sky blue 6 B, which have sulfonic groups in positions 2 and 4 of the naphthalene rings (C.I. 23860 and 24410). Two other symmetrical and less sulfonated dyes (C.I. 22120 and 23685) were also inactive. The other available dyes of this group were asymmetrical and had sulfonic substitution in the 3, 6 positions in only one of the naphthalene rings (C.I. 30280 and 22445). The ethanol extracted red fraction of trypan blue was also inactive; no significant variation in liver and kidney RNase was found in the saline-injected rats.

Examination of the tissue sections revealed uptake of all of the dyes, with the exception of acid red 33 and the red fraction, by both reticular and parenchymal cells of the liver and proximal convoluted tubules of the kidney. The two last mentioned dyes were rapidly excreted in the

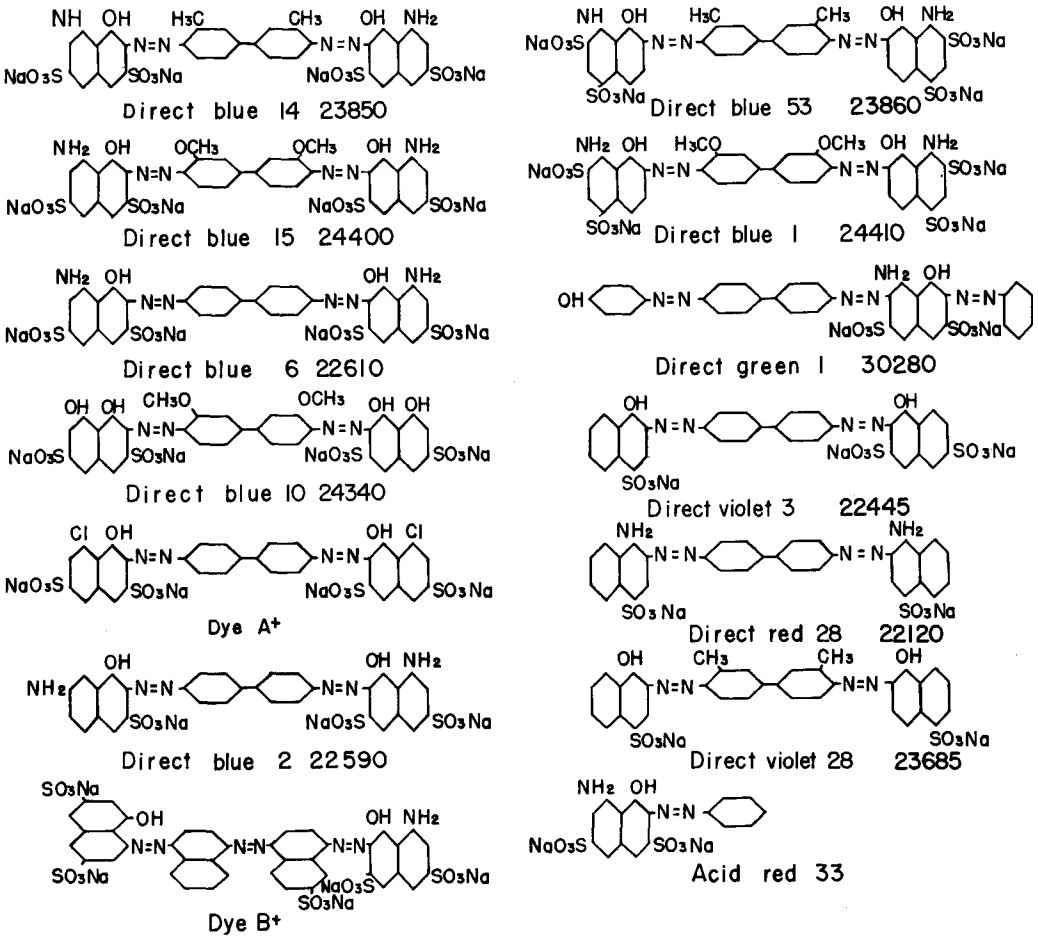

FIGURE 1

Structural formulae and Color Index names and numbers of the dyes studied (*cf.* Table I).

urine. As no quantitative observations could be performed on the dye uptake, no correlation of dye uptake with effect on RNase activity could be made at present.

Time Course of Liver and Kidney RNase Activity after Single Injections of Trypan Blue: Table II gives the combined results of two experiments on rats receiving 10 mg of trypan blue per 100 gm. body weight. In one of the experiments biopsies were performed at times 0, 1, 3, and 8 days after the injection; in the other they were made at times 0, 3, 15, and 30 days. It can be seen that saline-injected animals presented non-significant variations of RNase activity of kidney and liver. In the dye-treated animals, however, the behavior of both organs was different. In kidney, activity was already high 1 day after injection and remained at high levels up to the 30th day, when the experiment was terminated. In the liver, on the other hand, significant increases were obtained at 1 and 3 days only, activities dropping to initial levels at 8 days and thereafter.

Effect of Repeated Trypan Blue Administration on RNase Activity of Liver: To find out whether RNase activities of liver could be maintained at high levels by repeated trypan blue injections, an experiment was performed on 20 rats, of which 5 received saline injections throughout (Fig. 2). The remaining 15 received 5 mg. of tyrpan blue per 100 gm. body weight at time 0. At 3 days and thereafter, 5 rats of this group received saline, while the remaining 10 received a second injection

TABLE I

Effect of Acid Azo Dyes on Liver and Kidney Alkaline Ribonuclease Activity

Color index (2nd ed.) name	Common name	C.I. number (2nd ed.)	Manufacturer	Dose	Liver RNase*	Kidney RNase*
				(*mg./100 gm.*)		
Direct blue 14	Trypan blue	23850	Hartman-Leddon	1.7–7.0	161.0 ± 5.3(17)‡	242.4 ± 19.0(17)‡
Direct blue 15		24400	Sandoz Ltd.	30.0	140.0 ± 14.0(6)	150.0 ± 13.9(6)
Direct blue 6	Niagara blue 2B	22610	Hartman-Leddon	7.0–30.0	139.6 ± 12.2(10)	203.5 ± 30.9(10)
Direct blue 10		24340	I. Chem. Ind., ENIA	30.0	163.1 ± 8.9(7)	320.0 ± 41.5(7)
Dye A			Prep. Dr. J. Falzone	30.0	138.0 ± 10.0(8)	179.1 ± 17.3(8)
Direct blue 2		22590	CIBA Ltd., I.C.I.	7.5–15.0	160.7 ± 7.9(7)	279.0 ± 54.6(7)
Dye B			Prep. Dr. J. Falzone	25.0	179.3 ± 9.7(7)	237.0 ± 42.3(7)
Direct blue 53	Evans blue	23860	National Aniline	7.0–30.0	88.4 ± 6.3(5)	97.5 ± 6.8(8)
Direct blue 1	Niagara sky blue 6 B	24410	I.C.I.	7.0–30.0	98.6 ± 3.3(9)	94.5 ± 3.1(9)
Direct green 1		30280	I.C.I.	25.0	88.4 ± 5.4(7)	121.0 ± 9.3(7)
Direct violet 3		22445	I.C.I.	25.0	113.9 ± 20.3(5)	120.6 ± 15.9(5)
Direct red 28	Congo red	22120	Grübler	7.0	112.3 ± 14.3(8)	105.8 ± 17.9(8)
Direct violet 28	Azo blue	23685	Grübler	7.0	113.6 ± 20.3(8)	92.3 ± 10.7(8)
Acid red 33		17200	ENIA	30.0	106.6 ± 4.6(5)	119.6 ± 12.6(5)
	Trypan blue ethanol extract red fraction			10.0	95.7 ± 6.2(5)	99.5 ± 9.6(5)
	Saline				95.2 ± 3.9(14)	95.2 ± 7.4(14)

* In percentages of initial biopsies, 3 days after single injection. Liver and kidney data refer, in most cases, to the same experimental animals.
‡ Mean ± standard error (number of animals).

TABLE II

*Alkaline RNase Activity in Rat Liver and Kidney at Different Time Periods after Intraperitoneal Injection of Trypan Blue**

Treatment	1 day	3 days	8 days	15 days	30 days
			Liver		
Saline injected	119.7 ± 7.2(6)	109.9 ± 6.7(12)	96.0 ± 15.3(6)	112.9 ± 6.2(6)	
Trypan blue injected	138.7 ± 8.1(7)	165.5 ± 11.0(12)	105.1 ± 4.5(7)	125.5 ± 13.2(5)	108.3 ± 5.7(5)
			Kidney		
Saline injected	87.6 ± 7.0(6)	94.1 ± 7.2(12)	96.6 ± 8.0(6)	92.2 ± 13.6(6)	
Trypan blue injected	265.1 ± 35.1(8)	294.7 ± 26.7(15)	276.4 ± 27.3(8)	217.4 ± 27.6(7)	191.0 ± 26.2(7)

* In percentages of initial biopsies; liver and kidney data refer to the same animals. Mean percentages ± standard error (number of samples); each animal sampled four times.

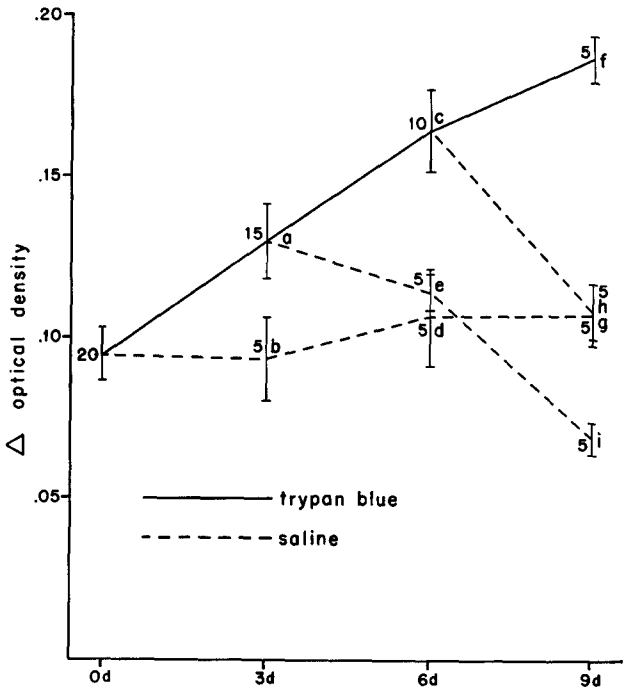

FIGURE 2

Alkaline RNase activity (ordinates) after single or repeated trypan blue doses. Abcissae: time in days. Standard errors given by vertical bars. Number of animals sampled at each time period is shown on the curves. Letters indicate means referred to in text and in Table III.

of dye. On the 6th day, of these remaining 10, another group of 5 was put on saline, whereas the remaining 5 received a 3rd injection of the dye. All the animals were biopsied at times 0, 3, and 6 days; on the 9th day the rats were sacrificed.

Statistical analysis of the results (Table III) shows that at 3 days the effect of a single injection of the dye was at the limit of significance (comparison *a-b*). On the 6th day, animals receiving 2 doses of dye (*c*) had significantly higher liver enzyme activities than those receiving 1 dose (*e*) or saline (*d*). These last two groups did not differ significantly. On the 9th day, rats receiving 3 doses of dye (*f*) differed significantly from saline-injected rats (*h*) and from animals injected once (*i*) or twice (*g*) with trypan blue. Whereas the 2-doses group (*g*) did not differ from control animals (*h*), the group injected with 1 dose of dye and with saline thereafter (*i*) dropped its RNase activity to levels below the control ones. This explains the significant differences between this group and the others on the 9th day (comparisons *g-i*, *f-i*, and *h-i*).

Effect of Trypan Blue on Serum RNase Activity: Serum was assayed in view of a suggested relationship between plasma and kidney cortex RNase

TABLE III

Results of Statistical Analysis (Mann-Whitney Test) (12) of Data Summarized in Fig. 2

Time	Comparison	Probability
3 days	a–b	$>.05$ $<.10$
	c–e	$<.02$
6 days	e–d	$>.4$
	c–d	$= .02$
	f–h	$= .004$
	f–i	$= .004$
	f–g	$= .004$
9 days	g–h	$>.3$
	g–i	$= .004$
	h–i	$= .004$

* Explanation of symbols is given in Fig. 1 and in the text.

TABLE IV

Effect of Trypan Blue Administration on Serum RNase Activity*

Treatment	1 day	3 days
Saline	74.6 ± 14.0 (6)‡	70.4 ± 7.8 (6)
Trypan blue	72.8 ± 4.0 (8)	62.1 ± 7.3 (7)

* 7 mg./100 gm. body weight. Same animals were sampled at times 0, 1, and 3 days.

‡ Mean ± standard error (number of animals).

TABLE V

*Effect of Ethionine Administration on Trypan Blue–Induced Increase in Liver and Kidney RNase Activity**

Treatment and total dose‡	Liver	Kidney
Saline	96.8 ± 6.0 (11)§	100.0 ± 11.8 (10)
Ethionine (37 mg./100 gm.)	78.4 ± 8.0 (5)	85.5 ± 18.6 (5)
Trypan blue (15 mg./100 gm.)	200.8 ± 13.6 (12)	350.1 ± 50.7 (12)
Trypan blue + ethionine	116.1 ± 6.3 (13)	193.7 ± 18.6 (13)
Trypan blue + ethionine + methionine (37 mg./100 gm.)	110.3 ± 5.3 (6)	249.8 ± 27.8 (6)
Trypan blue + ethionine + methionine (185 mg./100 gm.)	157.9 ± 11.5 (6)	310.3 ± 87.3 (6)

* Results in percentages of initial biopsies; liver and kidney data refer to same animals.

‡ Drugs administered in 2 doses, at 36 hour interval; experiments terminated 72 hours after first injection. 2nd dose was half of first.

§ Mean ± standard error (number of animals).

activity (3). It can be seen in Table IV that both saline and trypan blue administration reduced blood serum RNase activity to levels below the initial ones.

Effect of Ethionine Administration on the Response of Liver and Kidney RNase to Trypan Blue: Analysis of the results summarized in Table V indicates that ethionine in a total dose of 0.22 mmoles per 100 gm. body weight intraperitoneally administered together with trypan blue markedly inhibited the increase in liver RNase activity. The effect on kidney was of smaller magnitude, although also statistically significant. Ethionine alone, in the rather small sample of animals studied, did not significantly influence liver and kidney RNase activity. Methionine given at the level of 1.25 mmoles per 100 gm. but not at 0.25 mmoles per 100 gm. significantly counteracted the effect of ethionine on the liver, while in the case of the kidney the effect was not significant at both dosage levels.

DISCUSSION

The above results show that after administration of certain azo dyes distinct increases are found in alkaline RNase activities of total homogenates of liver and kidney. Interpretation of the findings is not unequivocal in view of the complexity of the system studied—the whole animal—and lack of knowledge concerning cytological fate and metabolism of the administered dyes. It may be stated that repeated attempts failed to reproduce the results in liver slices incubated with Ringer-phosphate-glucose or with 1:1 serum-Ringer medium containing trypan blue for up to 3 hours at 37°C. with oxygenation. However, examination of sections of the slices failed to reveal any uptake of the dye.

Structural Specificity of the Dyes: Attention is called to the fact that one essential requirement for activity was sulfonation in positions 3 and 6 of the naphthalene rings. Some of the dyes used are known to be variously bound to plasma albumin, the binding of the 2, 4 isomers being stronger than that of 3, 6 isomers (12, 13). The hypothesis could therefore be held, at least for trypan blue and Niagara blue 2B on the one hand, and Evans blue and Niagara sky blue 6B on the other, that their different behavior is due to the fact that less free dye is available to enter cells because of the stronger binding to albumin of the two last mentioned compounds. It is also conceivable that the intracellular pathways and fate of albumin-combined dye could be different from those of the free dye. Ultrastructural investigations of the intracellular localization of some of the dyes used would be of interest (14, 15). Study of other colloids taken up by cells could help to clarify the understanding of the phenomenon. So far, experiments not here reported have shown that the administration of colloidal thorium dioxide (Thorotrast, testagar) is without effect on liver and kidney RNase activity.

Different Behavior of Kidney and Liver RNase toward Trypan Blue: Our results show that the effect of trypan blue on kidney cortex RNase activity lasts much longer than does the effect of the dye

on the liver enzyme. This may be due to different ability of these organs to metabolize the dye. No data could be found on the enzymatic breakdown of the dyes used. However, the carcinogenic monoazo dyes are known to have their azo groups reduced at a more rapid rate by liver than by kidney slices or homogenates (*cf.* 16). Repeated administration of trypan blue was able to maintain RNase increase in liver for at least 9 days; it may be stated here that, except for a chance variation no other explanation can be given of the behavior of the 1-dose group sampled on the 9th day (Fig. 2).

Cytological Localization of Trypan Blue: No detailed discussion will be given of the classical controversies on intracellular localization of acid colloidal dyes in liver (17) and kidney (18), and their binding to proteins (19). On the basis of centrifugal fractionation of sucrose homogenates of liver, it has been suggested that trypan blue is bound to the mitochondrial fraction (20). Caution should be exercised, however, in interpreting this result, for most of the dye is found in the reticulum cells, which contribute but little to the mitochondrial fraction of the total homogenate. In view of the non-homogeneity of the mitochondrial fraction (see below), this work would be profitably controlled by biochemical analysis of the fractions.

The more recent findings of Straus (21) on the concentration of horseradish peroxidase in kidney droplets enzymatically similar to de Duve's lysosomes (22; see also 23–25), as well as other morphological data, (*e.g.* 26, 27), suggest that trypan blue (presumably bound to protein) is segregated in a similar cellular fraction. In an electron microscopic study, Schmidt (15) has recently reported that trypan blue administered to mice is found in kidney cells as granules, frequently clustered and without any relationship to either the mitochondria or the Golgi region.

Straus (28) has also found that "acid" RNase activity of kidney homogenates is increased after egg-white administration to rats, together with increase of other hydrolytic enzymes belonging to the lysosome fraction. The data available on the intracellular distribution of "alkaline" RNase are, however, conflicting (see 29), so that the relationship of our results to Straus's data must await further information. Study of the effect of trypan blue on other enzymatic systems of liver and kidney is under way.

Mechanism of the Effect of Trypan Blue on Kidney RNase: It was previously suggested (*cf.* 6) that an increased kidney cortex RNase activity could be due to (*a*) heightened reabsorption or (*b*) decreased inactivation of the enzyme; to this may be added (*c*) the *in situ* synthesis of the protein. As no increase in serum RNase activity was found after trypan blue administration, and as variations in glomerular filtration rate or reabsorptive activity are very improbable, the first mechanism may be ruled out. The ethionine experiment suggests that the trypan blue–induced RNase increase could be due to synthesis of the enzyme. However, the effect of ethionine on protein uptake by the kidney should be studied before any definite choice can be made between the second and third mechanisms.

De Novo Synthesis of RNase Induced in Liver by Trypan Blue: Although the interpretation of the ethionine experiment as regards kidney must await further information, results on liver suggest that trypan blue induces an increased synthesis of RNase in that organ. It is known that ethionine administration is able to inhibit a number of adaptive and non-adaptive increases in enzyme activity (30, 31). It should be pointed out here that no evidence could be obtained for the uptake of liver RNase from the plasma: (*a*) no correlation was found in normal rats between serum and liver RNase activity, and (*b*) no increase in liver RNase activity was obtained at various periods after bilateral nephrectomy in the rat, in spite of high levels of serum enzymatic activity (unpublished data). Results of the ethionine experiment would also be hard to reconcile with the hypothesis that trypan blue interferes with catabolic systems that intracellularly would degrade RNase. It may also be stated that preliminary experiments showed no variation in liver supernatant RNase inibitor (32) after trypan blue administration (unpublished data).

The authors are grateful to Dr. J. Falzone for information and for the gift of dyes; to ENIA S.A., Ciba Ltd., Sandoz Ltd., and Imperial Chemical Industries for several samples of dyes; and to Prof. E. S. Berquò for advice on statistical analysis of the results.

This work was aided by Conselho Nacional de Pesquisas and The Rockefeller Foundation.

Received for publication, August 31, 1960.

REFERENCES

1. Enzymes of polynucleotide metabolism, (O. V. St. Whitelock, editor), *Ann. New York Acad. Sc.*, 1959, **81,** 511.
2. BRACHET, J., Biochemical Cytology, New York, Academic Press, Inc., 1957.
3. RABINOVITCH, M., and DOHI, S. R., *Ciência e Cultura*, 1957, **9,** 195.
4. DOHI, S. R., TERZIAN, J. F., WIDMAN, A., BRENTANI, R., FAUSTO, N., LIBERMAN, B., and RABINOVITCH, M., *Am. J. Physiol.*, 1959, **196,** 924.
5. RABINOVITCH, M., *Proc. Soc. Exp. Biol. and Med.*, 1959, **100,** 865.
6. MAACK, T., BRETANI, R., and RABINOVITCH, M., *Nature*, 1960, **186,** 158.
7. ALLEN, T. H., and ORAHOVATS, P., *Am. J. Physiol.*, 1950, **161,** 473.
8. RABINOVITCH, M., and DOHI, S. R., *Arch. Biochem. and Biophysics*, 1957, **70,** 239.
9. PIRROTE, M., and DESREUX, V., *Bull. Soc. Chim. belge*, 1952, **61,** 167.
10. ROTH, J. S., *Nature*, 1953, **171,** 127.
11. SIEGEL, S., Non Parametric Statistics, New York, McGraw-Hill, 1956.
12. RAWSON, R. A., *Am. J. Physiol.*, 1943, **138,** 708.
13. BRENNER, S., *S. Afr. J. Med. Sc.*, 1952, **17,** 61.
14. TRUMP, B. F., *Anat. Rec.*, 1960, **136,** 293 (abstract).
15. SCHMIDT, W., *Z. Zellforsch.*, 1960, **52,** 598.
16. MILLER, J. A., and MILLER, E. C., *Adv. Cancer Research*, 1953, **1,** 339.
17. PFUHL, W., *in* Handbuch der Mikroskopischen Anatomie des Menschen, (W. v. Möllendorff, editor), 1930, **5,** (part 2), 312.
18. v. MÖLLENDORFF, W., *in* Handbuch der Mikroskopischen Anatomie des Menschen, (W. v. Möllendorff, editor), 1930, **7,** (part 1), 150.
19. FAUTREZ, J., *Arch. biol.*, 1939, **50,** 369.
20. SEYBOLD, G., and KALEE, E., *Z. Naturforsch.*, 1954, **9b,** 173.
21. STRAUS, W., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 1037.
22. DE DUVE, C., Les lysosomes, *Exp. ann. biochim. med.*, 1958, **20,** 197.
23. STRAUS, W., *J. Biophysic. and Biochem. Cytol.*, 1958, **4,** 541.
24. STRAUS, W., *J. Biophysic. and Biochem. Cytol.*, 1959, **5,** 193.
25. DE DUVE, C., *Exp. Cell. Research*, 1959, suppl., **7,** 169.
26. ESSNER, E., *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 329.
27. FARQUHAR, M. G., and PALADE, G. E., *J. Biophysic. and Biochem. Cytol.*, 1960, **7,** 297.
28. STRAUS, W., *J. Biophysic. and Biochem. Cytol.*, 1957, **3,** 933.
29. REID, E., and NODES, J. T., *Ann. New York Acad. Sc.*, 1959, **81,** 618.
30. GELBOIN, H. V., MILLER, J. A., and MILLER, E. C., *Cancer Research*, 1958, **18,** 608.
31. GOLDSTEIN, L., and KENSLER, C. J., *J. Biol. Chem.*, 1960, **235,** 1086.
32. ROTH, J. S., *Biochim. et Biophysica Acta*, 1956, **21,** 34.