TISSUE DISTRIBUTION OF ^{125}I-TOLUIDINE BLUE IN THE RAT

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The search for a highly specific, isotopically labeled material which would enable localization of parathyroid tissue by external scintillation detection has been conducted in a number of laboratories (*1–11*). Selenium-75-selenomethionine has been used with some success (*1–8*) to localize hyperactive glands, although localization of normal glands by this procedure has not been possible (*8*).

Recently, it has been reported that intravenously injected toluidine blue—a thiazin dye closely related to methylene blue, valuable as a nuclear stain, is concentrated preferentially by parathyroid and pancreas tissue (*9–15*). High concentration in heart has also been reported (*9,10*). Since the results of these studies suggest that toluidine blue labeled with a gamma-emitting isotope might prove useful for external detection of parathyroid tissue by scintillation methods, we have labeled toluidine blue with ^{125}I and have studied the distribution of both labeled and unlabeled dye in several tissues of the rat after intravenous injection.

MATERIAL AND METHODS

Toluidine blue 0 (color index No. 52040), 93% pure, was purchased from Fisher Scientific Co. Iodine-125-toluidine blue was prepared in this laboratory. The ^{75}Se-selenomethionine was Sethotope diagnostic injection obtained from E. R. Squibb and Sons, Inc.

Absorption spectra of solutions of 0.01% toluidine blue 0 in 0.9% NaCl and in absolute ethanol were measured in a Cary Model 15 recording spectrophotometer. The alcohol solution gave a single absorption peak at 630 mμ, the alpha-band; the saline solution showed an additional peak at 590 mμ, the beta-band, initially containing about 10% of the total material. When the saline solution was allowed to stand overnight, the beta-peak became lower while the alpha-peak increased in height. The appearance of the beta-band in solutions of metachromatic dyes such as toluidine blue has been ascribed to formation of ion polymers following hydration of dye molecules (*16*). The specific absorption of a saline solution of the dye was equal to that of an alcohol solution at concentrations below 0.1 mg% but fell off markedly at higher concentrations, probably due to association of dye molecules, while that of an alcohol solution remained constant up to 1.0 mg% toluidine blue. The alpha-peak at 630 mμ was chosen for colorimetric analysis.

Preparation of ^{125}I-toluidine blue. Toluidine blue 0, 93% pure, was labeled with ^{125}I by a procedure adapted from that used by Lemmon, Tarpey, and Scott (*17*) and Horeau and Süe (*18*) for the iodination of thyroxine. One such preparation will be described in detail. In a large Pyrex test tube were combined 14.0 ml (3 mCi) of Na^{125}I solution (Mallinckrodt Nuclear oral preparation containing 0.2% ascorbic acid) and 0.5 ml of 2 *M* KI (1.0 meq); the mixture was evaporated to approximately 1.0 ml by heating at 70°C in an electric tube heater under a stream of air; after the addition of 2.0 ml of 0.2 *M* $NaIO_3$ and 0.6 ml of glacial acetic acid (10.0 meq), the mixture was chilled in ice; 13.3 ml of concentrated NH_4OH (200 meq) were added drop-wise with constant stirring on a Vortex mixer; 31.5 mg (0.1 mmole) of toluidine blue in 5 ml of ethanol was added drop-wise with thorough mixing; after the last traces of toluidine blue had been washed in with ethanol the mixture was concentrated by heating at 70°C under a stream of air for 1 hr; 0.5 ml of 1 *N* I^0 in 2 *M* KI (0.5 meq of I^0) were added with stirring every 15–30 min, left standing at room temperature for 14 hr, then heated at 70°C under a stream of air to remove most of the ammonia. When very alkaline, the reaction mixture was emerald green

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in color; near neutrality it was clear blue with a black precipitate of toluidine blue. After the addition of sufficient glacial acetic acid to lower the pH to 4.5–5.0, the aqueous reaction mixture was washed three times with 20-ml portions of petroleum ether followed by three 20-ml portions of ethyl acetate to remove free iodine, three times with 20-ml portions of chloroform to remove a minor component of the dye mixture, then 10 times with 20-ml portions of n-butanol to extract the dye into the organic phase. The deep-blue colored n-butanol extract of labeled toluidine blue was evaporated nearly to dryness on a hot plate in a stream of air; the greenish black residue was taken up in 50 ml of absolute ethanol and evaporated to dryness three times to remove n-butanol; the final residue of ^{125}I-toluidine blue was dissolved in 7.5 ml of 0.9% NaCl for intravenous injection into rats.

Thin-layer chromatography. Aliquots of each phase of the ^{125}I-toluidine blue preparations were applied to Gelman SG instant thin-layer media and developed 10–25 min in the ascending mode in chloroform-acetone-2-propanol-6.4% sulfurous acid (30:40:20:10). The dye was visible on the chromatogram as blue or purple spots; free iodine appeared yellow. The spots were cut out of the chromatogram, cut into 3–5-cm square pieces and assayed for radioactivity in a crystal scintillation well counter.

In vivo uptake of toluidine blue and ^{125}I-toluidine blue. Female white rats of Wistar origin 200–250 gm were used. Unlabeled toluidine blue in a dose of 10–12-mg/kg body weight or 100 μCi (0.2 mg) ^{125}I-toluidine blue was injected by tail vein. Animals were sacrificed by cardiac exsanguination under ether anesthesia at 5, 15, or 30 min after injection and pieces of thyroid, neck muscle, pancreas, liver, stomach, and, in one experiment, heart muscle were removed and weighed. In the experiments with unlabeled dye, the tissues were homogenized in cold 0.9% NaCl, and aliquots were analyzed for nitrogen by a modification of the ultramicro method of Koch and McMeekin (*19*). The remaining homogenates were centrifuged 10 min at 12,350 g and the precipitates were extracted three times with equal volumes of absolute ethanol. The concentration of dye in the combined extract from each tissue sample was determined by measurement of optical density at 630 mμ. Parathyroid glands from 3–6 rats were removed to chilled containers by the procedure previously described (*20*), pooled, frozen, and extracted by maceration with a pointed glass stirring rod in warm absolute ethanol and toluidine blue measured by absorption at 630 mμ. Nitrogen content of the residue was determined as above.

In the animals given ^{125}I-toluidine blue, whole parathyroid, thyroid, and pieces of neck muscle, pancreas, and liver were counted after nitrogen content was determined. Results were expressed in terms of percent of injected dose per milligram tissue nitrogen.

Uptake of toluidine blue by rat tissue homogenates in vitro. Rats were killed with ether, and pieces of thyroid, pancreas, and liver were removed, weighed, and homogenized in chilled Potter-Elvehjem homogenizers in ice cold saline. Aliquots (1.0 ml) were incubated in an oscillating water bath at 37°C for 5 min with 2.0 ml of 0.005–0.05% toluidine blue in 0.9% NaCl. At the end of incubation, the contents were poured quickly into cold centrifuge tubes, centrifuged 10 min at 12,350 g, the supernatant solutions removed, and the precipitates washed once with 2.0 ml of cold 0.9% NaCl and the supernatant solutions combined. The drained, weighed precipitates were extracted three times with 2.0-ml portions of absolute ethanol in the oscillating water bath at 37°C for 2 hr, the supernatant solutions collected as before, and both saline and alcohol extracts combined and made up to a total volume of 10.0 ml with absolute ethanol. Colorimetric analysis of this solution at 630 mμ showed recovery of dye greater than 90% of that disappearing from the medium.

Uptake of ^{75}Se-selenomethionine and ^{125}I-toluidine blue in rats fed a low-calcium diet. Rats which had been fed a synthetic diet containing 0.04% calcium lactate for 26–28 days were used for comparison of uptake of ^{125}I-toluidine blue and ^{75}Se-selenomethionine. The corresponding control animals were fed the same diet supplemented with 1.0% calcium lactate. Either 100 μCi (6.8 nmole) of ^{75}Se-selenomethionine or 100 μCi (0.2 mg) of ^{125}I-toluidine blue was injected via the tail vein followed by sacrifice and removal of parathyroid, thyroid, neck muscle, pancreas, and liver at 15 or 60 min after injection. Whole tissue pieces were counted after which the nitrogen content was determined as before.

RESULTS

Labeling of toluidine blue with ^{125}I proceeded easily. The labeled dye was purified by solvent extraction to yield highly purified ^{125}I-toluidine blue which behaved similarly to the original unlabeled dye on thin-layer chromatography (Table 1). The chloroform extract of the ^{125}I-toluidine blue reaction mixture contained a blue dye which migrated differently from purified ^{125}I-toluidine blue (Table 1). The chloroform extract retained 12% of the total radioactivity in the Na^{125}I used for labeling while the n-butanol extract containing purified I-toluidine blue retained 53% of the original activity. The material extracted into chloroform is apparently a con-

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF FRACTIONS OF ^{125}I-TOLUIDINE BLUE

Sample	Rf	Color	% radioactivity* A	% radioactivity* B
Unlabeled toluidine blue	0.78	Deep blue		
1 N I°/2 M KI	0.73	Yellow		
$Na^{125}I$			92.8†	
Ethyl acetate extract of ^{125}I-toluidine blue	0.95	Light yellow & blue	96.7	3.1
Chloroform extract of ^{125}I-toluidine blue	0.95	Light blue	88.8	11.8
n-butanol extract of ^{125}I-toluidine blue	0.81	Deep blue	100.0	52.6
Aqueous remainder after n-butanol extraction				5.8

Ascending development 17 min (10 cm) in chloroform-acetone-2-propanol-6.4% sulfurous acid (30:40:20:10) on Gelman SG ITLC medium.

* A = Portion of total radioactivity of sample found in designated spot. B = Portion in each fraction of total activity of $Na^{125}I$ used for labeling.

† Activity in segment of chromatogram cut 7.5–10.0 cm from origin.

taminant of the original toluidine blue (*21*). After purification by extraction into n-butanol followed by evaporation of the n-butanol and redissolving in physiological saline the ^{125}I-toluidine blue could be injected intravenously into rats without apparent harmful effect.

Figure 1 shows the distribution of unlabeled toluidine blue in several tissues at times up to 33 min after intravenous injection of the dye into rats. At 5 min after injection, the concentration in parathyroid per unit of tissue nitrogen was nearly three times that in thyroid and over 20 times that in neck muscle; at 15 and 30 min the amount of dye in parathyroid was too small to measure. The concentration in pancreas was nearly five times that in liver, but only 1½ times that in stomach. At 15 min, the concentration of dye in pancreas, although still nearly four times that in liver, had dropped to a level less than that exhibited by the stomach. Dye concentrations in stomach at all times were three to four times those in liver; in cardiac muscle at 15 and 30 min, they were six times that in neck muscle or liver.

These data confirm visual observations made during the experiment. Soon after injection of the dye, parotid, thyroid, and parathyroid glands, pancreas, and kidney were deeply stained. After 15 min, parathyroid and thyroid glands and pancreas had lost most of their coloration while parotid gland, stomach (particularly the mucosa), and kidney were still deeply stained. The dye was rapidly cleared from the blood by the kidneys, as shown by the falling blood level (Fig. 1) and our observation of deeply stained urine. When the homogenized tissues were allowed to stand, the dye was seen in the supernatant while the insoluble tissue components appeared unstained except for a thin band at the top of the precipitate. This was seen with all tissues studied.

Uptake of ^{125}I-toluidine blue was very similar to that of the unlabeled dye (Fig. 2). The concentration in parathyroid 6 min after injection was three times that in thyroid and nearly 30 times that in neck muscle, falling off rapidly between 6 and 15 min as before. The comparisons in Table 2 show that the concentration of ^{125}I-labeled and unlabeled toluidine blue were nearly the same in parathyroid, thyroid, neck muscle, and liver, but markedly higher for unlabeled than labeled dye in both pancreas and stomach.

The studies of in vitro binding of toluidine blue failed to show organ specificity (Fig. 3A and B). Comparison of the two curves of Fig. 3A shows that a given weight of any one of these three tissues binds about three times as much dye from a 0.05% solution as from a 0.01% solution. Data for all three

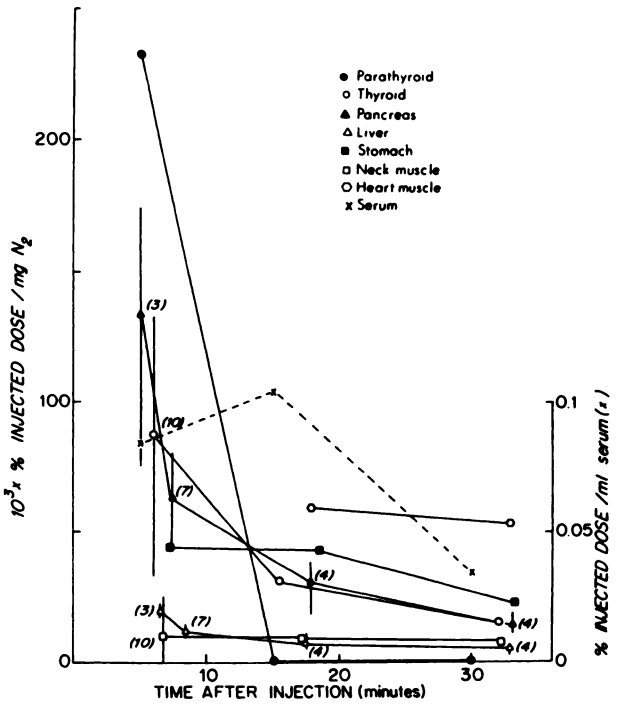

FIG. 1. Uptake of unlabeled toluidine blue by rat tissues at increasing times after intravenous injection. Data are from three separate experiments. Points represent means of number of observations shown in parenthesis; points without numbers in parenthesis represent pooled samples from 3–4 animals; vertical bars represent standard deviations for means of six or more observations, ranges for groups of less than six observations. Injected dose 9–13 mg/kg body weight.

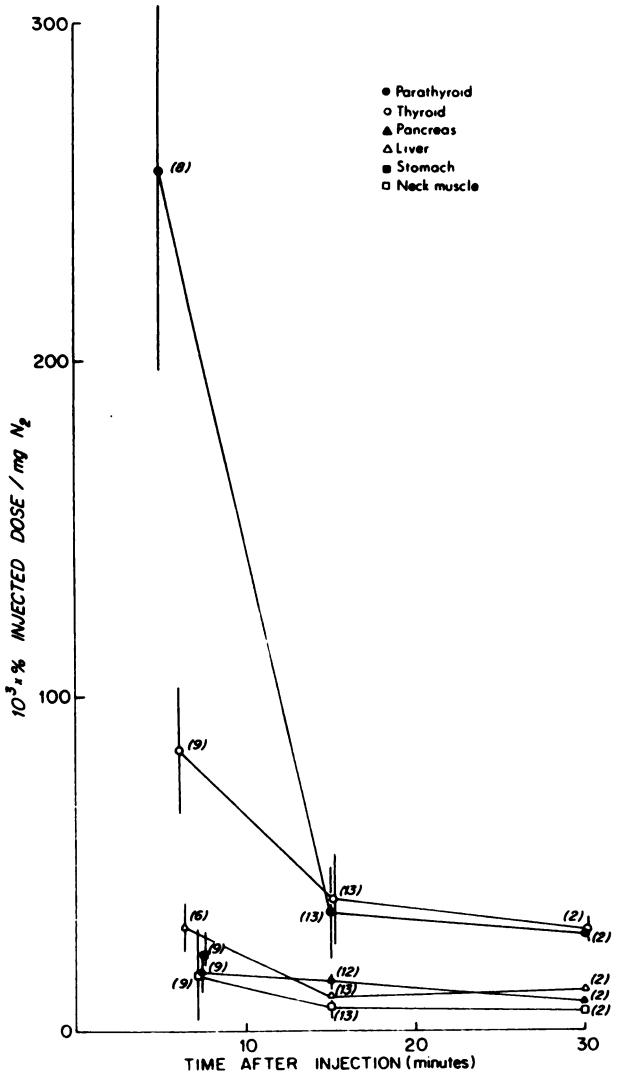

FIG. 2. Uptake of ^{125}I-toluidine blue by rat tissues at increasing times after intravenous injection. Labeled dye was extracted into ethanol and counted. Data are from three separate experiments. Points represent means of number of observations shown in parenthesis; vertical bars represent standard deviations for groups of six or more observations, range for less than six. Injected dose 0.08–0.8 mg (0.1 mCi)/kg body weight.

tissues incubated with 0.05% toluidine blue fit onto the same curve. Figure 3B shows that the amount of dye bound by a given homogenate depends on the ratio of toluidine blue to wet weight of tissue in the incubation medium rather than on the type of tissue. Both curves indicate a tendency toward saturation of binding capacity as the ratio of dye-to-tissue weight is increased.

The uptakes of ^{75}Se-selenomethionine and of ^{125}I-toluidine blue in the animals fed a nearly calcium-free diet and those in control animals are compared in Table 3. While the uptake of ^{75}Se-selenomethionine by parathyroid was 43% higher at 15 min in the experimental group, the uptake of ^{125}I-toluidine blue appeared to be decreased by 13% in the experimental group. However, 60 min after injection the uptake of ^{75}Se-selenomethionine by experimental animals was only half that by control animals. These data suggest that feeding a low-calcium diet may accelerate transport of amino acids into parathyroid and accelerate subsequent synthesis and release of protein, perhaps parathormone. However, as might be expected, the uptake of ^{125}I-toluidine blue by parathyroid is not affected by the calcium content of the diet.

On the other hand, a low-calcium diet influences the uptake of ^{125}I-toluidine blue by thyroid, which was 41% lower in experimental animals 15 min after injection, while no effect was seen on the uptake of ^{75}Se-selenomethionine at either time. This change in thyroid uptake is not understood.

No significant effect on uptake of either labeled compound at either time was seen in muscle or liver. Pancreatic uptake of ^{75}Se-selenomethionine 60 min after injection was increased 50% in the experimental group compared with controls; there was no significant effect of low-calcium diet on uptake of either material at 15 min. The reasons for this difference at 60 min are not understood.

DISCUSSION

Our observations (Figs. 1 and 2, Table 2) confirm those of others (*9–15*) that parathyroid glands accumulate toluidine blue to very high levels soon after intravenous injection. The concentration of dye in parathyroid glands was about three times that in thyroid and twice that in pancreas at 5 min after injection but had fallen to levels as low as, or lower,

TABLE 2. COMPARISON OF UPTAKE OF ^{125}I-LABELED AND UNLABELED TOLUIDINE BLUE

Tissue	No. of animals	Time after injection (min)	Uptake of dye ($10^3 \times$ % injected dose/mg N_2) ^{125}I-labeled	Uptake of dye ($10^3 \times$ % injected dose/mg N_2) Unlabeled
Parathyroid	8	5.2	256.24	
	7	5.0		275.52
Thyroid	11	6.3	78.61	
	10	6.0		87.54
Muscle	11	7.3	8.55	
	10	6.7		9.89
Pancreas	11	7.5	11.14	
	10	7.4		62.59
Liver	11	7.5	9.92	
	10	8.4		12.10
Stomach	11	7.7	13.20	
	3	7.2		43.83

Data represent means of observations from number of animals given and are from two or three experiments.

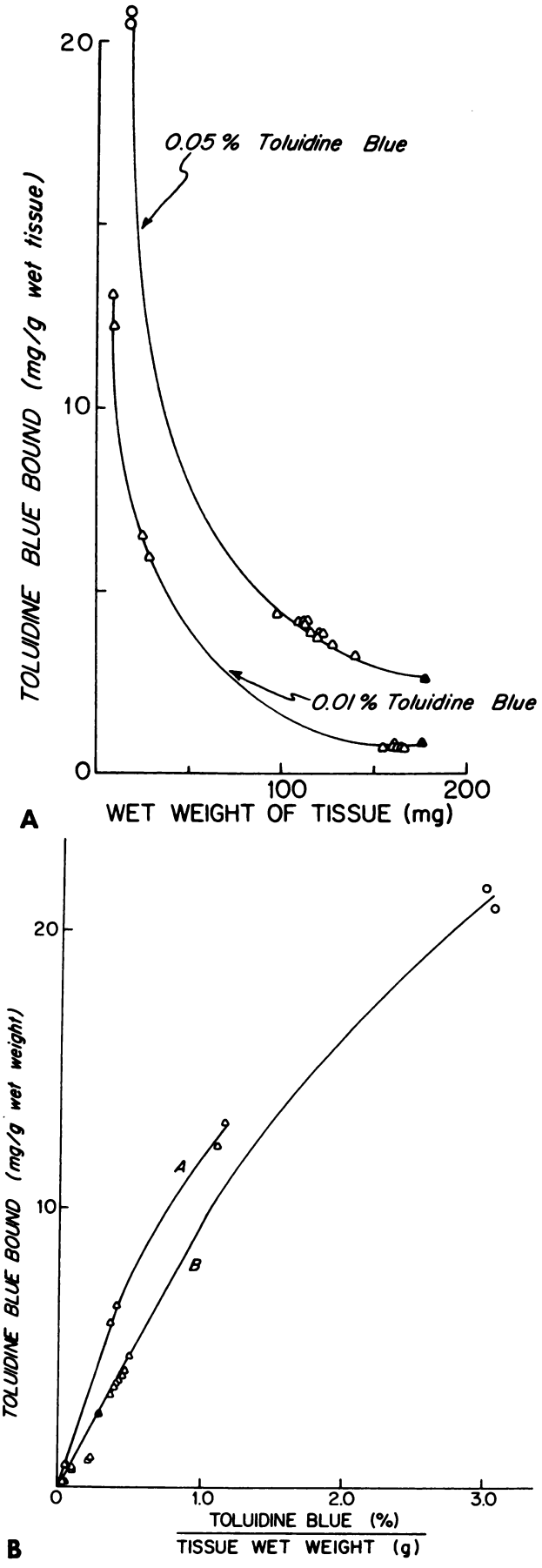

FIG. 3. Binding of unlabeled toluidine blue by rat tissue homogenates in vitro at two concentrations of toluidine blue in incubation medium. A shows specific binding of dye plotted as function of wet weight of precipitate from tissue homogenate in centrifuge tube after extraction of dye into ethanol. B shows specific binding of dye plotted as function of ratio of concentration of dye in incubation medium to wet weight of precipitate from extracted tissue homogenate. Curves A and B represent separate experiments. At abscissa value of 0.06 on Curve A, nine observations are superimposed.

than those in thyroid at 15 min after injection. The distribution ratio of toluidine blue between parathyroid tissue and blood serum was 130 at 5 min but close to zero 15 min after injection.

Other workers, however, have observed more persistent staining of dog parathyroid glands by intravenously injected toluidine blue (*9,10,13*). Hurvitz, Hurvitz and Morgenstern (*13*) found significant staining of dog parathyroid glands 15 min after injection and maximum staining of these glands after intravenous injection with a distribution ratio of 65 between parathyroid tissue and blood serum at this time. The basis for this discrepancy between the behavior of injected toluidine blue in parathyroid glands from rats and dogs is not understood.

We found, as did others (*9–13*), that the dye was concentrated to high levels by thyroid, pancreas, and stomach. In thyroid, pancreas, liver, stomach, and skeletal muscle, the dye was found at much lower concentrations than in parathyroid at 5–8 min after injection. At 15 and 30 min after injection heart muscle was stained much more deeply than any other tissues studied; heart muscle was not studied at earlier times. Our observations of heart muscle are in general agreement with those of other workers (*9,10*).

Basic dyes, such as toluidine blue, combine electrostatically with acidic components of tissue, such as nucleic acids, acid mucopolysaccharides, and some lipids (*22,23*). The amount of basic dye bound by a tissue section under standard conditions has been used as a measure of the quantity of acid mucopolysaccharides in the tissue (*24*). Our studies indicate that toluidine blue is bound in nonspecific fashion, presumably to acidic components of tissues.

Figures 3A and B show that when homogenates of thyroid, pancreas, and liver were incubated with toluidine blue in vitro the quantity of dye bound per unit weight of wet tissue was more dependent on the ratio of dye to tissue weight than on the type of tissue. That the quantity of dye extracted into ethanol is a fair measure of the quantity originally bound by the tissue is supported by the recovery in the ethanol extract of greater than 90% of that disappearing from the incubation medium in the in vitro binding experiments.

TABLE 3. COMPARISON OF RESPONSE OF TISSUE UPTAKE OF ^{75}Se-SELENOMETHIONINE AND ^{125}I-TOLUIDINE BLUE TO CALCIUM-DEFICIENT DIET

		Time after injection (min)	Specific uptake (10^3 × % injected dose/mg N_2)	
			^{75}Se-selenomethionine	^{125}I-toluidine blue
Parathyroid	Control	15	27.79 ± 4.72	28.56 ± 8.20
	Experimental	15	40.33 ± 5.05	24.80 ± 4.58
	Control	60	39.62 ± 8.94	
	Experimental	60	20.85 ± 7.85	
Thyroid	Control	15	14.02 ± 1.24	48.90 ± 10.52
	Experimental	15	16.04 ± 3.76	28.86 ± 2.84
	Control	60	16.74 ± 5.91	
	Experimental	60	9.65 ± 2.28	
Muscle	Control	15	13.30 ± 2.31	5.56 ± 1.38
	Experimental	15	10.08 ± 2.24	5.70 ± 1.76
	Control	60	4.41 ± 1.16	
	Experimental	60	5.23 ± 0.55	
Pancreas	Control	15	16.34 ± 2.65	9.93 ± 0.63
	Experimental	15	18.71 ± 6.20	10.08 ± 1.02
	Control	60	19.88 ± 7.32	
	Experimental	60	29.33 ± 2.40	
Liver	Control	15	9.63 ± 1.23	11.22 ± 2.06
	Experimental	15	9.85 ± 2.04	12.47 ± 1.28
	Control	60	12.45 ± 3.56	
	Experimental	60	10.13 ± 1.00	

Whole pieces of tissue were counted. Figures given are means of 4–6 observations; standard deviations are shown.

The tissues studied appeared to treat ^{125}I-toluidine blue very similarly to the unlabeled dye. For tissues other than pancreas and stomach the portion of injected dose of labeled dye determined by counting radioactivity retained per unit weight of tissue nitrogen was closely similar to the figure obtained by extraction of unlabeled dye from tissues with ethanol (Table 2). In both pancreas and stomach, however, the portion of injected dose taken up by tissue was much greater with unlabeled dye (injected dose 0.08–0.8 mg/kg body weight). The mechanism underlying this discrepancy is uncertain.

The data indicate no striking differences between the binding capacities for toluidine blue of cell-free homogenates of thyroid, pancreas, and liver. The explanation of the marked differences in ability of these three tissues to bind toluidine blue in vivo most likely is to be sought outside the cells, probably in the interstitial space. Important factors may be the blood flow through each tissue, the quantity of anionic binding sites in the interstitial space, and the rapidity of exchange of the dye between interstitial space and lymph and of clearance of the tissue by lymph.

Based on results in the rat, which show rapid disappearance of both labeled and unlabeled toluidine blue from the parathyroid glands, we must conclude that a gamma-emitting analog of toluidine blue would most likely be unsuitable for use in external scanning for localization of these glands. Larose, Whitaker, and Reba (*11*) using a different experimental technique, also in the rat, concluded that radioiodinated toluidine blue given by pulse intravenous injection would be an unsatisfactory scanning agent for parathyroid and pancreas for reasons of finding insufficiently high parathyroid-to-thyroid and pancreas-to-liver ratios of the compound at 3–60 min (peak concentration ratios of 1.13 and 1.18, respectively, at 3 min). Our results show a parathyroid-to-thyroid ratio of about 3 at 5 min, but the parathyroid level of toluidine blue falls too rapidly to allow for imaging. The pancreas-to-liver ratio was about 5 at 5 min and 4 at 15 min, but the significant levels of toluidine blue in the stomach would be expected to obscure the pancreas during scanning.

The quantitative studies of toluidine blue distribution in the dog (*9,10*) differ significantly from those in rats in that there is maintenance of high levels of toluidine blue in the parathyroid and high parathyroid-to-thyroid ratios during 3 hr of observation. The basis of these species differences has not been evaluated.

The studies in humans in which toluidine blue has been infused intravenously during neck exploration (*10,12,14,15*) have been generally successful in allowing for easier visualization and dissection of parathyroid tissue. When given in this fashion, the dose must be carefully controlled to avoid toxicity of the compound. However, these studies suggest that if toluidine blue could be labeled with a short-half-

life, gamma-emitting radionuclide in high specific activity, then scintillation scanning during continuous infusion of the radiopharmaceutical might be possible. However, since we have demonstrated that calcium depletion and resultant increased parathyroid gland metabolic activity do not increase toluidine blue uptake (Table 3), scanning with this agent would not be expected to differentiate normal from abnormal glands on the basis of difference in isotopic accretion. This would leave size alone as a determinant of abnormality, but currently available instrumentation probably lacks sufficient resolution to distinguish all but the largest adenomas from normal sized glands.

SUMMARY

Iodine-125-toluidine blue was prepared from toluidine blue and Na^{125}I and purified by solvent extraction. The ^{125}I-toluidine blue was found to be chromatographically pure and to behave similarly to unlabeled toluidine blue in rat tissues. Uptake of toluidine blue by parathyroid glands 5 min after intravenous injection was twice that by pancreas and three times that by thyroid. After 15 min, uptake by parathyroid tissue had fallen to levels as low or lower than those of pancreas and thyroid in which uptake had also declined. Uptake of toluidine blue by heart muscle at 15 min after intravenous injection was 1½ times that in stomach, twice that in pancreas and thyroid, and six times that in liver and skeletal muscle. Parathyroid uptake of ^{125}I-toluidine blue was not increased in rats which had been fed a low-calcium diet while uptake of ^{75}Se-selenomethionine was increased.

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