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THE RATES OF RELEASE AND BINDING OF THYROXINE BY BOVINE SERUM

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SUMMARY

1. Estimates have been made of the rates of release and binding of thyroxine by bovine serum proteins. The method involves measuring the rates at which columns of Amberlite resin take up thyroxine from serum.

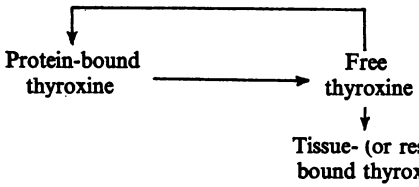
2. At 37° C the dissociation rate (the rate of release of bound thyroxine into free solution) is about 2·2 % sec^{-1}.

3. The binding of thyroxine by serum is a complex process, the majority of the hormone being 'loosely' bound within 0·1 sec but the over-all process taking some 20 sec to completion.

4. A mechanism is proposed for the uptake of thyroxine by the liver. It involves a simple competition between the plasma and the liver binding sites for the free thyroxine released into solution by the dissociation reaction.

INTRODUCTION

Almost all of the thyroxine in plasma is bound by specific thyroxine-binding proteins; only about 0·05 % is present in free solution and this fraction is in equilibrium with the protein-bound store (Robbins & Rall, 1960); it is generally thought that only the free hormone is able to enter the tissues (Ingbar & Freinkel, 1960; Hillier, 1969). The dissociation of thyroxine from binding protein is very probably a unimolecular reaction (Robbins & Rall, 1960); if this is so then a constant fraction of the bound thyroxine will be released as free hormone per unit time, and, at equilibrium, a similar quantity of free thyroxine will be rebound. When the plasma enters a tissue this equilibrium will be disturbed; the free thyroxine can either be rebound by the protein or be bound by the tissue binding sites. The amount of thyroxine taken up by the tissue will depend therefore upon the rate of dissociation reaction and also upon the relative speeds of the two binding processes which compete for the released free hormone. The situation can be represented diagrammatically as shown at the top of the next page.



If the dissociation reaction is very slow only the free thyroxine entering the tissue will be available for uptake. On the other hand if dissociation is very rapid the free thyroxine absorbed will be rapidly replenished, and considerable amounts of the protein-bound thyroxine could ultimately be taken up by the tissues during the few seconds that the plasma spends in the capillaries. A knowledge of the rates of dissociation and binding is therefore essential for understanding tissue thyroxine uptake. In a previous study of the perfused rat liver (Hillier, 1969) it was shown that dissociation is sufficiently rapid to allow significant amounts of thyroxine to be taken up by the liver from the protein-bound store. The method, however, was not very suitable for use with whole serum or for accurate estimations of the dissociation rate. In this paper another method is described using Amberlite resin. This resin rapidly and firmly binds small ions like free thyroxine but does not bind plasma proteins, and in this respect it is similar to a tissue. As the plasma passes through a column of resin the free hormone is rapidly removed and, by examining the resin uptake of thyroxine from serum, the rates of dissociation and binding can be estimated.

METHODS

The object in all experiments was to estimate the rate of thyroxine uptake by Amberlite resin. The resin was packed into long flexible plastic tubing and the columns of resin so formed were perfused at a fixed rate. In this way time intervals could be expressed in terms of lengths of column and the amount of thyroxine taken up during any time interval could be estimated by cutting out the appropriate segment of resin and measuring its radioactivity.

The resin columns. Amberlite resin (IRA, 400; standard grade; chloride form; 14–52 mesh) was used in the form in which it was received, after an initial washing with distilled water. Lengths of flexible plastic tubing (40–100 cm long, 2 or 4 mm diameter) were stopped at one end by a loose cotton wool plug, and water, containing suspended resin, was sucked through the tube causing the resin to pack behind the cotton wool. In this way columns of any length could be easily prepared free of air bubbles. In some experiments crushed resin was used. The dry material was crushed using a pestle and mortar and then suspended in water. Any particles that did not settle in about 30 sec were decanted off and after decanting 4 or 5 times a sample of uniformly crushed resin could be obtained free from any powdery material.

Perfusion was performed using a Buchler Polystaltic Pump. The resin columns were initially perfused very rapidly (10 ml./min) in order to pack the resin down

firmly; they were also rapidly perfused at the end of an experiment to wash out any contaminating radioactive fluid.

Krebs–Ringer solution or bovine serum was used for perfusion and the temperature was 21–23° C unless otherwise stated. In all of the uptake experiments a standard procedure was used; 5 ml. serum was perfused rapidly (about 7 ml./min) so that the resin was exposed to the serum for as short a time as was practicable (always less than 60 sec). By using only these short times the loss of adsorbed hormone back into the serum could be kept within reasonable limits (see Results), thereby allowing the small thyroxine flux from the resin to the serum to be ignored.

The time that the fluid spent within the columns was estimated by injecting blue dye into the fluid above the resin and timing its passage. This was done after the radioactive perfusion had been completed and it allowed time intervals to be expressed in terms of lengths of resin. In most experiments 1 sec was equivalent to about 3–5 cm of resin. For the rapid mixing experiments illustrated in Fig. 4, however, faster rates and smaller tubing were used to give values up to 25 cm/sec.

Thyroxine was usually added to the serum at least 15 min before use, to allow ample time for the binding process to come to equilibrium. However, in those experiments in which it was proposed to mix free thyroxine with serum (Figs. 3 and 4) the free hormone, in 0·1 ml. water, was injected directly into the resin column during the serum perfusion using a fine syringe needle. The injected volume was too small to affect the perfusion rate materially and it was assumed to mix with the serum at rates too fast to be limiting to the binding processes being studied. The concentrations of thyroxine added to the serum were always such as not to increase the endogenous level by more than 20 %.

Removal of radio-iodide contamination. The radioactive solutions of thyroxine contained 2–6 % inorganic radio-iodide. Iodide was taken up from serum by uncrushed resin with a $t_{\frac{1}{2}}$ of 0·5 sec (i.e. at about the same rate as free thyroxine) and it was necessary therefore to remove it. This was done by adding a few drops of the neat radio-thyroxine solution to 1 ml. bovine serum and passing this solution through 5 ml. resin at a rate of about 1 ml./min. This effectively removed 90 % of the iodide but very little of the thyroxine since this was firmly bound to the proteins. This procedure was repeated 3 or 4 times and it reduced the contaminating iodide to insignificant values. This 1 ml. serum, containing pure thyroxine, was then added to about 30 ml. ordinary serum which was used later in the uptake experiments.

In those experiments using free thyroxine (Figs. 3 and 4) this procedure could not be adopted. Here the level of iodide contamination was accurately estimated by chromatography using butanol:acetic acid:water (78:10:12) and Whatman 3 MM paper. The results of the uptake experiments were then appropriately corrected.

Serum was obtained from a local slaughter house. A bullock was stunned mechanically and about 2 gallons of blood was collected into a plastic bucket by bleeding from the jugular veins. The clotted blood was sliced open and the serum that exuded over the next hour was collected and centrifuged; the cleaned serum was stored at −20° C until use. In one experiment human plasma was studied. It was obtained from the National Blood Transfusion Service and had overrun the expiry date for transfusion by a few days.

Radioactive compounds were obtained from the Radiochemical Centre, Amersham. Inorganic ^{131}I, [^{125}I]L-3.5.3′.-tri-iodothyronine, [^{131}I]L-thyroxine and [^{125}I]L-thyroxine were all used.

RESULTS

The uptake of free thyroxine. In a series of some fifty experiments an examination was made of the resin uptake of thyroxine and tri-iodothyronine from Krebs–Ringer solution. The uptake was an exponential process, and, for uncrushed resin, both hormones were taken up with a $t_{\frac{1}{2}}$ of about 0·6 sec. By crushing the resin (to increase its surface area) the $t_{\frac{1}{2}}$ could be reduced to 0·1 sec and all intermediate values could also be obtained. Most of these experiments were done by simply adding thyroxine to the perfusion fluid but an identical picture was obtained if thyroxine was injected directly into the perfusion stream some way down the column. The concentration of thyroxine was not important and similar values were obtained with thyroxine at a concentration of 1×10^{-4} and 1 μg/ml. These experiments were all performed at room temperature (22° C); at 37° C the uptake was slightly faster ($t_{\frac{1}{2}}$ 0·35 sec for uncrushed resin).

The release of thyroxine from resin. An essential point in the analysis is that thyroxine, once adsorbed by the resin, is not thereafter released. This was checked by loading columns with thyroxine and measuring the rate at which it was released back into the perfusate. With ordinary Krebs–Ringer solution as the perfusion fluid, release was almost undetectable. With whole serum, however, release was substantial. In the uptake experiments reported later standardized conditions were used, viz. 5 ml. serum was passed at a rate of 7 ml./min and, under these conditions, it was found that about 18 % of the thyroxine was lost into the perfusate (mean of ten experiments). This means that, in the uptake experiments, the reported uptake values are underestimated by about 9 %. No correction was made for this source of error; it was assumed that the passage of thyroxine from the serum to the resin was unidirectional.

The resin uptake of thyroid hormones from serum. Results of an experiment with uncrushed resin are illustrated in Fig. 1. Over the first 10 sec the resin took up thyroxine from serum at a rate of $0{\cdot}164 \pm 0{\cdot}007$ (S.E.) % sec^{-1}. At the end of 10 sec therefore about 1·6 % of the total thyroxine had been extracted by the resin. Since the proportion of free hormone is only about 0·05 % it can be concluded that more than 95 % of the extracted thyroxine had come from the protein-bound store. In a similar experiment with human plasma (six observations) thyroxine was taken up at a rate of $0{\cdot}145 \pm 0{\cdot}012$ % sec^{-1}. Tri-iodothyronine, which is less firmly bound by serum protein, was taken up three times as fast as thyroxine.

Uncrushed resin takes up free thyroxine with a $t_{\frac{1}{2}}$ of 0·6 sec. If this resin were managing to capture only a fraction of the dissociated thyroxine (i.e. if there is a strong competition from the serum binding sites) then it would be expected that more hormone would be taken up from serum by

crushed resin, which captures the free thyroxine faster. This possibility was investigated. Columns were prepared of resin crushed to varying degrees of fineness; half of each column was used to estimate the $t_{\frac{1}{2}}$ for the uptake of free thyroxine from Krebs–Ringer solution and the other half used to estimate the thyroxine uptake rate from serum. The results, illustrated in Fig. 2, show that thyroxine was taken up from serum more rapidly by resins which adsorbed free thyroxine faster. If the uptake of free thyroxine were infinitely fast ($t_{\frac{1}{2}} = 0$) then all of the dissociated hor-

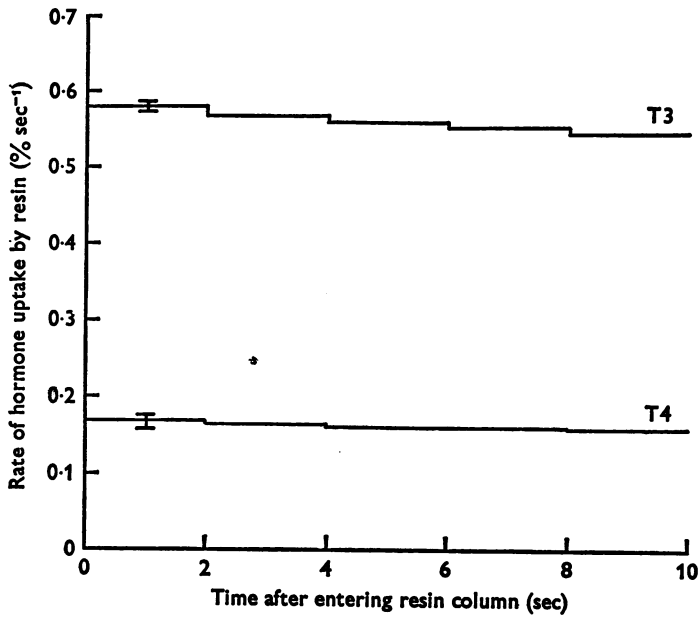


Fig. 1. The rate at which Amberlite resin takes up thyroid hormones from bovine serum over the first 10 sec of contact between the resin and the serum. T4-thyroxine, mean of ten experiments; T3-tri-iodothyronine, mean of three experiments. The vertical lines indicate 1 S.E. either side of the mean. Temperature 22° C.

mone would be captured by the resin and the uptake rate would equal the dissociation rate. It was not, however, possible to estimate the dissociation rate by extrapolation of the curve in Fig. 2 and the value of 0·9 % sec^{-1} was derived from results presented in the next section.

It can be noted that 50 % of the dissociated thyroxine is captured by resin which adsorbs free thyroxine with a $t_{\frac{1}{2}}$ of 0·12 sec and it could be suggested therefore that, under these conditions, the serum protein is also effectively managing to 'capture' the free thyroxine at a similar rate (although the binding reaction here is not a simple exponential process—see later). If this assumption is made, and if it is also assumed that the relative proportions of the dissociated thyroxine captured by the

resin and serum vary inversely with their respective $t_{\frac{1}{2}}$ for free thyroxine capture, then it is possible to predict the rate of thyroxine uptake from serum for any resin of given $t_{\frac{1}{2}}$. This was done and the curve drawn in Fig. 2 predicts the thyroxine uptake rate from serum having a dissociation rate of 0·9 % sec^{-1} and effectively capturing free thyroxine with a $t_{\frac{1}{2}}$ of 0·12 sec. The curve provides a reasonable fit to the experimental points at least over the observed range and supports the idea that the serum and resin binding sites compete for the dissociated hormone.

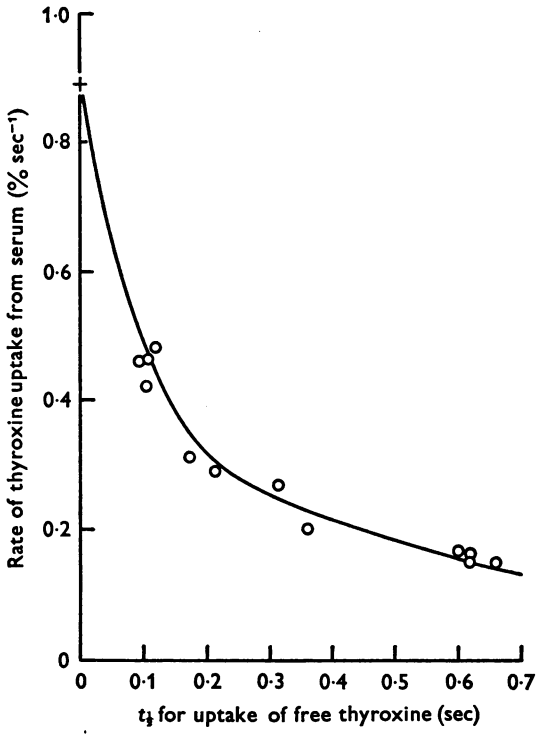


Fig. 2. The rate at which thyroxine is taken up from bovine serum by resins capturing free thyroxine at various speeds. Each point represents a single experiment. The value of 0·9 % sec^{-1} for the uptake at zero $t_{\frac{1}{2}}$ (the dissociation rate) is derived from data in Fig. 3 and the curve used to fit the points is derived in the text. Temperature 22° C.

Measurement of the dissociation rate. From the previous experiments it can be seen that resin does not capture all of the dissociated thyroxine but only a fraction of it. If this fraction were known then estimates could be made of the dissociation rate. Experiments were performed therefore to determine the relative proportions of the dissociated thyroxine captured by the resin and by the serum. This was done by injecting free thyroxine into resin columns perfused with serum and measuring, at successive time intervals, the proportion of the hormone taken up by the columns.

It has been shown that for uncrushed resin the mean rate of thyroxine uptake over the first 10 sec is 0·164 % sec^{-1}, or, expressing it differently, the transfer of thyroxine from the serum to the resin has a rate constant of 0·0164 per 10 sec or, very nearly, 0·00164 sec^{-1} and this rate is indicated in Fig. 3 by the slope of line *A*. This is the rate of uptake for thyroxine 'completely bound' by serum, i.e. for thyroxine that has been mixed with serum for at least 15 min. When free thyroxine is injected into a column perfused with serum (Fig. 3) it takes some 20 sec or so before the uptake

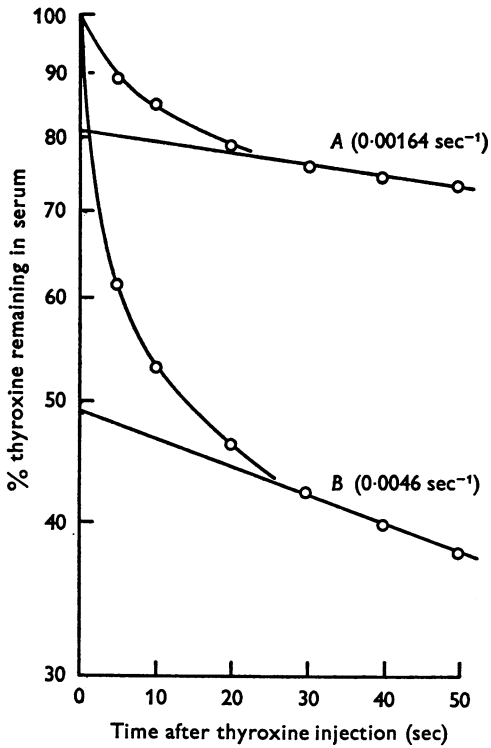


Fig. 3. A tracer dose of thyroxine was injected into resin columns perfused by serum at 22° C. The % of the hormone remaining in the serum is plotted on a log. scale at various times after the injection. Curve *A* is for uncrushed resin (mean of six experiments), curve *B* is for a sample of crushed resin (six experiments). The figures in brackets are rate constants for the transfer of thyroxine from the serum to the resin and indicate the slope of the two straight lines.

rate settles to this value, indicating that the hormone is completely bound by the serum only after about 20 sec. During this time interval some 16–20 % of the thyroxine is captured by the resin (a value obtained from the intercept of line *A* with the ordinate axis). If it is assumed that this

pulse injection of free thyroxine behaves like the free thyroxine released by the dissociation reaction, then it can be concluded that only 16–20 % of the dissociated hormone is in fact captured by the column and that 80–84 % is recaptured by the serum proteins. Since the thyroxine uptake rate from serum is 0·164 % sec^{-1} and only 16–20 % is captured, the dissociation rate is 0·82–1·02 % sec^{-1}. An exactly similar experiment was performed with a sample of crushed resin. This took up thyroxine from serum at a rate of 0·46 % sec^{-1} (i.e. with a rate constant of 0·0046 sec^{-1}, indicated in Fig. 3 by the slope of line *B*). When free thyroxine was injected into columns of the resin perfused with serum it again took some 20 sec for the uptake rate to settle to this value and during this time 48–53 % of the hormone was captured by the resin. These results indicated a dissociation rate of 0·87–0·96 % sec^{-1}, a value similar to the previous estimate. The mean value of 0·90 % sec^{-1} was taken as the dissociation rate at 22° C and this is the value used in Fig. 2.

The rate of thyroxine binding by serum. In the previous experiment it was noted that free thyroxine injected into serum was taken up at an equilibrium rate only after 20 sec. It can be concluded therefore that 20 sec are required before thyroxine is 'completely' bound by serum. In a series of six experiments thyroxine was injected into serum-perfused columns and the resin uptake rate was measured over the few seconds after mixing. A typical result is illustrated in Fig. 4. The plot on the ordinate axis is of the amount of the hormone taken up by the resin per 0·1 sec expressed as a percentage of the amount of hormone still present in solution at the beginning of the time interval. For free thyroxine injected into Krebs–Ringer solution between 10 and 11 % of the hormone was extracted by the resin every 0·1 sec and this value remained constant during the few seconds that it was measurable (the uptake being an exponential process). When thyroxine was injected into serum, however, the picture was quite different. Binding appeared to take place in two main stages. Within the first 0·1 sec or so there was a precipitous fall in the uptake rate down to a value of about 0·3 % per 0·1 sec; after the first sec the uptake rate fell more gradually and approached the equilibrium value exponentially with a $t_{\frac{1}{2}}$ of about 3·5 sec. This sort of behaviour would be expected if thyroxine were initially bound very rapidly by 'weak' binding sites having high association and dissociation rates and capable therefore of rapidly releasing the hormone back into free solution, and following this being bound by 'stronger' binding sites with slower association and dissociation rates. The experimental method was not sufficiently precise to allow accurate estimates of the uptake rate over the very short time intervals after mixing but the results tended to suggest that the falling curve over the first second could be resolved into two stages: an initial very rapid decline in uptake with a $t_{\frac{1}{2}}$

of less than 50 msec and a slower, probably exponential, fall with a $t_{\frac{1}{2}}$ of 0·1–0·2 sec.

The effect of temperature. The resin uptake of thyroxine from serum was very temperature-sensitive (Fig. 5). For uncrushed resin at 37–38° C the uptake rate was about 0·45 % sec^{-1}. At this higher temperature the resin captured 21·0 ± 2·7 (S.E.) % of the free thyroxine injected into serum

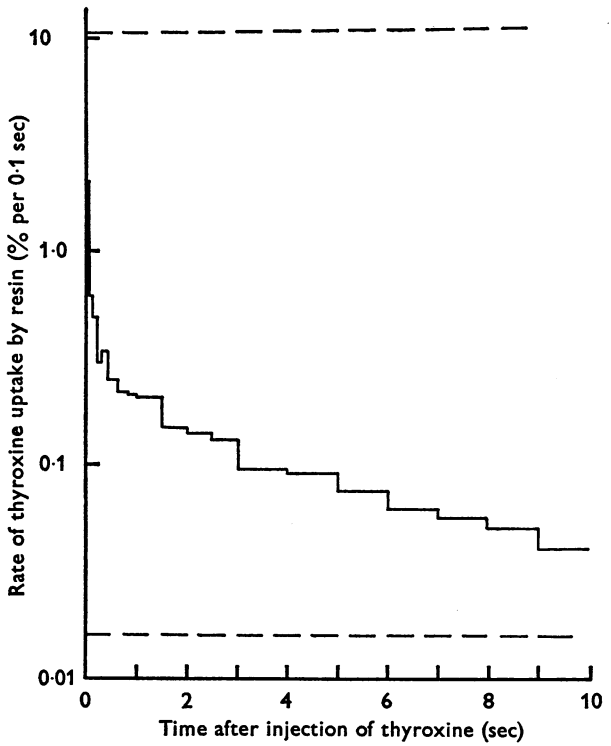


Fig. 4. The rate of thyroxine uptake by uncrushed resin at 22° C (the amount of thyroxine taken up each 0·1 sec expressed as a percentage of the amount of hormone still in solution at the start of the time interval). The continuous line shows the uptake rate of a tracer dose of free thyroxine injected into a resin column perfused by bovine serum. The upper dashed line shows the uptake rate for free thyroxine and the lower dashed line shows the uptake rate for thyroxine 'completely' bound by bovine serum.

(five experiments) and this indicated a dissociation rate of about 2·2 % sec^{-1}. The substantial increase in thyroxine uptake at higher temperatures, illustrated in Fig. 5, is due therefore to an increase in the dissociation rate and not to a greater ability of the resin to trap the released hormone. It can also be noted from Fig. 5 that the dissociation rate is especially sensitive to temperature changes over the physiological range.

DISCUSSION

In the Introduction a mechanism was proposed for the uptake of thyroxine by tissues which involved competition between the tissues and plasma binding sites for the dissociated, free hormone; and it has generally been assumed that thyroxine uptake proceeds by some mechanism of this sort (Ingbar & Freinkel, 1960; Hillier, 1969). Does this mechanism provide an adequate, quantitative description of thyroxine uptake by the liver?

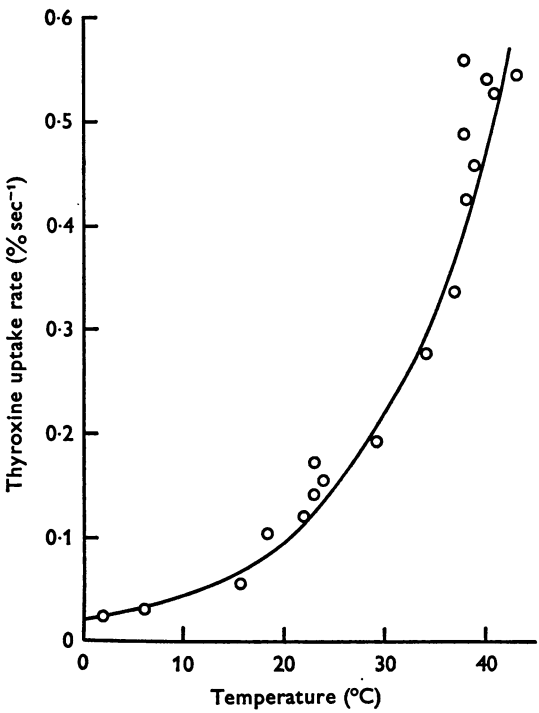


Fig. 5. The rate of thyroxine uptake from bovine serum by uncrushed resin at various temperatures. Each point represents a single experiment. The line was drawn by eye.

The isolated perfused rat liver at 22° C takes up free thyroxine with a $t_{\frac{1}{2}}$ of about 1 sec (Hillier, 1969) and on the basis of the above hypothesis the uptake rate from bovine serum should be about 0·1 % sec^{-1} (by extrapolation of the curve in Fig. 2). In fact, in the perfused liver, the thyroxine uptake rate from (rat) serum was estimated at between 0·06 and 0·11 % sec^{-1}, which is similar to the predicted value. This suggests that under these conditions liver cells and resin take up thyroxine from serum by a similar process, involving a competition for the released hormone between the

liver (or resin) and the serum protein; the liver is envisaged as acting simply as a passive adsorbing agent.

In vivo, however, the liver takes up thyroxine very much faster and several estimates are available for the clearance of thyroxine by this tissue. The most reliable of these involves direct measurement by cannulation of the hepatic vein and brachial artery in man (Dowling, Appleton & Musa, 1968) and this method gives a mean value of 32 ml. plasma/min. The mean hepatic plasma flow was 760 ml./min, indicating that the livers were extracting some 4·2 % of the hormone passing through in the plasma (the range was 1·9–7·5 % in five subjects). Probably almost all of this exchange occurs within the sinusoids and on average the blood spends about 7 sec within these vessels (Bradley, 1963). During these 7 sec about 15 % of the protein-bound thyroxine is released into the free state (assuming that human and bovine plasma are similar). The liver therefore could achieve the observed thyroxine clearance by capturing some 28 % of this dissociated hormone. In fact the perfused preparation at 22° C captures only 10 % but it is possible that *in vivo*, under physiological conditions of perfusion, the efficiency of this capturing process is greater. If this is so then the simple model proposed in the Introduction could provide an adequate explanation of the great speed with which the liver is able to absorb thyroxine from the blood.

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