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THE EFFECT OF 2,4-DINITROPHENOL ON THE 'UPTAKE' OF LABELLED THYROID HORMONES BY RED BLOOD CELLS AND RAT DIAPHRAGMS

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We have previously reported (Escobar del Rey & Morreale de Escobar, 1958) that at short intervals after the administration of radiothyroxine and 2,4-dinitrophenol to thyroidectomized, L-thyroxine-maintained rats, normal levels of radioactivity were found in the peripheral tissues, coexisting with a sharply decreased plasma PB^{131}I. (The following abbreviations are used: 2,4-dinitrophenol, DNP; ^{131}I-labelled L-thyroxine, ^{131}I-T_4, and L-triiodothyronine, ^{131}I-T_3; protein-bound iodine, PBI; thyroxine-binding globulin, TBG; thyroxine-binding albumin, TBA.) Moreover, an increased percentage of the blood radioactivity was in the red-blood-cell fraction in the DNP-treated rats. It has recently been shown (Morreale de Escobar & Escobar del Rey, 1960, 1961) that in intact DNP-treated rat the concentration of iodine-containing compounds in the tissues is maintained within limits comparable to those of untreated controls, whereas the plasma concentration is continuously low: the resulting increased tissue-to-plasma ratio of iodine-containing compounds is highest at short intervals of time after the administration of DNP, decreasing as the latter disappears from the circulation and increasing again after a new injection of the drug.

These findings suggested that the administration of DNP *in vivo* alters the distribution of thyroid hormones between plasma and tissues in favour of the latter. This paper reports results obtained when investigating whether such an effect is detectable by *in vitro* techniques and what mechanisms are likely to be involved. While this work was being carried out, reports appeared (Christensen, 1959, 1960*a*) describing a thyroxine-releasing effect of DNP on the thyroid-hormone–plasma-protein complex, with a dialysis technique. This paper shows that DNP does alter *in vitro* the distribution of radiothyroxine between plasma or plasma-free incubation

media and tissues, a direct effect of DNP on the cells being, however, postulated.

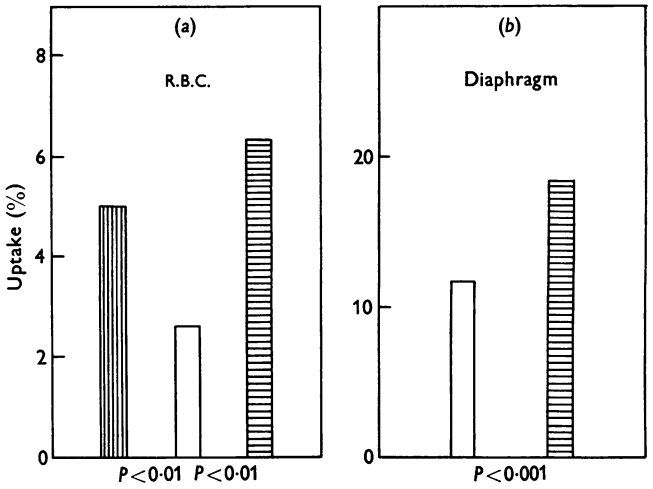

Fig. 1. Effect of DNP on the ^{131}I-T_4 'uptake' by rat R.B.C. and hemidiaphragms. (*a*) ^{131}I-T_4 was added in tracer amounts to whole blood of rats injected three times with 4 mg DNP at 8 hr intervals, to blood of untreated rats and to control blood to which 0·04 mg DNP/ml. was added. Incubation lasted 3 hr at 37° C. (*b*) ^{131}I-T_4 was added to a phosphate-saline buffer (Hogness *et al.* 1957) in tracer amounts; DNP in a 0·04 mg/ml. concentration. Incubation lasted 3 hr at 37° C. ▥ DNP-treated rats; □ control rats; ▤ control rats + DNP added *in vitro*.

METHODS

'*Uptake*' *of labelled thyroid hormones by red blood cells*: Determination of the 'uptake' was carried out by following in general a procedure similar to that proposed by Hamolsky, Stein & Freedberg (1957). The labelled hormones were added in tracer amounts, unless stated otherwise, to heparinized human or rat whole blood, the mixture was incubated at 37° C in stoppered tubes (Hamolsky, Golodetz & Freedberg, 1959) for 30 min–3 hr, the cells separated by centrifugation, washed thrice with ten volumes of isotonic saline and counted in a NaI (Tl) well-type scintillation counter. Both L-Thyroxine labelled with ^{131}I in the 3′ and 5′ and triiodo-L-thyronine in the 3′ positions, were supplied by Abbott Laboratories of Oak Ridge (U.S.A.), purified, whenever necessary, immediately before use on an anion-exchange resin and checked by chromatography.

RESULTS

Under such experimental conditions it was repeatedly observed that DNP, whether added *in vitro* or injected *in vivo*, increased the 'uptake' of labelled thyroxine both by human and rat R.B.C., typical results being shown in Fig. 1. It also increases the 'uptake' of labelled triiodo-L-thyronine, though proportionally less than that of ^{131}I-T_4: using whole rat blood incubated for 30 min with tracer amounts of ^{131}I-T_4 or ^{131}I-T_3, the

mean 'uptakes' were 3·4 % in the absence and 9·3 % in the presence of 0·1 mg DNP/ml. ($P < 0·001$) when L-T_4 was used, 20·0 and 34·5 % respectively ($P < 0·001$) for L-T_3.

Such an effect of DNP could well be mediated by interference by the drug on the thyroid-hormone–plasma-protein complex, by a direct action on cells, or by both. In order to investigate this point an attempt was first made to study the effect of DNP on the ^{131}I-T_4 'uptake' by washed R.B.C. incubated in protein-free media. The procedure, however, was found unreliable. In fact, it seems that R.B.C. integrity is not maintained; despite use of quite different isotonic buffers and media there was always some degree of haemolysis at the end of incubation and the control 'uptake' figures were in the order of magnitude described for R.B.C. stroma (Crispell & Coleman, 1956). Moreover, in the absence of binding sites in the media, adsorption of labelled thyroxine to glass-ware and specially to the large surface presented by the R.B.C. introduced more uncontrollable variables.

Owing to these difficulties indirect evidence was sought. Since it was found that repeated washings and incubation in media containing low concentrations of plasma proteins eliminated such difficulties, the problem was investigated by using R.B.C., separated from plasma, washed once with ten volumes of saline and twice with ten volumes of saline containing 1 % rat plasma and then incubated with the labelled hormone and DNP for 30–60 min in saline containing from 1 to 5 % rat plasma. After incubation the R.B.C. were separated and washed as usual. Increased ^{131}I-T_4 'uptakes' by the DNP-treated R.B.C. were always found. This, however, did not clarify the point, and an attempt was made to circumvent the necessary presence of protein in the system by (*a*) using very high thyroxine concentrations, which should be more than enough to saturate specific binding sites on the plasma proteins, and thus to minimize the effect of any further increase of the already high 'free' thyroxine fraction; (*b*) maintaining the plasma protein concentration constant and altering the concentration of R.B.C. If DNP acted by increasing the 'free' thyroxine level in the incubation medium, the line resulting when the 'uptake' is plotted against the R.B.C. content should be parallel and with a higher intercept than that obtained in the absence of the drug.

Figure 2(*b*) shows results obtained when R.B.C. treated as indicated above were incubated 60 min in saline containing 2·5 % rat plasma and increasing thyroxine concentrations. DNP (0·10 mg/ml.) increased the ^{131}I-T_4 'uptake' even at the highest hormone concentration employed. Curves obtained with an incubation medium containing only 1·0 % rat plasma were parallel.

Three series were prepared with saline containing 1·25, 2·5 and 5·0 % rat plasma as incubation medium and ^{131}I-T_4 in tracer amounts; the

'uptake' was determined for three different R.B.C. concentrations for each series, namely 12, 24 and 45% of the total volume. When the 'uptake' data were plotted against the corresponding R.B.C. content for each plasma-protein concentration, three parallel lines were obtained. The line with the highest intercept corresponded to the series with the lowest plasma-protein content, and, presumably, the highest proportion of 'free' thyroxine. When 0·10 mg DNP/ml. was added, the line obtained had a higher intercept than that corresponding to the same plasma-protein concentration, but the slope was steeper.

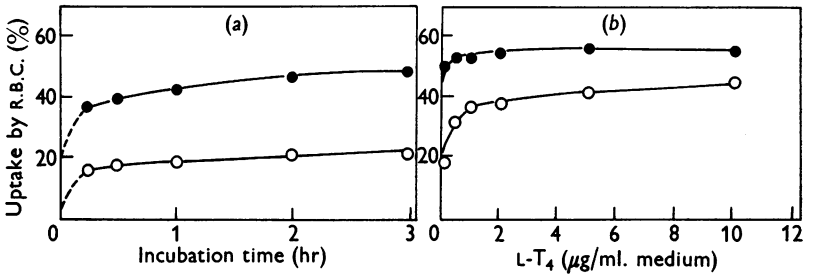

Fig. 2. Effect of DNP on the 'uptake' of ^{131}I-T_4 by washed R.B.C. incubated in saline containing rat plasma. (*a*) Influence of 0·10 mg DNP/ml. on the 'uptake' by R.B.C. incubated for 20 min–3 hr in saline containing 5% rat plasma and tracer doses of labelled thyroxine. (*b*) Influence of 0·10 mg DNP/ml. on the 'uptake' by R.B.C. incubated 60 min in saline containing 1% rat plasma and increasing concentrations of L-thyroxine. ○ control; ● DNP added.

The increased 'uptakes' obtained with DNP at the time intervals studied might have been due to the fact that the drug merely accelerated attainment of a maximum which would eventually be reached in the absence of the drug. Figure 2(*a*) shows results obtained with the above procedure, using saline containing 5% rat plasma as incubation medium, tracer amounts of ^{131}I-T_4 and increasing incubation periods. As may be seen, DNP actually maintains the 'uptake' at a higher level. It was concluded from this type of procedure that the data could not be explained by an action of DNP on the thyroid-hormone–plasma-protein complex only, a direct action on the R.B.C. being more likely.

Further experiments were carried out as follows. Whole rat blood was incubated for 30 min at 37° C with or without DNP (usually 0·1 mg/ml.), the R.B.C. were then separated by centrifugation, washed once with ten volumes of saline and twice with ten volumes of saline containing 1% rat plasma. They were incubated for another half-hour at the same temperature in saline containing 2·5% rat plasma and ^{131}I-T_4 in tracer amounts, after which the cells were separated and washed as usual. It was found that the 'uptake' of ^{131}I-T_4 by R.B.C. which had been pre-incubated with

DNP was always appreciably higher than that corresponding to untreated cells: i.e. 26% for controls versus 40% for DNP-pre-incubated R.B.C. ($P < 0.001$). Figure 3 shows that an increased 'uptake' was already appreciable at very low concentrations of DNP (0·0025 mg/ml.) during pre-incubation. These results also suggest a direct action of the drug on the R.B.C.: it seems rather unlikely that the traces of DNP, if any, which might be left in the incubation medium containing the labelled hormone after repeated washings might be responsible for the observed effect by acting on the small amounts of plasma proteins present.

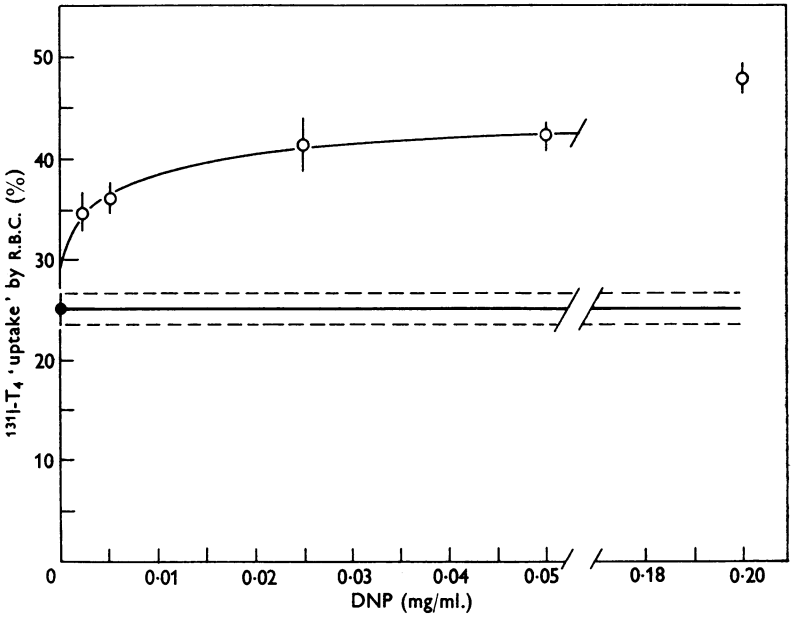

Fig. 3. Influence of DNP, 0·0025; 0·005; 0·025; 0·050 and 0·200 mg/ml., on the ^{131}I-T_4 'uptake' by R.B.C. pre-incubated 30 min with the drug and repeatedly washed before incubation for 30 min in contact with the labelled hormone in saline containing 2·5% rat plasma. Means ± S.D. of three samples are given. ● no DNP; ○ DNP.

'Uptake' of labelled L-*thyroxine by rat hemidiaphragms*

In general the procedure described by Hogness, Lee, Berg & Williams (1957) was followed. Young adult male or female Wistar rats were exsanguinated under ether narcosis through the inferior vena cava after injection of heparin. The diaphragms were rapidly excised, cleaned of adhering tissue, divided into halves, rinsed in incubation medium, blotted on filter paper and weighed on a torsion balance to the nearest milligram. They were transferred quickly to the incubation vessels, one hemidiaphragm serving as control, the other for DNP treatment, right and left being alternated. The incubation medium was either saline, saline-phosphate buffer (Hogness *et al.* 1957), Krebs-Ringer-phosphate or K-R-P + glucose (1 mg/ml.), pH 7·2–7·4, to which ^{131}I-T_4 was added in tracer amounts unless stated otherwise. DNP was usually added at a concentration of 0·05–0·1 mg/ml. Incubation was carried out

for 30 min–3 hr at 37° C in stoppered vessels, after which the tissue was rinsed thrice for 30 sec in ten volumes of cold incubation medium, blotted on filter paper and counted. All data are given as percentage of added ^{131}I-T_4 remaining with the hemidiaphragm per 100 mg wet weight. For some experiments the animals were perfused at killing with 100–150 ml. isotonic saline.

Figure 1 shows typical results. DNP increases the 'uptake' of ^{131}I-T_4 by rat hemidiaphragms in the absence of added plasma proteins. Similar results were obtained when using hemidiaphragms from saline-perfused rats: 11·4 % for controls versus 17·0 % for DNP-treated tissues ($P < 0·001$). It was observed that the response of the 'uptake' to DNP was of the same intensity irrespective of the incubation medium used. The response to DNP was, however, abolished by boiling the hemidiaphragms. Figure 4 shows the results obtained with hemidiaphragms from perfused rats, incubated for 3 hr in K-R-P + glucose containing tracer amounts of ^{131}I-T_4, increasing concentrations of stable hormone and 0·05 mg DNP/ml. As may be seen, DNP increases the 'uptake' of ^{131}I-T_4 by the diaphragms at all hormone concentrations studied, even for 1000 μg/100 ml. buffer. Such a concentration should be sufficient to saturate specific binding sites on plasma proteins if the incubation medium were totally substituted by plasma. Since the experimental conditions used make the presence of plasma proteins in the incubation medium rather unlikely, these results again support the idea that DNP exerts a direct action on the tissue.

The technique was later modified by pre-incubating the hemidiaphragms with DNP for 2·5 hr, followed by four washings with a tenfold volume of incubation medium and a second incubation for 2 hr in the presence of tracer amounts of ^{131}I-T_4. The 'uptakes' of labelled hormone by the hemidiaphragms which had been pre-incubated with DNP were higher than those corresponding to the controls, whether the incubation medium used was saline + 1 % plasma (15·9 % for controls and 21·5 % for DNP-pre-incubated, $P < 0·01$) or K-R-P + glucose (18·3 and 23·7 % respectively, $P = 0·02$). An increased ^{131}I-T_4 'uptake' by DNP-pre-incubated hemidiaphragms was also obtained with a protein-free K-R-P + glucose buffer and tissue from thoroughly perfused rats. These results are difficult to interpret on the basis of an action of DNP on the binding of thyroxine to plasma proteins.

The response to DNP observed in these pre-incubation experiments was less intense than that obtained with diaphragms directly incubated with DNP and ^{131}I-T_4 in the first incubation medium; this was to be expected, considering the long pre-incubation period and more frequent handling of the tissue.

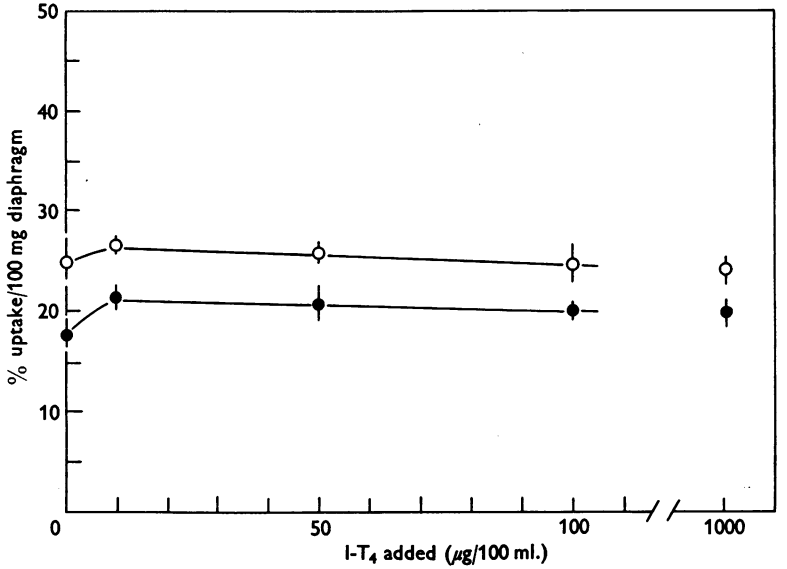

Fig. 4. The effect of DNP 0·05 mg/ml. on the ^{131}I-T_4 'uptake' by hemidiaphragms from thoroughly perfused rats, incubated for 3 hr in Krebs–Ringer-phosphate + glucose (1 mg/ml.) buffer, pH 7·2–7·4, containing increasing concentrations of L-thyroxine. The mean ± S.D. is given. ● no DNP; ○ DNP.

Electrophoretic studies

For this purpose human serum was mostly used, though some determinations were also carried out with rat sera. Untreated sera, and sera to which DNP was added in concentrations (0·05–0·10 mg/ml.) which induce an increase in the ^{131}I-T_4 'uptake' by R.B.C. to double or more of the control value, were separated by electrophoresis on Whatman No. 1 or No. 3 paper in a closed system apparatus. Either sodium 5,5-diethylbarbiturate-5,5-diethylbarbituric acid buffer, ionic strength 0·05, pH 8·6, or sodium 5,5-diethylbarbiturate-sodium acetate-sodium chloride buffer, molal ratio 2 : 1 : 1, adjusted to pH 8·6 with 1 N-HCl, ionic strength 0·1, was used. The latter buffer (Levey & Roberts, 1958) gave a very good separation of the α_1-globulin from albumin and was always used for rat serum. ^{131}I-T_4 was added in tracer amounts or with increasing concentrations of hormone in order to determine by a relative distribution method (Dowling, Freinkel & Ingbar, 1956) whether the presence of DNP altered the binding capacity of the plasma proteins. In one case DNP was added to the electrophoretic buffer and the distribution of the radioactivity between TBG and TBA was compared with that of the same plasma run simultaneously in buffer not containing the drug. Ingbar (1960) has recently

shown an 'unbinding' effect of salicylate by this procedure, which had not been detected by the standard techniques.

The yellow spot corresponding to DNP was found to migrate much ahead of the albumins both with human and rat sera. We were unable to show with either type of sera and the procedures described above any difference in the distribution of ^{131}I-T_4 among plasma proteins or in their binding capacity when DNP was present. To ascertain that the procedures employed were adequate to disclose abnormalities in binding of ^{131}I-T_4 to plasma proteins, butyl-4-hydroxy-3,5-diiodobenzoate (BHDB) was added to rat serum and the same alterations as those described by Van Arsdel & Williams (1956) were observed. BHDB at concentrations similar to those of DNP also increases the 'uptake' of ^{131}I-T_4 and ^{131}I-T_3 by rat R.B.C. (Escobar del Rey & Morreale de Escobar, unpublished).

DISCUSSION

From the results described here it is concluded:

(1) That DNP alters the distribution of thyroxine between plasma or protein-free media and cells in favour of the latter, as shown by *in vitro* techniques.

(2) It is suggested that this effect of DNP is responsible for the distribution of iodine-containing compounds found *in vivo* in rats (Morreale de Escobar & Escobar del Rey, 1961).

(3) It seems unlikely that such an effect is mainly and/or solely mediated by an alteration of the binding of the hormones to plasma proteins and it is postulated that some as yet undetermined cellular mechanism is directly involved.

The latter point is strongly supported by the data presented here. On the one hand, an effect of DNP on the thyroid-hormone–plasma-protein complex has not been demonstrable by means of electrophoretic techniques which were sufficiently sensitive to disclose another known alteration (Van Arsdel & Williams, 1956). On the other, an intense effect of the drug on the 'uptake' of thyroid hormones by rat R.B.C. and diaphragms has repeatedly been shown under experimental conditions which exclude a mechanism operating only by an action on plasma proteins.

However, Christensen (1959, 1960*a*), using a dialysis procedure, has found that the rate of transfer of ^{131}I-T_4 across the membrane is increased when DNP is added to the plasma, and interpreted this finding as an indication of a thyroxine-releasing effect of the drug on the thyroxine–plasma-protein complex. Closer inspection of his data indicates that the increase in rate of transfer of radioactivity across the membrane is rather weak as compared with the intense effect of equal doses of DNP on ^{131}I-T_4 and ^{131}I-T_3 'uptake' by R.B.C. reported here. Christensen (1960*b*) has in

fact reported a good correlation between the intensity of the changes in 'free' thyroxine and in ^{131}I-T_3 R.B.C. 'uptake', using serum from patients with various thyroidal conditions and pregnant women. With salicylate (Christensen, 1960*c*) the dose resulting in a 100 % increase above normal of the ^{131}I-T_3 'uptake' by R.B.C. augments the rate of transfer of labelled I-T_4 across the dialysis membrane 200 % above normal. On the contrary, in the case of DNP, doses (0·005 mg/ml.) which have been found by us to increase the R.B.C. 'uptake' of ^{131}I-T_4 and ^{131}I-T_3 about 100 % or more affect the rate of transfer only by 9 %; a 38 % increase in the rate of transfer of radiothyroxine was elicited by 0·05 mg DNP/ml., a dose which results in a 300 % increase in the ^{131}I-T_4 'uptake' by R.B.C. It has been reported in a recent publication (Christensen, 1961) that the addition to the dialysis system of tris-hydroxymethylaminomethane (Tris), barbital and urea, the latter in doses too low for denaturation of proteins, resulted in increased rates of transfer of ^{131}I-T_4 across the membrane, up to 322 % above normal with urea. As Christensen (1961) points out, it is difficult to interpret some of the results obtained with this latter compound as the result of a 'thyroxine-releasing' effect, and other possible causes cannot be excluded, i.e. that the membrane is affected. Rates of transfer of ^{131}I-T_4 across the dialysis membrane in the procedure used by Christensen are strictly comparable only when all experimental conditions are held constant, the serum being the only variable.

Therefore, there is direct evidence that DNP exerts an intense influence on the 'shift' of thyroid hormones towards tissues even in the absence of plasma proteins; an 'unbinding' effect on the thyroid-hormone–plasma-protein complex, on the contrary, appears as rather weak, if any, by equilibrium dialysis (Christensen, 1959) and is not demonstrable by electrophoretic procedures. We believe the facts discussed above lend experimental support to the last of our conclusions. No comments may be reliably formulated from the present data about the nature of the mechanisms involved at a cellular level which result in an increased 'uptake' of thyroxine.

At present one cannot be sure that a mechanism detected *in vitro* is the one operating *in vivo*. However, in the present instance the same effect was observed with similar intensity whether DNP and the labelled hormone were both administered *in vivo* (Escobar del Rey & Morreale de Escobar, 1958), the drug *in vivo* and the hormone *in vitro*, or both *in vitro*. Moreover, the tissue-to-plasma ratio of iodine-containing compounds increases for DNP-treated rats (Morreale de Escobar & Escobar del Rey, 1961) with an intensity of the same order of magnitude as the *in vitro* effect of DNP. It was thus concluded (Morreale de Escobar & Escobar del Rey, 1961) that it is very likely that the distribution pattern of thyroid hormone found for

intact animals is brought about by a 'shift' of the hormone towards the tissues caused by a direct effect of DNP on cells.

The bearing of such conclusions on the mechanism leading to intracellular concentration and metabolism of iodine-containing compounds, and the possible role of such parameters in a thyroid-pituitary feed-back system are discussed elsewhere (Morreale de Escobar & Escobar del Rey, 1961).

SUMMARY

1. Both injection of DNP and addition of the drug to whole blood *in vitro* increase the 'uptake' of ^{131}I-T_4 and ^{131}I-T_3 by rat and human R.B.C. The 'uptake' of ^{131}I-T_4 by washed R.B.C. incubated with the labelled hormone in saline containing 1–2·5 % rat plasma and increasing concentrations of stable thyroxine (up to 1000 μg/100 ml.) is also higher when DNP is present in the medium. When R.B.C. are pre-treated with DNP and washed repeatedly before incubation with ^{131}I-T_4, the resulting 'uptake' is higher than the uptake by controls even with very low initial concentrations of DNP, 0·0025 mg/ml.

2. Addition of DNP to protein-free incubation media results in an increased 'uptake' of ^{131}I-T_4 by rat hemidiaphragms, even when the tissues were obtained from perfused rats and incubated with high concentrations of L-thyroxine (up to 1000 μg/100 ml. buffer). Pre-incubation of the hemidiaphragms with DNP, followed by repeated washings of the tissue before incubation with ^{131}I-T_4 also increases the 'uptake'.

3. The use of several electrophoretic procedures, both with human and rat sera, did not disclose any effect of DNP on the distribution of ^{131}I-T_4 among plasma proteins or in their binding capacity.

4. It is concluded that DNP alters the distribution of thyroid hormones between plasma or incubation media and tissues in favour of the latter. It is postulated that a direct effect of DNP on the cells is involved and not mainly and/or solely an alteration of the thyroid-hormone–plasma-protein complex. It is considered likely that this 'shift' of the thyroid hormone towards tissues is responsible for the distribution pattern of iodine-containing compounds found *in vivo*.

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