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UPTAKE OF N-ETHYLMALEIMIDE AND 1-FLUORO-2, 4-DINITROBENZENE IN RELATION TO THE IRREVERSIBLE INHIBITION OF GLUCOSE TRANSFER IN THE HUMAN ERYTHROCYTE

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SUMMARY

1. The uptake of the inhibitors N-ethylmaleimide (NEM) and 1-fluoro-2,4-dinitrobenzene (DNFB) by human red cells has been correlated with the inhibition of glucose exit.

2. With both inhibitors there was an initial rapid uptake by the cells with little inhibition; this was followed by a phase when inhibition was developing rapidly but uptake continued at a steady rate even after the development of inhibition had flattened off.

3. The rate of uptake of DNFB during the rapid development of inhibition corresponded to about 4×10^8 molecules/cell for 100% inhibition, irrespective of the temperature of incubation. This cannot be used as an estimate of the number of glucose transfer sites in the cell membrane because of the lack of specificity.

4. In an examination of lipids from red cells incubated with [^{14}C]DNFB, labelling associated with lipids was eluted with peaks in chloroform–methanol 4:1 and 1:4 respectively. Thus, although DNFB is normally regarded as a protein reagent, involvement of lipids in the transfer of glucose could not be excluded.

INTRODUCTION

Inhibition of the facilitated transfer of glucose through the human erythrocyte membrane produced by the reagents NEM (Dawson & Widdas, 1963) and DNFB (Bowyer & Widdas, 1956, 1958) has been shown to be irreversible and non-competitive. These reagents are capable of making covalent compounds with molecules having free –SH, $-NH_2$ or even –OH groups and have been extensively used as 'protein' reagents. Although the inhibitory reaction of these compounds on the transfer of

glucose in the erythrocyte has a number of anomalous features when studied kinetically (Sen & Widdas, 1962), the most probable explanation of the inhibition is that the reagents react with those membrane components with which glucose is presumed to form a complex during transfer across the membrane.

Kinetic studies do not normally distinguish between a large number of membrane components working slowly and a small number with a rapid turnover. Measuring the uptake of inhibitors is one means of obtaining an estimate of the numbers of molecules concerned, and this information is of obvious importance in any projected work to isolate the membrane components.

The availability of NEM and DNFB labelled with ^{14}C enables some of these problems to be examined further and, in the present work, the relation of the uptake of inhibitor by the human red cell to the inhibition produced has been studied. It has been shown that irreversibility is correlated with the persistence of dinitrophenol (DNP) labelling and a proportion of the labelling has been shown to be present in the lipid-soluble fractions of the cell membrane. A preliminary report of part of this work was made to the Physiological Society (Forsling, Remfry & Widdas, 1964).

METHODS

The inhibitor suspensions were 4·25 and 5·75 mM for NEM and 1·4 mM for DNFB, and contained a quantity of ^{14}C-labelled reagent. The labelled reagents were [^{14}C]*N*-ethylmaleimide, specific activity 1 mc/m-mole, as supplied by Schwarz Bioresearch Inc., and [U-^{14}C]DNFB, specific activity 4·37 mc/m-mole, as supplied by the Radiochemical Centre. Human erythrocytes, obtained on the day of the experiment by venepuncture, were incubated with 76 mM glucose and then with the inhibitor. As inhibition progressed, samples were removed for determination of (i) the percentage inhibition after the method of Bowyer & Widdas (1958) and Dawson & Widdas (1963), and (ii) the quantity of inhibitor bound by the erythrocytes.

Binding of radioactivity was determined in the following way. Samples of 1·0 ml. from the suspension of cells were added to 9 ml. ice-cold buffer, mixed well and centrifuged. An aliquot of the supernatant (0·2 ml.) was taken, mixed with a small volume (0·1 ml.) of 7·6 mM cysteine solution and dried on a planchet for counting. The cells were resuspended in 5 ml. buffer, spun down and further samples of the supernatant taken. The procedure was repeated so that the cells were washed 3 times. Finally the cells were suspended in 1 ml. saline and samples of 0·2 ml. were taken for counting and haemoglobin estimation. The number of cells in any sample was derived from the haemoglobin content relative to a standard made up of 50 mm^3 blood/100 ml.

Lipid extracts of inhibitor-treated cells were obtained by adding 50 ml. packed cells to 500 ml. redistilled butanol, the mixture standing overnight at 4° C. The extract was taken to a small bulk under a reduced nitrogen pressure at 45° C and subsequently applied to a column of silicic acid (Malinkrodt 100 mesh) and hyflo-supercel (Hopkins & Williams) in chloroform–methanol 9:1, and then separated by elution with chloroform–methanol mixtures in a manner similar to that described by Hanahan, Dittmer & Warashina (1957). Eluent fractions were collected and aliquots taken for phosphorous estimation, as described

by Taussky & Shorr (1953), and for determination of radioactivity using a thin-window Geiger counter. Those fractions containing peaks of activity were pooled, taken to dryness, and further separated on thin-layer chromatograms. Thin-layer plates were prepared as described by Stahl (1965), and the developing solvent used was chloroform–methanol–water (80:30:3). Radioactive spots were located by scanning the plates placed on a trolley moving under a thin-window Geiger counter. The output of the rate meter was registered on a chart recorder with a paper speed identical with that of movement of the trolley. Conventional lipid stains were used, as described by Skipski, Peterson & Barclay (1962).

RESULTS

[^{14}C]*N-ethylmaleimide.* Two experiments were carried out with this inhibitor. The mean supernatant concentrations were estimated to be 4·25 and 5·75 mM, although these changed by approximately 1 mM during $3\frac{1}{2}$ hr incubation at 21° C owing to the increasing cell uptake.

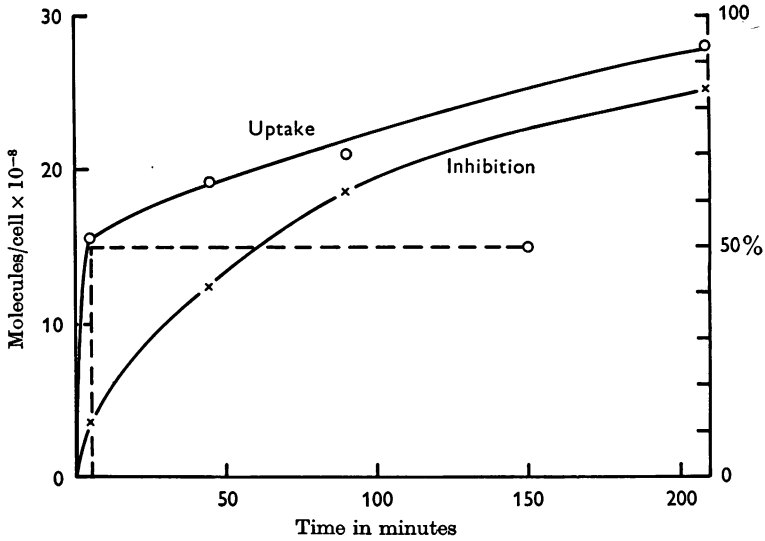

Fig. 1. NEM uptake related to inhibition of glucose exit. Points ○ and scale on left—uptake; points × and scale on right—percentage inhibition; point ○ and interrupted line—uptake of sample washed after 5 min and incubated without inhibitor. NEM 4·25 mM, 21° C.

Both results showed similar features to that in Fig. 1, which was for 4·25 mM NEM. There was a sharp initial uptake of inhibitor in the first 5 min of incubation with relatively little inhibition of glucose transfer. During the next hour, inhibition increased rapidly while uptake of inhibitor was at a slower steady rate.

A group of cells washed after 5 min incubation and reincubated in an NEM-free medium for $2\frac{1}{2}$ hr retained inhibitor at the same level as the

5 min sample. This excluded the possibility that the initial uptake was due to differential solubility in the cell membrane or cell contents.

If most of the rapid initial uptake was regarded as having no direct relevance to the glucose transfer system and the further uptake between 5 and 100 min related to the increase in inhibition of glucose exit during this time, it could be estimated that an uptake of 1×10^9 molecules of NEM/cell would be sufficient to give complete inhibition of glucose transfer.

[^{14}C]1-*fluoro*-2,4-*dinitrobenzene*. Since Bowyer & Widdas (1956) found incubation with 1·4 mM DNFB to be capable of producing almost complete inhibition of glucose exit without serious haemolysis, it was decided to use this concentration for uptake studies.

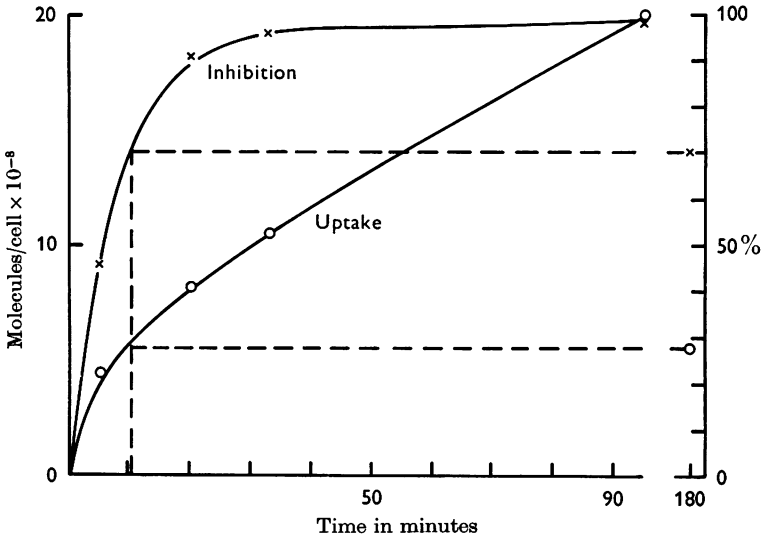

Fig. 2. DNFB uptake related to inhibition of glucose exit. Points ○ and scale on left—uptake; points × and scale on right—percentage inhibition; interrupted line shows sample washed after 10 min and incubated without inhibitor. DNFB 1·4 mM, 28° C.

Experiments on uptake and inhibition were carried out with incubation at four temperatures, 15° C, 21° C, 28° C and 33° C, to give different overall reaction rates. Apart from the time factor, the results were all generally similar to Fig. 2, which shows the results for 28° C.

With DNFB the rapid initial uptake was less well marked than for NEM and inhibition developed rapidly over the first 30 min (at this temperature). This graph shows that from 30 to 90 min there was a progressive steady uptake, although inhibition had flattened off.

As with NEM, a sample withdrawn at an early stage (10 min), when washed and reincubated until 180 min, had labelling and degree of inhibi-

tion appropriate to the initial incubation time. These results suggested that the irreversible inhibition was due to the formation of DNP-derivatives and that these were stable and not lost from the cells during subsequent incubation.

At 15° C 3 hr incubation time was necessary to achieve 95 % inhibition, whereas at 33° C this was reached in 15 min. A similar twelvefold change in time scale accompanied uptake and there was a relation between inhibition and uptake, independent of temperature of incubation, as shown in Fig. 3.

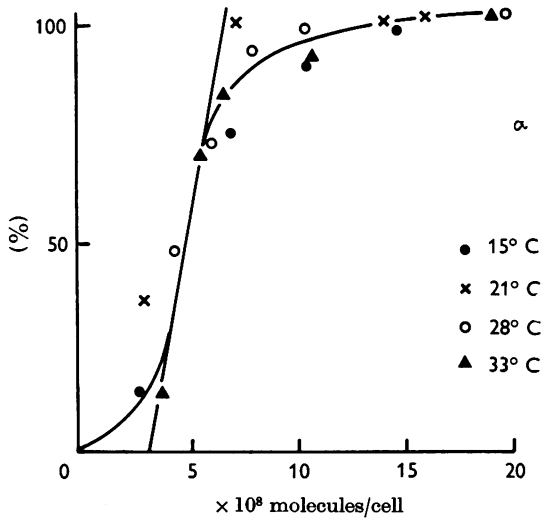

Fig. 3. Temperature-independent relation between inhibition and DNFB uptake. Results from experiment at 15° C—points ●, at 21° C—points ×, at 28° C—points ○, and at 33° C—points △. The line of greatest slope corresponds to 4×10^8 molecules/cell for 100 % inhibition.

This relationship, which is sigmoid, can be considered in three parts: an early phase when uptake is occurring rapidly but little inhibition is resulting, a phase when inhibition is developing rapidly for little uptake, and a late phase when inhibition has flattened out but uptake is proceeding.

The second phase, which has the steepest slope, would suggest that an uptake of 4×10^8 molecules of DNFB/red cell would be sufficient to produce full inhibition of glucose transfer. Since the uptakes associated with the early and later phases would presumably contribute to the uptake in this steep region, however, this figure must be regarded as an over-estimate.

Lipid-soluble extracts of DNFB-treated erythrocytes. When cells were incubated with [^{14}C]DNFB under conditions similar to those used for uptake studies, but without repeated washings, and subsequently extrac-

ted with butanol, about 1–3% of the labelling could be detected in the butanol-soluble lipids. The extracted lipids were separated on silicic acid columns and labelled fractions further chromatographed on thin-layer plates.

With eluting solvents of chlorofrom–methanol in the ratios 9:1, 4:1, 3:2 and 1:4, it was found that free DNFB was eluted early in chloroform–methanol 9:1. The position of elution of marker lipids such as cholesterol, cardiolipin, cerebrosides, phosphatidyl ethanolamine, phosphatidyl serine, lecithin, lysolecithin and sphingomyelin were also noted, but in mixtures of these some overlapping was observed.

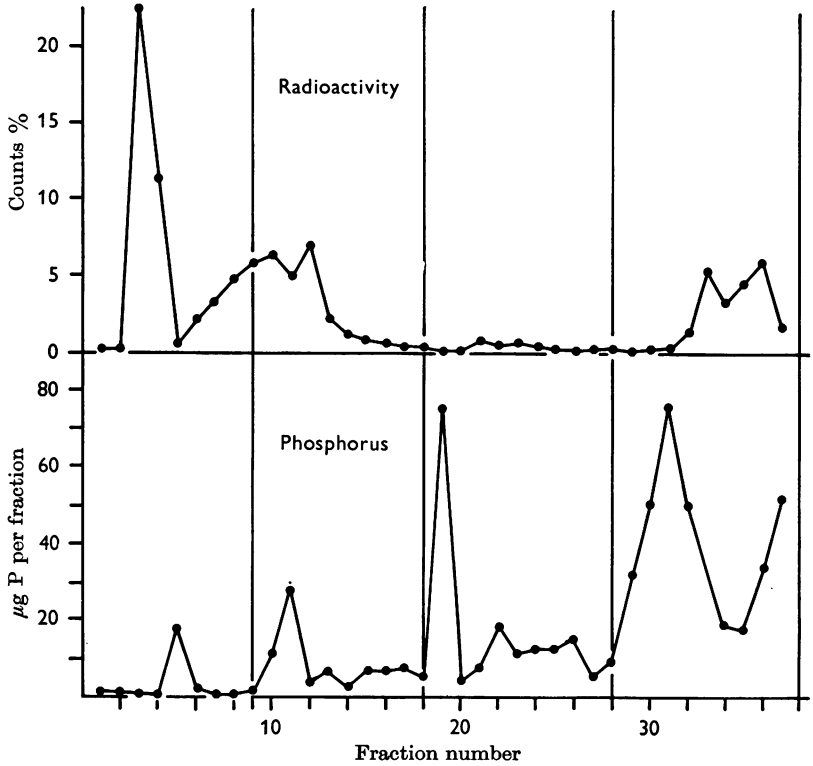

Fig. 4. Silicic acid chromatogram of lipids from red cells incubated with [^{14}C]-DNFB. Upper line—elution pattern of radioactive labelling; lower line—elution of lipid phosphorus. Elution solvents were chloroform–methanol 9:1, 4:1, 3:2 and 1:4.

Figure 4 shows the fractionation of red cell lipids on a silicic acid column. The elution of lipid phosphorus is compared with that of [^{14}C]DNFB-derivatives carrying label. Although the majority of the label is eluted early, there are two other regions of substantial elution with peaks in chloroform–methanol 4:1 and 1:4 respectively.

Thin-layer chromatography resolved the labelling into a number of spots associated with iodine-staining lipids, but their chemical identity was uncertain because of the possibility that a DNP-derivative would have a different R_F value from the standard markers. (In further work, using acid hydrolysis, the only DNP-derivative confidently identified (Forsling, 1967) was ethanoline-DNP, presumably derived from phosphatidyl ethanolamine, and this was eluted earlier from the column than a phosphatidylethanolamine standard.)

Allowing for the fact that the lipid soluble labelling was only a small fraction of the total uptake, it was estimated that, if this labelling was associated with the inhibition of glucose transfer, only 10–20 million molecules of DNFB would be needed per cell for full inhibition. The non-free DNFB amounted to 4×10^6 and 3×10^6 molecules/cell in the early and late peaks respectively.

However, it was also shown that sheep red cells incubated with [^{14}C]DNFB had a comparable though smaller uptake of labelling in the cell lipids, and since such cells lack a facilitated transfer for glucose such an estimate would be largely invalidated.

DISCUSSION

The kinetic studies with the irreversible inhibitors NEM and DNFB have shown a number of anomalies. Bowyer & Widdas (1956) found a high Q_{10} for the reaction of DNFB and showed that incubation in the presence of glucose accelerated the rate of development of inhibition. Dawson & Widdas (1963) similarly found a high Q_{10} for the reaction of NEM.

Another anomaly is that, with DNFB, exit into a glucose-free medium is inhibited more than entry of glucose (Bowyer & Widdas, 1958; Sen, 1960). This is consistent with a competitive inhibitor and the possibility of DNFB forming a compound which acts as a membrane-fixed competitive inhibitor was considered, but Sen & Widdas (1962) could find no change in the half-saturation concentration for glucose, as might have been expected.

The uptake studies throw little light on these anomalies. There is a rapid initial uptake which may be associated with reactions occurring on the outside of the membrane and which is associated with some inhibition of glucose transfer. It would appear, however, that inhibition of glucose transfer occurs chiefly during a second uptake phase, whereas the later phase of uptake, after inhibition has started to flatten off, would presumably represent reactions with intracellular proteins and other compounds.

The uptake studies, however, do confirm that, associated with a particular degree of inhibition, there is a stable binding of labelling derived from DNFB which presumably represents DNP-derivatives. Both with NEM

and DNFB there is no subsequent leaching or loss of radioactivity in a subsequent incubation, and therefore the technique could reasonably be used to label components involved in the facilitated transfer of glucose.

Unfortunately the reagents may be attacking many different compounds and this lack of specificity for the facilitated transfer system is a serious drawback.

That the inhibition by DNFB is not just a general stiffening of the membrane, as proposed for tannic acid by Hunter (1964), is suggested by the findings of Dawson & Widdas (1963) that the activation energy for the maximal transfer rate is not increased in NEM-inhibited cells, nor the penetration of malonamide reduced. Forsling (1967) has also shown that the penetration of malonamide, which follows diffusion-type kinetics, is not affected by incubation with DNFB to a point where glucose exit was 99% inhibited.

Although there is therefore no reason to question the hypothesis that NEM and DNFB inhibit glucose transfer by directly combining with the membrane components concerned in the facilitated transfer of glucose, the lack of specificity makes it impossible to use the uptake measurements to estimate the amount of such components present.

The estimate of 4×10^8 molecules/cell, made from the slope of uptake of DNFB against inhibition (Fig. 3), must be a gross over-estimate. LeFevre (1961), incubating ghosts with low concentrations of [^{14}C]glucose, calculated that the number of sites involved in glucose transfer was unlikely to be greater than 500,000/cell. Weed, van Steveninck & Rothstein (1964) used a number of mercurial compounds which inhibit glucose transfer and which are known to react strongly with sulphydryl groups. They found 90% of the mercurial associated with haemoglobin, 6% with glutathione and 4% with the membrane. Glucose transfer was inhibited when 1·2% of the membrane groups had reacted, and from this they calculated the maximum number of sites in the intact cell to be 700,000/cell.

Against these estimates, the uptake of DNFB, even in the lipid fractions, is in great excess and differences observed between human and sheep cells would be sufficient to cover the uptake of DNFB by the components involved in facilitated glucose transfer. It is clear therefore that, if used to label sites involved in glucose transfer, such reagents as NEM and DNFB must be used alongside techniques for careful subfractionation and separation of the extracts.

Stein (1964) used DNFB labelled with ^{3}H and ^{14}C to make a study of peptide labelling in erythrocyte membranes by a technique designed to differentiate centres concerned in glucose transfer. Peptide labelling of the order of 2×10^6 molecules/erythrocyte ghost was obtained.

If the lipid-soluble labelling obtained in the present experiments had

been below the estimates of LeFevre (1961) and Weed *et al.* (1964) referred to above, a strong indication would have been given that proteins were involved in the glucose transfer system. As it stands, this point is unresolved by these experiments and the results are not inconsistent with a component of the lipid-soluble fraction being important, as is suggested from other work (LeFevre, Habich, Hess & Hudson, 1964; Mawdsley & Widdas, 1967).

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