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A FLUORESCENT CHEMICAL MARKER FOR THE LIVER CELL PLASMA MEMBRANE

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SUMMARY

The binding of a fluorescent stilbene isothiocyanate disulfonic acid (SITS) by intact liver cells and cell fractions was studied. Whole cells and isolated cell fractions bind SITS to varying degrees. The binding is markedly inhibited by Hg^{2+} and iodoacetamide. *p*-Chloromercuribenzoate inhibits binding of SITS by whole cells, microsomes and cytoplasm but enhances uptake by isolated mitochondria. Phospholipase C slightly inhibited SITS uptake by whole cells and cell fractions. When intact liver cells were labeled with SITS, washed, and homogenized to obtain the cell fractions, nearly all the SITS was found in the mitochondrial fraction and none was detected in the cell cytoplasm and purified nuclei. These results are interpreted to mean that the plasma membrane contains the bound SITS (which cannot penetrate the cell membrane) and that these fragmented plasma membranes centrifuge mainly with the mitochondria. SITS therefore serves as a specific chemical marker for the plasma membranes of intact cells.

A key observation which made this study possible was our finding that strong KOH markedly intensifies the fluorescence of SITS so that it can easily be detected. The spectral and chromatographic properties of SITS were also examined.

INTRODUCTION

MADDY[1] prepared the fluorescent compound 4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulfonic acid (SITS) and examined its reaction with isolated red blood cells. His studies indicate that this compound reacts specifically with the red-cell membrane and does not penetrate the cell. If these observations apply to other mammalian cells then SITS is potentially an excellent marker for the plasma membranes of intact cells and as such may facilitate the fractionation and purification of these membranes. A "membrane marker" may also help with studies concerning the

Abbreviations: PMCB, *p*-chloromercuribenzoate; SITS, 4-acetamido-4′-isothiocyanate-stilbene-2,2′-disulfonic acid.

turnover rate of plasma membranes and on the physical and chemical changes which occur in the plasma membrane of the cell as a result of the action of various agents such as enzymes, hormones and drugs. With these aims in mind we examined the binding of SITS by isolated whole liver cells, liver mitochondria, microsomes, cytoplasm and by plasma membranes isolated from rat liver.

METHODS AND MATERIALS

SITS was obtained from the British Drug Houses. Whole cells of rat liver were prepared by a procedure similar to BRANSTER AND MORTON[2] except that the liver was gently mashed with a rubber stopper. Plasma membranes were prepared by the method of NEVILLE[3] as modified by EMMELOT *et al.*[4]. The "physiological" salt solution consisted of the following (a) 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4, 0.2 g KH_2PO_4; these were dissolved in 800 ml distilled water; (b) 0.1 g $CaCl_2$ in 100 ml water and (c) 0.1 g $MgCl_2 \cdot 6H_2O$ in 100 ml water. The three solutions were autoclaved separately, cooled and mixed (Solution A).

The citrate buffer was prepared as follows: 0.9 g NaCl, 0.042 g KCl, 0.01 g $NaHCO_3$, 0.1 g glucose and 0.569 g citric acid were dissolved in water, the pH adjusted to 7.0 with NaOH and the volume brought to 100 ml with water (Solution B).

Liver or isolated liver cells were disrupted with a teflon homogenizer in 0.25 M sucrose and mitochondria, microsomes and cell cytoplasm were prepared by differential centrifugation in the usual way[5] using a Spinco Model L preparative ultracentrifuge.

Spectral analyses and absorbance measurements were carried out on a Hilger spectrophotometer using 3-ml quartz cells (1-cm light path).

Naja naja venom (cobra) was used as a source for phospholipase A and *Clostridium welchii* toxin was used as a source for phospholipase C.

Iodoacetamide and *p*-chloromercuribenzoate (PCMB) were obtained from the British Drug Houses.

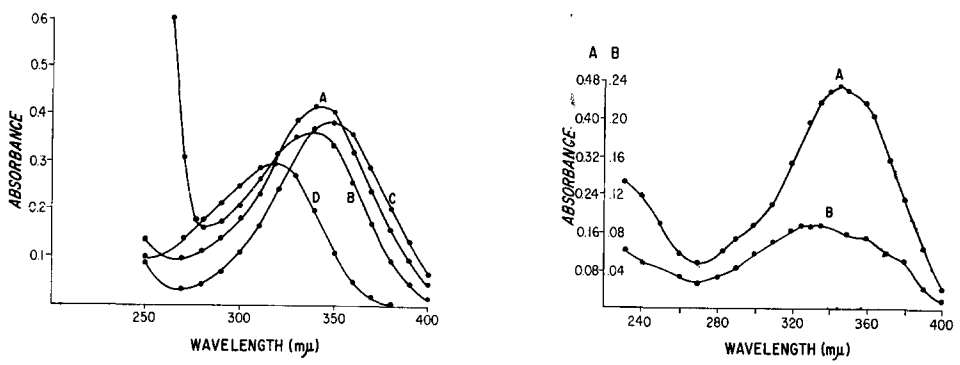


Fig. 1. The absorption spectra of SITS in aqueous medium under different conditions. A, spectrum of 10 μM SITS in Solution A, pH 7.0. B, spectrum of 10 μM SITS in 8% trichloroacetic acid. C, spectrum of 10 μM SITS in 14% KOH. D, spectrum of 10 μM SITS in 90% H_2SO_4.

Fig. 2. The absorption spectra of SITS in organic solvents. A, spectrum of 0.1 mM SITS in methanol–tetrahydrofuran (2:3; v/v). B, spectrum of approx. 10 μM SITS in tetrahydrofuran.

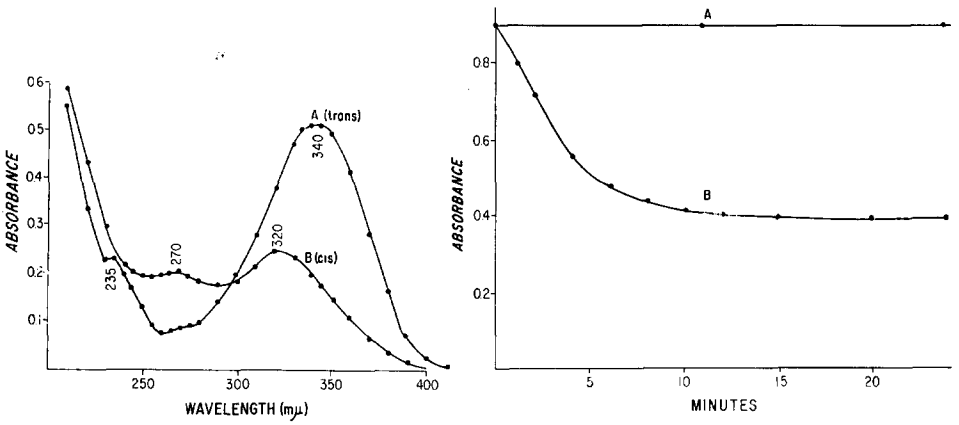


Fig. 3. The effect of light on the absorption spectrum of SITS. A, spectrum of non-exposed 125 μM SITS in Solution A, pH 7.0. B, spectrum of same solution of SITS given in A but exposed to daylight for 30 min.

Fig. 4. The change in absorbance of a solution of SITS exposed to subdued light and to daylight. A, the absorbance of 20 μM SITS in Solution A, pH 7.0, exposed to subdued light from an incandescent lamp (75 W) at a distance of about 10 ft. B, the absorbance of the same solution in A but exposed to daylight.

Spectral analysis of SITS

The spectra of SITS in aqueous medium (under acid, neutral and alkaline conditions), in a polar organic solvent (tetrahydrofuran–methanol, 1:1; v/v) and in a relatively non-polar solvent (tetrahydrofuran) are depicted in Figs. 1 and 2. The effect of light on the spectrum of SITS is shown in Figs. 3 and 4. The absorption maxima and molar extinction coefficients of SITS under various conditions are given in Table I.

In acid solution (trichloroacetic acid and H_2SO_4) the fluorescence of SITS as seen under ultraviolet light at 366 mμ is diminished whereas in strong alkaline solution there is a considerable increase in the fluorescence with a change from blue to

TABLE I

SPECTRAL PROPERTIES OF SITS

Solvent	*Absorption bands (mμ)*		*Molar extinction coefficient*	
	Major	*Minor*	*Major*	*Minor*
Water, pH 7.0	340	235	42 300	18 800
Water, pH 7.0 (light exposed)*	320	270	19 600	16 500
Water, 8% trichloroacetic acid	340	—	36 400	—
Water, 90% H_2SO_4	320	—	30 300	—
Water, 14% KOH**	350	—	39 200	—
Methanol–tetrahydrofuran	345	360 shoulder	—	—
Tetrahydrofuran	325, 335	360, 380 shoulders	—	—

* Light causes a photochemical isomerization of the *trans* to the *cis* form[1,6].

** In KOH the fluorescence of SITS is very markedly intensified and changes from blue to yellow-green.

an intense yellow-green. The molar extinction coefficients are decreased in acid solution and also when the *trans* isomer is photochemically changed to the *cis* isomer. The absorption maximum is also influenced by solvent and by light. Exposure of SITS to daylight not only causes a decrease in the molar extinction coefficient but also shifts the absorption maximum from 340 mμ to 320 mμ and gives rise to a new band at 270 mμ. H_2SO_4 shifts the peak from 340 mμ to 320 mμ whereas KOH shifts the peak from 340 mμ to 350 mμ. In a relatively non-polar solvent such as tetrahydrofuran where intermolecular and intramolecular reactions between SITS can occur the spectrum is broader and more complex and displays two major bands at 325 mμ and 335 mμ. The spectral properties which we observed are not in exact agreement with those reported by MADDY[1] since he gives the absorption maximum for the *trans* isomer at 335 mμ and for the *cis* isomer at 265 mμ. We find these peaks at 340 mμ and 270 mμ, respectively.

The photochemical effect of daylight on the absorption of SITS was followed with time and is shown in Fig. 4. 43% of the absorption at 340 mμ is lost after 15 min exposure. The loss of absorbance is rapid within the first 5 min and gradual over the next 20 min.

LEWIS, MAGEL AND LIPKIN[6] studied the absorption and re-emission of light by *cis*- and *trans*-stilbenes and observed that the *trans* isomer was strongly fluorescent whereas the *cis* isomer had very little fluorescence. The *trans* isomer had a wide absorption band between 270 mμ and 330 mμ and the *cis* isomer showed absorption over the 260–300-mμ range. The molar extinction coefficient of the *trans*-stilbene at 290 mμ was 24 000 and for the *cis*-stilbene was 10 000.

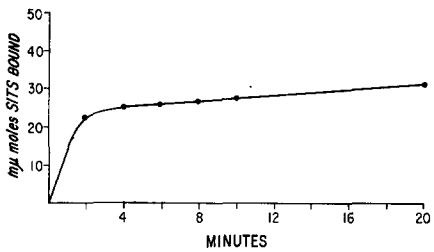
DETAR AND CARPINO[7] studied the spectral properties of 2-substituted *cis*- and *trans*-stilbenes and found that the absorption maxima for the *trans* compounds in the ultraviolet were shifted 15–40 mμ to a longer wavelength as compared to the *cis* isomers and the value of the extinction coefficients were 1.5–3 times greater for the *trans* isomers.

Chromatographic properties of SITS

MADDY[1] reported that the *cis* and *trans* isomers of SITS were resolved by paper chromatography in a solvent consisting of 0.2 M NH_4OH. In our hands this solvent gave elongated spots with only partial resolution of the two isomers. We found that *n*-propanol–NH_4OH–water (6:3:2; by vol.) gave a complete separation of the two isomers and yielded discrete spots. The R_F value of the *cis* and *trans* isomers were 0.73 and 0.41, respectively. *n*-Butanol–pyridine–water (10:8:5; by vol.) also gave discrete spots but the resolution was not as good. In an aqueous borate buffer (pH 8.0) the *cis* isomer moved slower than the *trans* isomer but the spots were elongated and the resolution was poor. Both the *cis* and *trans* isomers were seen as blue fluorescent spots (on dry chromatograms) under ultraviolet light (366 mμ). The SITS as purchased (from the British Drug Houses) was essentially all in the *trans* form.

Uptake of SITS by isolated whole liver cells and cell fractions

The purity and viability of the isolated whole liver cells were checked by phase-contrast microscopy, and by fluorescence microscopy after staining with acridine orange.



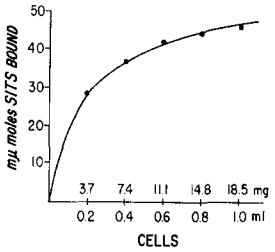


Fig. 5. The time course uptake of SITS by whole liver cells of the rat. 0.4 ml of whole cell suspension (7.4 mg dry wt. of cells) in Solution A was treated with 0.5 ml of 0.1 mM SITS for 2, 4, 6, 8, 10, 20 and 30 min at room temperature. The reaction was stopped at the time indicated by adding 1.0 ml of 40% trichloroacetic acid. The solutions were brought to a total volume of 5.0 ml and centrifuged. The absorbances of the supernatants were determined at 340 mμ and the uptake of SITS calculated from a calibration curve. Controls were also run containing the same amount of cells but no SITS. The values for these controls were subtracted from the respective values of tubes containing both cells and SITS.

Fig. 6. The uptake of SITS by whole cells as a function of the amount of cells. 0.2–1.0-ml aliquots of whole cell suspension in Solution A (0.4 ml = 7.4 mg dry wt. cells) were brought to a volume of 1.0 ml with Solution A. To each solution was added 0.5 ml of 0.1 mM SITS and incubated for 20 min at room temperature. To each tube was then added 1.0 ml of 40% trichloroacetic acid and water to make a total volume of 5.0 ml. The tubes were centrifuged and the absorbances of the supernatants determined at 340 mμ. Controls were also run containing the same amount of cells but no SITS. The values for these controls were subtracted from the respective values of tubes containing both cells and SITS.

The relationship of the time of incubation *versus* SITS uptake and the amount of intact whole cells *versus* SITS uptake are shown in Figs. 5 and 6, respectively. These data are plotted in Fig. 7 to show the per cent of added SITS which is taken up by the cells. There is an initial rapid 2-min uptake (Fig. 5) followed by a slower prolonged uptake over a period of 30 min. Within the first 2 min about 45% of the added SITS is bound. During the next 18 min an additional 17% of the added SITS is taken up. This observation might indicate at least two different binding sites on the membrane for SITS. Figs. 6 and 7 also show that the uptake of SITS is not a linear function of the amount of cells.

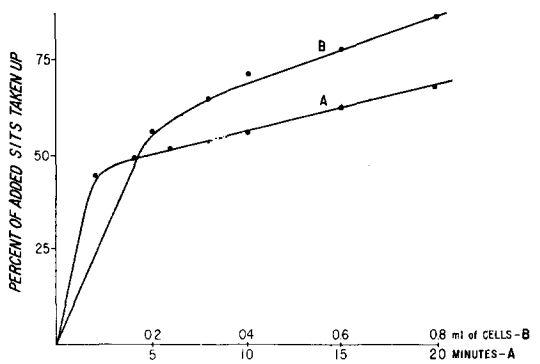


Fig. 7. The relationship between the percentage of added SITS which is taken up by whole cells *versus* the amount of cells and time of incubation. The data in Figs. 5 and 6 are shown here as the percentage of the added SITS which is taken up by the cells.

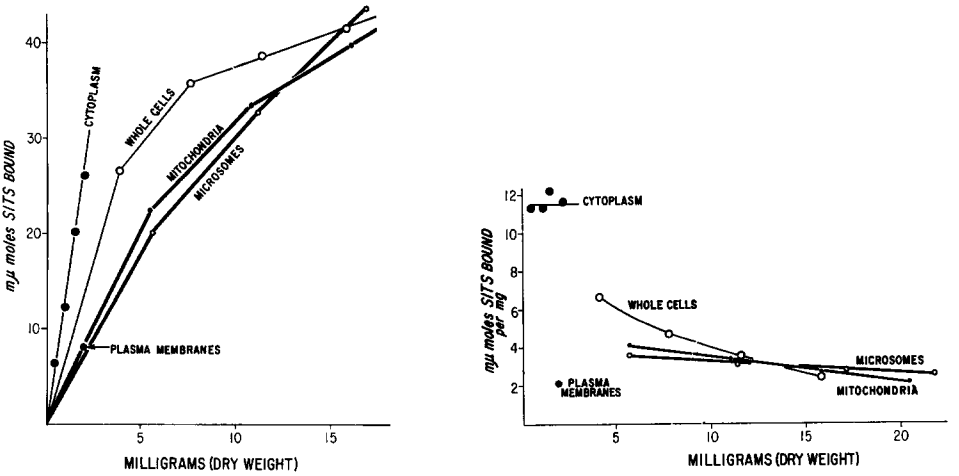


Fig. 8. The uptake of SITS by whole cells and cell fractions of rat liver. The data are obtained from the experiments given in Figs. 5 and 6 and Tables II and III and are plotted here to show the millimicromoles of SITS taken up *versus* the amount of sample.

Fig. 9. The relationship between the amount of SITS bound per unit weight of cell fraction *versus* the amount of cell fraction. The data are taken from Figs. 5 and 6 and Tables II and III and show the uptake of SITS per mg of cell fraction *versus* the dry weight of cell fraction.

The relationship between the millimicromoles of SITS taken up by the whole liver cells and cell fractions *versus* the amount of each cell fraction is shown in Fig. 8 whereas the relationship between the millimicromoles of SITS taken up per mg of sample *versus* the amount of sample is shown in Fig. 9. The cytoplasm takes up more SITS on a unit weight basis than do the whole cells or the cell fractions. This is expected since the cytoplasm has proteins in solution and hence more of the sites on the protein are available for reaction with SITS. The whole cells take up more SITS than the isolated cell mitochondria, microsomes or plasma membranes. The value for the isolated cell membranes is the lowest but represents only one sample. This low value is partly due to the fact the isolated membranes tended to clump together and although they were homogenized before testing, they were difficult to disperse. One must consider this value to be preliminary and a minimal one.

Effect of various agents on the uptake of SITS

The effect of several agents on the uptake of SITS was also studied. In order to see if sulfhydryl groups were involved, the effect of Hg^{2+}, PCMB and iodoacetamide was examined. To test the role of lipids in the uptake, the effect of *Clostridium welchii* phospholipase C and cobra venom phospholipase A was tested. The effect of succinate was tested on the isolated mitochondria in order to see if mitochondrial swelling affected the SITS uptake. The data are given in Tables II and III.

It can be seen that Hg^{2+} and iodoacetamide inhibited the uptake of SITS by all the systems studied and that PCMB markedly inhibited SITS uptake by liver cells, microsomes and cytoplasm but not by mitochondria. Indeed, PCMB enhanced SITS uptake by mitochondria. This finding was unexpected and raised the question of whether the mitochondrial membrane had any sulfhydryl groups or whether there

TABLE II

THE EFFECT OF IODOACETAMIDE AND PCMB ON THE BINDING OF SITS TO MEMBRANES OF WHOLE CELLS, MITOCHONDRIA AND MICROSOMES OF RAT LIVER

0.4 ml whole cells (7.4 mg dry wt.) suspended in Solution A was incubated at room temperature for 20 min, then treated with 0.5 ml 0.1 mM SITS (50 mμmoles) for 30 min. The iodoacetamide and PCMB dissolved in Solution A, were added to 0.4 ml whole cells which were incubated for 20 min, then 50 mμmoles of SITS (in Solution A) were added and the cells incubated for 30 min. The reaction was stopped by adding 1.0 ml of 40% trichloroacetic acid, and water added to make a total volume of 5.0 ml. The tubes were centrifuged for 2 min at 4600 rev./min and the absorbances of the supernatants measured at 340 mμ in order to determine the SITS uptake. Controls were also run of cells without SITS and their absorbances subtracted from the sample containing cells and SITS. Controls were also run to see if iodoacetamide and PCMB alone had measurable absorbance at 340 mμ and to see if they affected the absorbance of SITS. These results were negative. The SITS uptake was calculated from a calibration curve which was linear over the range of 5 mμmoles to 100 mμmoles of SITS. 0.4 ml (11 mg dry wt.) of microsomes and 0.4 ml (3 mg dry wt.) of mitochondria were used in these experiments. All procedures were carried out in subdued incandescent light under which conditions SITS was stable (see Fig. 4, and Curve A).

	mμmoles of SITS bound		
	Whole cells	*Microsomes*	*Mitochondria*
Control	33 ± 0.5	33 ± 0.6	9 ± 1.5
Iodoacetamide: 2 μmoles	27 ± 0.5	24 ± 1.2	6 ± 0.6
PCMB: 1 μmole	12 ± 0.5	16 ± 0.4	15 ± 1.1

were sulfhydryl groups on the membrane which did not react with SITS. It is also possible that PCMB caused the mitochondria to be more permeable to SITS or to swell and release components which could react with SITS.

The effect of iodoacetamide, PCMB and Hg^{2+} support the involvement of sulfhydryl groups in the binding of SITS but iodoacetamide is also known to react with histidine groups. The chemical work done on the interaction of cyanates and thiocyanates with proteins has shown that these agents react with terminal amino groups, sulfhydryl groups, tyrosyl groups, and possibly guanidinium groups[8–12].

Phospholipase A gave variable effects with whole cells and intact mitochon-

TABLE III

THE EFFECT OF PHOSPHOLIPASES AND MERCURIC ION ON THE BINDING OF SITS TO WHOLE CELLS AND CELL FRACTIONS OF RAT LIVER

In these experiments the following procedure was used: 0.4 ml whole cell suspension (approx. 5.0 mg dry wt.); 0.2 ml mitochondria (11 mg dry wt.); 0.2 ml microsomes (11.4 mg dry wt.); and 0.3 ml cytoplasm (1.6 mg dry wt.). The cells or cell fractions were first incubated with the lipase or Hg^{2+} for 20 min at room temperature and then 50 mμmoles of SITS were added and incubation continued for 15 min more. 1 ml of 40% trichoroacetic acid was then added and water to make a total volume of 5.0 ml. The remaining experimental details are the same as those given in Table II.

	mμmoles of SITS bound			
	Whole cells	*Mitochondria*	*Microsomes*	*Cytoplasm*
Control	20 ± 0.4	33 ± 0.5	33 ± 0.5	20 ± 0.5
Phospholipase C: 1 unit	18 ± 0.2	29 ± 0.5	31 ± 0.6	18 ± 0.5
Phospholipase A: 1 μg	22 ± 0.7	32 ± 0.6	31 ± 0.6	18 ± 0.5
Hg^{2+}: 1 μmole	13 ± 0.7	19 ± 0.4	22 ± 0.5	15 ± 0.4

dria but produced a small but significant inhibition of SITS uptake with microsomes and cytoplasm. Phospholipase C gave a small inhibition with whole cells and all the cell fractions.

Cellular localization of SITS taken up by whole cells

The above studies show (as expected) that all the cell fractions can bind SITS.

In order to test whether the SITS taken up by whole cells was confined only to the plasma membrane two approaches were employed. In the first approach the isolated cells were treated with SITS, the cells washed, homogenized, and the cell fractions obtained and examined for SITS. The results of these experiments (Table IV) showed repeatedly that the major amount of the SITS was localized in the mitochondrial fraction, some was also located in the nuclear and microsomal fraction and essentially none occurred in the cell cytoplasm. When the nuclear fraction was purified there was no detectable amount of SITS present.

The question arose whether the SITS was indeed on the membrane of the mitochondria (and microsomes) or whether it was only on the plasma membrane but this was contaminating the mitochondrial and microsomal fractions. A preliminary

TABLE IV

LOCALIZATION OF SITS IN THE VARIOUS LIVER CELL FRACTIONS

In Expts. 1 and 1A, whole cells, prepared from two rat livers were treated with 2 ml of 1 mM SITS at room temperature for 15 min. The cells were divided into two halves and washed four times with Solution A. From one half, subcellular fractions were prepared in 0.25 M sucrose (Expt. 1). Subcellular fractions were also prepared from the other half in bicarbonate medium (pH 7) during an abortive attempt to prepare plasma membranes (Expt. 1A). In Expt. 2 the cells from another liver were prepared and treated as in Expt. 1. In Expt. 3 a rat liver was perfused *in situ* with 50 ml of Solution B, then with 10 ml of Solution B containing 5.0 mg SITS. The liver was let stand 0.5 h at room temperature and then washed by perfusion with 80 ml of Solution B. The whole cells were prepared in the usual way, the cells were washed, homogenized, and the subcellular fractions prepared in 0.25 M sucrose. To measure the SITS, each fraction was adjusted to the same volume and 1-ml aliquots were treated with 1 ml of 70% KOH and then diluted to 5.0 ml with water. The absorbances were determined at 350 mμ. The intense yellow-green fluorescence of SITS which is produced in strong alkaline solution was detected visually under ultraviolet light at 366 mμ.

Expt. No.		$A_{350\ m\mu}$*	*KOH-enhanced SITS fluorescence at 366 mμ*
1 and 2	Nuclei	0.53 (0.64)	Moderate
	Mitochondria	0.76 (0.56)	Very strong
	Microsomes	0.27 (0.22)	Very weak
	Cell cytoplasm	0.17 (0.07)	None
1A	Nuclei	0.08	None
	Mitochondria	0.54	Very strong
	Microsomes	0.14	Very weak
	Cell cytoplasm	0.25	None
3	Nuclei	**	**
	Mitochondria	0.43	Very strong
	Microsomes	0.15	Very weak
	Cell cytoplasm	0.06	None

* The values for Expt. 2 are given in parentheses.
** The nuclear fraction in this experiment was lost.

attempt to subdivide the mitochondrial fraction by density-gradient centrifugation was unsuccessful.

In the second approach intact liver was perfused with SITS. A rat was killed, the abdomen was opened and the liver was perfused *in situ* first with Solution A by inserting a needle in the superior vena cava and draining from the inferior vena cava, and then with the same solution containing SITS. In two experiments liver was let stand for 15 min and 30 min, respectively. The liver was washed with Solution A to remove excess SITS and a suspension of whole cells was prepared. The cells were washed and then fractionated into subcellular components. The major part of the SITS occurred in the mitochondrial fraction and again no SITS was detected in the cell cytoplasm (Table IV, Expt. 3). These findings confirmed the previous experiment in which the whole cells were prepared and then labeled *in vitro* with SITS.

The quantitation of the uptake of SITS by whole cells or cell particles is easy to carry out since the reaction is stopped by trichloroacetic acid and centrifugation gives a clear supernatant which can be measured at 340 mμ. However, the measurement of SITS which has been bound to the cell particles is much more difficult since the SITS is covalently bound to the cell particles which must be solubilized in order to carry out absorbance measurements. Attempts were made to solubilize the particles with strong acid (H_2SO_4), strong base (KOH and tetraethylammonium hydroxide) and with deoxycholate. Strong acid was not successful since the absorption and fluorescence of SITS was markedly diminished. The deoxycholate solubilized the particles but there was still sufficient opalescence to make readings at 340 mμ useless. The strong KOH gave the best results, but the solutions of the particles were still opalescent and hence readings of absorbances at 350 mμ did not give accurate quantitative measurements of SITS uptake. However, the major advantage of using KOH was the intense yellow-green fluorescence it produced from solutions of SITS under ultraviolet light for this fluorescence proved a very sensitive indicator for the presence of SITS in the samples. Some of the control cell fractions (without SITS) produced a pale yellow color when treated with strong KOH but their fluorescence was very weak.

An attempt was made to prepare plasma membranes from a suspension of whole cells, labeled with SITS, in water adjusted to pH with $NaHCO_3$ by the method of EMMELOT *et al.*[4]. Unfortunately a plasma membrane fraction was not obtained but in the subcellular fractions which were also prepared essentially all the SITS was present in the mitochondrial fraction and none was detected in the nuclear fraction (*cf.* Table IV, Expts. 1 and 1A).

It is interesting that the plasma membranes could not be isolated from isolated whole cells under any of these conditions but they were isolated from intact liver by EMMELOT'S method[4]. Attempts on our part to isolate plasma membranes by EMMELOT'S method from intact whole cells or from liver which was first perfused with Solution A, Solution B, or isotonic saline have been unsuccessful. The plasma membrane appears to be altered under these conditions such that it does not appear in the density gradient in the usual place.

DISCUSSION

Cell membranes are currently receiving much attention by a variety of workers in different disciplines of science[13–18]. The structure of the membrane at the molecular

level is still a highly controversial and speculative subject due to the inherent difficulties in the interpretation of electron micrographs and X-ray diffraction patterns of membrane-containing systems. The limitations incurred by fixation methods which invariably alter the "true" structure of membranes to varying degrees, coupled with a lack of the basic chemistry involved with fixatives and cellular components have posed major problems in this field. Certain membranes such as myelin, the red-cell membrane, mitochondrial and microsomal membranes are easier to prepare than the outer cell membrane (called the plasma membrane). The method of NEVILLE[3] or its modification by EMMELOT *et al.*[4] are two of the very few procedures employed at the present time to isolate plasma membranes but in our hands the yields were low (about 2 mg freeze-dried membranes from approximately 40 g rat liver*). This low yield indicates that an appreciable part of the plasma membrane must have been present in the other cell fractions obtained by centrifugation.

When liver cells are disrupted to prepare membranes and cell particles the plasma membranes must obviously be ruptured first and are probably ruptured at more than one point giving rise to fragments of different sizes. Where the fragments end up upon differential centrifugation has not been studied very carefully since they are not easily seen by the light microscope and also are hard to identify by electron microscopy. It seems reasonable to expect that with the present procedures for the preparation of subcellular components some plasma membrane fragments may occur in the nuclear, mitochondrial and nuclear fractions, but the extent of plasma membrane contamination in these fractions remains an unsolved problem. Mitochondrial and microsomal fractions obtained from rat liver are not homogeneous when examined by electron microscopy and the per cent contamination by other cell fragments, especially fragmented plasma membranes would be difficult indeed to assess unless there were some way to specifically label the plasma membrane. If the microsomal membrane is continuous with the plasma membrane at several points[18] mutual contamination is inevitable and quantitative isolation impossible!

The work of MADDY[1] on the binding of SITS to the red-cell membrane suggested that this compound might also bind to the plasma membranes of other mammalian cells without penetrating the cells. Our experiments with liver cells supported this since when isolated cells were labeled *in vitro* with SITS or when the cells were labeled *in situ* by perfusing a liver with SITS and then preparing whole cells, the subsequent fractionation of these labeled cells showed that no SITS occurred in the cell cytoplasm but rather occurred primarily in the mitochondrial fraction. Some SITS was located in the microsomal and nuclear fractions but not in purified nuclei. Our studies showed (as expected) that SITS could bind to all isolated cell fractions and the cell cytoplasm which contained soluble proteins bound more SITS on a unit weight basis than did mitochondria, nuclei and microsomes. If the SITS had penetrated the plasma membrane of intact cells it would be expected that the cytoplasm would contain SITS but this was not the case. It was thus concluded that the SITS did not penetrate the plasma membrane and thus was bound only to this membrane. It followed that the SITS located in the mitochondrial, nuclear and microsomal fractions obtained by fragmenting whole cells previously labeled with SITS, originated from plasma membrane fragments which contaminated these fractions.

* The low yield is in part due to the loss of membrane by repeated washing of the preparation.

The studies reported here show that the binding of SITS to the plasma membrane of isolated cells involves sulfhydryl groups since this binding is inhibited markedly by PCMB, iodoacetamide, and Hg^{2+}. The binding of SITS to isolated cell fractions is also inhibited by iodoacetamide and Hg^{2+} but PCMB has a curious effect since it inhibited SITS uptake by isolated whole cells, microsomes and cell cytoplasm but enhanced SITS uptake by isolated mitochondria. This latter effect may mean that either sulfhydryl groups are not accessible on the mitochondrial membrane or that PCMB causes the mitochondria to become leaky and let SITS penetrate or PCMB allows proteins inside the mitochondria to leak out and react with SITS. Succinate also caused an enhancement of SITS uptake by mitochondria and succinate is known to cause mitochondria swelling. The sulfhydryl groups on mitochondrial membrane have been determined by KLOUWEN[19] and RILEY AND LEHNINGER[20].

The effect of phospholipases on SITS uptake indicates that phospholipids may play only a very minor role in the binding of SITS since the inhibition by these enzymes was very small. In this regard MADDY[1] has reported that lipids of the red blood cells do not significantly bind SITS since extracts of labeled cells with lipid solvents were free from SITS fluorescence.

The results of these experiments give strong supporting evidence that SITS is bound only to the plasma membrane of isolated whole liver cells, a finding which is in agreement with the work of MADDY[1] on isolated red blood cells.

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