sults. In serum medium alone, only cholesterol esters are depleted, whereas it is apparent from the experiment using labelled cholesterol that both free and ester are taken up. The most probable explanation of the results is that both free and ester cholesterol are taken up by cells. Cholesterol esters are then hydrolyzed by the cells and are deposited as free cholesterol. Any excess is then excreted back into the medium as free cholesterol. We have observed the functioning of this excretion process by using cells grown on C^{14} labelled cholesterol and later transferred to unlabelled medium(8). The role of the serum proteins in this excretion process is being investigated.

Summary. 1. Some of the factors influencing uptake of cholesterol by a strain of mammalian cells growing *in vitro* have been examined. 2. Cholesterol uptake by cells is dependent upon the type of serum used in the medium. Cells grown on rabbit serum had a higher cholesterol content than cells grown on human adult serum or human placental cord serum. Rabbit serum, however, had the lowest serum cholesterol level. 3. Cholesterol content of cells was not influenced by concentration of serum cholesterol in the growth medium provided the relative proportions of cholesterol and serum protein were not changed. 4. When emulsions of free (non-protein bound) cholesterol were added to serum medium, relatively small increases in cholesterol content of the medium resulted in large increases in cholesterol content of the cells. 5. By use of C^{14} labelled cholesterol, it was shown that unbound cholesterol was taken up preferentially as compared with cholesterol in the protein-bound form. 6. Cholesterol content of cells was not influenced by addition of stearic acid or linoleic acid to the growth medium. 7. Cells utilized both free and esterified serum cholesterol. The ester was apparently hydrolyzed intracellularly since the cell cholesterol was almost entirely in the unesterified form. 8. It is concluded that the main factor controlling cellular cholesterol uptake may be the relationship between serum cholesterol and the binding power of the serum proteins, rather than the cholesterol level itself.

1. Bailey, J. Martyn, Gey, George O., Gey, Margaret K., PROC. SOC. EXP. BIOL. AND MED., 1959, v100, 686.

2. Rutstein, D. D., Ingenito, E. F., Craig, J. M., Martinelli, M., *Lancet,* 1958, 545.

3. Azarnoff, D. L., PROC. SOC. EXP. BIOL. AND MED., 1958, v98, 680.

4. Pearson, S., Stern, S., McGavack, R., *Anal. Chem.,* 1953, v25, 813.

5. Zlatkis, A., Zak, B., Boyle, A., *J. Lab. Clin. Med.,* 1953, v41, 486.

6. Bailey, J. Martyn, Maymandi-Nejad, A., *ibid.,* 1961, in press.

7. Wykoff, H. D., Parsons, J., *Science,* 1957, v25, 347.

8. Bailey, J. Martyn, Gey, George O., *Fed. Proc.,* 1959, v18, 17.

Received January 18, 1961. P.S.E.B.M., 1961, v107.

Interaction of Compound 48/80 and Stilbamidine with Strandin.* (26525)

ALFRED F. HARRIS, ABRAHAM SAIFER AND SHEILA K. WEINTRAUB
(Introduced by Bruno W. Volk)
Isaac Albert Research Institute, Jewish Chronic Disease Hospital, Brooklyn, N. Y.

Histamine release from mast cells may be effected by a variety of substances, among which are chlorpromazine(1), protamine(2), the thiazine dye, toluidine blue O(2), compound 48/80(2) and stilbamidine(2,3). All of these compounds interact markedly with heparin and chondroitin sulfate. MacIntosh, reviewing the behavior of a number of histamine releasers, has found a parallel between histamine release and heparin combining power(2).

Recent work in this laboratory has indi-

* Supported by a grant from National Tay Sachs' Assn.

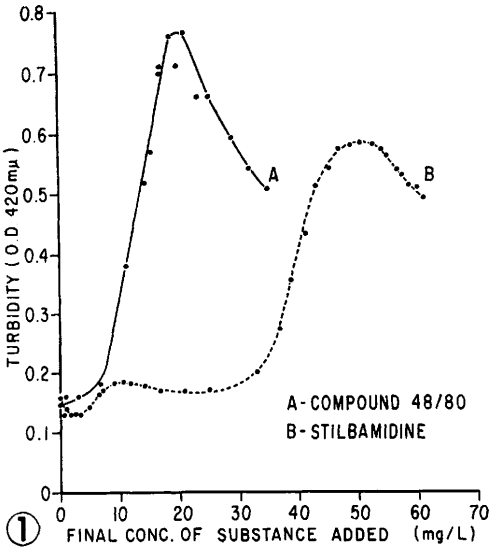
cated that chlorpromazine(4), protamine(5) and toluidine blue O(6) all interact with the brain ganglioside strandin, to produce respectively; a soluble product, turbidity leading to precipitation, and metachromasy. In view of these facts we wished to ascertain whether compound 48/80 and/or stilbamidine would also interact with strandin.

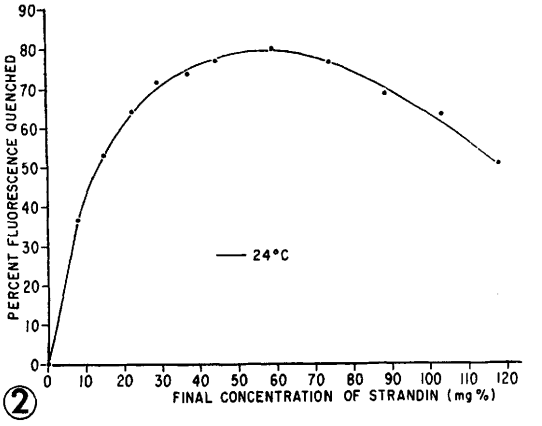
Materials and methods. 1. The brain ganglioside, strandin, was obtained from the cortical gray matter of a case of Tay Sachs' disease by the method of Folch, Lees and Sloane-Stanley(7). Tissue was maintained at -8°F in the deep freeze prior to extraction. Strandin was analyzed by the neuraminic acid method of Saifer and Gerstenfeld(8), assuming an average value of 30.5 mg of neuraminic acid per 100 mg of strandin(9). 2. Materials were obtained as follows: Compound 48/80 from Burroughs Wellcome and Co. Inc.† 3. Stilbamidine (4,4'-stilbene dicarboxamidine) di (β-hydroxyethane sulfonate), from Delta Chemical Works, N. Y. 4. Chondroitin sulfate (CSA) from Nutritional Biochemicals Corp., Cleveland, Ohio. 5. Germanin(3,3'-ureidobis (8 - (benzamido-p-toluido)-1,2,5-napthalene trisulfonic acid)) from Delta Chemical Works, Inc., N. Y. 6. Lysozyme (2× crystallized) from Worthington Biochemical Corp., Freehold, N. J. 7. Protamine Sulfate (Salmine) C.P., from Mann Research Laboratories, N. Y. 8. Poly-l-lysine from Schwarz BioResearch, Inc., Mount Vernon, N. Y. 9. Toluidine Blue O (TBO) (Total dye content of 94%) from National Aniline Division, Allied Chemical and Dye Corp., N. Y. 10. Acriflavine, N.F. (2:8 diamino-N-methyl acridinium chloride) from Abbott Laboratories, North Chicago, Ill. 11. All other chemicals used were of reagent grade. 12. Measurements of turbidity were made using the Bausch and Lomb Spectronic-20 Spectrophotometer set at a wavelength of 420 mμ. Metachromasy was measured with the same instrument, using as index of its intensity the Optical Density Ratio (O.D.R.) which is equal to (O.D. 550/O.D. 635)(6). 13. Ultraviolet absorption spectra were measured using the Beckman DU Spectrophotometer. 14. Fluorescence measurements were made with the Coleman Electronic Photofluorometer Model 120. For excitation the optical filter 12-221 (B_1) was used. For transmission of the fluorescent light of acriflavine, the yellow optical filter 14-212 (PC-2) was used. For transmission of the fluorescence of stilbamidine, the blue optical filter 12-223 (B3) was used. The molecular weight of strandin in water solution is not known with certainty(10). Compound 48/80 is likewise a material of uncertain molecular weight (11). Hence in dealing with these compounds we were unable to use molarity as a measure of concentration, and were forced to utilize such expressions as mg/L or mg%. In the case of stilbamidine isethionate (M.W. = 516.59) we have employed the millimolar expression for concentration in conjunction with the metric concentration, since the latter is required for purposes of comparison with compound 48/80.

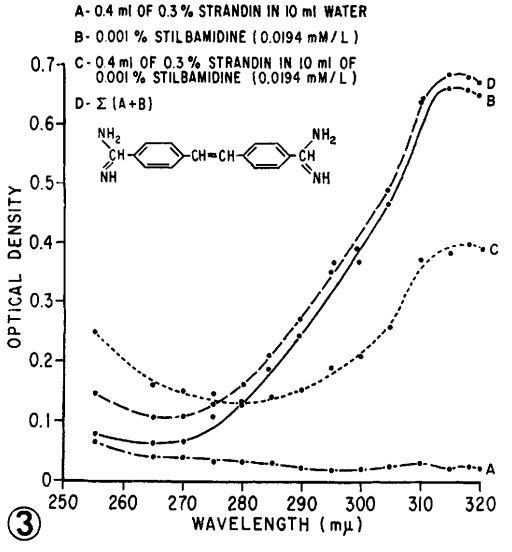
Results. A) *Direct interaction of strandin with histamine liberators.* As indicated in Fig. 1A, the turbidimetric titration of 3 ml of 0.37% strandin with 1% of compound 48/80 at 6°C results in a marked increase in optical density at 420 mμ. The increase becomes maximal upon addition of 20 mg/L of the substance. The increase is of the order of 0.610 optical density units, and further addition of compound 48/80 resulted in a reduction in turbidity. The pH change was from 5.7 to 3.5. To ascertain whether this change in pH was significant, 3 ml of 0.37% strandin were titrated with 0.1N HCl down to pH 3.5 at 6°C. There was no change in turbidity. In the case of stilbamidine (Fig. 1B), the interaction was of less magnitude. A maximal turbidity increase of 0.420 optical density unit occurs on addition of 50 mg/L (0.097mM/L) of 1% stilbamidine to 3 ml of 0.37% strandin, followed by a slight decline on addition of further amounts of stilbamidine. The pH change which accompanies the titration is from 5.7 to 5.0 units.

Further evidence of direct interaction was obtained by studying the effect of strandin on

† We wish to thank Dr. W. P. Colvin, Med. Dept., Burroughs Wellcome and Co., for providing a supply of this compound.







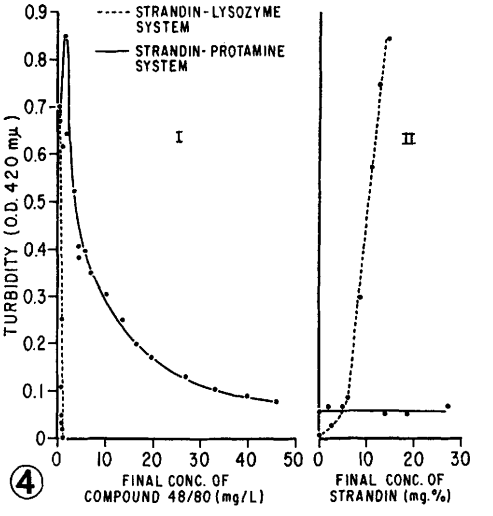


FIG. 1. Turbidimetric titration of strandin with stilbamidine and compound 48/80 at 6°C. 3 ml of 0.37% strandin titrated with indicated amounts of stilbamidine (1.0%) (19.4 mM/l) and compound 48/80 (1.0%). pH at start was 5.7; at end of stilbamidine titration, 5.0 and at end of compound 48/80 titration, 3.5.

FIG. 2. Fluorescence quenching of stilbamidine by strandin. 5 ml total volume containing 10 mg % of stilbamidine (0.194 mM/l) plus indicated amounts of strandin.

FIG. 3. Effect of strandin on UV absorption spectrum of stilbamidine. Concentrations as indicated in legend. Initial pH of stilbamidine solution was 6.8; after addition of strandin, 6.1.

FIG. 4. Repression of strandin-protein turbidity by compound 48/80 and reversal of lysozyme system by strandin. 3 ml total volume in each case, containing 0.1 ml of 1% protamine sulfate, and 0.3 ml of 0.3% strandin, plus indicated amounts of compound 48/80 and 0.1 ml of 1% lysozyme plus 0.1 ml of 0.3% strandin plus indicated amounts of compound 48/80. pH change for the protamine system was from 3.8 to 3.3; for the lysozyme system, from 6.8 to 6.2.

the fluorescence of stilbamidine. The fluorescence of stilbamidine (10 mg%) (0.194mM/L) is quenched by addition of varying amounts of a 0.74% strandin solution (Fig. 2). The effect is a function of the strandin concentration since, with increasing concentration of strandin, the effect rises to a maximum and then decreases. Initial pH of the stilbamidine solution was 7.0. Upon addition of 118 mg% of strandin at 24°C, pH dropped to 6.8. While compound 48/80 does not exhibit fluorescence, it does possess the capacity to reverse the quenching of fluorescence of acriflavine by strandin(6).

Strandin causes a marked alteration in the UV spectrum of stilbamidine (Fig. 3C). The theoretical curve resulting from the algebraic addition of absorption of strandin (0.4 ml of 0.30% in 10 ml of water) and of 0.001% (0.0194 mM/L) of stilbamidine is shown in curve 3D. This curve shows a slightly greater absorption than that of stilbamidine alone (curve 3B). The difference to be stressed, however, is that between curves 3D and 3C, since the latter represents the observed effect of strandin (0.4 ml of 0.30%) on 10 ml of 0.001% stilbamidine. A rise occurs in optical absorption in the region below 280 mμ and a profound drop between 280 mμ and 320 mμ, giving further indication of interaction between the 2 compounds.

We were unable to demonstrate a significant effect of strandin on the UV spectrum of compound 48/80.

B) *Indirect evidence of interaction with strandin.* 1. *Interference with Protein-Strandin interaction.* The interaction between strandin and the basic proteins, lysozyme and protamine, has been demonstrated(5). A number of physicochemical factors which influence this interaction have also been described. We have found that both the protamine-strandin turbidimetric interaction product and the lysozyme-strandin product are sensitive to the action of compound 48/80 (Fig. 4). The lysozyme system is the more sensitive, and within the concentration limits studied, it is clearly reversed by readdition of strandin. The pH change for the lysozyme-strandin system, titrated with compound 48/80 was from 6.8 to 6.2; on retitration with strandin, pH dropped to 6.1 indicating that lowering the pH was not the cause of the preceding effect. Reversal was not found to be the case with the protamine system which had been suppressed by 48/80. In the case of the protamine system there is an initial rise in turbidity upon addition of the first aliquot of 48/80. A phenomenon similar to this was observed in the interaction of cetyltrimethylammonium bromide with the protamine-strandin system(12).

In dealing with stilbamidine we found that results similar to those shown in Fig. 4 were obtained with both lysozyme and the synthetic polypeptide, poly-l-lysine. The situation with protamine was entirely different. We found here that addition of stilbamidine caused a rise in turbidity to a maximum, followed by a slight fall. Readdition of strandin caused a rise in turbidity to an even greater value than did addition of stilbamidine alone.

2. *Interference with toluidine blue O-strandin metachromasy.* Titration of the strandin-toluidine blue O (TBO) system (a 20 ml solution containing 1.06×10^{-5}M TBO and 0.45 ml of strandin (.30%)) with 0.5% stilbamidine (9.68 mM/L) causes a pronounced reduction in metachromasy, as measured by optical density ratio (O.D.R.) so that it has dropped from a value of 1.17 to 0.47 upon addition of 0.04 ml of stilbamidine. Upon adding 0.2 ml of stilbamidine solution, O.D.R. reaches a value of 0.40 unit and the corresponding pH change is from 7.2 to 6.3. Attempts to reverse this phenomenon by further addition of strandin were not successful.

Somewhat similar results were obtained on titration of the strandin-TBO system with 0.5% compound 48/80, except that upon addition of the first few aliquots of compound 48/80, a slight rise in metachromasy occurred, followed thereafter by a profound fall. This system could not be reversed by readdition of strandin. The pH change was from 6.0 to 6.3.

3. *Effect of strandin on polysulfonate-stilbamidine interactions.* Mota *et al.*(13) have indicated that addition of compound 48/80 or of stilbamidine to a solution of heparin, li-

quoid or cellulose sulfate will result in precipitate formation. We have found that 48/80 and stilbamidine also precipitate chondroitin sulfate (CSA) and Germanin. The latter compound possesses 2 features in common with heparin; it inhibits clotting of blood and has pronounced metachromatic properties (14).

We found that in the case of compound 48/80, strandin caused only minor reduction in turbidity of either the heparin, the CSA or the Germanin systems. A change of about 49% occurred when a stilbamidine-Germanin system (containing 1.19 millimoles/L of Germanin and 1.13 millimoles/L of stilbamidine in a total volume of 3 ml) was titrated with 1.0 ml of 1.0% strandin. Allowance was made, in calculation, for the dilution effect, and pH was found to drop from 6.1 to 5.0. For this system it was found that lowering the pH by titration with 0.1N HCl caused an *increase* in turbidity which at pH of 5.0 was not very marked. It was therefore concluded that the pH change attendant on this reaction was not significant. In the case of a CSA-stilbamidine system (containing 0.17% of CSA and 0.48 millimoles/L of stilbamidine in a total volume of 3 ml), the reduction in turbidity attendant on addition of 1 ml of 1% strandin was also 49%. Allowance was made for the dilution effect of the addendum, and the pH change was from 6.1 to 5.7. Titration of this system with 0.1N HCl revealed that until a pH of 3.3 had been reached, no change in turbidity occurred. Upon reaching pH 3.3, a rise in turbidity with time occurs. Thus the pH change is again of little consequence.

Discussion. We have demonstrated that some substances (which are also histamine liberators) such as compound 48/80 and stilbamidine, interact with the brain ganglioside, strandin. From a chemical point of view the reaction falls into the general class of reactions determined by the anionic character of strandin, giving rise to a series of phenomena which are also characteristic of the sulfated mucopolysaccharides. The interaction of these substances with strandin probably proceeds *via* the carboxyl groups of the neuraminic acid moiety of strandin, the secondary nitrogen atom of the compound 48/80 polymeric chain, and the guanidine groups of the stilbamidine molecule.

In view of the evidence indicating direct interaction between the cationic compounds (CC) and strandin (S), we may postulate the following reversible equilibrium (disregarding charge):

$$CC + S \rightleftarrows (CC\text{–}S) \quad \ldots\ldots\ldots\ldots\ldots\ldots\ 1$$

Evidence supporting this picture of the reaction comes from the turbidimetric studies at 6°C, in the case of both compound 48/80 and of stilbamidine; from quenching of fluorescence of stilbamidine by strandin, and from the effect of strandin on the UV-spectrum of stilbamidine. The tendency of the interaction product to dissociate, indicating weak bonding (which is in turn designated by the relative size of the arrows in the above equation), is substantiated by the weak retention of such cationic substances by strandin during an equilibrium dialysis experiment we have performed.

The effect of strandin on interaction of stilbamidine with Germanin and CSA, and the interference of both compound 48/80 and stilbamidine with strandin-TBO metachromasy and with strandin-protein interaction may be interpreted in terms of competitive equilibria. This interpretation is not, however, applicable to the interaction of stilbamidine with the protamine-strandin system, in which case an increase in turbidity results which may be due to a common guanidine group being present in both stilbamidine and in the arginine of the protamine molecule.

These data show that some cationic substances which are known histamine liberators not only interact with heparin as reported by MacIntosh(2), but also with the brain ganglioside, strandin. Whether all cationic histamine liberators will also interact with strandin, requires further investigation.

Summary. 1. Both compound 48/80 and stilbamidine interact directly with strandin at 6°C to form turbidimetric interaction products. 2. The fluorescence of stilbamidine is quenched by strandin, the effect being a func-

tion of the strandin concentration. 3. Strandin exerts a marked effect upon the UV spectrum of stilbamidine, optical density rising above the theoretical below 280 mμ, and falling well below the theoretical curve above 280 mμ. 4. Compound 48/80 causes a reduction in turbidity of the strandin-lysozyme and the strandin-protamine systems. In the case of the lysozyme system the reaction was markedly reversed by readdition of strandin. This was not the case with the protamine system. With stilbamidine similar results were obtained with both lysozyme-strandin and poly-l-lysine-strandin. In the case of protamine-strandin, stilbamidine caused an increase in turbidity of the system. 5. Both compound 48/80 and stilbamidine suppress the metachromasy of the toluidine blue O-strandin system. 6. Strandin was found effectively to suppress the interaction between chondroitin sulfate and stilbamidine, and the interaction between Germanin and stilbamidine. 7. A mechanism involving a reversible equilibrium between these compounds and strandin has been proposed to explain the results obtained.

1. LeBlanc, J., PROC. SOC. EXP. BIOL. AND MED., 1958, v97, 238.

2. MacIntosh, F. C., Histamine and Intracellular Particles, in Ciba Foundation Symposium on Histamine, edited by Wolstenholme, G.E.W. and O'Connor, C. M., Little Brown and Co., Boston, 1956, pp. 20-35.

3. Riley, J. F., The Mast Cells, E. and S. Livingstone Ltd., Edinburgh and London, 1959, pp. 59-63.

4. Harris, A. F., Saifer, A., Volk, B. W., PROC. SOC. EXP. BIOL. AND MED., 1960, v104, 542.

5. Harris, A. F., Saifer, A., *J. Neurochem.*, 1960, v5, 383.

6. ———, *ibid.*, 1960, v5, 218.

7. Folch, J., Lees, M., Sloane-Stanley, G. H., *J. Biol Chem.*, 1957, v226, 497.

8. Saifer, A., Gerstenfeld, S., *J. Lab. Clin. Med.*, 1957, v50, 17.

9. Folch, J., Lees, M., *Amer. J. Dis. Child.*, 1959, v97, 730.

10. Klenk, E., Neuraminic Acid in Ciba Foundation Symposium on the Chemistry and Biology of the Mucopolysaccharides, Little Brown and Co., Boston, 1958, p. 305.

11. Baltzly, R., Buck, J. S., deBeer, E. J., Webb, F. J., *J. Am. Chem. Soc.*, 1949, v71, 1301.

12. Harris, A. F., Saifer, A. Unpublished observations.

13. Mota, I., Beraldo, W. T., Junqueira, L. C. U., PROC. SOC. EXP. BIOL. AND MED., 1953, v83, 455.

14. Harris, A. F., Sobel, A. E., *Arch. Biochem. Biophysics*, 1958, v74, 345.

Received January 23, 1961. P.S.E.B.M., 1961, v107.

Fat Excretion in Dogs Lacking Both Bile and Pancreatic Juice.* (26526)

BENNETT J. COHEN (Introduced by Ralph R. Sonnenschein)
Department of Physiology and the Vivarium School of Medicine, University of California, Los Angeles

When dogs are deprived of bile or pancreatic juice they excrete 40 to 60% of ordinary dietary triglycerides over a wide range of intakes(1). However, degree of steatorrhea has not been measured in dogs when both bile and pancreatic deficiency occur simultaneously. This investigation was undertaken to make this determination.

Methods. Experiments were performed on normal dogs and on dogs lacking pancreatic juice, bile or both. Surgical procedures were performed aseptically on mongrel females weighing 9 to 18 kg with due regard for the requirements of proper animal care. Pancreatic deficiency was produced by removing all of the pancreas except the uncinate process(2). Subsequent necropsy and histological study showed no connection between duodenum and pancreatic remnant. None of the dogs became diabetic. External bile fistulas were prepared by a modified Rous-McMaster technic(3). The combined deficiency was produced by simultaneous pancreatectomy and preparation of the bile fistula. A return

* Supported by grant from Nat. Inst. of Arthritis and Met. Dis.