TABLE I. Experiment 3, November, 1965: Serum Neutralizing Antibody Responses and Serial Pharyngeal Virus Isolations of Rhinovirus Type 14 Strain NIH 1059 after Nebulization-Intranasal Instillation of 10,000 $TCID_{50}$.

		Days after virus administration												
		−7	−0.1	0	0.2	1	2	4	6	8	10	14	21	28
Chimp. 304	Virus isolates[a]	—	—	+	+	—	+	—	+	+	+	+	+	—
	Antibody titer			<3			<3	<3	<3	<3	<3	<3	<3	90
Chimp. 308	Virus isolates	—	—	+	+	—	+	+	+	+	+	+	—	—
	Antibody titer			<3			<3	<3	<3	<3	<3	181	256	256
Chimp. 309[b]	Virus isolates	—	—	—	—	—	—	—	—	—	—	—	—	—
	Antibody titer			181			181	90	128	128	128	90	90	256
Chimp. 315[b]	Virus isolates	—	—	+	+	—	—	—	+	+	+		—	—
	Antibody titer			<6			<6	<6	<6	<6	45	1444	2048	512

[a] (—) indicates that the throat specimen was passaged once in human embryonic diploid tissue culture and no rhinovirus 14 was isolated; (+) indicates rhinovirus 14 isolation.

[b] Chimpanzees 309 and 315 were previously inoculated with rhinovirus 14.

interference either by injection or by respiratory infection. It should also be possible to carry out the detailed studies on the pathogenesis of rhinovirus infections which are impossible in man.

Summary. In three serial experiments, chimpanzees free of specific neutralizing antibody were infected with rhinovirus types 14 or 43. Although infected animals shed virus from 6 to 21 days and produced high levels of specific antibody, clinical illness was not discernible. Providing serum neutralizing antibody was still present, chimpanzees rechallenged with the same agent shed virus for less than 5 hours. To the author's knowledge this is the first report of successful experimental infection by human rhinoviruses of species other than man.

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A Chemical Method for the Estimation of Brain Sulfhydryl Groups (32876)

SABIT GABAY, ANTONIO M. CABRAL,[1] AND ROBERT HEALY[2]

Biochemical Research Laboratory, Veterans Administration Hospital, Brockton, Massachusetts, 02401 and Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118

Numerous methods for determining concentrations of sulfhydryl groups (–SH) in tissue homogenates have been developed. In addition to methods measuring total –SH content (1,2); more specific procedures for –SH amino acids (3), glutathione (4,5), total non-protein —SH (6) and protein–SH (7) have

[1] Present address: Department of Pharmacology, Emory University, Atlanta, Georgia.

[2] Fellow of Tobacco Industries Research Council, New York. Present address: Buffalo General Hospital, Buffalo, New York.

been established. Techniques in these various methods include amperometry, spectrophotometric titration, colorimetry, and determination of side group ionization ratio. The usefulness of these various methodologies was ably reviewed by Benesch and Benesch (8) who concluded that no single method for estimating —SH in proteins should be relied on exclusively.

Ellman (1) described a chemical method using 5,5′-dithiobis-(2-nitrobenzoic acid),[3] for estimating total -SH content in tissues other than brain. This method was subsequently modified by Jocelyn (7) to differentiate between nonprotein -SH and protein -SH in rat liver homogenates.

The present study deals with the estimation of brain -SH groups in rat brain homogenates by a chemical method, based on DTNB, which enables one to measure protein -SH in the presence of nonprotein -SH groups. Some of the salient advantages of this method were also evaluated.

Materials and Methods. All reagents were prepared in doubly glass-distilled water and solutions were kept refrigerated until just prior to use.

Phosphate buffers, pH 6.8 and 7.6, in two ionic strengths($I = 0.2$ and $I = 0.05$) at each pH were made up as described by Datta and Grzybowski (9). 'Phosphate saline' was prepared as prescribed by Pinto (4).

The DTNB was obtained from Aldrich Chemical Co. (Milwaukee, Wisconsin) and used without further purification. Two solutions, designated as "solution A" and "solution B," were always freshly prepared prior to their use: Solution A—1 m*M* DTNB was prepared in phosphate buffer, pH 6.8, $I = 0.2$; and Solution B—10 m*M* DTNB in phosphate buffer, pH 7.6, $I = 0.2$.

Reduced glutathione, chromatographically homogenous, was purchased from Schwarz Bio-Research, Inc. (Orangeburg, New York) and stock solutions of 60 μmoles per 100 ml of 'phosphate saline' were prepared daily. The concentration of subsequent dilutions used throughout these experiments were estimated on the basis of a titration with *p*-chloromercuribenzoate (*p*-CMB) according to Boyer's method (10).

Crystalline bovine serum albumin was obtained from Sigma Chemical Co. (St. Louis, Missouri) and stock solutions (24 mg/ml of 'phosphate saline') were prepared as needed.

The animals used in this study consisted of adult rats (Carworth Farms, New York) weighing 250 ± 10 gm. For each experiment 2–4 animals were killed by decapitation with a guillotine. Brains were removed within 1 min, rinsed with ice-cold KCl (0.9%) and placed on a flat slab of frozen KCl. Brains were quickly dried by touching them to a clean piece of filter paper and were weighed on a Roller-Smith type balance, transferred to a chilled Potter-Elvejhem glass homogenizer (chamber clearance: 0.005 to 0.007 inches)

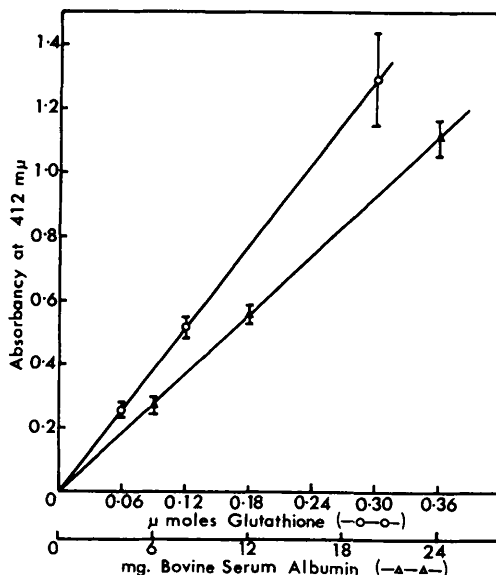

FIG. 1. Change in absorbancy at 412 mμ when varying concentrations of glutathione or bovine serum albumin were mixed with DTNB. Glutathione in 1 ml of 'phosphate saline' was added to solution A (pH 6.8) and the absorbancy read 1 min after mixing. Bovine serum albumin in 1 ml of 'phosphate saline' was added to solution B (pH 7.6) and the absorbancy read 20 min after mixing. Points plotted are mean values of 14 assays of glutathione and 10 assays of bovine serum albumin. Vertical bars represent standard deviations.

[3] Abbreviation used throughout: DTNB di-(5-carboxy-4-nitrophenyl) disulfide (I) and 5-5′-dithiobis-(2-nitrobenzoic acid) (II) are synonymous. For their use, previously, see for I: (Ref. 7), and for II: (Ref. 1).

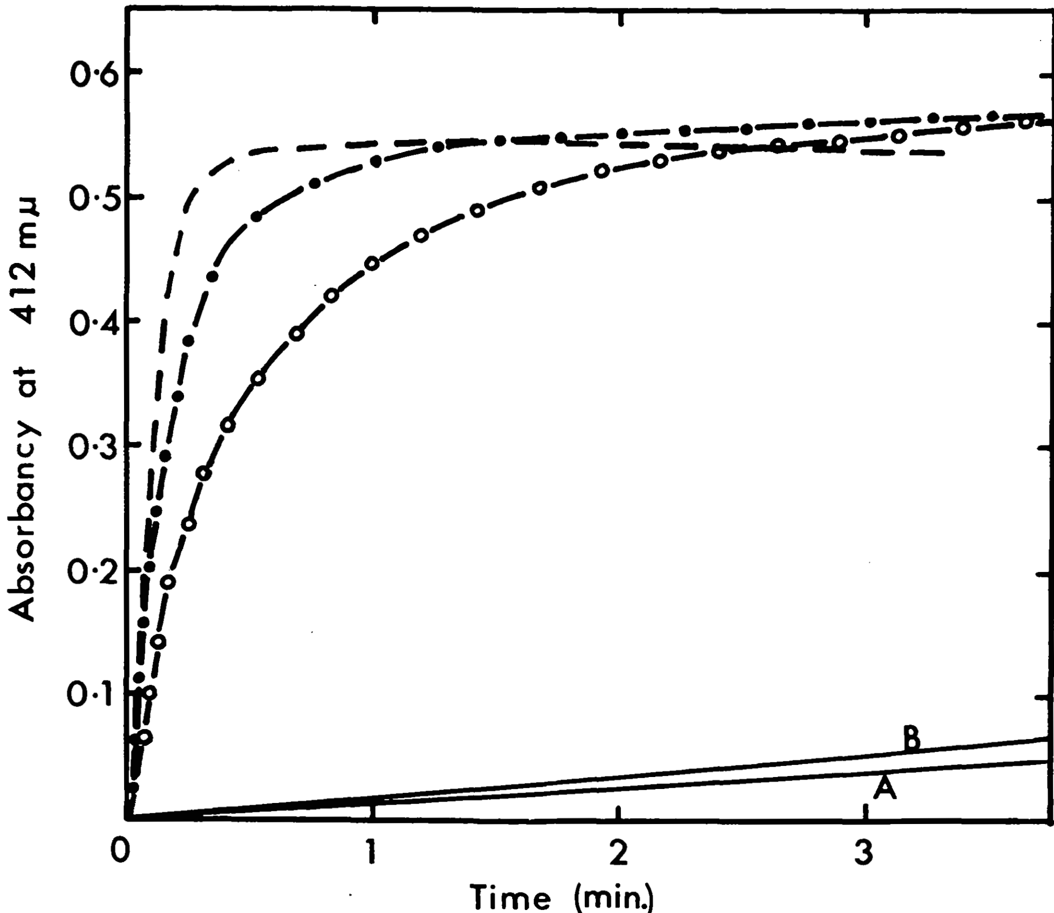

FIG. 2. Progress of color development when nonprotein –SH is estimated in the presence of protein –SH with 1 mM DTNB, pH 6.8 (solution A). Glutathione (0.06 μmoles: — — —) and bovine serum albumin (6 mg: line A, and 12 mg: line B) in 1 ml of 'phosphate saline' were tested separately. The 0.06 μmoles of glutathione was then tested in the presence of 6 mg (●) and 12 mg (○) of bovine serum albumin by diluting appropriate amounts of the two stock solution (see "Materials and Methods") to 1 ml with 'phosphate saline'.

and homogenized with a motor-driven Teflon pestle for 2 min in small volumes of 'phosphate saline' kept at 4°C. The volume was then adjusted to give a 20% (w/v) homogenate and centrifuged at $2000g_{max}$[4] in a refrigerated centrifuge (PR-2 International, Head No. 823A) for 10 min at 5°. The supernatant was subsequently diluted to 10% w/v (hereafter designated as "10% homogenate") with ice-cold 'phosphate saline' and used immediately.

Experimental Procedure. A typical reaction mixture consisting of a final volume of 3.0 ml contained 1.5 ml of phosphate buffer (I = 0.05), pH 6.8 or 7.6; 1 ml of 'phosphate saline' containing appropriate amounts of either glutathione, albumin or the 10% homogenate; and, as DTNB, 0.5 ml of either solution A or B. Specifics of the reaction components are given in the legends to Figs. 1 and 2. The absorbance was recorded in a Beckman model DU or DB spectrophotometer at 412 mμ. A reference cuvette contained an identical reaction mixture to which no glutathione, albumin or 10% homogenate had been added. Turbidities of reaction mixtures containing homogenates were estimated by

[4] Gravitational forces are given as maximum.

measuring the absorbance at the same wavelength of 0.2 ml of 10% homogenate to which 2.0 ml of phosphate buffer, pH 6.8 (I = 0.05) and 0.8 ml of 'phosphate saline' were added. The turbidity reference contained 1.0 ml of 'phosphate saline' and 2.0 ml of buffer.

Solutions of glutathione (0.06, 0.12, and 0.30 μmoles in 1 ml of 'phosphate saline', respectively) were prepared and mixed with solution A following the procedure described by Jocelyn (7). The absorbance was recorded 1 min after mixing and the mean values of 14 individual assays were used to establish a standard curve for nonprotein –SH. Similarly, solutions of bovine serum albumin (6, 12, and 24 mg in 1 ml of 'phosphate saline,' respectively) were prepared and mixed with solution B. The absorbance was recorded 20 min after mixing and the mean values of 10 separate assays were used to establish a standard curve for total –SH (Fig. 1).

The progress of color development of nonprotein –SH in the presence of protein –SH with solution A (Fig. 2) was followed with a Beckman DB attached to a recorder (Photovolt Microcord 44, New York).

Samples (0.2 ml) of freshly prepared homogenates were mixed with either solution A or B and their respective absorbancies recorded 2 min and 20 min after mixing. Nonprotein –SH and total –SH were estimated in terms of μmoles equivalents of glutathione. Protein –SH was then estimated by subtracting nonprotein –SH from total –SH,

Results and Discussion. Since its utility was first described by Ellman (1), DTNB has been used by numerous workers as a sulfhydryl reagent (7,11,12). For example, it was employed to differentiate between nonprotein –SH and protein –SH in liver homogenates (7). Figure 1 depicts the standard curves obtained when DTNB was reacted with increasing amounts of glutathione and bovine serum albumin as described in its legend. A comparison of the two molar absorptivities[5] indicates that DTNB, at pH 7.6, titrates only 0.67 SH groups per molecule of bovine serum albumin. This is in excellent agreement with the results reported in the literature (13,14). Thus, it was assumed that DTNB reacted equally well with both protein –SH and nonprotein –SH depending on the reaction time and the pH. As a cautionary note, it should be added that protein –SH measurements are based upon the results obtained using bovine serum albumin as a model. The variety of sulfhydryl reactivity has an important bearing on kinetic and binding studies; thus, bovine serum albumin might not be an adequate model for all proteins. It is, therefore, suggested that a study of the kinetics of the "DTNB–protein–SH reaction," such as described herein, would be desirable for each case.

Jocelyn (7) suggested reading the absorbance for nonprotein –SH 1 min after mixing the reagents to minimize the slow increase in absorbance which he attributed to a slow interaction between solution A and protein –SH. Figure 2 (lines A and B) indicates that this interaction does indeed occur. However, it was found (reproducibly) that the presence of protein –SH delays the color development in the reaction between nonprotein –SH and Solution A, and that the delay increased when the amount of protein –SH was increased. It was observed that, by reading the absorbance for nonprotein –SH in the presence of protein –SH 2 min after mixing the reagents, the color developed from this mixture was the same as that developed by the nonprotein –SH fraction per se. Increases in absorbance due to turbidity of homogenates amounted to 0.150 ± 0.020 which were subtracted from the observed absorbances.

As evident from Table I, estimates of nonprotein –SH and total –SH in rat brain reported by a number of investigators using various methods are in good quantitative agreement with those obtained in this study. The concentration of protein –SH, calculated as the numerical difference between total –SH and nonprotein –SH, could then be a valid estimate.

The utilization of DTNB at pH 6.8 and 7.6 to estimate the nonprotein and protein –SH fractions of rat brain seems to provide a relatively simple, accurate, and rapid method for a differential assay of the two fractions without requiring specialized instruments or techniques. Furthermore, this spec-

[5] The ϵ of glutathione and bovine serum albumin were calculated to be 13,200 and 8,910, respectively.

TABLE I. Comparison of Estimates of Nonprotein and Total–SH in Rat Brain Using Various Methods.

Method of assay	Nonprotein–SH (μmoles/gm of wet tissue)	Total–SH (μmoles/gm of wet tissue)	Ref.
1. DTNB	2.6 ± 0.4[a] ($n = 26$)[b]	3.8 ± 0.6 ($n = 26$)	Present paper
2. Amperometry	—	4.9	(2)
3. Glyoxalase	2.2–3.5	—	(16)
4. Column chromatography followed by colorimetry	1.9–2.3	—	(15)

[a] Standard deviation.
[b] n = number of animals.

trophotometric method has an advantage of not requiring measurements in the region between 250 and 280 mμ where interference by ultraviolet-absorbing substances is often a complication.

Summary. A spectrophotometric method using DTNB for the estimation of nonprotein –SH in the presence of protein –SH groups in rat brain homogenates is described. Details of the optimum conditions for the color development which were found to be dependent on the reaction time and pH are also given. Estimates of nonprotein –SH and total –SH in rat brain reported by a number of investigators using various methods are in good quantitative agreement with those obtained in this study. Thus, findings led us to conclude that the relative specificity, rapidity, and accuracy of this chemical method are comparable with the existent methods requiring specialized instruments or techniques. This spectrophotometric method has a further advantage of not requiring measurement in the region between 250 and 280 mμ where interference by ultraviolet-absorbing substances is often a complication.

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