anism of action of these non-specific activators (1) seems, however, to be open to question.

Summary. Total-body x-irradiation produces within a few hours a dose-dependent increase in the tryptophan peroxidase-oxidase system of rat liver. The increase does not occur in adrenalectomized rats, and hence cannot be construed as a direct effect of x-irradiation.

1. Knox, W. E., *Brit. J. Exp. Path.*, 1951, v32, 462.
2. Knox, W. E., and Mehler, A. H., *J. Biol. Chem.*, 1950, v187, 419.
3. Leblond, C. P., and Segal, G., *Am. J. Roentgenol.*, 1942, v47, 302.
4. Mason, M., and Berg, C. P., *J. Biol. Chem.*, 1951, v188, 783.

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Isolation and Chemical Composition of Complement-Fixing Antigens From Leptospires. (20776)

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The purpose of this report is to present a comprehensive account of a fractionation procedure applied to the leptospiral cell and to show the comparative yield of the complement-fixing cell fractions from selected serotypes of leptospires. Fraction 1, a complement-fixing substance, has been isolated and its chemical and serological properties described(1-3).

The process of fractionation of the leptospiral cell has been extended to include the cellular debris remaining after extraction of Fraction 1. The cell residue served as the starting material from which were isolated three serologically active fractions.

Methods. Cultures of leptospires. Three serotypes of leptospires were employed, namely. *Leptospira icterohemorrhagiae* AB, *L. canicola* and *L. bataviae*. Leptospires were propagated and harvested as previously described(1,3). *Isolation of complement-fixing antigens.* The protocol for fractionation of the leptospiral cell is outlined in Fig. 1. Washed viable organisms were disrupted as previously described(1,3). After shaking with one-half volume 4:1 chloroform-n-amyl alcohol, the mixture was centrifuged at 4000 rpm for 20 minutes, at 0°C. The preparation separated into (A) an aqueous phase, (B) an interfacial cell-protein gel or chloroform "cake" and (C) a chloroform layer. The aqueous phase yielded, on further processing, a genus-specific antigen previously described (1-3). The cell protein debris (B) was separated from the chloroform layer, suspended in and thoroughly dialyzed against 0.15 M NaCl solution and disintegrated by sonic vibration. The final volume of this preparation was 1 to 2% of the original culture volume. The crude cell-protein antigen was fractionated as follows: The crude material was precipitated from its aqueous suspension by the addition of one-half volumes of each of 0.2 N H_2SO_4 and freshly prepared 2% sodium tungstate solution. A heavy flocculent precipitate appeared soon after the addition of tungstate solution. After 30 minutes, the acidified mixture (pH about 2.0) was centrifuged at low speed, in the cold, and yielded: 1) A soluble supernatant, which on subsequent dialysis against 0.15 M NaCl solution and pervaporation yielded a non-dialyzable, complement-fixing substance, identified as Fraction 4; 2) An insoluble sedimentable portion which was then suspended in water to one-half of the volume of the original crude suspension. Three volumes of absolute ethanol were added, the mixture shaken and held at about 0°C for 24 to 48 hours. After low speed centrifugation, the latter mixture separated into: a) A clear to slightly opalescent alcoholic phase which, after thorough dialysis against distilled water and pervaporation, yielded a non-dialyzable water-insoluble material designated Fraction

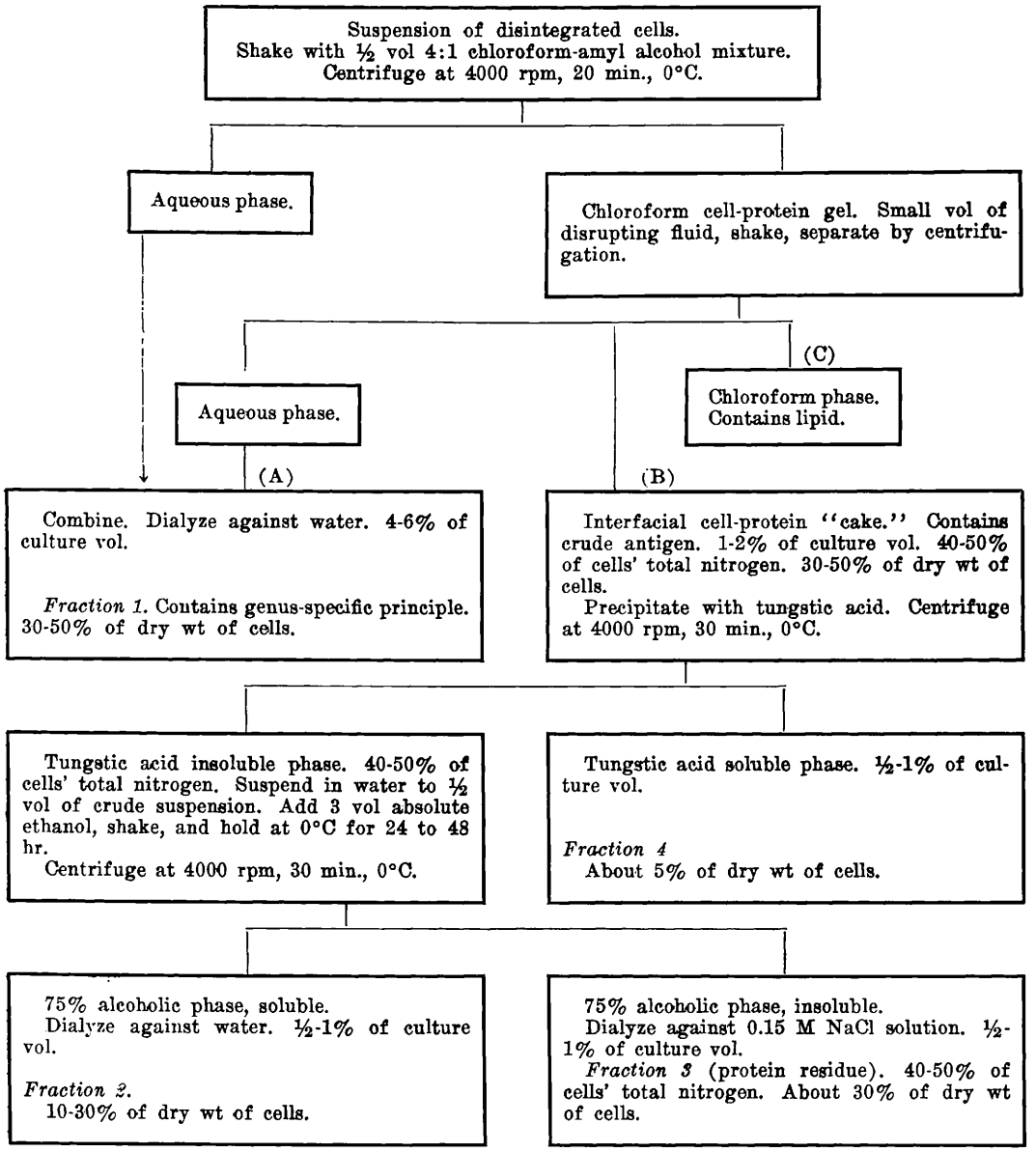

FIG. 1. Fractionation of washed viable leptospires.

2; b) The 75% ethanol insoluble portion appeared as a packed white sediment. Suspended in and dialyzed against 0.15 M NaCl solution, the final product has been designated Fraction 3, or the protein residue antigen. The final volumes of each of the above fractions of the crude antigen were 0.5 to 1% of the original culture volume. The terminology Fractions 1, 2, 3 and 4 was arbitrarily applied to the complement-fixing fractions, representing, in the order indicated, decreasing amounts of serologically reactive material derived from the leptospiral cell. *Analytical procedures.* The application of analytical methods was the same as previously reported(1,3). Quantitative estimation of total carbohydrate was accomplished with an orcinol-sulfuric acid reagent(4). Ultraviolet absorption spectra were determined of reaction products of orcinol-sulfuric acid reagent with Fraction 1

TABLE I. Yield and Composition of Four Arbitrary Complement-Fixing Fractions from *L. icterohemorrhagiae* and *L. canicola*.

	L. icterohemorrhagiae 1	*L. icterohemorrhagiae* 2	*L. canicola* 3
Fraction 1			
Yield (%)	43	19	39
Reactivity*	.10	.30	.30
% of total reactivity†	43	71	52
Composition (%)			
Nitrogen	6.2	8.8	9.5
Pentose	7.2	8.6	8.3
Desoxyhexose	5.9	6.0	4.1
DNA	8.9		12.1
Glucosamine	4.0		5.7
Phosphorus	1.0		1.1
Hexose	2.4		4.0
Total carbohydrate‡	26	32	26
Copper reducing substances§	21		
Fraction 2			
Yield (%)	12	24	29
Reactivity	.21	.07	.19
% of total reactivity	26	21	22
Composition (%)			
Nitrogen	3.8	2.8	.8
Pentose	4.8	2.7	2.7
Desoxyhexose		.3	Tr.
Glucosamine	Tr.		
Total carbohydrate	19	15	14
Fraction 3			
Yield (%)	30	23	28
Reactivity	.05	.01	.18
% of total reactivity	17	4	20
Composition (%)			
Nitrogen	10.8	8.6	11.4
Protein‖	(67.7)	(53.8)	(71.3)
Pentose	1.5	1.0	2.2
Fraction 4			
Yield (%)	4	1	10
Reactivity	.33	.21	.15
% of total reactivity	14	3	6
Composition (%)			
Nitrogen	2.8	4.4	2.2
Pentose	8.3	7.4	6.0
Desoxyhexose	5.3	6.0	4.1
Recovery			
% of organism	89	68	106

* Reactivity $= 1/x$, when x is minimal wt (μg) of material/tube yielding max. homologous hyperimmune serum titer.

† % of total activity $= \dfrac{\dfrac{\text{Yield (\%)}}{x}}{\Sigma \dfrac{\text{Yield (\%)}}{x}}$.

‡ Total carbohydrate as glucose, estimated by the orcinol-sulfuric acid procedure(4).

§ Copper reducing substances as glucose, after hydrolysis with 1N H_2SO_4 or 6N HCl, at 100°C, for 6-8 hr.

‖ ( ) Total nitrogen multiplied by 6.25.

Tr. = Trace.

and Fraction 2 antigens. The color development of sulfhydryl-sulfuric acid (CyR10) reagent(5) with Fraction 2 antigens was measured spectrophotometrically.

Results. The crude cell-protein antigen contained 30 to 50% of the dry weight and 40 to 50% of the nitrogen of the whole leptospiral organism. The active substance in the crude material was stable to heating at 100°C for 1 hour, sonic vibration, chloroform-amyl alcohol extraction, and precipitation with tungstic acid.

Relative yields and composition of the 4 fractions are shown in Table I. Calculations of serologically active substances were based on the minimal weight (μg) of each cell fraction which reacted with homologous hyperimmune rabbit serum at maximal titer (a dilution of 1:240 to 1:480 for *L. icterohemorrhagiae* and *L. bataviae*, respectively, and 1:120 to 1:240 for *L. canicola*). The yield of the complement-fixing fraction antigens of each of these serotypes of leptospires was 2- to 4-fold greater than that of the sonic-vibrated, lyophilized whole cell antigen of Randall *et al.*(6). The complement-fixing fractions were non-dialyzable materials, all of which contained pentose. *L. icterohemorrhagiae, L. canicola* and *L. bataviae* were each found to contain 4.4, 5.2 and 5.4% non-dialyzable pentoses by weight. Approximately 80% of the total pentose was recovered in Fractions 1 and 2. After acid hydrolysis Fraction 1 and 2 antigens gave positive ninhydrin reactions.

In the estimation of total carbohydrate, the orcinol-sulfuric acid method depends upon the absorption band at 425 mμ, a property characteristic of hexoses and pentoses. Fractions 1 and 2 illustrate this absorption band. Fraction 2 displays in addition a chemically unidentified maximum at 385-390 mμ (Fig. 2, A). Total carbohydrate, calculated as glucose, varied between 26 and 32% for three preparations of Fraction 1. After acid hydrolysis total carbohydrate was about the same as in unhydrolyzed materials. One preparation of Fraction 1 of *L. icterohemorrhagiae* contained 21% copper-reducing substance on hydrolysis. In addition to pentose and desoxyhexose(3), Fraction 1 contained glucosamine, hexose and other carbohydrate residues.

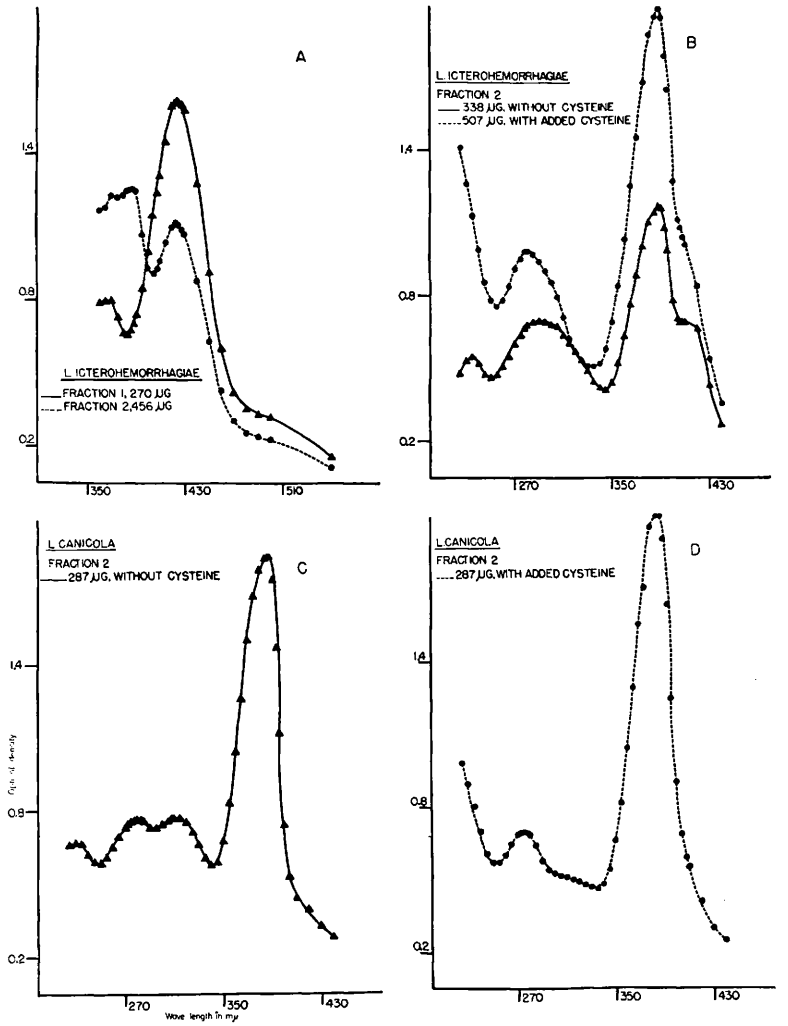

FIG. 2. A. Ultraviolet absorption spectra of materials after heating fractions 1 and 2 with an orcinol-sulfuric acid reagent(4), at concentrations of 270 and 456 µg, respectively, in 11 ml of reaction mixture. B, C, D. Absorption spectra of materials after heating fraction 2 antigens with sulfhydryl-sulfuric acid (CyR10) reagent(5). Spectra were measured on the Beckman DU spectrophotometer.

The chemical composition of Fraction 2 was distinguished from that of Fraction 1 by its lower content of nitrogen, pentose and desoxyhexose, by the apparent absence of glucosamine, and by the presence of an unidentified ultraviolet absorption peak at 385-390 mµ. It contained less total carbohydrate, as measured by the acid-orcinol procedure. At concentrations of 500 µg Fraction 2 reacted strongly with Dische's naphthol carbohydrate reagent and gave specific color complexes with sulfhydryl-sulfuric acid reagent(5). The absorption maxima of the cysteine-sulfuric acid color complexes with Fraction 2 lie approximately at the same wave lengths (Fig. 2, B, D). An absorption band was located at 265-275 mµ, and a second very sharp peak at 385-390 mµ. The latter peak occurred in the reaction mixture in the absence of cysteine (Fig. 2, B, C), but the extinction coefficient was slightly lower. On a weight basis, the (cysteine) extinction coefficient at 388 mµ was greatest for *L. canicola,* less for *L. icterohemorrhagiae* and least for *L. bataviae.* The active

principle was insoluble in water at pH less than 7, and insoluble in absolute ethanol; but was readily soluble in water at pH greater than 7, and in 75% ethanol. Fraction 2 was isolated virtually free of protein only after denaturation of the crude cell-protein material with tungstic acid.

Extensive complement fixation tests have been applied to the crude cell-protein, intermediate products, and cell fraction antigens derived from the residue materials(7). The antigens of the leptospiral cell illustrated complement fixation reactivity with homologous hyperimmune rabbit serum at concentrations of antigen of 1 to 3 μg.

Discussion. A fractionation procedure has resulted in the isolation of 4 non-dialyzable, pentose-containing, serologically reactive fractions from the leptospire cell. A proportionately large amount of a complex, genus-specific complement-fixing antigen is isolated. A considerable quantity of crude cell-protein material remaining after removal of Fraction 1 is denatured with tungstic acid. The acid treated crude substance then serves as the starting material from which are isolated 3 additional cell fractions all of which show good complement fixation activity.

The active principle of Fraction 1 appears to be identified with the non-dialyzable pentose. Purification steps which result in the concentration of virtually all of the pentose polysaccharide moiety also retain virtually all of the complement-fixing reactivity(3). Preparations have been adsorbed with 20% Attaclay SF(8), resulting in the separation of the pentose polysaccharide moiety. The latter preparations illustrate reduced complement fixation activity depending on the amount of pentose separated.

Fractions 1 and 2 are chemically distinct compounds which are spectrophotometrically distinguishable from each other. Both preparations show relatively similar reactivity as complement fixation antigens. However, Fraction 2 contains about one-fourth of the amount of pentose found in Fraction 1. The pentose carbohydrate in Fraction 2 may be different. A possibility exists that the active principles in these fractions may be different. However, it has not yet been possible to determine the number of complement-fixing principles in the leptospire cell.

The cysteine-sulfuric acid (CyR10) reaction with Fraction 2 preparations of selected serotypes of leptospires indicates that this color complex contains an unidentified sharp peak at 385-390 mμ which may serve as a possible aid in the identification of the serotype.

Fraction 3 is predominantly protein, containing about 40% of the total nitrogen of the cell. Complement fixation reactivity may be accounted for by the pentose in this preparation, which is possibly a contaminant remaining after extraction of Fraction 2. It appears, therefore, that the protein residue is poorly reactive in the complement fixation test or has been seriously damaged during the fractionation procedure. Fraction 4 is a "non-protein-nitrogen" material and is apparently contaminated with carbohydrate residues found in Fraction 1.

Summary. A chemical separation procedure which yields four arbitrary cell fractions has been developed and applied to three serotypes of leptospires. The four complement-fixing fractions are non-dialyzable, pentose-containing materials, the serological activities of which have been measured semi-quantitatively. One or more complement-fixing principles, of nature not yet known, are concentrated in two chemically distinct, relatively free of protein, polysaccharide-containing cell fractions. The protein constituent (Fraction 3) of the leptospiral cell was poorly reactive in the complement fixation test. Spectrophotometric differences among preparations of Fraction 2 from three serotypes may prove to be an aid in the differentiation of serotypes.

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1. Schneider, M. D., PROC. SOC. EXP. BIOL. AND MED., 1953, v82, 655.
2. ———, *Bact. Proc., Soc. Am. Bact.*, 1953, p48.
3. ———, *J. Inf. Dis.*, in press.

4. Fernell, W. R., and King, H. K., *Analyst,* 1953, v78, 80.
5. Dische, Z., and Shettles, L. B., *J. Biol. Chem.,* 1948, v175, 595.
6. Randall, R., Wetmore, P. W., and Warner, A. R., *J. Lab. and Clin. Med.,* 1949, v34, 1411.
7. Unpublished data from this laboratory.
8. Hiatt, C. W., Rothstein, N., and Yager, R. H., PROC. SOC. EXP. BIOL. AND MED., 1951, v78, 703.

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Adrenal Response of Rats to Salicylamide and Sodium Salicylate with and Without Para-Aminobenzoic Acid.*† (20777)

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The administration of either acetylsalicylic acid or sodium salicylate to rats has been shown to cause a marked drop in the content of ascorbic acid and to a lesser extent of cholesterol in the adrenals(1-4), an effect which is not found in the hypophysectomized animal(1,2). Cronheim *et al.*(1) gave salicylamide to a few animals and found its effect on the adrenals to be somewhat less than that of sodium salicylate. In later experiments these investigators administered para-aminobenzoic acid (PABA) along with sodium salicylate but failed to get an augmented effect(5). They suggested that their inability to demonstrate an additional effect with PABA was probably due to the fact that the animals were sacrificed too soon after the administration of the drug, namely, 2 hours.

Since the analgesic effect of salicylamide in the experimental animal has been shown to be greater than that of acetylsalicylic acid (6-8), it was decided to carry out a more extensive investigation on its adrenal effects, studying not only the intensity of its action but also the time required for the adrenal cholesterol and ascorbic acid to return to normal concentrations. For comparative purposes experiments were carried out with both salicylamide and sodium salicylate, alone or with an equivalent amount of PABA.

Methods. Albino rats were used throughout. All animals purchased from a commercial dealer were placed on our stock diet of Rockland rat pellets for at least a week before they were used in the experimental work. No food was allowed for 16 hours prior to the ad-

TABLE I. Effect of Oral Salicylamide with and without Para-Aminobenzoic Acid on Adrenal Cholesterol and Ascorbic Acid of Male Rats.

Concentration following drug administration, %				
Ascorbic acid		Cholesterol		
2 hr	4 hr	2 hr	4 hr	Remarks
.324 ± .053* (3)	.310 ± .037* (10)	3.25 ± .29* (3)	2.91 ± .37* (9)	Controls receiving gelatin
.164 ± .021 (4)	.274 ± .088 (14)	2.70 ± .61 (4)	2.51 ± .65 (14)	Salicylamide, 300 mg/kg
.180 ± .029 (4)	.272 ± .097 (15)	2.93 ± .43 (4)	2.51 ± .72 (15)	*Idem.* + equimolar quantity of PABA

* Stand. dev.
No. of animals in parentheses.

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