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FINAL PROGRESS REPORT

UNITED STATES ATOMIC ENERGY COMMISSION

"Isolation of a Factor Preventing X-ray Induced Leukemia"

Period covered: 1 June 1964 through 31 May 1971

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Summary: This final report includes a summary of previous reports and work completed through 31 December 1969 and a detailed report of the final phases of the project. The experiments reported are of 2 types: (1) Those designed to isolate the factor (RFP, for radiation leukemia protection) and test its ability to prevent thymic lymphosarcoma in x-irradiated C57BL/6 mice and (2) Attempts to correlate other therapeutic effects of tissue extracts serum and their fractions with RFP activity.

Serum and spleen extract, injected after irradiation, consistently lower the incidence of thymic lymphosarcoma. Several Cohn fractions, and especially IV-4 do likewise. Serum and spleen extracts can be separated into active and inactive fractions on Sephadex. Cohn fractions III and IV-4 can apparently be fractionated but not enough testing has been done to permit firm conclusions as to the identity of RFP.

Sheep spleen extracts do not protect against the lethal effects of single exposures to 550-800 R. However, several acute changes produced by 180-300 R are reversed by injection of serum after exposure. The changes reversed include elevated serum and organ levels of lactate dehydrogenase, alkaline phosphatase, RNA polymerase, and to a lesser extent, DNase II, and decreased levels of trypsin-esterase binding activity and DNA polymerase. Serum also hastens recovery of thymus weight. Experiments with serum fractions have been less successful. Although albumin is definitely devoid of activity, the active fraction has not been positively identified.

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As this is a final report, all the studies performed are included. Work already reported in COO-1391-1, 6, 13, 17, and 21 is presented in summary form, and studies not previously reported are described in greater detail. The bibliography is complete to date.

The work itself comprises the actual attempts at isolation of the Radiation Leukemia Protection (RLP) factor with the attendant mouse tests, and various ancillary experiments. Most of the latter were designed as attempts to correlate changes following x-irradiation (such as changes in serum enzyme levels) with protective action against leukemia.

A. Isolation of RLP

1. Protocol for x-irradiation, injection and care of mice. This was given in COO-1391-1. Deviations from the original protocol have included (a) entering 25-50 males and females instead of 100 of each and (b) use of younger mice - down to about 30 days. The first change was made when funding failed to keep pace with financial requirements and the second when it became obvious that the incidence of leukemia in saline-injected controls was low (in older mice). For the purposes of this work the term "leukemia" is synonymous with "thymic lymphosarcoma", whether the latter is disseminated or remains localized. The significance of differences between leukemia incidence in control and treated animals has been consistently calculated by the method of Kimball (Bull. Internat. Statist. Inst. 36:193, 1958) on the incidences corrected for deaths due to extraneous causes - except that in the last group of experiments, the significance was initially and periodically calculated on the crude incidence (lymphosarcoma/survivors at first lymphosarcoma) and experiments that didn't appear promising were terminated early. Experiments carried to term (one year) were subjected to the Kimball analysis. For the sake of consistency, the incidences in the later experiments (this report) are given as lymphosarcoma/survivors at first lymphosarcoma, although the "P" values at term are calculated on the corrected incidences. In general the significances of differences calculated on the crude and refined data were comparable.

2. General conclusion. It may be stated that injection of mice after irradiation with 40% homogenate of sheep spleen, or with whole serum, exerts a protective effect manifested by a lowered incidence of thymic lymphosarcoma in the injected animals. This statement applies to C57Bl/6 and BALB/c mice. RF mice show no change. Bovine spleen extracts, but not bovine serum albumin are also protective. The evidence for the conclusions is set forth in Hodes et al., Cancer Research 26, Pt. I, 1780-1786 (1966) (COO-1391-5).

3. Fractionation of sheep spleen and serum on Sephadex G-200. Preliminary experiments with spleen extracts suggested that fractionation on Sephadex should be preceded by separation into a fraction precipitable with 30-40% saturated ammonium sulfate. Many experiments were done following this scheme. This was unfortunate because it later became apparent that RLP precipitates between 0-60% saturation with ammonium sulfate. After it became apparent that serum was as good a source of RLP as spleen, ammonium sulfate fractionation of serum was also followed by chromatography on Sephadex G-200.

RLP activity appeared mainly in the early fractions (void volume) of sheep spleen extracts. The RLP activity appeared in the same place in both the 0-40% and 40-60% saturated ammonium sulfate pools. On the other hand, the activity in serum eluted from the columns in later fractions and more diffusely, especially from the 40-60% saturated ammonium sulfate pool. Of course all the fractions were heterogeneous on polyacrylamide gel electrophoresis.

The appearance of activity in more than one fraction, and the shift in activity from heavy (spleen) to light (serum) was thought to indicate that RLP may be a complex substance, and that either more than one protein is necessary for activity, or that RLP is a small molecule carried by a large protein, such as α_2M .

It has proved easier to rule out certain fractions as RLP than to determine exactly which protein bears the activity. Thus albumin and γ -globulin are devoid of RLP activity.

The Sephadex fractionation work is summarized in Clewell et al., Cancer Res. 29:1333 (1969) (COC-1391-22).

4. Experiments with Cohn fractions of serum. By the time the Sephadex studies were completed, Berenblum et al., Nature 217:857 (1968) had reported that α_2M possessed certain attributes expected of RLP (increased regeneration of bone marrow and thymus cells after x-irradiation of mice and injection with α_2M), and we had found that serum contains RLP. Other schemes of fractionation were sought. It was decided to utilize the commercial Cohn fractions of sheep, and to subdivide these further by DEAE-cellulose chromatography according to Oh and Sanders (Anal. Biochem. 15:232, 1966). Because individual tests require 13 or more months from start to finish, including 12 months of observation of the mice, it was decided to begin the fractionation of the Cohn fractions before the tests on the whole Cohn fractions themselves were complete. If a particular Cohn fraction proved negative, the experiments comprised therein could

be discarded. It was thought this scheme would be cheaper and quicker than waiting a year or two for definitive results on the Cohn fractions themselves. As reported above albumin (Cohn V) and α -globulin (Cohn II) from bovine sources had already been shown to be negative. In preliminary single tests (Table 1, COO-1391-21), Cohn fractions I and IV were shown to produce significant lowering of the incidence of lymphosarcoma, whereas IV-4 was negative. α_2 -globulin (IX solution) produced a highly significant difference once, but on a second try the control incidence was too low to permit a meaningful comparison.

The final results of the tests on crude Cohn fractions are outlined in Table 1. Cohn fractions I, III, III-O, IV-1, IV, and α_2 -globulin each proved positive on a single test. Repeat tests on III-O, IV-1, and α_2 -globulin were negative. Repeat tests were not performed on III, III-2 (negative), III-3 (negative), IV (positive) or I (positive), and were positive on IV-4.

(a) Cohn III on DEAE cellulose. A typical pattern obtained on DEAE-cellulose was presented as Fig. 1 of COO-1391-17 and the gel and immunoelectrophoresis patterns as Figs. 2 and 3 of that report.

The results of the RLP testing of the fractions is summarized in Table 2 of this report. Although fractions 2, 5 and 12 of the sheep material each showed a striking difference between control and injected animals on one occasion, this was not reproducible. Fraction 3 produced a significant difference twice, and failed to do so once (although fewer leukemias appeared in the treated group). No consistent significant differences were found in the experiments with bovine Cohn III. In general, it appears that RLP activity if present at all may appear in the early fractions, for here the incidence of leukemia in the control animals is often higher than in the treated. However, the significance of the difference more often than not falls short of the $P=.05$ level.

(b) Cohn IV-4. The preliminary results of the anti-leukemia testing were presented in Table 4 of COO-1391-21 and an elution diagram as Fig. 1 of that report. The final results are presented here in Table 3 of the current report. Note that although unfractionated IV-4 yielded significant differences in 2 out of 3 tests (Table 1), only fractions 13 and 14 demonstrated RLP activity, and a repeat of 13 could not be properly evaluated because of the low leukemia incidence

in PBS injected controls.

5. Experiments with miscellaneous materials. These were usually performed as pilot tests. They are presented here briefly for the sake of completeness.

Sheep hemoglobin (COO-1391-21, Table 5) gave protection in one experiment and failed to do so on 4 repeated attempts. Denatured hemoglobin, hemin, and globin were without effect. Fibrinogen also afforded no protection, and the same negative effect was found with mouse tissue extract (COO-1391-21, Table 6). This latter result was surprising.

Summary of attempts to isolate RLP

The initial findings of leukemia prevention in mice (C57Bl/6 or BALB/c) when these were inoculated after x-irradiation with extracts of sheep spleen or serum were confirmed and extended to bovine preparations. RLP precipitates from solutions between 0 and 60% saturated with ammonium sulfate. The RLP of spleen is eluted from Sephadex G-200 with or near the void volume, whereas that of serum eluted near albumin. Several commercial Cohn fractions prepared from sheep serum demonstrated RLP activity on one or more occasions. These were I, III, III-O, IV, IV-1, and IV-4. The latter was positive in 2 of 3 tests. Certain subfractions of Cohn III and IV-4 were also positive, but no firm conclusions can be drawn owing to insufficient repeated testing (excessive mortality, low incidence of leukemia in saline-injected controls) or inconsistency among the results of the tests.

B. Possible correlates of RLP action and the search for a shorter assay.

The necessarily extended lag period between preparation of material to be tested for RLP activity and the final correlation of the data from a single experiment (a minimum of 13 months) led us to seek a shorter assay. Some of the changes occurring after x-irradiation are immediate, and reversal of these was sought. As outlined below these studies led to the finding that some of these changes can be reversed by injections of whole serum after irradiation. Attempts to localize the activity to particular fractions of serum have not yet been successful. Not enough fractions have been examined in detail to warrant conclusions about the probable success of such experiments, nor to provide correlations with the RLP activity (or absence thereof) in any given fraction except bovine serum albumin. This latter is without effect.

1. Effect of sheep spleen extract on x-irradiated cultures of Chinese hamster cells (COO-1391-6). No difference in chromosomal aberrations among cells receiving 1X, 5X or 0X extract were noted at 8, 12, 25 and 30 hours post irradiation.
2. Effect of sheep spleen extracts on bone marrow of C57Bl/6 mice (COO-1391-6). Femoral bone marrows were examined on days 2, 3 and 7 after each of 4 weekly exposures to 180 R and inoculated with extract. No differences were noted between control and treated animals.
3. Effect of sheep spleen extracts on death rate following exposure to sublethal doses of x-ray (COO-1391-5 and 9). Some 25 experiments on Swiss, C57Bl and G3H/ab were performed. Single doses of 550-800 R were followed or preceded by injections of sheep spleen extract. Only 3 experiments yielded differences significant at the 5% level and these findings were not reproducible.
4. Effect of sheep spleen extracts on Rauechar leukemia (COO-1391-5). There were no significant differences in the incidence of Rauechar leukemia in two groups (8 each) of mice injected with virus and sheep spleen extract.
5. Effect of sheep serum and its fractions on the enzyme levels of various organs of x-irradiated mice (COO-1391-11, 13, 19). This study comprised a large number of experiments of varying duration performed on Swiss and C57/Bl mice. They may be summarized as follows: Injection of serum, but not boiled serum, albumin, γ -globulin, or FHS, caused a lowering of the elevated LDH and alkaline phosphatase levels (and to a lesser extent and more variably of the DNase II levels) of liver, spleen and serum of x-irradiated mice. Levels of these enzymes in thymus were unchanged. There was sometimes a highly significant interaction between the time post-irradiation and the enzyme level, and significant lowering was limited to a particular day or days. An attempt was made to correlate the enzyme-lowering effect on day 7 with the RLP activity of the Sephadex fractions obtained from sheep serum. The correlation with fractions was only fair, although whole serum correlated well (for certain enzymes and organs).

The results of these experiments are complex, and the

reader is referred to the original reports and publications for details.

DNA and RNA polymerase activities of several organs were studied. In general, RNA polymerase behaved like LDH and alkaline phosphatase, rising after exposure to 300 R and falling when serum was injected (COO-1391-17, 18). DNA polymerase activity by contrast, falls after irradiation, and serum injection tends to raise the level toward normal. This correlates well with the findings of Cividalli and Knyssynski (Radiation Res. 30:148, 1967) that sheep spleen injections restore the thymus weights of irradiated animals.

6. The α_2M level of serum and serum fractions (COO-1391-17, 19). Berenblum (personal communications and Nature 217:857, 1968) suggested that α_2M is or carries RLP and that α_2M is able to restore thymus and bone marrow function after x-irradiation. For this reason α_2M , measured as trypsin esterase binding activity (TEBA) was sought in sheep serum and its fractions.

The highest level of activity was found in Cohn III. Cohn IV-1 and IV each had about 50% the activity of III, whereas IV-4 was almost devoid of activity. Sheep and mouse serum had comparable levels of activity. The activity fell on storage.

The α_2M (TEBA) was found in the first Sephadex fractions as expected. This did not correlate with the RLP activity of serum (COO-1391-17; compare Tables 1 through 3, COO-1391-5). Therefore either α_2M is not RLP or the differences reported in the tables occurred by chance and are erroneous. Similarly, Cohn IV-4 demonstrated RLP but little or no TEBA.

The TEBA activity (and trypsin inhibitory capacity, TIC) of DEAE-cellulose column eluates of whole serum have been investigated during the past year and are presented as Appendix I to this report. This study was undertaken because α_2M is readily damaged, and we thought studies should be done on fresh, whole serum.

The changes in TEBA of mouse serum were studied after x-irradiation and serum injection (COO-1391-17;19). Irradiation causes a lowering of TEBA in serum, whereas injection of sheep serum (but not albumin) causes the level to rise again.

7. Effects of serum injection on thymus weights and leukocyte counts of x-irradiated mice (C00-1391-17, 18, 21). The findings of Cividalli and Knyszynski cited in (3) above led to a study of the effects of serum and serum fraction injections on mice exposed to a single x-ray dose of 300 R and a single injection of test substance. Serum but not albumin caused a return in thymus weight toward normal. This finding is of interest because the test of Cividalli and Knyszynski requires 4 exposures to 180 R and 4 injections of "ELP", whereas our results were obtained after a single exposure to 300 R and one injection of ELP. However, the differences were not apparent during the first 10 days.

Several DEAE-cellulose fractions of Cohn III were tested. Fractions 5, 11, 12, 13 did not produce significant differences in thymus weight on days 6-21. Fraction 13 caused a significant difference in wbc on day 14 in one experiment.

Injection of serum caused little difference in the low peripheral wbc between treated and untreated mice for 9-14 days. After this, treated mice showed an accelerated return toward normal.

Table 1
Results obtained in the leukemia prevention test
with commercial Cohn fractions (not chromatographed)

Expt. No.	Material injected	Sex	Lymphosarcoma	P	Duration (days)
			Survivors at first lymphosarcoma		
39	III PBS	F	8/37 21/39	.011	367
62	III-0 ^a PBS	M	12/33 26/34	.0003	363
66	III-0 ^a PBS	M	16/33 23/36	N.S.	366
49	III-2 ^a PBS	M	7/27 12/32	N.S.	363
63	III-3 ^a PBS	F	19/39 20/39	N.S.	363
60	IV-1 PBS	M	24/48 31/50	N.S.	363
64	IV-1 PBS	M	17/33 19/25	.046	363
61	IV-4 PBS	F	10/18 29/35	.014	363
65	IV-4 PBS	F	17/31 30/40	.021	363
83	IV-4 PBS	M+F	14/36 12/45	N.S.	363
84	IV PBS	M+F	9/11 16/47	.03	363
107	I PBS	M+F	6/50 24/40	<.0001	364
109	α_2 -globulin PBS	M+F	5/33 32/50	<.001	363
110	α_2 -globulin PBS	M+F	5/51 7/50	N.S.	366

^a bovine; remainder are ovine in origin

Table 2

Results obtained in the leukemia prevention test
with Cohn fraction III chromatographed on DEAE-cellulose columns

Expt. No. ^a	Material injected	Lymphosarcoma	P ^b	Duration (days)
		Survivors at first lymphosarcoma		
111	Fr. 1 ^b PBS	*		—
112	Fr. 2 ^b PBS	*		—
113	Fr. 3 PBS	9/44 20/41	.008	365
114	Fr. 4 PBS	15/42 16/43	N.S.	365
115	Fr. 5 PBS	5/26 14/36	N.S.	365
116	Fr. 6 PBS	18/45 15/37	N.S.	365
117	Fr. 7 PBS	11/32 18/37	N.S.	365
118	Fr. 8 PBS	21/44 20/39	N.S.	365
122	Fr. 1 PBS	23/45 22/46	N.S.	365
123	Fr. 2 PBS	19/50 22/49	N.S.	365
124	Fr. 3 PBS	15/45 19/41	N.S.	365
125	Fr. 4 PBS	16/44 20/43	N.S.	365

Table 2 continued

126	Fr. 5 PBS	16/47 25/45	.05	365
128	Fr. 7 PBS	4/49 1/47	N.S.	365
129	Fr. 8 PBS	7/46 10/42	N.S.	365
130	Fr. 9 PBS	1/47 6/40	N.S.	365
132	Fr. 11 PBS	2/47 3/42	N.S.	365
135	Fr. 14 PBS	1/34 1/49	N.S.	365
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140	Fr. 1 PBS	11/48 14/47	N.S.	365
142	Fr. 3 PBS	9/39 14/37	N.S.	365
143	Fr. 4 PBS	18/44 10/38	N.S.	365
144	Fr. 5 PBS	17/48 14/44	N.S.	365
145	Fr. 6 PBS	12/37 18/44	N.S.	365
146	Fr. 7 PBS	13/41 23/41	N.S.	365
149	Fr. 10 PBS	11/50 13/43	N.S.	365
150	Fr. 11 PBS	21/48 23/45	N.S.	365

Table 2 continued

151	Fr. 12 PBS	6/46 24/43	.0002	365
152	Fr. 13 PBS	17/39 23/40	N.S.	365
153	Fr. 14 PBS	14/47 19/48	N.S.	365
175	Fr. 1 PBS	11/44 ^b 16/37	N.S.	299
176	Fr. 3 PBS	8/40 15/33	.016	365
177	Fr. 5 PBS	15/43 21/44	N.S.	365
178	Fr. 7 PBS	8/50 ^b 9/50	N.S.	281
179	Fr. 9 PBS	14/45 ^b 9/42	N.S.	269
180	Fr. 11 PBS	12/42 12/45	N.S.	268
207	Fr. 1 PBS	11/36 ^b 8/31	N.S.	306
208	Fr. 1 PBS	14/33 19/25	.03	365
209	Fr. 2 PBS	21/39 ^b 15/31	N.S.	260
210	Fr. 2 PBS	15/30 20/30	N.S.	365
211	Fr. 3 PBS	18/41 20/36	N.S.	364
212	Fr. 3 PBS	20/24 29/36	N.S.	347

Table 2 continued

213	Fr. 4 PBS	6/26 15/31	.04	354
214	Fr. 4 PBS	6/26 9/38	N.S.	345
215	Fr. 5 PBS	3/24 6/28	N.S.	346
216	Fr. 5 PBS	10/31 13/37	N.S.	305
217	Fr. 6 PBS	10/43 11/45	N.S.	307

^a 111-180, Sheep Cohn III; 207-217 beef Cohn III.

^b Terminated early owing to low incidence of lymphosarcoma in control group.

^c Based on corrected values, method of Kimball except for experiments terminated early. In the latter case, χ^2 was computed from the "lymphosarcoma/survivors at first lymphosarcoma" values.

Table 3

Results obtained in the leukemia prevention test
with Cohn fraction IV-4 chromatographed on DEAE cellulose

Expt. No.	Material injected	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
154	Fr. 1 PBS	18/43 16/44	N.S.	365
155	Fr. 2 PBS	15/44 24/48	N.S.	365
156	Fr. 2 PBS	17/46 11/38	N.S.	365
157	Fr. 4 PBS	15/36 22/46	N.S.	367
158	Fr. 5 PBS	19/36 22/39	N.S.	354
159	Fr. 6 PBS	16/38 14/41	N.S.	365
160	Fr. 7 PBS	8/24 12/29	N.S.	365
161	Fr. 8 PBS	15/43 22/36	N.S.	356
162	Fr. 9 PBS	10/28 16/33	N.S.	354
163	Fr. 10 PBS	19/36 21/42	N.S.	343
164	Fr. 11 PBS	14/47 17/46	N.S.	341
165	Fr. 12 PBS	16/44 21/43	N.S.	365

Table 3 continued

166	Fr. 13 PBS	9/45 18/45	.026	365
167	Fr. 14 PBS	9/45 15/42	.046	365
181	Fr. 1 PBS	11/47 12/45	N.S.	266
182	Fr. 2 PBS	13/49 11/45	N.S.	306
183	Fr. 3 PBS	11/40 15/41	N.S.	294
184	Fr. 4 PBS	6/46 8/47	N.S.	293
185	Fr. 5 PBS	26/43 25/41	N.S.	279
186	Fr. 6 PBS	22/41 24/41	N.S.	278
187	Fr. 7 PBS	20/36 20/34	N.S.	267
188	Fr. 8 PBS	24/34 6/19	N.S.	213
189	Fr. 9 PBS	19/32 23/37	N.S.	251
190	Fr. 10 PBS	27/45 13/36	N.S.	251
191	Fr. 11 PBS	21/50 20/46	N.S.	274
192	Fr. 12 PBS	22/42 10/42	N.S.	273

Table 3 continued

194	Fr. 1 PBS	14/45 22/45	(ϕ , .01)	365
195	Fr. 2 PBS	21/45 21/46	N.S.	250
201	Fr. 8 PBS	14/29 15/32	N.S.	360
203	Fr. 10 PBS	12/32 13/37	N.S.	358
206	Fr. 13 PBS	3/26 6/40	N.S.	365

Appendix I

The Distribution of Trypsin Esterase Binding Activity (TERA) and Trypsin Inhibitory Capacity (TIC) in Sheep Serum.

The trypsin esterase binding activity (TERA) of serum is confined to α_2 -macroglobulin (α_2M), whereas the trypsin inhibitory capacity (TIC) is not. Because α_2M has been suggested as a candidate for NLP, the radiation leukemia protection factor (1) or its carrier (2), and because α_2M may be responsible for the reversal of certain acute effects of x-irradiation, we have investigated various fractionation schemes for serum protein. Here we report the distribution of α_2M as determined by its TERA and the distribution of the TIC in whole sheep serum fractionated on DEAE-cellulose.

Materials and Methods

Sample and column preparation. Sheep blood, purchased from Hoerster and Son, Lamb Packers, was allowed to clot and centrifuged at 1800 rpm for 40 min at 2°. The serum was removed and 25 ml applied to a 66 x 3.5 cm column of DEAE-cellulose equilibrated with 0.01 M sodium phosphate buffer, pH 7.8. The column was eluted by the stepwise procedure of Oh and Sanders (3), utilizing 0 to 0.2 M NaCl in 0.01 M sodium phosphate buffer, pH 7.8 to 65. One column volume of 0.01 M buffer was passed through the column after application of the sample to remove unadsorbed protein.

Sephadex G-200 chromatography was accomplished on a 24 x 1 cm column to which 1 ml of the concentrated, pooled fractions from the DEAE-cellulose column were applied.

Immunoelectrophoresis. This was performed in a Galman apparatus, using sodium barbital buffer, pH 8.6 and 1.5% agarose at 3 mmx per slide for 1.5 hours. Slides were stained with amido black 10 B and destained with 3% acetic acid. The antiserum was prepared in rabbits injected twice at weekly intervals with serum emulsified in Freund's adjuvant and once with whole serum. The rabbits were bled the fourth week.

Gel electrophoresis. The method of Strauss and Kaesberg (4) was used. The columns were stained with a mixture of Coomassie blue and amido black 10 B, and destained electrophoretically.

Enzyme assays on column fractions. A modification of the method of Canrot, previously described (3), was used. The samples were 30 μ l.

Acute experiments with a column fraction. Eight week old male Swiss mice were purchased from the Laboratory Supply Co. One x-ray dose of 300 R was followed within the hour by injection of 0.25 ml of either phosphate-buffered saline (PBS), whole sheep serum or pooled fraction X ($A_{280} = 1.0$) from the column depicted in Fig. 2. This fraction exhibited considerable TEBA (Table 1). Control groups were: (1) Non-irradiated, noninjected mice, matched for age, (2) nonirradiated mice, injected with sheep serum and (3) mice irradiated and injected with PBS. Alkaline phosphatase activity was determined in triplicate on 30 μ l samples as reported previously (6) on each of the 6 mice in every group, one day after x-irradiation.

Results

The elution patterns varied somewhat and resulted in 9-13 peaks of absorbance at 280 nm on chromatography on DEAE-cellulose (Figs. 1 and 2). The variation was due to a variable separation at the high-salt portion of the gradient. The approximate size distribution of the proteins in the fractions is evident from the Sephadex elution patterns (Fig. 3) of the fractions obtained from the DEAE column of Fig. 2. The fractions containing the larger molecules (i.e., eluting with or near the void volume) were I, IV, VI, VII and IX. The bulk of the TEBA eluted in VI and VII, and the TIC in IV and VI.

The TEBA generally eluted with the later fractions and over a narrower range than the TIC (Table 1). The fractionation on DEAE-cellulose resulted in more and sharper peaks than are obtained from DEAE-Sephadex (7) or Sephadex G-200 (8), but neither the α_2 M nor the TIC was confined to a single peak. Although the TEBA is limited to those fractions exhibiting a precipitin line in the α_2 M area, examination of both the immunoelectrophoretic (Fig. 4) and polyacrylamide gel (Fig. 5) patterns shows that a clear separation from other proteins has not been obtained in any of the pooled fractions.

The single experiment on lowering of serum alkaline phosphatase level is shown in Table II. Fraction X restored the elevated enzyme concentration to normal, whereas whole serum was less effective.

Discussion

The experiments described here are a continuation of a series designed to separate the therapeutically active proteins from sheep serum and to determine whether the x-ray protective activity of serum is confined to α_2M . As α_2M is rather labile and little is known of the stability of purified radiation protective factors, we chose to fractionate serum rapidly by the procedure of Oh and Sanders (3). The procedure has apparently not been applied to sheep serum previously, although Aalund *et al.*, (7) have studied the behavior of sheep serum on DEAE-Sephadex. They identified a protein as α_2M by its position on immunoelectrophoresis and its elution from Sephadex G-200. This α_2M was distributed on the high-salt side of the elution pattern, in consonance with our experience using DEAE-cellulose. Aalund and his coworkers gave no data on TEBA or TIC.

The work reported from this laboratory has so far not been able to confirm that α_2M is either RLP or the factor responsible for the other therapeutic effects of serum, although the single experiment of Table II is not inconsistent with this conclusion. As stated elsewhere in this report, fractions free of α_2M have demonstrated RLP or other therapeutic activities, suggesting that other proteins are responsible. Some of these may be carried by α_2M .

Studies on sheep α_2M have been hampered by lack of specific antibody. Despite the statement by James (9), we have been unable to find a precipitin line between sheep serum and antihuman α_2M . Our identifications of the protein have been by position on the chromatogram and immunoelectrophoretic patterns and trypsin-esterase binding activity.

The problem of comparing elution patterns from different species has been approached by Baumstark (10). Human and swine sera were compared by chromatography on DEAE-cellulose and Sephadex G-200, and individual proteins identified by immunodiffusion and immunoelectrophoresis. Gradient elution was used, and the fractionation from DEAE-cellulose is not as sharp as that obtained here by the method of Oh and Sanders (3). α_2M eluted in much the same area as in our study. The method has been applied by Sanders and Oh (11) to human Cohn III with similar results: α_2M elutes with the second half of the gradient.

The identification of α_2M by means of its TEBA seems clear enough. The identification of the proteins exhibiting TIC is more difficult. This activity is shown by various α_1 and α_2 proteins, not all of which have been clearly identified (12).

Conclusions

Whole sheep serum can rapidly and conveniently be separated into a number of fractions by the method of Oh and Sanders. While not homogeneous, the fractions are enriched in various of the protein components of serum. α_2 Macroglobulin, identified by its position on immunoelectrophoresis and trypsin esterase binding activity, is eluted in the high salt half of the eluate from DEAE-cellulose, whereas the trypsin inhibitory activity of the serum is more widely scattered. A fraction with high TEBA lowered the alkaline phosphatase level of mice on day 1 after exposure to 300 R of x-irradiation.

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Table I

Trypsin esterase binding activity (TEBA) and trypsin inhibitory capacity (TIC)
of DEAE-cellulose fractions of sheep serum 1

Fraction No	TEBA	$\Delta A_{410}/\text{min/ml}$	TIC
I	0.000		0.000
II	0.000		0.450
III	0.000		0.000
IV	0.000		1.480
V	0.041		0.460
VI	0.208		1.911
VII	0.172		0.720
VIII	0.016		0.300
IX	0.006		0.000

¹ The fractions were obtained from the column of Fig. 2

Table II

The effect of 300 R on alkaline phosphatase activity
of mouse serum 1 day after exposure

Treatment	Alk. phosphatase ($\Delta\Delta\Delta 10/\text{min/ml}$)
Age Control	0.144
Non-irradiated + Sheep Serum	0.160
X-Ray + PBS	0.242
X-Ray + Sheep Serum	0.208
X-Ray + Fraction X ¹	0.137

¹ From the column of Fig. 1

Fig. 1

Fig. 2

Fig 9

Fig. 4

Fig. 5

Legenda for Figures

- Fig. 1. Elution of sheep serum proteins from DEAE-cellulose. Column dimensions, 3.5×66 cm. Sample size, 25 ml. Fraction size, 6 ml. Flow rate, 220 ml/hr.
- Fig. 2. Elution of sheep serum proteins from DEAE-cellulose. Column dimensions, 7×66 cm. Sample size, 50 ml. Fraction size, 25 ml. Flow rate, 400 ml/hr.
- Fig. 3. Elution of pooled fractions from DEAE-cellulose from Sephadex G-200. The fractions were pooled from the column of Fig. 2 as indicated, concentrated, and applied to the column. Column size, 1×24 cm. Sample size, 1 ml. Fraction size, 0.5 ml. Flow rate, 7.5 ml/hr.
- Fig. 4. Polyacrylamide gel electrophoresis of pooled fractions from DEAE-cellulose. The fractions from the column of Fig. 1 were pooled and concentrated. The electrophoresis was performed in an B-C vertical gel apparatus. Staining was with Coomassie blue: Amido black 10B and was followed by electrolytic destaining.
- Fig. 5. Immuno-electrophoresis of pooled fractions from DEAE-cellulose. This was performed on the material used for the patterns of Fig. 4, as described in methods.

INDIANA UNIVERSITY
CONTRACT No. AT(11-1)-1391

FINAL PROGRESS REPORT

UNITED STATES ATOMIC ENERGY COMMISSION

"Isolation of a Factor Preventing X-ray Induced Leukemia"

Period covered: 1 June 1964 through 31 May 1971

Principal Investigator: M. E. Hodes, M.D., Ph.D.
Professor of Medicine and Biochemistry

Summary: This final report includes a summary of previous reports and work completed through 31 December 1969 and a detailed report of the final phases of the project. The experiments reported are of 2 types: (1) Those designed to isolate the factor (RLP, for radiation leukemia protection) and test its ability to prevent thymic lymphosarcoma in x-irradiated C57Bl/6 mice and (2) Attempts to correlate other therapeutic effects of tissue extracts serum and their fractions with RLP activity.

Serum and spleen extract, injected after irradiation, consistently lower the incidence of thymic lymphosarcoma. Several Cohn fractions, and especially IV-4 do likewise. Serum and spleen extracts can be separated into active and inactive fractions on Sephadex. Cohn fractions III and IV-4 can apparently be fractionated but not enough testing has been done to permit firm conclusions as to the identity of RLP.

Sheep spleen extracts do not protect against the lethal effects of single exposures to 550-800 R. However, several acute changes produced by 180-300 R are reversed by injection of serum after exposure. The changes reversed include elevated serum and organ levels of lactate dehydrogenase, alkaline phosphatase, RNA polymerase, and to a lesser extent, DNase II, and decreased levels of trypsin-esterase binding activity and DNA polymerase. Serum also hastens recovery of thymus weight. Experiments with serum fractions have been less successful. Although albumin is definitely devoid of activity, the active fraction has not been positively identified.

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- COO-1391-10 PREVENTION OF X-RAY-INDUCED LEUKEMIA IN MICE. II. FRACTIONATION OF SPLEEN EXTRACTS CONTAINING THE RADIATION LEUKEMIA PROTECTION FACTOR. By Don B. Clewell, J. Donald Hubbard, Pao-Lo Yu and M. E. Hodes.
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- COO-1391-12 EFFECT OF SUBSTANCES WITH RADIATION LEUKEMIA PROTECTIVE ("RLP") ACTION ON ENZYME LEVELS IN MICE AFTER X-RAY. By Lily C. Yip, N. Blair, P. Yu and M. E. Hodes. Proc. Am. Assoc. Cancer Res. 9:79 (1968).
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- COO-1391-15 EXPERIMENTS ON THE MODE OF ACTION OF THE RADIATION LEUKEMIA PROTECTIVE ("RLP") ACTIVITY OF SERUM. L. Yip, P.-L. Yu, and M. E. Hodes. Proc. Am. Assoc. Cancer Res. 10:102 (1969).
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- COO-1391-19 TRYPSIN ESTERASE BINDING AND ITS RELATION TO THE RADIATION-LEUKEMIA PROTECTION (RLP) FACTOR. Lily C. Yip and M. E. Hodes Proc. Soc. Exptl. Biol. & Med. 133, 1285 (1970).
- COO-1391-20 Lily C. Yip and M. E. Hodes. Abstract submitted and presented to the International Cancer Congress, Houston, Texas, May 1970.
- COO-1391-21 Progress report: February 1, 1969 through December 31, 1969.
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As this is a final report, all the studies performed are included. Work already reported in COO-1391-1, 6, 13, 17, and 21 is presented in summary form, and studies not previously reported are described in greater detail. The bibliography is complete to date.

The work itself comprises the actual attempts at isolation of the Radiation Leukemia Protection (RLP) factor with the attendant mouse tests, and various ancillary experiments. Most of the latter were designed as attempts to correlate changes following x-irradiation (such as changes in serum enzyme levels) with protective action against leukemia.

A. Isolation of RLP

1. Protocol for x-irradiation, injection and care of mice. This was given in COO-1391-1. Deviations from the original protocol have included (a) entering 25-50 males and females instead of 100 of each and (b) use of younger mice - down to about 30 days. The first change was made when funding failed to keep pace with financial requirements and the second when it became obvious that the incidence of leukemia in saline-injected controls was low (in older mice). For the purposes of this work the term "leukemia" is synonymous with "thymic lymphosarcoma", whether the latter is disseminated or remains localized. The significance of differences between leukemia incidence in control and treated animals has been consistently calculated by the method of Kimball (Bull. Internat. Statist. Inst. 36:193, 1958) on the incidences corrected for deaths due to extraneous causes - except that in the last group of experiments, the significance was initially and periodically calculated on the crude incidence (lymphosarcomas/survivors at first lymphosarcoma) and experiments that didn't appear promising were terminated early. Experiments carried to term (one year) were subjected to the Kimball analysis. For the sake of consistency, the incidences in the later experiments (this report) are given as lymphosarcoma/survivors at first lymphosarcoma, although the "P" values at term are calculated on the corrected incidences. In general the significances of differences calculated on the crude and refined data were comparable.

2. General conclusion. It may be stated that injection of mice after irradiation with 40% homogenate of sheep spleen, or with whole serum, exerts a protective effect manifested by a lowered incidence of thymic lymphosarcoma in the injected animals. This statement applies to C57Bl/6 and BALB/c mice. RF mice show no change. Bovine spleen extracts, but not bovine serum albumin are also protective. The evidence for the conclusions is set forth in Hodes et al., Cancer Research 26, Pt. 1, 1780-1786 (1966) (COO-1391-5).

3. Fractionation of sheep spleen and serum on Sephadex G-200. Preliminary experiments with spleen extracts suggested that fractionation on Sephadex should be preceded by separation into a fraction precipitable with 30-40% saturated ammonium sulfate. Many experiments were done following this scheme. This was unfortunate because it later became apparent that RLP precipitates between 0-60% saturation with ammonium sulfate. After it became apparent that serum was as good a source of RLP as spleen, ammonium sulfate fractionation of serum was also followed by chromatography on Sephadex G-200.

RLP activity appeared mainly in the early fractions (void volume) of sheep spleen extracts. The RLP activity appeared in the same place in both the 0-40% and 40-60% saturated ammonium sulfate pools. On the other hand, the activity in serum eluted from the columns in later fractions and more diffusely, especially from the 40-60% saturated ammonium sulfate pool. Of course all the fractions were heterogeneous on polyacrylamide gel electrophoresis.

The appearance of activity in more than one fraction, and the shift in activity from heavy (spleen) to light (serum) was thought to indicate that RLP may be a complex substance, and that either more than one protein is necessary for activity, or that RLP is a small molecule carried by a large protein, such as α_2M .

It has proved easier to rule out certain fractions as RLP than to determine exactly which protein bears the activity. Thus albumin and γ -globulin are devoid of RLP activity.

The Sephadex fractionation work is summarized in Clevell *et al.*, Cancer Res. 29:1333 (1969) (COO-1391-22).

4. Experiments with Cohn fractions of serum. By the time the Sephadex studies were completed, Beranblum *et al.*, Nature 217:857 (1968) had reported that α_2M possessed certain attributes expected of RLP (increased regeneration of bone marrow and thymus cells after x-irradiation of mice and injection with α_2M), and we had found that serum contains RLP. Other schemes of fractionation were sought. It was decided to utilize the commercial Cohn fractions of sheep, and to subdivide these further by DEAE-cellulose chromatography according to Oh and Sanders (Anal. Biochem. 15:232, 1966). Because individual tests require 13 or more months from start to finish, including 12 months of observation of the mice, it was decided to begin the fractionation of the Cohn fractions before the tests on the whole Cohn fractions themselves were complete. If a particular Cohn fraction proved negative, the experiments comprised therein could

be discarded. It was thought this scheme would be cheaper and quicker than waiting a year or two for definitive results on the Cohn fractions themselves. As reported above albumin (Cohn V) and α -globulin (Cohn II) from bovine sources had already been shown to be negative. In preliminary single tests (Table 1, COO-1391-21), Cohn fractions I and IV were shown to produce significant lowering of the incidence of lymphosarcoma, whereas IV-4 was negative. α_2 -globulin (IX solution) produced a highly significant difference once, but on a second try the control incidence was too low to permit a meaningful comparison.

The final results of the tests on crude Cohn fractions are outlined in Table 1. Cohn fractions I, III, III-0, IV-1, IV, and α_2 -globulin each proved positive on a single test. Repeat tests on III-0, IV-1, and α_2 -globulin were negative. Repeat tests were not performed on III, III-2 (negative), III-3 (negative), IV (positive) or I (positive), and were positive on IV-4.

(a) Cohn III on DEAE cellulose. A typical pattern obtained on DEAE-cellulose was presented as Fig. 1 of COO-1391-17 and the gel and immunoelectrophoresis patterns as Figs. 2 and 3 of that report.

The results of the RLP testing of the fractions is summarized in Table 2 of this report. Although fractions 2, 5 and 12 of the sheep material each showed a striking difference between control and injected animals on one occasion, this was not reproducible. Fraction 3 produced a significant difference twice, and failed to do so once (although fewer leukemias appeared in the treated group). No consistent significant differences were found in the experiments with bovine Cohn III. In general, it appears that RLP activity if present at all may appear in the early fractions, for here the incidence of leukemia in the control animals is often higher than in the treated. However, the significance of the difference more often than not falls short of the $P=.05$ level.

(b) Cohn IV-4. The preliminary results of the anti-leukemia testing were presented in Table 4 of COO-1391-21 and an elution diagram as Fig. 1 of that report. The final results are presented here in Table 3 of the current report. Note that although unfractionated IV-4 yielded significant differences in 2 out of 3 tests (Table 1), only fractions 13 and 14 demonstrated RLP activity, and a repeat of 13 could not be properly evaluated because of the low leukemia incidence

in PBS injected controls.

3. Experiments with miscellaneous materials. These were usually performed as pilot tests. They are presented here briefly for the sake of completeness.

Sheep hemoglobin (COO-1391-21, Table 5) gave protection in one experiment and failed to do so on 4 repeated attempts. Denatured hemoglobin, hemin, and globin were without effect. Fibrinogen also afforded no protection, and the same negative effect was found with mouse tissue extract (COO-1391-21, Table 6). This latter result was surprising.

Summary of attempts to isolate RLP

The initial findings of leukemia prevention in mice (C57Bl/6 or BALB/c) when these were inoculated after x-irradiation with extracts of sheep spleen or serum were confirmed and extended to bovine preparations. RLP precipitates from solutions between 0 and 60% saturated with ammonium sulfate. The RLP of spleen is eluted from Sephadex G-200 with or near the void volume, whereas that of serum eluted near albumin. Several commercial Cohn fractions prepared from sheep serum demonstrated RLP activity on one or more occasions. These were I, III, III-O, IV, IV-1, and IV-4. The latter was positive in 2 of 3 tests. Certain subfractions of Cohn III and IV-4 were also positive, but no firm conclusions can be drawn owing to insufficient repeated testing (excessive mortality, low incidence of leukemia in saline-injected controls) or inconsistency among the results of the tests.

B. Possible correlates of RLP action and the search for a shorter assay.

The necessarily extended lag period between preparation of material to be tested for RLP activity and the final correlation of the data from a single experiment (a minimum of 13 months) led us to seek a shorter assay. Some of the changes occurring after x-irradiation are immediate, and reversal of these was sought. As outlined below these studies led to the finding that some of these changes can be reversed by injections of whole serum after irradiation. Attempts to localize the activity to particular fractions of serum have not yet been successful. Not enough fractions have been examined in detail to warrant conclusions about the probable success of such experiments, nor to provide correlations with the RLP activity (or absence thereof) in any given fraction except bovine serum albumin. This latter is without effect.

1. Effect of sheep spleen extract on x-irradiated cultures of Chinese hamster cells (COO-1391-6). No difference in chromosomal aberrations among cells receiving 1X, 5X or 0X extract were noted at 8, 12, 25 and 30 hours post irradiation.
2. Effect of sheep spleen extracts on bone marrow of C57Bl/6 mice (COO-1391-6). Femoral bone marrow were examined on days 2, 3 and 7 after each of 4 weekly exposures to 180 R and inoculated with extract. No differences were noted between control and treated animals.
3. Effect of sheep spleen extracts on death rate following exposure to sublethal doses of x-ray (COO-1391-5 and 9). Some 25 experiments on Swiss, C57Bl and C3H/ab were performed. Single doses of 350-800 R were followed or preceded by injections of sheep spleen extract. Only 3 experiments yielded differences significant at the 5X level and these findings were not reproducible.
4. Effect of sheep spleen extracts on Rauscher leukemia (COO-1391-5). There were no significant differences in the incidence of Rauscher leukemia in two groups (8 each) of mice injected with virus and sheep spleen extract.
5. Effect of sheep serum and its fractions on the enzyme levels of various organs of x-irradiated mice (COO-1391-11, 13, 19). This study comprised a large number of experiments of varying duration performed on Swiss and C57/Bl mice. They may be summarized as follows: Injection of serum, but not boiled serum, albumin, γ -globulin, or PBS, caused a lowering of the elevated LDH and alkaline phosphatase levels (and to a lesser extent and more variably of the DNase II levels) of liver, spleen and serum of x-irradiated mice. Levels of these enzymes in thymus were unchanged. There was sometimes a highly significant interaction between the time post-irradiation and the enzyme level, and significant lowering was limited to a particular day or days. An attempt was made to correlate the enzyme-lowering effect on day 7 with the HLP activity of the Sephadex fractions obtained from sheep serum. The correlation with fractions was only fair, although whole serum correlated well (for certain enzymes and organs).

The results of these experiments are complex, and the

reader is referred to the original reports and publications for details.

DNA and RNA polymerase activities of several organs were studied. In general, RNA polymerase behaved like LDH and alkaline phosphatase, rising after exposure to 300 R and falling when serum was injected (COO-1391-17, 18). DNA polymerase activity by contrast, falls after irradiation, and serum injection tends to raise the level toward normal. This correlates well with the findings of Cividalli and Knyasynski (Radiation Res. 30:148, 1967) that sheep spleen injections restore the thymus weights of irradiated animals.

6. The α_2M level of serum and serum fractions (COO-1391-17, 19). Berenblum (personal communications and Nature 217:857, 1968) suggested that α_2M is or carries RLP and that α_2M is able to restore thymus and bone marrow function after x-irradiation. For this reason α_2M , measured as trypsin esterase binding activity (TEBA) was sought in sheep serum and its fractions.

The highest level of activity was found in Cohn III. Cohn IV-1 and IV each had about 50% the activity of III, whereas IV-4 was almost devoid of activity. Sheep and mouse serum had comparable levels of activity. The activity fell on storage.

The α_2M (TEBA) was found in the first Sephadex fractions as expected. This did not correlate with the RLP activity of serum (COO-1391-17; compare Tables 1 through 3, COO-1391-5). Therefore either α_2M is not RLP or the differences reported in the tables occurred by chance and are erroneous. Similarly, Cohn IV-4 demonstrated RLP but little or no TEBA.

The TEBA activity (and trypsin inhibitory capacity, TIC) of DEAE-cellulose column eluates of whole serum α_2M have been investigated during the past year and are presented as Appendix I to this report. This study was undertaken because α_2M is readily damaged, and we thought studies should be done on fresh, whole serum.

The changes in TEBA of mouse serum were studied after x-irradiation and serum injection (COO-1391-17;19). Irradiation causes a lowering of TEBA in serum, whereas injection of sheep serum (but not albumin) causes the level to rise again.

7. Effects of serum injection on thymus weights and leukocyte counts of x-irradiated mice (COO-1391-17, 18, 21). The findings of Cividalli and Knyssynski cited in (5) above led to a study of the effects of serum and serum fractions injections on mice exposed to a single x-ray dose of 300 R and a single injection of test substance. Serum but not albumin caused a return in thymus weight toward normal. This finding is of interest because the test of Cividalli and Knyssynski requires 4 exposures to 180 R and 4 injections of "RLP", whereas our results were obtained after a single exposure to 300 R and one injection of RLP. However, the differences were not apparent during the first 10 days.

Several DEAE-cellulose fractions of Cohn III were tested. Fractions 5, 11, 12, 13 did not produce significant differences in thymus weight on days 6-21. Fraction 13 caused a significant difference in wbc on day 14 in one experiment.

Injection of serum caused little difference in the low peripheral wbc between treated and untreated mice for 9-14 days. After this, treated mice showed an accelerated return toward normal.

Table 1

Results obtained in the leukemia prevention test
with commercial Cohn fractions (not chromatographed)

Expt. No.	Material injected	Sex	Lymphosarcoma		P	Duration (days)
			Survivors at first lymphosarcoma			
39	III PBS	F	8/37 21/39		.011	367
62	III-0 ^a PBS	M	12/33 26/34		.0003	365
66	III-0 ^a PBS	M	16/33 23/36		N.S.	366
49	III-2 ^a PBS	M	7/27 12/32		N.S.	365
63	III-3 ^a PBS	F	19/39 20/39		N.S.	365
60	IV-1 PBS	M	24/48 31/50		N.S.	365
64	IV-1 PBS	M	17/33 19/25		.046	365
61	IV-4 PBS	F	10/18 29/35		.014	365
65	IV-4 PBS	F	17/31 30/40		.021	365
83	IV-4 PBS	M+F	14/36 12/45		N.S.	363
84	IV PBS	M+F	9/11 16/47		.05	365
107	I PBS	M+F	6/50 24/40		<.0001	364
109	α_2 -globulin PBS	M+F	5/33 32/50		<.001	365
110	α_2 -globulin PBS	M+F	5/51 7/50		N.S.	366

^a bovine; remainder are ovine in origin

Table 2

Results obtained in the leukemia prevention test
with Cohn fraction III chromatographed on DEAE-cellulose columns

Expt. No. ^a	Material injected	Lymphosarcoma	P ^c	Duration (days)
		Survivors at first lymphosarcoma		
111	Fr. 1 ^b PBS	*		--
112	Fr. 2 ^b PBS	*		--
113	Fr. 3 PBS	9/44 20/41	.008	365
114	Fr. 4 PBS	15/42 16/45	N.S.	365
115	Fr. 5 PBS	5/26 14/36	N.S.	365
116	Fr. 6 PBS	18/45 15/37	N.S.	365
117	Fr. 7 PBS	11/32 18/37	N.S.	365
118	Fr. 8 PBS	21/44 20/39	N.S.	365
122	Fr. 1 PBS	23/45 22/46	N.S.	365
123	Fr. 2 PBS	19/50 22/49	N.S.	365
124	Fr. 3 PBS	15/45 19/41	N.S.	365
125	Fr. 4 PBS	16/44 20/43	N.S.	365

Table 2 continued

126	Fr. 5 PBS	16/47 25/45	.05	365
128	Fr. 7 PBS	4/49 1/47	N.S.	365
129	Fr. 8 PBS	7/46 10/42	N.S.	365
130	Fr. 9 PBS	1/47 6/40	N.S.	365
132	Fr. 11 PBS	2/47 3/42	N.S.	365
135	Fr. 14 PBS	1/34 1/49	N.S.	365
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140	Fr. 1 PBS	11/48 14/47	N.S.	365
142	Fr. 3 PBS	9/39 14/37	N.S.	365
143	Fr. 4 PBS	18/44 10/38	N.S.	365
144	Fr. 5 PBS	17/48 14/44	N.S.	365
145	Fr. 6 PBS	12/37 18/44	N.S.	365
146	Fr. 7 PBS	13/41 23/41	N.S.	365
149	Fr. 10 PBS	11/50 13/43	N.S.	365
150	Fr. 11 PBS	21/48 23/45	N.S.	365

Table 2 continued

151	Fr. 12 PBS	6/46 24/43	.0002	365
152	Fr. 13 PBS	17/39 23/40	N.S.	365
153	Fr. 14 PBS	14/47 19/48	N.S.	365
175	Fr. 1 PBS	11/44 ^b 16/37	N.S.	299
176	Fr. 3 PBS	8/40 15/33	.016	365
177	Fr. 5 PBS	15/43 21/44	N.S.	365
178	Fr. 7 PBS	8/50 ^b 9/50	N.S.	281
179	Fr. 9 PBS	14/45 ^b 9/42	N.S.	269
180	Fr. 11 PBS	12/42 12/45	N.S.	268
207	Fr. 1 PBS	11/36 ^b 8/31	N.S.	306
208	Fr. 1 PBS	14/33 19/25	.03	365
209	Fr. 2 PBS	21/39 ^b 15/31	N.S.	260
210	Fr. 2 PBS	15/30 20/30	N.S.	365
211	Fr. 3 PBS	18/41 20/36	N.S.	364
212	Fr. 3 PBS	20/24 29/36	N.S.	347

Table 2 continued

213	Fr. 4 PBS	6/26 15/31	.04	354
214	Fr. 4 PBS	6/26 9/38	N.S.	345
215	Fr. 5 PBS	3/24 6/28	N.S.	346
216	Fr. 5 PBS	10/31 13/37	N.S.	305
217	Fr. 6 PBS	10/43 11/45	N.S.	307

^a 111-180, Sheep Cohn III; 207-217 beef Cohn III.

^b Terminated early owing to low incidence of lymphosarcoma in control group.

^c Based on corrected values, method of Kimball except for experiments terminated early. In the latter case, χ^2 was computed from the "lymphosarcoma/survivors at first lymphosarcoma" values.

Table 3

Results obtained in the leukemia prevention test
with Cohn fraction IV-4 chromatographed on DEAE cellulose

Expt. No.	Material injected	Lymphosarcoma	P	Duration (days)
		Survivors at first lymphosarcoma		
154	Fr. 1 PBS	18/43 16/44	N.S.	365
155	Fr. 2 PBS	15/44 24/48	N.S.	365
156	Fr. 2 PBS	17/46 11/38	N.S.	365
157	Fr. 4 PBS	15/36 22/46	N.S.	367
158	Fr. 5 PBS	19/36 22/39	N.S.	354
159	Fr. 6 PBS	16/38 14/41	N.S.	365
160	Fr. 7 PBS	8/24 12/29	N.S.	365
161	Fr. 8 PBS	15/43 22/36	N.S.	356
162	Fr. 9 PBS	10/28 16/33	N.S.	354
163	Fr. 10 PBS	19/36 21/42	N.S.	343
164	Fr. 11 PBS	14/47 17/46	N.S.	341
165	Fr. 12 PBS	16/44 21/43	N.S.	365

Table 3 continued

166	Fr. 13 PBS	9/45 18/45	.026	365
167	Fr. 14 PBS	9/45 15/42	.046	365
181	Fr. 1 PBS	11/47 12/45	N.S.	266
182	Fr. 2 PBS	13/49 11/45	N.S.	306
183	Fr. 3 PBS	11/40 15/41	N.S.	294
184	Fr. 4 PBS	6/46 8/47	N.S.	293
185	Fr. 5 PBS	26/43 25/41	N.S.	279
186	Fr. 6 PBS	22/41 24/41	N.S.	278
187	Fr. 7 PBS	20/36 20/34	N.S.	267
188	Fr. 8 PBS	24/34 6/19	N.S.	213
189	Fr. 9 PBS	19/32 23/37	N.S.	251
190	Fr. 10 PBS	27/45 13/36	N.S.	251
191	Fr. 11 PBS	21/50 20/46	N.S.	274
192	Fr. 12 PBS	22/42 10/42	N.S.	273

Table 3 continued

194	Fr. 1 PSS	14/45 22/45	($\bar{q}$, .01)	365
195	Fr. 2 PSS	21/45 21/46	N.S.	250
201	Fr. 8 PSS	14/29 15/32	N.S.	360 '
203	Fr. 10 PSS	12/32 13/37	N.S.	358
206	Fr. 13 PSS	3/26 6/40	N.S.	365

Appendix I

The Distribution of Trypsin Esterase Binding Activity (TEBA) and Trypsin Inhibitory Capacity (TIC) in Sheep Serum.

The trypsin esterase binding activity (TEBA) of serum is confined to α_2 -macroglobulin (α_2M), whereas the trypsin inhibitory capacity (TIC) is not. Because α_2M has been suggested as a candidate for RLP, the radiation leukemia protection factor (1) or its carrier (2), and because α_2M may be responsible for the reversal of certain acute effects of x-irradiation, we have investigated various fractionation schemes for serum protein. Here we report the distribution of α_2M as determined by its TEBA and the distribution of the TIC in whole sheep serum fractionated on DEAE-cellulose.

Materials and Methods

Sample and column preparation. Sheep blood, purchased from Hoerter and Son, Lamb Packers, was allowed to clot and centrifuged at 1800 rpm for 40 min at 2°. The serum was removed and 25 ml applied to a 66 x 3.5 cm column of DEAE-cellulose equilibrated with 0.01 M sodium phosphate buffer, pH 7.8. The column was eluted by the stepwise procedure of Oh and Sanders (3), utilizing 0 to 0.2 M NaCl in 0.01 M sodium phosphate buffer, pH 7.8 to 65. One column volume of 0.01 M buffer was passed through the column after application of the sample to remove unadsorbed protein.

Sephadex G-200 chromatography was accomplished on a 24 x 1 cm column to which 1 ml of the concentrated, pooled fractions from the DEAE-cellulose column were applied.

Immunoelectrophoresis. This was performed in a Gelman apparatus, using sodium barbital buffer, pH 8.6 and 1.5% agarose at 3 uamp per slide for 1.5 hours. Slides were stained with amido black 10 B and destained with 3% acetic acid. The antiserum was prepared in rabbits injected twice at weekly intervals with serum emulsified in Freund's adjuvant and once with whole serum. The rabbits were bled the fourth week.

Gel electrophoresis. The method of Strauss and Kaesberg (4) was used. The columns were stained with a mixture of Coomassie blue and amido black 10 B, and destained electrophoretically.

Enzyme assays on column fractions. A modification of the method of Garret, previously described (3), was used. The samples were 30 μ l.

Acute experiments with a column fraction. Eight week old male Swiss mice were purchased from the Laboratory Supply Co. One x-ray dose of 300 R was followed within the hour by injection of 0.25 ml of either phosphate-buffered saline (PBS), whole sheep serum or pooled fraction X ($A_{280} = 1.0$) from the column depicted in Fig. 2. This fraction exhibited considerable TERA (Table I). Control groups were: (1) Non-irradiated, noninjected mice, matched for age, (2) nonirradiated mice, injected with sheep serum and (3) mice irradiated and injected with PBS. Alkaline phosphatase activity was determined in triplicate on 30 μ l samples as reported previously (6) on each of the 6 mice in every group, one day after x-irradiation.

Results

The elution patterns varied somewhat and resulted in 9-13 peaks of absorbance at 280 nm on chromatography on DEAE-cellulose (Figs. 1 and 2). The variation was due to a variable separation at the high-salt portion of the gradient. The approximate size distribution of the proteins in the fractions is evident from the Sephadex elution patterns (Fig. 3) of the fractions obtained from the DEAE column of Fig. 2. The fractions containing the larger molecules (i.e., eluting with or near the void volume) were I, IV, VI, VII and IX. The bulk of the TERA eluted in VI and VII, and the TIC in IV and VI.

The TERA generally eluted with the later fractions and over a narrower range than the TIC (Table I). The fractionation on DEAE-cellulose resulted in more and sharper peaks than are obtained from DEAE-Sephadex (7) or Sephadex G-100 (8), but neither the α_2 M nor the TIC was confined to a single peak. Although the TERA is limited to those fractions exhibiting a precipitin line in the α_2 M area, examination of both the immunoelectrophoretic (Fig. 4) and polyacrylamide gel (Fig. 5) patterns shows that a clear separation from other proteins has not been obtained in any of the pooled fractions.

The single experiment on lowering of serum alkaline phosphatase level is shown in Table II. Fraction X restored the elevated enzyme concentration to normal, whereas whole serum was less effective.

Discussion

The experiments described here are a continuation of a series designed to separate the therapeutically active proteins from sheep serum and to determine whether the x-ray protective activity of serum is confined to α_2M . As α_2M is rather labile and little is known of the stability of purified radiation protective factors, we chose to fractionate serum rapidly by the procedure of Oh and Sanders (3). The procedure has apparently not been applied to sheep serum previously, although Aalund *et al.*, (7) have studied the behavior of sheep serum on DEAE-Sephadex. They identified a protein as α_2M by its position on immunoelectrophoresis and its elution from Sephadex G-200. This α_2M was distributed on the high-salt side of the elution pattern, in consonance with our experience using DEAE-cellulose. Aalund and his coworkers gave no data on TERA or TIC.

The work reported from this laboratory has so far not been able to confirm that α_2M is either RLP or the factor responsible for the other therapeutic effects of serum, although the single experiment of Table II is not inconsistent with this conclusion. As stated elsewhere in this report, fractions free of α_2M have demonstrated RLP or other therapeutic activities, suggesting that other proteins are responsible. Some of these may be carried by α_2M .

Studies on sheep α_2M have been hampered by lack of specific antibody. Despite the statement by James (9), we have been unable to find a precipitin line between sheep serum and antihuman α_2M . Our identifications of the protein have been by position on the chromatogram and immunoelectrophoretic patterns and trypsin-esterase binding activity.

The problem of comparing elution patterns from different species has been approached by Baumstark (10). Human and swine sera were compared by chromatography on DEAE-cellulose and Sephadex G-200, and individual proteins identified by immunodiffusion and immunoelectrophoresis. Gradient elution was used, and the fractionation from DEAE-cellulose is not as sharp as that obtained here by the method of Oh and Sanders (3). α_2M eluted in much the same area as in our study. The method has been applied by Sanders and Oh (11) to human Cohn III with similar results: α_2M elutes with the second half of the gradient.

The identification of α_2M by means of its TERA seems clear enough. The identification of the proteins exhibiting TIC is more difficult. This activity is shown by various α_1 and α_2 proteins, not all of which have been clearly identified (12).

Conclusions

Whole sheep serum can rapidly and conveniently be separated into a number of fractions by the method of Oh and Sanders. While not homogeneous, the fractions are enriched in various of the protein components of serum. α_2 Macroglobulin, identified by its position on immunoelectrophoresis and trypsin esterase binding activity, is eluted in the high salt half of the eluate from DEAE-cellulose, whereas the trypsin inhibitory activity of the serum is more widely scattered. A fraction with high TERA lowered the alkaline phosphatase level of mice on day 1 after exposure to 300 R of x-irradiation.

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Table I

Trypsin esterase binding activity (TEBA) and trypsin inhibitory capacity (TIC)
of DEAE-cellulose fractions of sheep serum ¹

Fraction No	TEBA	$\Delta A_{410}/\text{min/ml}$	TIC
I	0.000		0.000
II	0.000		0.450
III	0.000		0.000
IV	0.000		1.480
V	0.041		0.460
VI	0.208		1.911
VII	0.172		0.720
VIII	0.016		0.300
IX	0.006		0.000

¹ The fractions were obtained from the column of Fig. 2

Table II

The effect of 300 R on alkaline phosphatase activity
of mouse serum 1 day after exposure

Treatment	Alk. phosphatase ($\Delta A_{410}/\text{min}/\text{ml}$)
Age Control	0.144
Non-irradiated + Sheep Serum	0.160
X-Ray + PBS	0.242
X-Ray + Sheep Serum	0.208
X-Ray + Fraction X ¹	0.137

¹ From the column of Fig. 1

Fig. 1

Fig. 2

Fig 3

Fig. 4

Fig. 5

Legends for Figures

- Fig. 1. Elution of sheep serum proteins from DEAE-cellulose. Column dimensions, 3.5 x 66 cm. Sample size, 25 ml. Fraction size, 6 ml. Flow rate, 220 ml/hr.
- Fig. 2. Elution of sheep serum proteins from DEAE-cellulose. Column dimensions, 7 x 66 cm. Sample size, 50 ml. Fraction size, 25 ml. Flow rate, 400 ml/hr.
- Fig. 3. Elution of pooled fractions from DEAE-cellulose from Sephadex G-200. The fractions were pooled from the column of Fig. 2 as indicated, concentrated, and applied to the column. Column size, 1 x 24 cm. Sample size, 1 ml. Fraction size, 0.5 ml. Flow rate, 7.5 ml/hr.
- Fig. 4. Polyacrylamide gel electrophoresis of pooled fractions from DEAE-cellulose. The fractions from the column of Fig. 1 were pooled and concentrated. The electrophoresis was performed in an E-C vertical gel apparatus. Staining was with Coomassie blue: Amido black 10B and was followed by electrolytic destaining.
- Fig. 5. Immunoelectrophoresis of pooled fractions from DEAE-cellulose. This was performed on the material used for the patterns of Fig. 4, as described in methods.