to note that with primary and established rabbit kidney cell cultures, Verna and Eylar (5) developed a plaque assay method for titration of fibroma virus using a liquid growth medium overlay; these workers noted that conventional procedures using agar-containing overlay medium for animal virus plaque assay were unsatisfactory when applied to the fibroma virus due to poor cell maintenance.

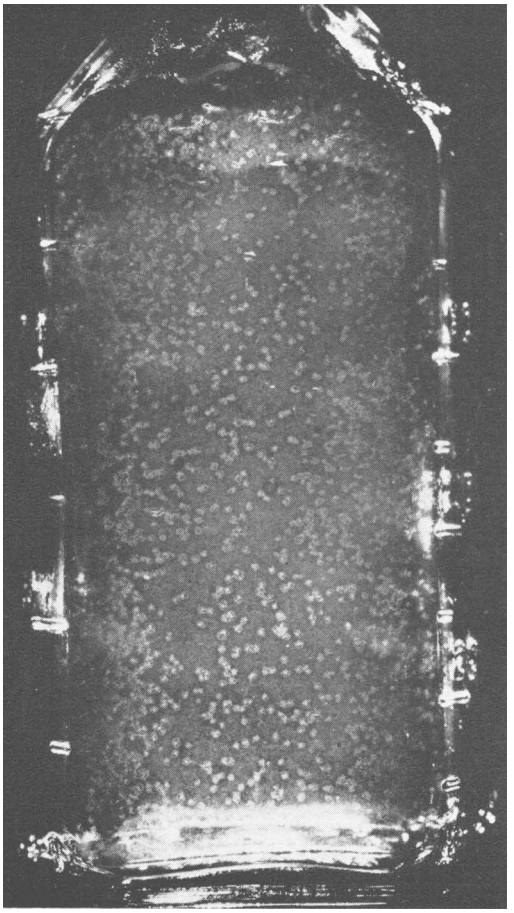

FIG. 4. Myxoma virus plaques on a monolayer of $RK_{13}$ cells in a 3-oz prescription bottle, 6 days after infection, in the presence of sodium bicarbonate in the agar overlay medium, × 2.

*Summary.* Two morphologically distinct types of MV plaques were produced on monolayers of $RK_{13}$ cells under agar; the appearance of these plaques depended upon the presence or absence of sodium bicarbonate in the agar overlay medium. The numbers of plaques produced by a given suspension of myxoma virus were identical, regardless of the presence or absence of sodium bicarbonate. Moreover, the yield of virus from infected $RK_{13}$ cells was not altered by addition or omission of sodium bicarbonate. The specificity of the two plaque types was established by pathogenicity tests and neutralization of plaque forming units by anti-myxoma serum.

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## Polyacrylamide Gel Electrophoresis: Hormonal and Species Specificity Of Antibody Binding of Bovine $I^{131}$ Thyrotropin.* (31254)

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Previous publications have described the resolution of $I^{131}$ $TSH^3$(1) and $I^{131}$ insulin (2) from their soluble antibody complexes by means of disc electrophoresis in polyacrylamide gel. Because of the possible usefulness

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of the method for immunoassay of thyrotropic hormone,‡ information was collected on the relative mobilities of biological activity and radioactivity, and also on the interference potency of various preparations of TSH in relation both to purity and species source.

*Materials and methods.* Pierce-Carsten bovine TSH(3), containing 15 to 35 USP units per mg, was iodinated to give a product with Sp.A. of 0.4 to 3.6 $\mu c/\mu g$(4). The estimated number of I atoms per molecule of TSH was 0.75 to 2.4. Antiserum (AS) was prepared by injecting a rabbit subcutaneously with Bates TSH(5) in Freund's adjuvant, followed by intravenous booster doses(6). Normal serum (NRS) was drawn from the same rabbit before immunization. For interference experiments the following unlabeled preparations of bovine TSH were used: USP (0.074 U/mg), Condliffe (*ca.* 29 U/mg), and Pierce (*ca.* 39 U/mg). Human TSH preparations consisted of crude material (0.03 to 0.11 U/mg), prepared as described previously(6,7), and its 12-fold purified derivative obtained by chromatography on Amberlite IRC-50 (CG-50) resin(7).

All solutions of TSH, $I^{131}$-TSH, and diluted serum were stored and used in isotonic buffer at pH 7.2 to 7.4 (Krebs-Ringer phosphate with or without $Ca^{++}$, or 0.15 M NaCl in 0.01 M adjusted $Na_2HPO_4$). The amount of human serum albumin (HSA) included to prevent adsorption on containing vessels during storage was 0.2 to 0.5%. Temperature was —15°C during storage and between 0 and 5°C during operational procedures.

Bioassays were by a modified McKenzie mouse method(8) or a modified *in vitro* beef thyroid slice procedure(9). In the case of the *in vitro* procedure the medium contained 0.025 to 0.25% HSA and 0.001 M neutralized propylthiouracil.

*Electrophoresis.* Two techniques were used. The simpler one utilized vertical homogeneous slabs of 4 to 7½% polyacrylamide gel with Raymond's apparatus and techniques (10). Buffer in the gel and electrode compartments was at pH 8.5 (0.125 M Tris, 0.02 M boric acid, and 0.001 M EDTA). Sample volume was 15 to 20 $\mu$l, current strength 160 ma, and running time 3.5 to 4 hours. The gel path of each sample's migration was cut into 0.5 cm fractions for counting of gamma activity in a well counter. For bioassay, each fraction was pulverized and eluted overnight in 5 ml of buffer containing 0.25% HSA.

The other technique was disc electrophoresis(11) in vertical cylindrical glass tubes 0.5 cm in diameter. A column of 7.5% acrylamide reagent, 5.5 cm in length, was polymerized chemically (hard gel). On it was layered 2.5% acrylamide reagent which was photopolymerized to form spacer gel. In most instances, from 50 to 75 $\mu$l of sample mixture was incorporated in an equal volume of 5% acrylamide reagent. After a count of radioactivity, this 2.5% acrylamide mixture was photopolymerized (sample gel) after it had been placed atop the spacer gel. In some experiments the sample was mixed with ⅓ volume of saturated sucrose and introduced in liquid form rather than as 2.5% gel. Electrophoresis was downward at pH 9.5 with a current strength of 2.5 ma per column. The average run was approximately 45 minutes, at which time the albumin band had moved at least 4 cm in the hard gel. After removal of the soft gel (sample gel + spacer gel) as a separate fraction, the main column was cut into 2 mm segments as described elsewhere (1). Elution of these segments for bioassay was accomplished overnight in 1.5 to 2.0 ml of isotonic buffered saline or Krebs-Ringer phosphate medium containing 0.1 to 0.25% HSA.

*Reaction mixtures.* In the slab technique reaction mixtures of $I^{131}$-TSH plus AS or NRS, and also mixtures containing interfering amounts of non-radioactive TSH, were allowed to react from 1 hour to overnight before electrophoresis. The non-radioactive TSH always was added to the AS before the $I^{131}$-TSH. In disc electrophoresis, on the other hand, the $I^{131}$-TSH and unlabeled TSH were mixed first, after which the AS was added and the mixture allowed to equilibrate overnight.

*Results.* Total radioactivity accounted for

‡ Various preparations of thyrotropin, regardless of purity, will be referred to as "TSH." The pure hormone—a presumed chemical entity—will be referred to as "thyrotropic hormone."

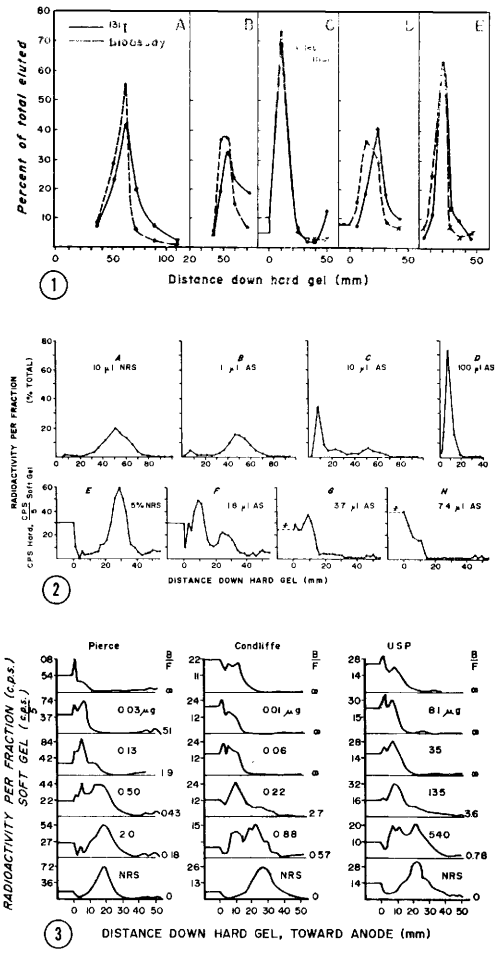


FIG. 1. Comparison of radioactivity and biological activity. Each pair of vertically aligned dots on the curves represents the midpoint of a section of gel eluted for bioassay and measurement of radioactivity. The total quantity of each eluted from the sections was taken as 100%. The small error introduced by using "less than" in place of discrete values does not affect the position of the curves.

A, 7.5% gel slab, 10 $\mu$g $I^{131}$-TSH, *in vivo* assay; B, 4% gel slab, *in vivo* assay; C, disc method, gelled sample, *in vitro* assay; D, disc method, gelled sample, *in vitro* assay; E, disc method, liquid sample, *in vivo* assay. The amount of $I^{131}$-TSH in B through E varied from 17 to 19 $\mu$g. HSA was 0.25 to 0.28% in A through D, 0.16% in E. A and B were the same lot of $I^{131}$-TSH, C another lot, and D and E another lot.

FIG. 2. Effect of antiserum on electrophoretic pattern. A through D: Slab technique with 6% gel. E through H: Disc electrophoresis, gelled sample, 45 min. The amount of $I^{131}$-TSH was constant; amounts of AS per microgram $I^{131}$-TSH are given in the Figure. Some of the soft gel (starred) in G and H was lost during removal. B/F values (see Table I for definition) F, 1.6; G, 13; H, 42.

FIG. 3. Interference by unlabeled preparations of TSH. Disc method, gelled samples. 0.11 $\mu$g $I^{131}$-TSH, 1.9% NRS, 2.5 $\mu$l AS, 0.01% HSA in all samples. No unlabeled TSH was added in the top row; quantities added to the others are shown in Figure. The faster albumin band had run nearly 50 mm with Pierce TSH and 60 mm with the other two. B/F is defined in Table I.

in uneluted segments of gel was reasonably close to 100% of that in the starting material. Recovery by elution was generally 80 to 90% for hard gel fractions but notably incomplete (*ca.* 10%) for soft (sample gel) in the disc technique.

*Mobility of biological activity in relation to radioactivity.* Fig. 1 demonstrates that in both techniques biological activity migrated fairly closely with the major peak of radioactivity, but in 4 of the 5 runs the curve of biological activity was located somewhat behind that of radioactivity. Dissociation was confirmed by comparison of the ratios of biological activity to radioactivity in subsections of the main peak. A significant difference in the ratios (95% confidence limits) among eluted pools was demonstrated in A, B, and D. The secondary peaks of radioactivity in the region occupied by prealbumin and albumin (Fig. 2E) did not contain activity detectible by bioassay.

*Effects of antiserum and of competing unlabeled TSH.* With the slab technique almost all of the radioactivity was localized in a single well-defined peak when nothing was added to $I^{131}$ TSH save NRS (Fig. 2A). With disc electrophoresis considerable activity was also present in the soft gel and lesser components with a mobility of prealbumin and albumin were appreciable. Reaction of $I^{131}$ TSH with increasing amounts of AS progressively obliterated the main peak of radioactivity and transferred a corresponding amount of activity to a slower-moving component or components (Fig. 2). In the case of disc electrophoresis (lower row) the effect of increasing proportions of AS was to transfer activity first into the upper hard gel, and with a gross excess to hold back essentially all activity in the soft gel. The albumin and prealbumin peaks were decreased slightly, if at all, by AS.

As would be expected, addition of an unlabeled preparation of TSH to a reaction mixture of $I^{131}$-TSH and antibody competed with

TABLE I. Quantities of Purified and Crude Bovine TSH Preparations Which Caused Half-Maximum Interference with Antibody-Binding of $I^{131}$-TSH* (See Fig. 3).

| | Pierce TSH | | Condliffe TSH | | USP TSH | |
|---|---|---|---|---|---|---|
| | $\mu g$ | mU, mean (95% conf. limits) | $\mu g$ | mU, mean (95% conf. limits) | $\mu g$ | mU |
| Soft gel radioactivity | .059 | 2.3 (1.89– 2.8) | .112 | 3.3 ( 2.5– 4.0) | 35 | 2.6 |
| Upper hard gel radioactivity | .31 | 12.1 (9.9 –14.9) | .73 | 21.2 (16.0–26 ) | *ca.* 760 | *ca.* 56 |
| Main (free) peak radioactivity | .26 | 10.1 (8.3 –12.5) | .54 | 15.7 (11.9–19.4) | 410 | 30 |
| B/F | .25 | 9.8 (8.0 –12.0) | .53 | 15.4 (11.7–19.1) | 430 | 32 |

* Bioassays were by the *in vivo* method. A blank value obtained with NRS was subtracted from the observed bound activity in soft and upper hard gel. The depression between main peak and upper hard gel peak was taken as the boundary between the two. For the 3 separate regions listed the half maximal values were interpolated from a line : log dose of unlabeled TSH *vs* amount of radioactivity. For the ratio B/F (bound radioactive antigen/free radioactive antigen) a log-log plot was used for interpolation.

the labeled TSH for binding with antibody and hence reduced the amount of activity in antibody-bound form. Such behavior was observed upon addition of several different preparations of bovine TSH (Fig. 3). It is apparent that increasing amounts of unlabeled TSH decreased the amount of activity in the soft gel and upper hard gel successively and shifted a corresponding amount into the peak representing free $I^{131}$-TSH.

An appraisal of the relative potencies of various unlabeled preparations of TSH in terms of their capacity to interfere with antibody-binding of $I^{131}$-TSH was made by estimating the amount, in units of biological activity, which reduced maximum binding by half. Table I lists such values for interference potency calculated in several ways. Attenuation of activity of bound material in the soft gel is seen to be the most sensitive index of interference. With this response variable all 3 preparations, whether crude or highly purified, are of the same potency. When displacement of bound activity from upper hard gel, rather than soft gel, is used for calculation, some disparity is evident. The same may be said for the calculation based on a shift of activity to the region of free TSH, and for the ratio of total bound activity to that in the main free peak (B/F). But whether such disagreement is significant cannot be judged with assurance because, aside from the estimated error of bioassay, other cumulative errors exist. Sources of such errors include the overlap of peaks and the uncertainties attending interpolation. However, the purified preparations were consistently as potent or more potent than the crude (USP) preparation. The reverse would be true if the interference effect were caused by non-specific protein instead of hormone.

Human TSH showed detectible interference effects, and, as was true for bovine TSH, a gross excess interfered completely. Again, crude and purified preparations were not significantly different in interference potency in relation to biological potency. Nevertheless, the potency of human TSH was much lower in terms of biological activity and the log-dose response curve was flatter than that of bovine TSH. Actual amounts necessary for half maximum interference, as indicated by reduction in soft gel activity, were relatively large. The estimated amount was 116 and 121 mU in separate experiments with the crude preparation, and 126 and 189 mU in the case of the purified form. These values compare with 2.3 and 3.3 mU for 2 purified preparations of bovine TSH (Table I).

*Discussion.* Although we did not attempt to adapt the polyacrylamide gel system for the bioassay of hormone in serum, its potential usefulness deserves comment. The amount of non-hormonal protein in the purified iodinated preparation of TSH which we employed might be as high as 80 to 95%. For this reason the finding of a rather close association of the great bulk of radioactivity

and biological activity within a single electrophoretic peak is impressive. However, such a correspondence might mean simply that the mobility of the main mass of labeled inert protein is close to that of hormone. Pierce(12) has reported that his preparation consists mostly of components having similar molecular size as determined by electrodialysis. Another possible explanation may be that the hormone is more susceptible to iodination than other non-specific protein. In any event, in spite of the probable existence of non-hormonal protein in amounts even greater than is suggested, both by the presence of minor peaks and the existence of slight but significant heterogeneity in measured ratios of radioactivity to biological activity in subsections of the main peak, nevertheless either crude or purified preparations showed an interference potency roughly comparable to biological activity. This fact contrasts markedly with previous results with Pierce TSH in a hemagglutination system wherein interference potency varied inversely with the purity of the TSH preparation(6); such a system obviously is non-specific for hormone.

The sensitivity of the interference phenomenon with the given immune system in disc electrophoresis compares very favorably with the bioassay method of McKenzie(13). With $I^{131}$-TSH preparations of 100 to 300 $\mu c/\mu g$, the specific activity usually used in immunoassay, the amount of hormonal activity giving half maximal interference would be about 0.002 to 0.013 mU. Minimal assayable amounts might approach 0.0005 mU. These estimates apply only for the assay of bovine hormone in a system of bovine $I^{131}$-TSH plus anti-bovine TSH serum. It is obvious that for a highly sensitive assay of human thyrotropic hormone our system of bovine reactants would be undesirable since the interference potency of human TSH proved only about 1/50 to 1/200 as great as that of bovine preparations with comparable biological activity. These findings agree with those of Utiger and coworkers(14) except that these workers obtained with human preparations slopes so low that 100% interference was not approached. Their demonstration of high hormone and species specificity for a system comprising human $I^{131}$-TSH plus anti-human TSH serum points to the distinct superiority of such a system for the assay of human hormone. Utiger(15) has recently reported success with such a system as applied in the assay of human serum by the Yalow-Berson(16) technique using paper chromato-electrophoresis for separation of free and antibody-bound labeled antibody.

*Summary.* In polyacrylamide gel electrophoresis of bovine $I^{131}$-TSH the radioactivity and biological activity migrate similarly but with slight dissociation. Increasing quantities of antiserum shift increasing quantities of radioactivity to slower moving regions. Bovine TSH preparations interfere with $I^{131}$-TSH binding to antibody in proportion to biological activity. Human TSH is much less active.

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