180. The therapeutic effects of 6MP and KTS against S-180/B_6 were greater in C57Bl/6 than in Swiss HaICR mice. The effects of 6MP and vitamin B_6 deficiency against ECA were evident only in Swiss HaICR sensitized to S-180. Thus, in this case, the effectiveness of therapeutic treatments was influenced by the degree of the host response to the tumor.

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Improvement of Human Growth Hormone Immunoassay Using ^{125}I.* (32460)

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Immunoassay of circulating human growth hormone (HGH) utilizing HGH-^{131}I as tracer has already been reported(1-5). Morgan(6) has also reported a radio-immunoassay for HGH using ^{125}I but the sensitivity appeared to be insufficient. The present report describes a radio-immunoassay technique utilizing a double antibody procedure and HGH-^{125}I which provides a high degree of sensitivity and permits determinations of HGH in unextracted small plasma samples.

Materials and methods. Antiserum was produced in rabbits by repeated administration of Raben's preparation of HGH(7) emulsified in complete Freund's adjuvant. Anti-rabbit gamma globulin serum was obtained from goat and was prepared commercially.‡ A highly purified preparation of HGH (Wilhelmi HS 612 A) served as standard and for the iodination procedure. Phosphate buffer (0.05 M, pH 7.4) containing 2‰ of bovine serum albumin was used as diluent both for the HGH-^{125}I and the antisera. Dilutions of the standards were made in bovine serum.

Preparation of HGH-^{125}I. HGH was labelled with ^{125}I§ by the method of Greenwood *et al*(8) (1.5-2.5 mC ^{125}I/20-30 μl; 50 μl phosphate buffer (0.5 M, pH 7.4); 2-4 μg HGH/20-40 μl; 100 μg Chloramine-T/25 μl; 250 μg $Na_2S_2O_5$/100 μl; 50 μl normal human serum; 1 mg KI/100 μl). HGH, Chloramine-T, $Na_2S_2O_5$ and KI were diluted in phosphate buffer (0.05 M, pH 7.4). After removal of unreacted ^{125}I on a Sephadex G-50 column (10 × 1 cm), the HGH-^{125}I was purified by chromatography on a column of Sephadex G-200 (50 × 2 cm). The first peak of radioactivity corresponded to damaged products and the second peak to "pure" labelled hormone (Fig. 1.). The effect of reaction times of Chloramine-T, ranging from 5 to 60 seconds, on the specific activity and the purity of HGH-^{125}I has been analyzed (Table I). In general, the reaction was stopped after 30-40 seconds because longer times appeared to increase the amount of radioactive impurities. For 15 iodination procedures at 8 different times (Table I), the estimated mean

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§ Code no. IMS-3, The Radiochemical Centre, Amersham, England.

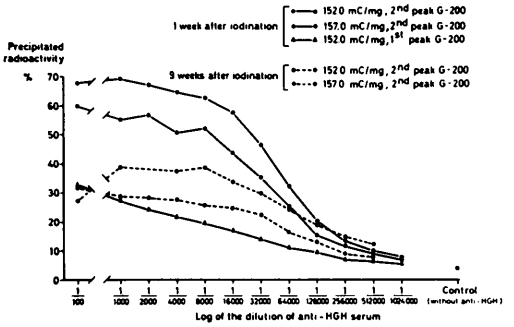


FIG. 1. Effect of the dilution of the anti-HGH serum on the percentage of the total radioactivity which was precipitated. The experimental procedure was the same as that summarized in Table II except for the amount of anti-HGH serum (0.2 ml) and the absence of unlabelled HGH. Date of the iodination procedure: 6.20.66. 0.2 mμg HGH-^{125} and 0.1 ml of undiluted precipitating serum were utilized.

specific activity of the product was 191.2 mC/mg with a mean yield (obtained specific activity divided by the theoretical specific activity) of 24.3%. 22.7% of the initial radioactivity remained adsorbed in the reaction vial after removal of the iodination solution. In all but two experiments more than 80% of this residual radioactivity were extractable by 3 KI washes of the reaction vial. The radioactivity of KI washes was considered to be due to unreacted ^{125}I since most of its content was not precipitable by trichloracetic acid, and it was recovered in the salt peak after chromatography on a Sephadex G-50 column. Therefore, the radioactivity remaining in the reaction vial after KI washes was assumed to be HGH-^{125}I. These preparations of HGH-^{125}I, stored at −20°C, progressively lost their reacting capacity with the anti-HGH serum (Fig. 1). Nevertheless, they could be used for up to 2 months without major modification of the slope of the standard curve.

TABLE I. Summary of Data of the Iodination Procedures.

Date	Theoret. spec. activity (mC/mg)	Total adsorbed radio-activity (%)	KI extractable adsorbed radioact. (%)	Reaction time (seconds)	Calculated spec. activity (mC/mg)	Yield (%)	Maximal precipit.* (%)
12-14-65	400.0	12.0	91.0	60	92.2	23.1	—
2- 7-66	750.0	13.7	0	5	275.0	36.7	63.7
"	567.0	18.1	11.0	20	400.0	70.5	"
2-21-66	625.0	12.9	87.0	30	217.0	34.7	—
"	625.0	14.6	83.8	45	257.0	41.1	—
4-18-66	625.0	28.1	92.2	30	104.0	16.7	63.0
"	625.0	30.0	91.3	60	78.5	12.6	49.5
5-23-66	1250.0	32.4	97.9	30	185.0	14.8	64.0
"	833.0	40.0	95.5	60	175.0	21.0	60.5
6-20-66	1250.0	29.0	80.0	45	157.0	12.6	60.7
"	833.0	35.5	80.0	45†	152.0	18.3	69.5
8-15-66	1250.0	13.9	86.4	35†	79.5	6.4	83.0
"	1250.0	11.6	86.7	35	167.4	13.4	"
10-11-66	1250.0	22.8	89.7	40	247.0	19.8	60.0
"	1250.0	26.1	84.3	50	281.0	22.5	"
Mean	892.2	22.7			191.2	24.3	

* In presence of 0.1 ml of undiluted precipitating serum.
† 50 μg choloramine-T/25 μl.

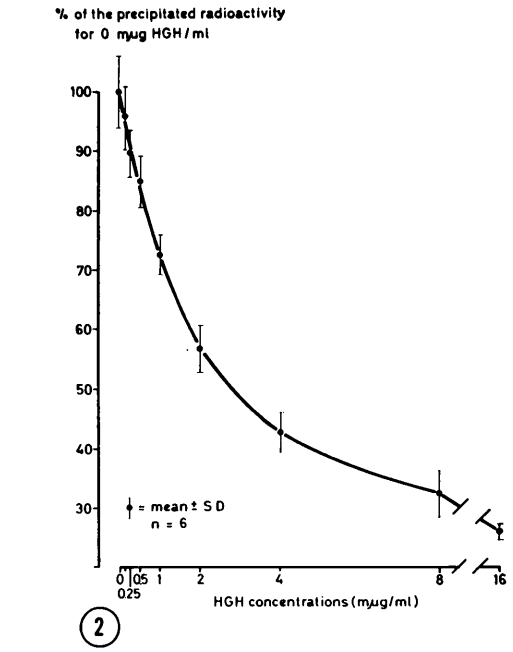
Radio-immunoassay procedure. The reactions and the incubation times used are indicated in Table II. The use of 0.1 ml of anti-HGH serum at an initial dilution of 1:32,000 and 0.1 ml of anti-rabbit gamma globulin goat serum (diluted 1:3) produced the precipitation of 15 to 30% of the labelled hormone and still permitted a steep initial slope of the standard curve. The indicated amount of precipitating serum was also chosen to ensure full precipitation within 24 hours. Normal rabbit serum (diluted 1:300) was added as carrier. In the experiments concerning the effect of disodium ethylenediaminetetraacetate (EDTA) on the plasma HGH levels, the same concentrations of this reagent have been used for both the standards and the unknown samples.

Results. The accuracy of the assay method, expressed by the coefficient of variation

TABLE II. Immunoassay of HGH Using Two Antibody Method and HGH-^{125}I.

0.3 ml phosphate buffer (0.05 M, pH 7.4, 2‰ bovine serum albumin) 0.1 ml HGH standard or assay plasma (dilution 1:3) 0.1 ml anti-HGH serum (dilution 1:32,000)
↓ 4°C for 24 hr ↓
0.1 ml HGH-^{125}I solution (0.2-0.4 mμg)
↓ 4°C for 7 days ↓
0.1 ml normal rabbit serum (dilution 1:300) 0.1 ml anti-rabbit gamma globulin serum (dilution 1:3)
↓ 4°C for 24 hr ↓ Centrifuge (3200 rpm for 40 min at 4°C) ↓
Supernatant (discarded) ↓ Precipitate Wash with 0.5 ml H_2O at 4°C Centrifuge (3200 rpm for 40 min at 4°C) ↓
Precipitate (counted in a Packard 410 Autogamma counter)

(standard deviation divided by the mean) has been determined by replicate determinations. The mean coefficient of variation, for the standard HGH concentrations ranging from 0 to 16 mμg/ml, was 6.3% (range : 4.4-12.4) (Fig. 2). In this experiment, we observed decreases of 4 and 10% in the precipitated radioactivity for HGH concentrations of 0.125 and 0.250 mμg/ml respectively. Recently, using minimal amount of the hormonal tracer and optimal dilutions of antisera (tested for each new preparation of HGH-^{125}I), a greater sensitivity and a decrease in the radioactivity of the precipitate of 8% for the first standard concentration (0.125 mμg/ml) were obtained. The coefficients of variation measured for some plasma samples containing different HGH levels were between 8.5 and 22.2% (Table III). Moreover, the recovery, tested by addition of 2 HGH concentrations (2 and 4 mμg/ml) to the same plasma, containing 3.3 mμg of endogenous HGH per ml, was 99.0 and 114.2% respec-



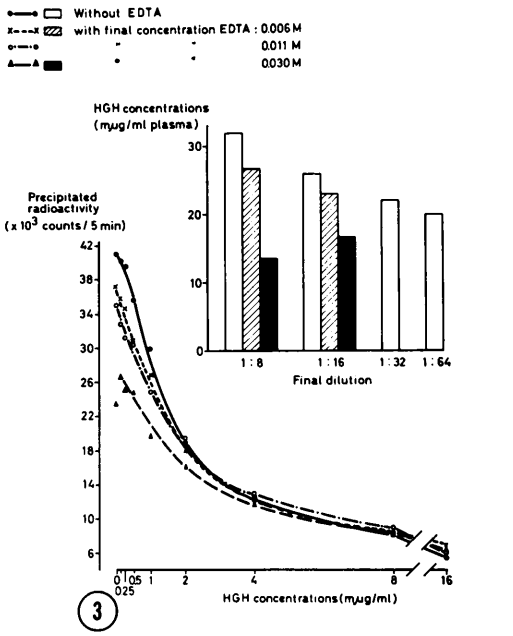


FIG. 2. Decrease of the precipitated radioactivity as a function of HGH concentrations in presence of 0.2 mμg HGH-^{125}I. The precipitationed radioactivity in absence of unlabelled HGH was assumed to be 100%.

FIG. 3. Effect of various concentrations of EDTA on the slope of the standard curve and on the estimated plasma HGH levels.

TABLE III. Accuracy and Recovery of HGH Immunoassay in Plasma Samples.

Sample	Clinical state	n	Mean (mμg/ml)	Standard deviation	Coefficient of variation (%)	Recovery (%)
1	Normal fasting	6	3.3	0.7	21.2	—
" + 2mμg HGH/ml	"	6	5.3	0.6	11.3	99.0
" + 4mμg HGH/ml	"	6	7.9	1.7	21.5	114.2
2	"	7	3.3	0.7	21.2	—
3	"	7	5.4	1.2	22.2	—
4	Acromegalic fasting	6	10.4	1.1	10.5	—
5	"	6	11.8	1.0	8.5	—
6	"	6	38.7	7.4	19.1	—

tively (Table III). Initially, undiluted plasma was used for HGH determinations. In some of these samples with HGH levels above 8-10 mμg/ml the obtained values appeared to be excessive. Addition of EDTA or dilution of these samples reduced the final concentration of HGH (Fig. 3). However, EDTA reduced the radioactivity in the precipitate and the initial slope of the standard curve (Fig. 3). Subsequently, the routine procedure decided upon to minimize the possible overestimation of HGH levels in undiluted plasma was a 1:3 dilution of the plasma sample (final dilution 1:24).

Discussion. The relatively short half-life of ^{131}I makes its use for standard immunoassay techniques for protein hormones somewhat inconvenient. The application of ^{125}I for the iodination procedure of HGH has therefore been investigated.

Fifteen consecutive iodination procedures over a 10 month period resulted regularly in satisfactory yields of a purified labelled preparation. At equal specific activities, the lower energy gamma emission of this isotope does not require a higher degree of iodination of the hormone than ^{131}I. As has been described for insulin(9), it seems possible that incorporation of a large number of iodine atoms to the HGH molecule affects the biological and immunological properties of this molecule. Nevertheless, this cannot alter the validity of the immunoassay procedure, because HGH level of unknown samples is estimated by reference to a standard curve of a highly purified preparation. Since there has been an attempt to standardize the labelling procedure (except for the reaction time of the Chloramine-T and, on two occasions, for the quantity of this reagent), part of the diversity of the obtained yield could be attributed to the batch of the labelled iodine employed. In spite of these differences, all preparations served successfully in the described immunoassay procedure.

The sensitivity and the accuracy of the standard curve permit the measurement of 0.125 mμg HGH/ml, which corresponds to 0.375 mμg of endogenous HGH per ml of undiluted plasma.

The dilution of some plasma samples decreased the absolute level of endogenous HGH after correction for the dilution factor. Similar results were obtained after addition of EDTA, a chelator of divalent metal ions (Ca^{++} and Mg^{++}) which are necessary for the attachment of some components of the complement to antigen-antibody complexes(10). Overestimations of plasma HGH levels could result from excess of free or antibody-bound labelled HGH in the supernatant, consecutive to an inhibition of either the first or the second antigen-antibody reaction. The effect of EDTA and the analogy of our results with those reported for insulin(11-14), especially by Morgan *et al*(13,14), suggest the hypothesis that complement is the inhibitor. The lack of such inhibition with techniques using a single antibody system may well indicate that this inhibitor interferes with the precipitating step of the double antibody system. The effect of EDTA in decreasing the amount of precipitated radioactivity in the absence of unlabelled HGH or with low concentrations, remains unexplained. A direct inhibitory effect of this compound on the antigen-antibody reaction is not excluded.

Further investigation is necessary to clarify the intrinsic mechanisms of the inhibition in the double antibody immunoassay procedures.

Summary. An iodination procedure for human growth hormone using ^{125}I and the double-antibody immunoassay utilizing this labelled preparation as tracer are described. The mean specific activity was estimated to be 190 mC/mg. The growth hormone determinations were made on 0.1 ml of 1:3 diluted plasma. The results showed satisfactory recovery, accuracy and a sensitivity of 0.375 mμg/ml.

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Ribonucleic Acid Synthesis and Nuclear Ribonucleic Acid Polymerase Activity in Livers of Mice Infected With Mouse Hepatitis Virus (MHV-3).* (32461)

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In the past few years we have been interested in the identification of early metabolic alterations in the livers of mice infected with the MHV-3 strain of murine hepatitis virus. A marked elevation in the blood plasma of many enzymes of hepatic origin was detected during the acute phase of the disease (1). At 36 hours after infection some mitochondrial alterations of liver cells were apparent, as shown by (a) uncoupling of oxidative phosphorylation and (b) release of some mitochondrial enzymes into the soluble phase of cytoplasm and from this to the bloodstream(2,3). In addition, recent work from this laboratory has shown that lysosomes are altered at an even earlier stage of infection (4). Very little is known, however, about the changes of nuclear structures and functions induced by hepatitis viruses. This paper deals with investigations on *in vivo* synthesis of ribonucleic acid (RNA) in liver tissue of mice infected with MHV-3, and on the *in vitro* activity of nuclear RNA polymerase.

Materials and methods. All experiments were performed with the Swiss strain of albino mice weighing 12-16 g. They were infected intraabdominally with 0.1 ml of a suspension of infected mouse liver containing about 10,000 LD_{50} of the Craig strain of MHV-3 virus. The viral titer, expressed as LD_{50}, was calculated according to Reed and Muench(5). The liver nuclear fraction was obtained as described by Barnabei *et al*(6). RNA polymerase activity was assayed in this fraction essentially according to Weiss(7,8). The composition of the incubating mixture is shown

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