SPECTROPHOTOMETRIC AND RADIOISOTOPIC REFERENCE METHODOLOGIES

FOR THE APPLICATION OF INDOCYANINE GREEN AS AN

INDICATOR OF LIVER FUNCTION

by

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INTRODUCTION

The liver is the largest organ of the body, and has a multitude
of functions. They include (1) storage of food materials, manufacture
of food materials and many other substances, detoxification, regulation
of blood volume and, most closely related to this paper, the excretion
of exogenous substances into the bile. The liver has enormous reserve
power. Some 80% can be removed or damaged before functional abnormality
can be detected. There are a number of liver function tests to help
assess the functional capacity of the liver.

When used for clinical diagnosis, some of these tests are not sel-
ective enough, some are influenced by extra-hepatic factors, so that
they must always be regarded as an aid to diagnosis, often in carefully
chosen combinations, and always in conjunction with the history and
physical. After the diagnosis has been established, some of these tests
are a useful guide to the therapy and prognosis of the patients. (1)
In a case of jaundice, for instance, the patient with hepatocellular
damage may have increased direct VandenBerg reaction and positive
flocculation tests, whereas the patient with hemolytic jaundice will
have liver function tests that are normal. His jaundice is due to excess-
ive destruction of red cells and will give an increased value for indirect
bilirubin. (2)

Current clinically useful liver function tests can be divided into
the following basic groups (1). 1. Tests indicative of parenchymal dys-
function, such as bilirubin in serum, urobilinogen in urine, serum globulin
and albumin, cephalin flocculation, and thymol turbidity. 2. Tests of

special protein synthesis, such as the prothrombin. 3. Tests related
to disturbed carbohydrate metabolism, checked by glucose and galactose
tolerances. 4. Tests of serum enzyme alteration, including the widely
used serum glutamic oxalacetic transaminase (SGO-T), serum glutamic pyruvic
transaminase (SGP-T) and serum alkaline phosphatase. 5. Tests related
to excretory function, such as Bromsulfophthalein, Rose Bengal, I-131
tagged Rose Bengal, (3) and Cardio Green clearances. The work in this
paper is in this latter category of liver function tests.

Bromsulfophthalien (BS) clearance is one of the most sensitive and
clinically useful tests for the estimation of functioning hepatic mass.
We will consider it in some detail as the best example of the established
clearance methods.

Following the introduction of the BSP test, there was some contro-
versy about which liver cells were responsible for its extraction from
the blood. Klein and Levinson, (4) Cantarow and Wirtz, (5) favored up-
take by the Kupfer cells. However, the studies by Shore and Zilverschmit
(6) showed that the removal of colloidal gold, which is phagocytized by
the reticuloendothelial system was not affected by the doses of India ink
that markedly inhibited BSP uptake. There is considerable evidence that
BSP and other compounds are taken up by the parenchymal cells and this
is now generally accepted as the mechanism of extraction. Krebs and Brauer
(7) demonstrated by autoradiography that the radioactive BSP is taken up
exclusively by the hepatic cells. It is now generally accepted also that
BSP is excreted as a conjugate of amino acids. Combes (8) was one of the
earliest workers to establish that BSP was conjugated with glycine, glutamic
acid and cysteine. Later investigators have modified these conclusions

somewhat without altering the basic fact that much of the BSP in the bile has been changed in the process of extraction and excretion.

The percent of dye cleared by the liver is another point that has been the subject of investigation. About 70-80% of the dye is removed by the liver, the remainder being removed by extrahepatic mechanisms(9).

After intravenous injection of the dye, a typical disappearance curve is seen in the normal human. Figure 1., after Mendenhall and Leevy (10), shows the early exponential phase which continues until 20 or 25 minutes after injection. At a later time, the shape of the curve indicates that the rate of removal of BSP decreases more rapidly than the concentration of BSP in the plasma.

The original technique of Rosenthal and White (11) consisted of injecting 2 mgm per kilogram of dye, and the estimation of the amount of dye retained at five minutes post-injection, and also at 30 minutes. They expected 20-25% of the dye to be retained at five minutes, and all of it to have disappeared at the end of thirty minutes. O'Leary, Greene and Rowntree (12) introduced the use of a test dose of 5 mgms/kg of body weight. Various investigators tried different drawing times to attempt to achieve the maximum in sensitivity (13, 14, 15, 16, 17, 18, 19). A more ambitious adaptation was that of MacDonald (20), who injected amounts varying from 2 to 10 mgms/kg, and then drew samples every five minutes for 30 minutes, using the points to plot a curve of the rate of disappearance of the dye. Helm and Machella (21) decided that a 5 mgm/kg dose and a 30 minute drawing time was optimal. They checked 50 known hepatic cases and 12 controls. Of the hepatic cases receiving the 5 mgm dose, 38 of them showed some retention, ranging from 4 to 64% at thirty minutes, and

Figure 1. Per cent Bromsulfalein retained.
(After Mendenhall and Levy)
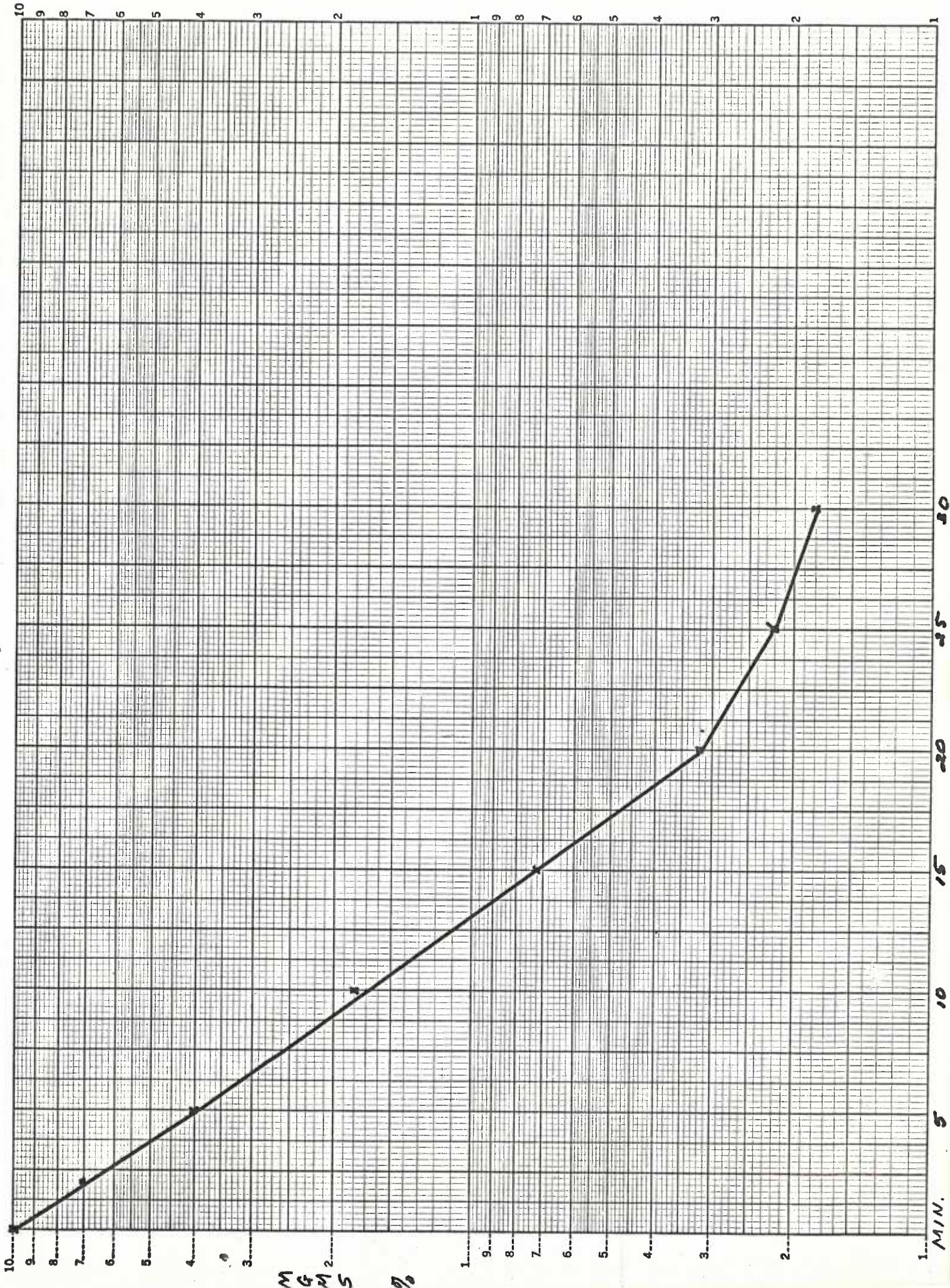
27 of them showed retention after 60 minutes. None of their control patients showed retention with 2 or 5 mgm doses at either 30 or 60 minutes after injection.

The BSP test has problems associated with it, the first one being the safety of the patient. Accidental extravasation of the dye into the tissue can cause painful sloughing of the tissue. Reactions, and at least one death have been attributed to its use (21). Cases of anaphylactic shock have also been reported following a second injection of BSP (23).

Drugs have an affect on the retention of BSP. Enovid and morphine increase the retention of the dye. (24) Recent BSP tests themselves increase the retention of test doses injected 2 or 3 days following an initial injection (25). Recent cholecystography, androgens and other anabolic steroids, bile acid derivatives, and antifungal agents (25) also give spuriously high results. Acocella, Nicolis, and Tenconi (26) found that BSP, bilirubin and Indocyanine Green were all retained longer during an infusion of Rifamycin S. V. They reasoned that since the conjugation systems of BSP and bilirubin are different and Indocyanine Green is not conjugated at all, it would seem that some other mechanism is involved in the increased retention. These authors feel that an interference at the level of excretion is more likely, although an effect on hepatic uptake cannot be excluded.

Non-hepatic disease conditions can also be the cause of increased BSP retention. Cardiac failure and acute cholecystitis (25) and the related group of hemorrhage, shock, trauma and operative procedures give the same effect. Hypovolemia, common to all this group, may explain this result.

Hemolysis and lipemia of serum may interfere with the reading of the BSP test (27). Seligson (28) found that errors from icterus, hemolysis, and lipemia were minimized by the use of an alkaline buffer to develop the color and an acid buffer for the blank.

In the procedure in common clinical use, the injection of 5 mgms of BSP per kilogram of body weight is considered to give a concentration of 10 mgms per 100 ml plasma at zero time, and on this assumption the calculation of percent retained at the drawing time is based (29). If this were to be true, each and every patient would need to have a plasma volume of 50 ml/kg. This, of course, is not the case. The 95% range of plasma volume in ml/kg as given by Hutchens (30) is 23.6-44.6 ml/kg in males, and 25.5-37.1 ml/kg in females. These normal ranges are fairly wide, as are those reported by Gibson and Evans, (31) who compared 49 men and 41 women in a comparative study of blood and plasma volumes in healthy people. They found variations explainable by a number of factors--weight, height, muscularity, obesity, degree of muscular acitivity, age, and pre- and post-menopausal conditions in women. Total blood volume varied between men and women, but the component in question, the plasma volume, showed less variation due to compensating hematocrit difference. They found that about two-thirds of the people tested fell within $\pm$10% of the normal values. Extremes of both sexes approached 35%.

Given this variation in normal people, it is not surprising to find variations are even wider in people who are not in good health. Obviously, people who have suffered blood loss will have a smaller volume immediately after loss, and will have a comparatively larger plasma volume until the cellular components have been replaced. Other variations can be caused by fever, electrolyte imbalance, cirrhosis and congestive heart failure (32).

In congestive heart failure, the increased BSP retention is found to parallel the severity of the congestion and to decrease as the patient's condition improves. This is more complex than a simple increase in plasma volume, the albumin-globulin ration and venous pressure being two other parameters showing positive correlation to the severity of the disease.

There is a difference in plasma volume in an erect patient and a recumbent one. The difference is especially marked in a cardiac patient, in whom 15-20% increase in blood volume is noticeable after lying down for an hour or two. (33) Berson compared blood and plasma volumes of patients with cirrhosis, anemia, heart failure, both before and after compensation, and polycythemia. The values were as expected, plasma volumes below normal in cirrhosis, polycythemia vera, and heart failure. Plasma volumes were up in patients with anemia. Variations, however, were wide. In cirrhosis, for example, plasma volumes of 40 to 90 ml/kg were found.

As the foregoing indicates, the most serious objection to the validity of the use of BSP as a liver function test lies in the difficulty of establishing the initial plasma concentration of the dye. The initial assumed value of 10 mgms per 100 ml has been shown to be in error by several authors (10, 34, 35).

Zieve and Hill (36) suggested a weight correction factor in evaluating BSP scores. They suggest using a mean weight of 162 lbs as a reference point, and adding 1% for people weighing less and subtracting up to 3% as weights reach up to 279 lbs. These and similar adjustments of amounts or results still do not take into account the possible range of plasma volume variation in normal people with varying degrees of obesity, or possible variation in body weight and liver mass ratio.

Mendenhall and Leevy (10) investigated false positive BSP tests by injecting six patients with histologically active liver disease in whom an abnormality of BSP hepatic removal capacity was not reflected in the standard 45 minute test. They concluded that the normal 45 minute BSP readings were due to low initial levels of the dye, attributable in part to expanded extra-cellular fluid volume. Skeletal muscle uptake, renal excretion, and accelerated hepatic extraction did not appear to be significant in these patients. They suggested obtaining early blood samples and the determination of the percentage disappearance rate of the dye. They believed that the percent disappearance rate gave a more accurate idea of the liver dysfunction than a single specimen would have. In order to calculate the percent disappearance rate, at least two post-injection samples were necessary, and the rate could then be calculated by this formula:

$$\text{Percent Disappearance Rate (PDR)} = (1-D)$$

$$\text{where } \log D = \frac{\log C_2 - \log C_1}{t_2 - t_1}$$

D = fraction retained per min.

C_1 & C_2 = conc at t_1 & t_2

t_1 & t_2 = time in minutes after injection

These authors injected 5 mgm/kg of dye in normal controls and in patients with known liver disease. The 45 minute levels of retention were normal in the patients with liver disease, as well as those of the normal patients. Plasma decay studies carried out at the same time showed abnormalities in both the removal pattern and the disappearance rate for the initial exponential period of plasma decay were decreased from a normal mean of 11.3% to 5.7% in patients with liver disease.

Since it is obvious that the plasma volume is a critical unmeasured factor in an accurate determination of liver function by the clearance type of test, it is surprising that no routine procedure has been established to incorporate the measurement of plasma volume at the time of

the liver function test.

It would, of course, be possible to do a blood volume determination by use of radio-iodinated human serum albumin, or similar isotopic tracer, or a colorimetric one such as Evans Blue just before, or just after the liver function test. This would cause some additional discomfort to the patient, due to the necessity for at least two additional venipunctures.

Hutchens and his group (37) devised a reference method of calculating the percent disappearance rate on the basis of one or two post-injection drawings. The rationale of the reference method is that the I-131 radio-iodinated human serum albumin will remain close to the 100% level during the test period while the BSP is being removed from the plasma by the liver. BSP is very rapidly removed from the plasma, but since the iodinated albumin is not, the amount of isotope remaining can be assumed to be the same as the amount injected, and after a suitable time interval the ratio of dye to isotope will indicate the amount of dye retained. Procedure was to mix BSP and I-131-labelled human serum albumin and inject it intravenously. Early work with the reference method used two post-injections drawings, and a calculation of percent disappearance rate. It will be seen that by establishing a pre-injection ratio between the reference dye or isotope and the liver function measuring dye, a single drawing at 10 minutes or any other convenient time within the range of linear extraction of the liver-function measuring dye, it is possible to establish the slope of the early exponential portion of the dye retention curve.

Percent Dye retained by reference calculation:

Theory: $\dfrac{\text{Dose BSP concentration}}{\text{Dose RISA concentration}}$ = Dose ratio$_o$ (at time zero)

$\dfrac{\text{Serum BSP concentration}_t}{\text{Serum RISA concentration}_t}$ = Serum ratio$_t$ (at time t)

$$\% \text{ BSP dye retained}_t = \frac{\text{Serum ratio}_t}{\text{Dose ratio}_o} \times 100$$

Practical application:

$$\% \text{ BSP dye retained}_t = \frac{\text{OD serum}_t \times \text{cpm/ml std}_o}{\text{OD std}_o \times \text{cpm/ml serum}_t} \times 100 \text{ (Formula \#1)}$$

In Hutchens unpublished data, (37) they report drawing two samples, one at 15 and the other at 45 minutes post-injection. Their percent disappearance rates were very near the mean of 11 given by Mendenhall and Leevy (10). The reference method calculation was used in the calculation of the percent dye remaining, and the plasma volume by the following calculation formula:

$$\frac{\text{mgm/ml BSP in dose}}{\text{mgm BSP in diulted}} \times \begin{array}{c} \text{cpm/ml in diluted} \\ \text{standard} \end{array} \times \text{ml given} = \text{plasma volume in mls.}$$

$$\frac{\text{standard}}{\substack{\text{cpm/ml serum in 15 minute} \\ \text{specimen}}} \qquad \text{(Formula \#2)}$$

Interestingly, their (37) mgms% values on the basis of plasma volume were nearly all above the 10 mgms% assumed to be 100% on the basis of the calculation of 5 mgms/kg of body weight. The range of BSP concentration at time zero was approximately 11 to 17 mgms%.

The diagnostic importance of a liver function test such as the BSP, if accurately done, with all the variables under control, cannot be disputed. Some of the difficulties of BSP seem insurmountable, such as the toxicity of the dye itself and the widely varying initial concentrations present as a result of using the 5 mgm/kg guide to amount of dye injected. The amount, and percentage of dye conjugated by the liver to the colorless form is another variable that is difficult to assess.

Indocyanine Green, which has been used successfully as a diagnostic agent for the estimation of liver function, was a possible substitute for BSP in the reference method of assessing liver function (39, 40, 41, 42, 43, 44).

The method of extraction of Indocyanine Green from the plasma by the liver is similar to that of BSP. Both are apparently extracted by the parenchymal cells. There the similarity seems to end. BSP is excreted into the bile at least partly in a conjugated form. Cherrick, et al, (38) used ascending chromatography of the bile of patients who had been given Indocyanine Green to determine if there were differences from the injected material. They found no evidence, using three different systems, that the dye had been secreted into the bile in a conjugated form. Their studies also indicated that Indocyanine Green is not cleared by the kidney and that peripheral tissue uptake of the dye is negligible. Wheeler, Cranston and Meltzer (43) were able to recover nearly 100% of the dye from the bile, and detected none in the urine. The appearance in the bile, however, is delayed. Apparently the dye is rapidly removed from the plasma, stored in the liver, and then, like some of the flavine and phthalein dyes, is gradually secreted into the biliary tree. Dye is recoverable from the bile of dogs for over 5 hours after injection, long after all the dye has disappeared from the plasma. Cerrick, et al (38) continued to sample bile from human subjects until up to 19 hours after injection, at which time only a trace was present.

Like BSP, Indocyanine Green appears to be rapidly and completely bound to protein, the albumin fraction carrying about 95% of the dye(38).

Some authors feel that the linearity of post-injection concentrations of Indocyanine Green is better than that of BSP. Wheeler, et al, (43) found that the plasma concentrations of Indocyanine Green were linear up to 30 minutes after injection of approximately 1 mg/kg of dye, whereas after an injection of 2.5 mg/kg of BSP, the plasma concentration was linear up to only about 10 minutes. Both of these curves were obtained by

plotting plasma concentrations against time on semi-log paper.

Another similarity in the history of the two dyes is the wide range of dosages tried and recommended by various investigators. As mentioned, Wheeler and his group (43) gave a comparatively large dose of Indocyanine Green, 1 mg/kg to dogs, Cherrick, et al, (38) gave .5 mg/kg for liver function in humans, Cooke (44) found a dosage of .1 mg/kg to be optimum for his procedure of testing liver function, and Hunton, et al, (39) used .25 mg/kg in humans.

Cherrick (38) used .5 mg/kg, and a 20 minute sampling time. He gives 20 minute retention of less than 5 to 6%, and an initial decay of greater than 15% per minute as normal. Cooke, (44) prefers a 10 minute sample with his dose of .1 mg/kg. Normals with his technique vary from 0 to 20% retention in 10 minutes. Hunton (40) used disappearance rate, and found the average in normal people to be about 26% per minute. He injected .25 mg/kg, and sampled at 5 and 10 minutes.

There are no reports of toxic effects of Indocyanine Green (38). Accidental extravasation of the dye produced no irritation and doses of up to 2 mg of dye per kilogram of body weight has produced no untoward reactions upon single or repeated intravenous injections or infusions.

Some substances interfere with the assessment of liver function by the use of Indocyanine Green (26). Sodium bisulfite reduces the absorption peak of Indocyanine Green (45). Sodium bisulfite is a common contaminant in heparin preparations, so heparinized blood is unsuitable for ICG determinations. Other things that interfere with BSP colorimetric determinations are not a problem with Indocyanine Green. At the wave length at which the dye is assayed, hemolysis and lipemia do not interfere.

The initial plasma level of Indocyanine Green is not as critical as the initial level of BSP, since the disappearance of the dye is a linear function over a fairly wide range of concentrations (38, 40, 45).

Wiegand, et al, (39) evaluated Indocyanine Green as a test of liver function, and concluded that it was an excellent and relatively simple means of assessing liver function. They used the dosage that is now recommended by the manufacturer, 0.5 mg/kg of body weight, and drew their samples at 5, 8, 11, 14 and 17 minutes post-injection. They found the values when plotted against time on semi-log paper fell in a straight line, allowing extrapolation back to zero time, if this is what is desired. They found the half-time to be 3.8 minutes in normal subjects and increased in patients with liver damage. The manufacturer in the brochure furnished with the dye recommends a single 20 minute specimen and describes the normal as being no more than 4% dye retained at 20 minutes.

The foregoing work on Indocyanine Green led us to investigate the use of this dye as a test of liver function, and the success of Hutchens (25) and his workers with the ratio method in the BSP-radio-iodinated human serum albumin application is the reason for its being selected for use with the mixture of Indocyanine Green and tagged albumin, or Indocyanine Green and Evans Blue, to determine simultaneously liver function and plasma volume. In t he reference method, the linearity of the disappearance of the dye is the only necessary condition, since the reference material, Evans Blue or radio-iodinated human serum albumin, gives the other information necessary for the calculation. By drawing only two samples, the percent disappearance rate can be calculated, if this is the only preferred form of assessment of liver function.

Evans Blue has been a classical agent for many years for determination

of plasma volume, and would be preferred for use in a laboratory which had no isotope capabilities. The radio-iodinated human serum albumin has advantages over Evans Blue, one of them being that hemolysis is not an interfering agent. As mentioned earlier, it has been successfully used by Hutchens (25).

We also wished to investigate the possibility of pre-mixing the two testing agents together so they would conveniently be ready at any time that a request for the procedure might be received.

MATERIALS AND METHODS

INDOCYANINE GREEN AND EVANS BLUE

I. MATERIALS:

    A. Injection Materials:

        1. Evans Blue Dye: Supplied by General Diagnostics Division
of Warner-Chilcott Co. Evans Blue is a brand of tetra-
sodium salt of 4, 4' - Bis (7-(1-amino-8-hydroxy-2,4-disulfo)-
naphtylazo -3,3' -bitolyl. Each vial contains 5 ml of a
0.5% solution (equivalent to 0.452% anydrous salt of dye
in water.) 25 mg per ampoul.

        2. Indocyanine Green: Supplied by Hynson, Westoott and Dunning
as Cardio Green[R]. It was used in the 10, 25, and 40 mgm
vials. It was reconstituted by adding water (supplied in
the package). Indocyanine Green is a water soluble tricarbo-
cyanine dye with the formula 3, 3, 3', 3'-tetramethyl-1,1'
sulfo butyl-4,5,4', 5' dicarbocyanine hydroxide sodium salt.
It contains approximately 5% NaI as a contaminant.

        3. 22% Human Albumin. Obtained from Ortho Pharmaceutical.

        4. Pooled human serum: Obtained from the Department of Clini-
cal Pathology pooled serum supply.

    B. Experimental Animals:

        1. Healthy dogs varying in weight from 28 to 52 lbs were obtained
from the Animal Care Division of the University of Oregon
Medical School.

    C. Instrumentation:

        1. Spectrophotometer: A Beckman D. U. was used. Light source
was a tungsten lamp.

    D. Human Volunteers

1. Four healthy adults with no indication of liver disease volunteered for the study.

II. METHODS:

A. Animal injections: All dogs were fasting for 12 hours before the injections. All were anesthetized with nemutal before the injections, and had a #13 catheter inserted in the jugular vein for withdrawing samples. This preparatory work was done by the personnel of the Animal Care Division of the University of Oregon Medical School. A pre-injection sample was drawn through the catheter to use for standard and blank determinations. The two dyes were mixed in the proportion of 10 mgms of Evans Blue to 5 mgms of Indocyanine Green and 1 ml of mixed dye, containing 5 mgm of Evans Blue and 2.5 mgm of Cardio Green, was injected quickly through the neck catheter. The syringe and catheter were rinsed three times by drawing blood back into the syringe and re-injecting it.

B. Human Injections: The four humans used in the study were fasting for 12 hours before the injections. None were anesthetized. A preinjection sample of blood was drawn for use in standard and blanks. All were injected with the same proportions of dyes as were the dogs. Each subject received 25 mgm of Evans Blue and 12.5 mgm of Indocyanine Green. Subjects exercised lightly between injections and drawing of samples.

C. Colorimetric Determinations: For the Evans Blue readings, the blue-sensitive filter was used, at a wavelength of 620 mu. For the Indocyanine Green readings the red-sensitive filter was used, at a setting of 810 mu. A matched set of Corex rectangular cells

was used throughout. The light path was 1 cm.

D. Calculations:

Plasma volume = 500 x volume dye injected x $\dfrac{O.D.S.}{O.D.U.}$

(Formula #3)

% Dye retained: (reference method):

$$\dfrac{O.D.U. \quad (810 \text{ mu}) \times O.D.S. \ (620 \text{ mu})}{O.D.S. \quad (810 \text{ mu}) \times O.D.U. \ (620)\text{mu}} \times 100 = \% \text{ dye remaining at time}_t$$

O. D. S.: Optical density of standard
O. D. U.: Optical density of unknown.

E. Plotting: All percent retention values were plotted on semi-log paper to allow maximum usefulness of the information. Since the % retention is an exponential function for early times, it will be linear on semi-log paper. Such plotting allows easy calculation of T½.

(Formula #4)

$$T\frac{1}{2} = \dfrac{l_n\, 2}{k} = \dfrac{.693}{k}$$

EXPERIMENTAL AND RESULTS

INDOCYANINE GREEN AND EVANS BLUE

A. COLORIMETRIC STABILITY OF CONCENTRATED INDOCYANINE GREEN

The manufacturers of Indocyanine Green state in their brochure that the dye is unstable in water and should be used the same day it is reconstituted. We diluted a vial of dye to the usual concentration of 5 mgm/ml and diluted an aliquot 1-500 with saline. 2 ml of the diluted dye was mixed with 2 ml of pooled human serum and read against a blank of 2 ml of serum and 2 ml of saline. The vial and remaining dye without added protein were kept at 4° C and another aliquot diluted after 2 hours. A third aliquot was diluted after 24 hours. Optical densities on these aliquots were 1.13 immediately after reconstitution, 1.11 2 hours later and .73 after 24 hours.

B. SPECTROPHOTOMETRIC CHARACTERISTICS OF INDOCYANINE GREEN

ST curves were run on Indocyanine Green to determine its spectral characteristics immediately after dilution with the provided diluent and after standing for up to four hours in the cuvettes at room temperature. Readings for the ST curves were begun at 450 mu, and were taken every 10 mu up to a maximum of 850 mu. Maximum absorbance of Indocyanine Green under these conditions was found to be at 810 mu. Optical density of a 1-500 dilution of Indocyanine Green at 620, the absorbance maximum of Evans Blue, was .064.

C. COLORIMETRIC STABILITY OF INDOCYANINE GREEN-SERUM MIXTURES

After the O. D. readings for the ST curves had been obtained, the cuvettes were allowed to stand at room temperature, covered with Parafilm to minimize evaporation, for two hours, and reread. Another two hour period of standing was allowed to elapse and the cuvettes read

again. At the end of the four hour period, the decrease in O. D. at 810 mu was .04.

A flask containing a 1-500 dilution of Indocyanine Green in saline, with 2 ml of pooled human serum added, was allowed to stand for 30 hours, at 4° C. Two ml of the dye dilution was mixed with 2 ml of serum and read against a blank of serum and saline. No decrease of O. D. was noted.

To determine if a stock solution of Indocyanine Green could be stabilized by the addition of protein, we added .1 ml of serum to a vial containing 25 mgm of Indocyanine Green in 5 ml of diluent. After three weeks at 4° C., the readings obtained on the protein-stabilized dye, diluted and mixed with serum as described above, were .003 O. D. units less than the freshly diluted dye.

D. COLORIMETRIC STABILITY OF EVANS BLUE

Evans Blue is stated by the manufacturer to be stable for several weeks in a saline dilution, so no serum was added to the saline dilution of this dye. 1 ml of Evans Blue was diluted to 250 ml with saline and allowed to stand at 4° C. for 30 hours. A 2 ml aliquot of the dye mixture was mixed with 2 ml of serum and read against a blank of 2 ml of saline and 2 ml of serum. No appreciable difference in O. D. was noted. The ST curve on Evans Blue alone showed a maximum absorption at 620 mu. The optical density at 810 mu was .150.

E. SPECTROPHOTOMETRIC CHARACTERISTICS OF A MIXTURE OF INDOCYANINE GREEN AND EVANS BLUE

A mixture of the two dyes was prepared, containing 2 ml of Evans Blue in 500 ml, and 1 ml of Indocyanine Green, the same concentrations of each dye as were used in the work on each singly. When the mixed dyes

were mixed with 2 ml of saline and read against a blank of 2 ml of serum and 2 ml of saline, the curve for Indocyanine Green obtained from the mixture of dyes could be superimposed on the curve for Indocyanine Green when diluted and read alone.

F. COLORIMETRIC STABILITY OF INDOCYANINE GREEN AND EVANS BLUE MIXTURE

Cuvettes containing 2 ml of mixed dyes and 2 ml of serum were read immediately upon mixing and then reread after 2 hours and 4 hours of standing at room temperature, as described in the section on Indocyanine stability. The change at 620 mu was .006 increase in O. D. At 810, the decrease in O. D. was .01.

We have reported in the colorimetric stability of Indocyanine Green section that the color of the dye could be stabilized by adding .1 ml of serum to 5 ml (25 mgm) of diluted dye. We wished to determine if a mixture of Evans Blue and Indocyanine Green could be similarly stabilized. We diluted the Indocyanine Green powder directly with the solution of Evans Blue. Indocyanine Green appears to be readily soluble in Evans Blue solution. The resultant solution contained 5 mgms of Evans Blue and 2.5 mgm of Indocyanine Green per ml.

We used 30% human albumin for this study, injecting into the vials the amounts of protein shown in Table I. Each vial contained 50 mgms of Evans Blue and 25 mgms of Indocyanine Green in a total volume of 5 ml. Immediately after mixing, .2 ml of the mixed dyes were diluted to 100 with .9% saline. 2 ml of the diluted mixture and 2 ml of pooled serum were read against a blank of 2 ml of saline and 2 ml of serum. Similar aliquots were removed from the vials 6 days later and 28 days later. The data is summarized in Table I.

TABLE I

VIAL NO.	MGMS PROTEIN/ML	IMMEDIATE READINGS		6 DAYS LATER		28 DAYS LATER	
		620 mu	810 mu	620 mu	810 mu	620 mu	810 mu
1	50	.350	1.04	.380	1.06	.350	1.06
2	100	.410	1.15	.419	1.10	.379	1.03
3	200	.412	1.17	.496	1.17	.380	1.04

G. BIOLOGICAL BEHAVIOR OF THE MIXED DYES

1. DOG INJECTIONS

Since our studies showed that Indocyanine Green has a greater opt-
ical density than Evans Blue, we decided to use one-half as much
Indocyanine Green as Evans Blue for dog injections. Five dogs
were to receive 10 mgms of Evans Blue and 5 mgms of Indocyanine
Green. The dogs ranged in weight from 15 to 23 kilograms. Pre-
injection samples of blood were drawn for use in standard and
blanks. 10 mgms of Evans Blue and 5 mgms of Indocyanine Green
gave a dosage of approximately .5 mgm/kg of Evans Blue and .25
mgm/kg of Indocyanine Green. The mixture of dyes was quickly
injected through the jugular vein catheter of the anesthetized
dogs and the dyes rinsed in by drawing blood back into the syringe
and reinjecting it three times, post-injection. Samples were
drawn through the jugular vein catheter at the following inter-
vals: 1 min, 2 min, 3 min, 5 min, 10 min, and 20 minutes. Dogs
number 4 and 5 also had 30 and 60 minute specimens drawn. Less
than 10 seconds was necessary to draw each specimen. The blood
was put into a tube, allowed to clot, and centrifuged. 2 ml of
serum was removed after centrifugation and mixed with 2 ml of
saline. A 1 mgm% standard of Evans Blue was prepared and a .5
mgm% standard of Indocyanine Green. A blank of 2 ml of pre-

injection serum and 2 ml of saline was used to read standards and unknowns against. All samples were read at 620 and 810 mu. The percent dye remaining was calculated by the ratio calculation (Formula #3) and the percent dye remaining at each time interval is shown in Table II.

TABLE II

TIME	DOG #1	#2	#3	#4	#5	AVERAGE ICG RETENTION
1 min		93	71	93	95	88
2 min	95	82	72	76	88	82
3 min		72	73	72	69	71.5
4 min	66	64	74	65		67
5 min		56	69	56	69	60
10 min	30	34	39	32	46	36
15 min		21	35-	21	32	27.5
20 min	17	14			25	19
30 min			16	10	12	13
60 min			7	4.5	4	5

Figure 2 shows the data from the injection of Dog #5 in graphic form. The average early Indocyanine Green disappearance $T_{1/2}$ for the five dogs was 7 minutes.

The plasma volumes of the five dogs were 57, 58, 67, 61 and 50 ml per kilogram, an average 59 ml per kilogram of body weight.

2. HUMAN INJECTIONS

Each human volunteer was injected intravenously with an immediately premixed mixture of 25 mgms of Evans Blue and 12.5 mgms of Indocyanine Green. On Subjects 1 and 2, samples were drawn at 5 minutes, 10 and 30 minutes. On Subjects 3 and 4, samples were drawn at 10, 15, 20 and 30 minutes. The blood was allowed to clot and serum removed after centrifugation. 2 ml of each sample was mixed with 2 ml of saline and read at 620 mu and 810 mu

Figure 2. Dog # 5

———— % dye retained by Formula 3.

——— ———Indocyanine Green in O. D. units at 810 mu

— — — — — Evans Blue in O. D. units at 610 mu

KEUFFEL & ESSER CO.
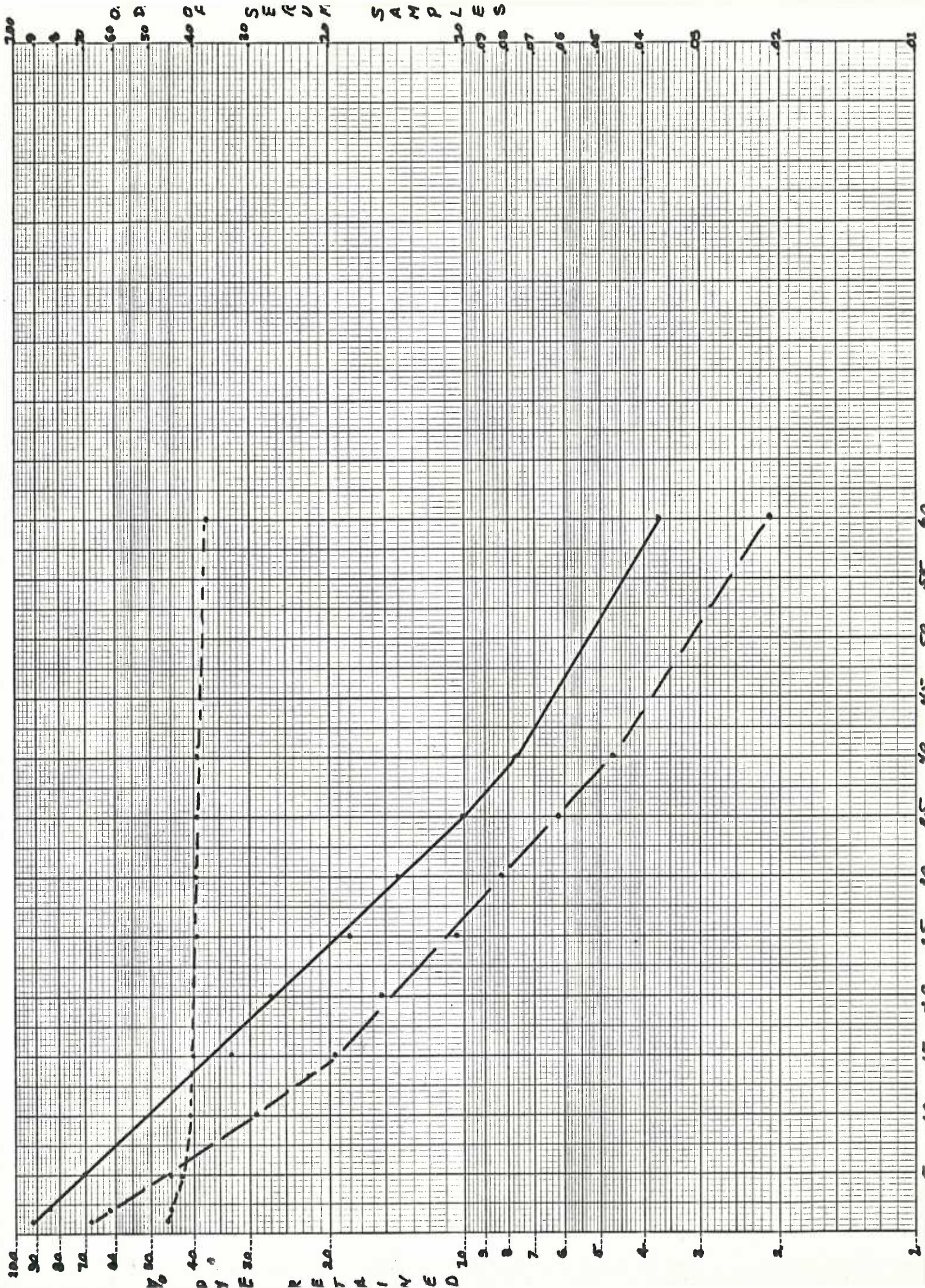
against a blank of serum and saline. Standards of 1 mgm% Evans
Blue and .5 mgm% of Indocyanine Green were used. Results are
shown in Table III.

TABLE III

TIME	SUBJECT				AVERAGE RETENTION
	1	2	3	4	
5 min	45.5%	43.8%			44.6%
10 min	18.1	14.2	13.2%	18.0%	15.9
15 min			4.4	8.4	6.4
20 min	4.5	3.1	2.2	4.9	3.9
30 min	1.9	.5	1.0	3.6	1.8

The average percent Indocyanine Green retained at 20 minutes
corresponds very closely to the results obtained by Cherrick, et
al (38), who quote an average value of 3.85% retained at 20 min-
utes.

Figure 3 shows the percent dye retained calculated using
Formula #5 and the concentration of Indocyanine Green and Evans
Blue in terms of optical density, as determined on the serum of
subject #1. The plasma volume of this subject was 39 ml/kg, well
within the normal range for males, as given by Hutchens (30).

Again it is noted that the disappearance curves based on
Indocyanine Green concentration and on percent retention figures
calculated using the reference formula #5 are parallel and approx-
imate exponential clearance between 5 and 20 minutes.

Figure 3.   Subject # 1

_____ % dye retained by Formula 3.

____ ___Indocyanine Green in O. D. units at 810 mu

_ __ __ _Evans Blue in O. D. units at 620 mu

100%
90
80
70
60
50
40
30
20
10
9
8
7
6
5
4
3
2
1

D Y E R E L A T I V E D

5   10   15   20   25   30
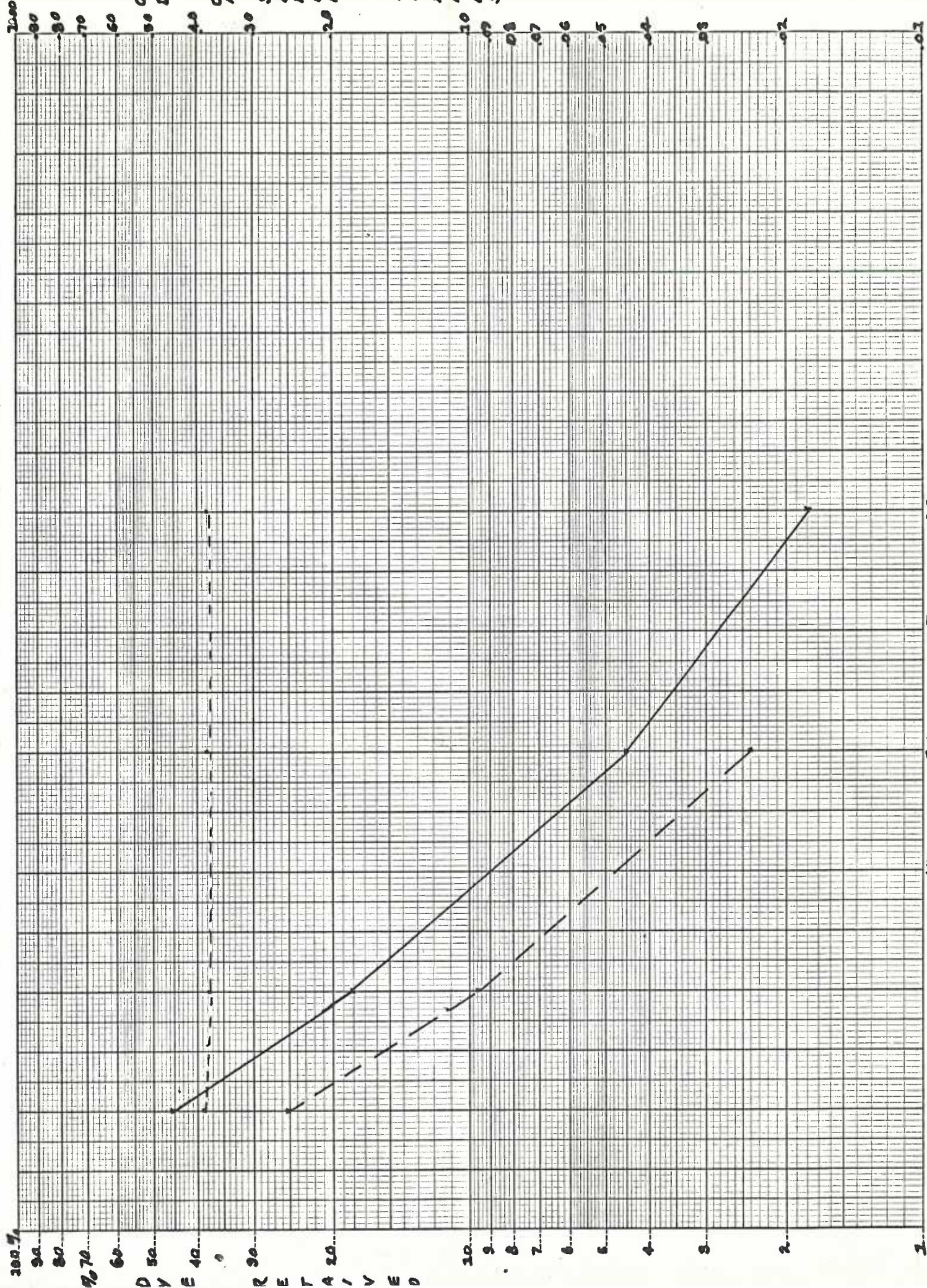
MATERIALS AND METHODS

RADIO-IODINATED HUMAN SERUM ALBUMIN AND INDOCYANINE GREEN

I. MATERIALS

   A. INJECTION MATERIALS

      1. Indocyanine Green: See Indocyanine Green-Evans Blue section.

      2. I-131-Labelled human serum albumin: As Albumotope[R] Squibb
         Radio-Iodinated Serum Albumin (human) USP. The sterile,
         pyrogen-free solution contains approximately 6% albumin or
         50 to 60 mgm per ml. On the day of receipt, there was ap-
         proximately 4 uc of activity per milligram of protein. The
         pH of the solution was adjusted by Squibb to 7.2-7.8.

      3. Bromsulfathalein dye: 5% aqueous solution of phenoltetra-
         bromphthaleindisodium sulfonate. From Hynson, Westcott
         and Dunning.

      4. Pooled normal human serum: See Indocyanine Green-Evans
         Blue section.

      5. NaI 131: From Squibb. Contained 1.9 mc/ml on day of use.
         Diluted with .9% saline to contain 1.9 uc/ml.

   B. DOGS

      See Indocyanine Green-Evans Blue section.

   C. Ion EXCHANGE COLUMNS

      Abbott Laboratories Ioresin[R] was used, 50% w/v in distilled
      water with benzyl alcohol, 0.5% as bacteriostat.

   D. INSTRUMENTATION

      1. Spectrophotometer: See Indocyanine Green-Evans Blue section.

      2. Radio-assay Assembly:

         a. Nuclear Radiation Analyzer, Model 1810, equipped with
            an American Electronics scintillation well counter and

a Nuclear Chicago Decade Scaler, Model 186.

    b. Paper Chromatography Strip Counter: Low-background automatic Chromatogram scanner, Model 880, Vanguard Instruments Co., with Texas Instruments Corporation Recti-riter, a linear recording milliammeter.

3. Paper Electrophoresis Assembly:

    a. Durrum cell: Spinco Model R, Series C. Beckman Instruments.

    b. Duostat: Spinco Regulated D. C. power supply.

    c. Beckman Paper Electrophoresis strips, No. 320046, (S&S 2043A mgl). The strips were standard size, 1 and 3/8 inches by 12 inches.

    d. Buffer: Buffer B-2. pH 8.6.

4. Planimeter: Lasico Planimeter, No. 127A. (Metric) Los Angeles Scientific Instrument Company, Los Angeles, California.

II. METHODS

A. PAPER ELECTROPHORESIS

Paper strips were placed in position in the cell, the Veronal buffer added to the proper level, the strips moistened, and the cell allowed to equilibrate for 20 minutes before the samples were placed on the strip with the Spinco applicator. A sample of .006 ml was placed on each strip and a constant current of 2.5 ma applied for a period of three hours. Following migration, the strips were dried in an oven at 95°, with the door ajar, for 20 minutes. When thoroughly dry, the strips were taped together for running through the scanner. Scanning was done on the instrument described, with the following setting on the Auto-scanner.

High voltage: 1325 volts. Carrier gas pressure: 7.5 lbs, and
80% flow. Carrier gas: 1.3% butane, 98.7% helium. Operation:
Sum. Range: This varied somewhat with the age of the I-131-
labelled human serum albumin, but was usually 10,000 or 30,000.
Time Constant: B. Collimation: 1 cm. Speed: 3/4 inch per
minute. Areas under each peak were determined by planimetry.

B. ION EXCHANGE CHROMATOGRAPHY

Columns were set up according to the directions given by Abbott
for the use of the resin, except that glass syringes, washed to
remove all iodine, were used as containers for the resin. The
barrels of the syringes were used instead of the plunger suggested
by the manufacturer to exert a gentle pressure to express the
final drops of eluate after the material had been passed through
the column. Columns were set up, rinsed with saline, and 2 ml
of the mixtures counted in the well counter. The mixtures were
put onto the columns, and, after all the material had gone through
by free flow, the vial used for the pre-passage counting was
rinsed with saline, and the rinsings put through the column.
One ml of saline was used for this rinse. Two additional ml of
saline were put directly onto the column and the final drop ex-
pressed from the resin by gentle pressure from the syringe barrel.
2 ml of well-mixed eluate were transferred to a vial and counted
in the well counter of the Nuclear Radiation Analyzer. A factor
of 5/.2 was applied to the resulting counts to correct for dilution.

C. TRICHLOROACETIC ACID PRECIPITATION

The procedure was that of Frank and Zizza (46). Each of the mix-
tures to be assayed was treated with 10% trichloroacetic acid in

the presence of 1 ml carrier human protein. 2 ml of each mixture was then poured into a 15 ml graduated centrifuge tube and the vial rinsed with 1 ml of saline, the rinsings being added to the tube. 1 ml of human serum was added to each tube and 6 ml of cold trichloroacetic acid added to the contents of each tube. All tubes were stoppered and shaken for 20 minutes, then allowed to stand at 4° C. for 30 minutes. Tubes were then centrifuged at high speed for 10 minutes, and 2 ml of supernatant fluid removed for counting. The resultant counts were multiplied by 5 to compensate for dilution.

D. DOG INJECTIONS

No animal was used more than once, to avoid the possibility of sensitization to the human albumin contained in the I-131-labelled human serum albumin. All animals were anesthetized with nembutal, and a #13 catheter inserted in the jugular vein. All animals were prepared by the Animal Care department of the Medical School. Injections were carried out as described for the Indocyanine Green-Evans Blue work, except where so noted in the Experimental and Results section.

E. CALCULATIONS

A reference formula was again used to calculate the percent of Indocyanine Green retained:

(Formula #5)

$$\% \text{ dye remaining}_t = \frac{\text{O. D. (Serum)} \times \text{Cts/min (Std)}}{\text{O. D. (Std)} \times \text{Cts/min (serum)}_t} \times 100$$

Plasma volumes were calculated by the formula:

(Formula #6)

$$\frac{\text{mgm ICG/ml in dose}}{\text{mgm ICG/ml in standard}} \times \text{cpm/ml in std} \times \text{ml dose injected} = \text{P.V. in mls.}$$

_____

Cpm/ml in 15 minute serum specimen

Dose: mixture of Indocyanine Green and I-131-labelled human serum albumin used for injection.

Standard: approximately 7 drops of Indocyanine Green-I-131-labelled albumin mixture diluted to 100 mls with .9% saline.

EXPERIMENTAL AND RESULTS

INDOCYANINE GREEN AND I-131-LABELLED HUMAN SERUM ALBUMIN

A. SPECTROPHOTOMETRIC CHARACTERISTICS OF INDOCYANINE GREEN

Spectrophotometric characteristics of Indocyanine Green have been dis-
cussed in the Indocyanine Green-Evans Blue Experimental and Results
section.

B. COLORIMETRIC STABILITY OF INDOCYANINE GREEN-I-131-LABELLED ALBUMIN
    MIXTURE

As indicated in the Indocyanine Green-Evans Blue section, as little
as .1 ml of human serum is sufficient to stabilize the color of a vial
of Indocyanine Green containing 25 mgms of dye in 5 ml of diluent.
We now wished to know if there was enough protein in the Radio-Iodinated
Human Serum Albumin as received from the supplier to stabilize Indo-
cyanine Green, and if the presence of the isotope would affect the
stability of Indocyanine Green.   .2 ml and .5 ml of Radio-Iodinated
Human Serum Albumin were added to vials containing 10 mgms of Indo-
cyanine Green, dissolved in the water diluent. Using the minimum
figure for albumin content given by the manufacturer, this would mean
we added 10 mgms and 25 mgms of protein to each vial. 1.8 ml of water
was used for the first vial and 1.5 ml for the dilution of the second,
so that the final solution in each case contained 5 mgm of dye per ml.
The Indocyanine Green-Iodinated Human Serum Albumin mixtures were
stored in the original sterile Indocyanine Green vials, aseptic tech-
nique having been used in adding the diluent and the Iodinated Human
Serum Albumin. 1 ml of the contents of each vial was diluted to 250
ml with .9% saline, and 2 ml of the diluted dye mixed with serum and
read against a serum-saline blank at 810 mu immediately after the

Indocyanine Green and the Radio-Iodinated Human Serum Albumin were mixed. The vials were then stored at 4° C. for 28 days and another 1 ml aliquot diluted to 250 ml and read as before. There was a decrease of .01 O.D. units in the aliquot stored for 28 days, as compared to the aliquot read immediately after mixing.

The Radio-Iodinated Human Serum Albumin is as effective as non-iodinated human serum albumin in stabilizing the color on Indocyanine Green.

C. ELECTROPHORETIC STUDIES ON INDOCYANINE GREEN-I-131-LABELLED HUMAN SERUM ALBUMIN MIXTURES

The next points to be investigated were the possible deiodination of Radio-iodinated Human Serum Albumin and denaturation of albumin in vitro in the presence of Indocyanine Green.

To ascertain whether deiodination had taken place, we attempted to demonstrate an increase of free I-131 in a mixture of Indocyanine Green and I-131-labelled human serum albumin. The following four mixtures were studied by paper electrophoresis:

Mixture 1. Indocyanine Green diluted with water diluent, and Radio-Iodinated human serum albumin added so that each ml of mixture contained approximately 24 uc of I-131, 2 mgms of albumin, and 5 mgms of Indocyanine Green.

Mixture 2. Radio-Iodinated Human Serum Albumin diluted with .9% saline so that each ml contained approximately 24 uc of I-131, and 2 mgms of albumin.

Mixture 3. 1 ml of diluted NaI-131 added to 4 ml of diluted Indocyanine Green so that each ml contained approximately 4 mgms of Indocyanine Green and .2 uc of NaI-131.

Mixture 4.   1 ml of diluted NaI-131 added to 3 ml of saline, and 1 ml

of I-131-labelled Human kSerum Albumin, so that each ml

contained approximately 2 mgms albumin and 20 uc of I-131

and .2 uc of NaI-131.

The carrier free NaI-131 in mixtures 3 and 4 was added to establish

the electrophoretic behavior of free I 131 when added to the mixture

of Indocyanine Green and I-131-labelled albumin.   A saline solution

of free NaI 131 was also electrophoresed.

.006 ml of each of the four mixtures was placed on paper electro-

phoresis strips, two strips being planted with each mixture, and

paper electrophoresis carried out as described.   A strip was planted

with NaI alone, to establish its rate of migration.   Tracings of the

four mixtures and the NaI 131 control strip are shown in Figure 4.

The arrow at upper right in each tracing indicates the point of ap-

plication of sample.   Comparison of the tracings of mixtures 1 and 2

shows no apparent differences; in each, the I 131 appears to be bound

to, and migrating with, the albumin.   A small peak appears to the left

of the albumin peak in each of these tracings.   Comparing the tracings

for mistures 3 and 4, free I 131 can be seen to the left of the albu-

min peak in mixture 4, indicating that the free I 131 migrates more

rapidly than does the albumin, the direction of migration being from

right to left in these figures.   Mixture 3 contains no albumin, but

does show a small peak in the position usually occupied by the albu-

min.   This may mean that a small amount of I 131 was bound to the

Indocyanine Green, and traveled less rapidly than the free I 131.

Free added I 131 migrated the same distance from the application point

in mixtures 3 and 4, and the small peaks mentioned in the tracings of

Figure 4 (a) Mixture 1.
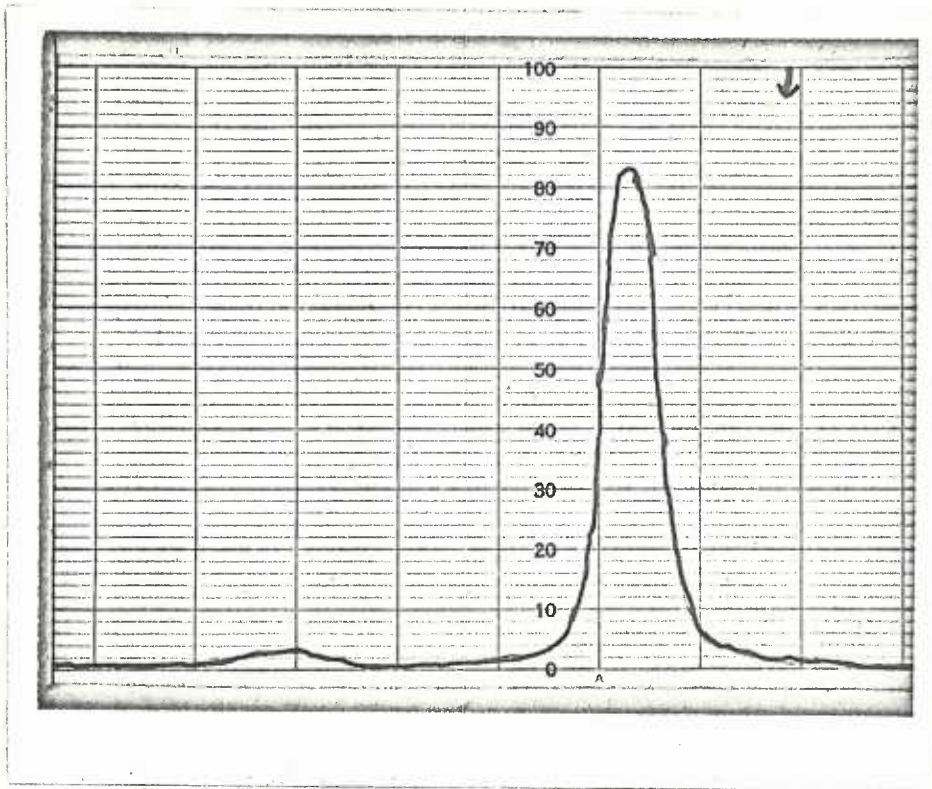

Figure 4 (b) Mixture 2.
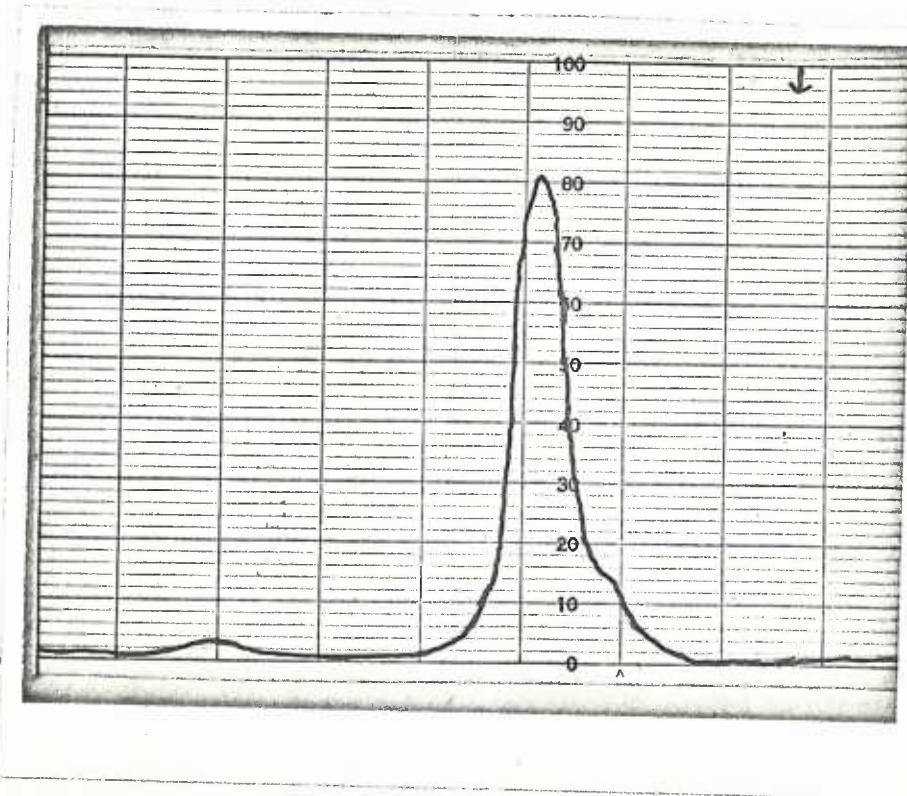
Figure 4 (a)

Figure 4 (b)

Figure 4 (c) Mixture 3.
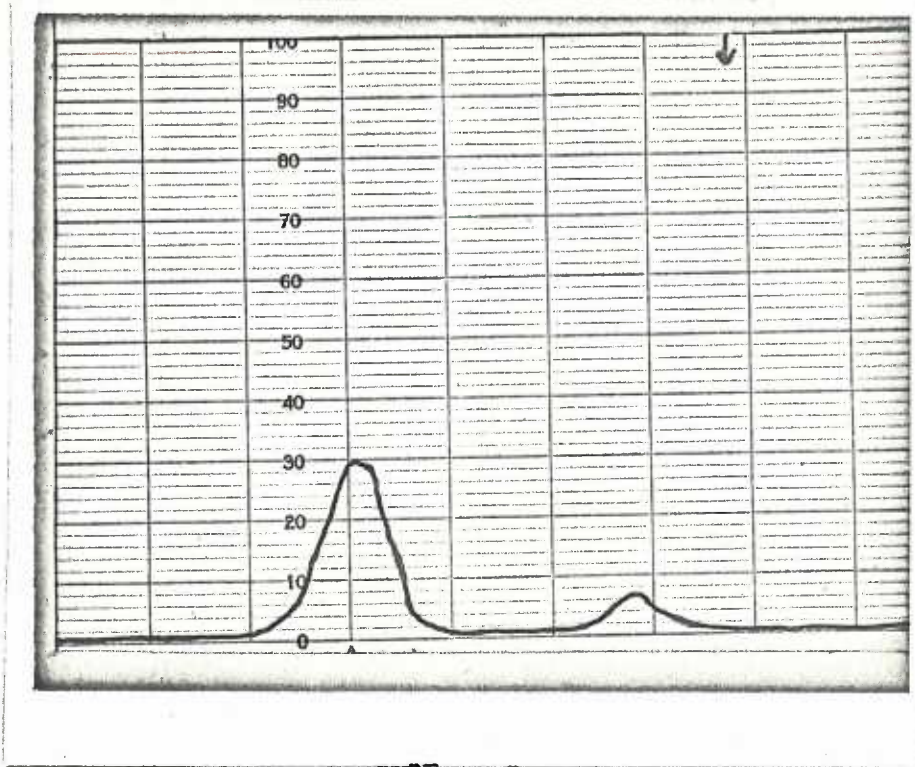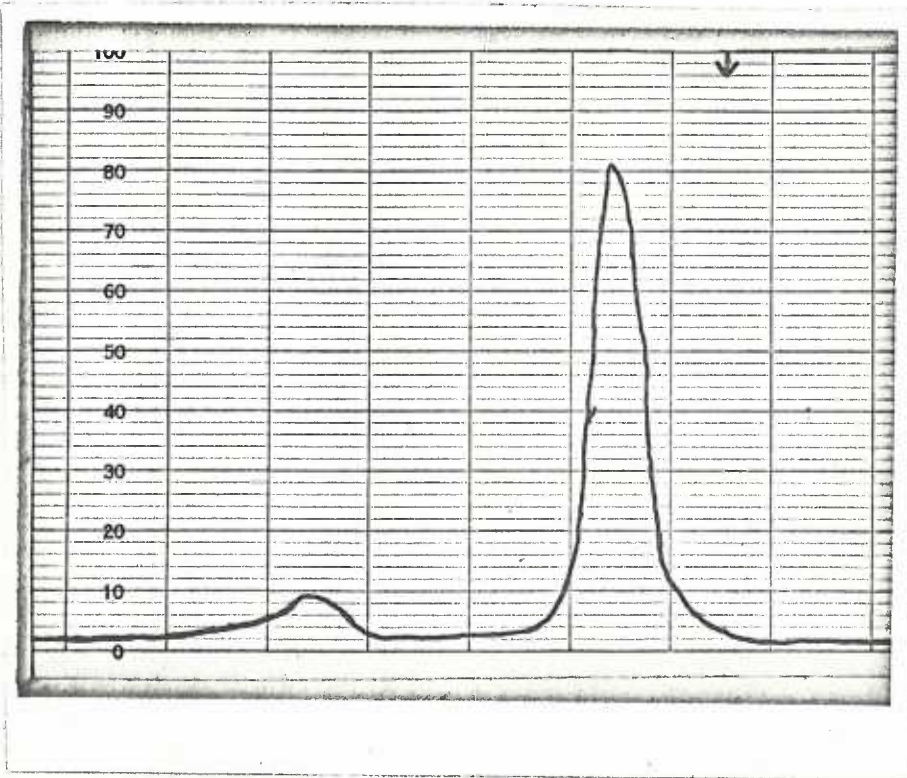
Figure 4 (d) Mixture 4.

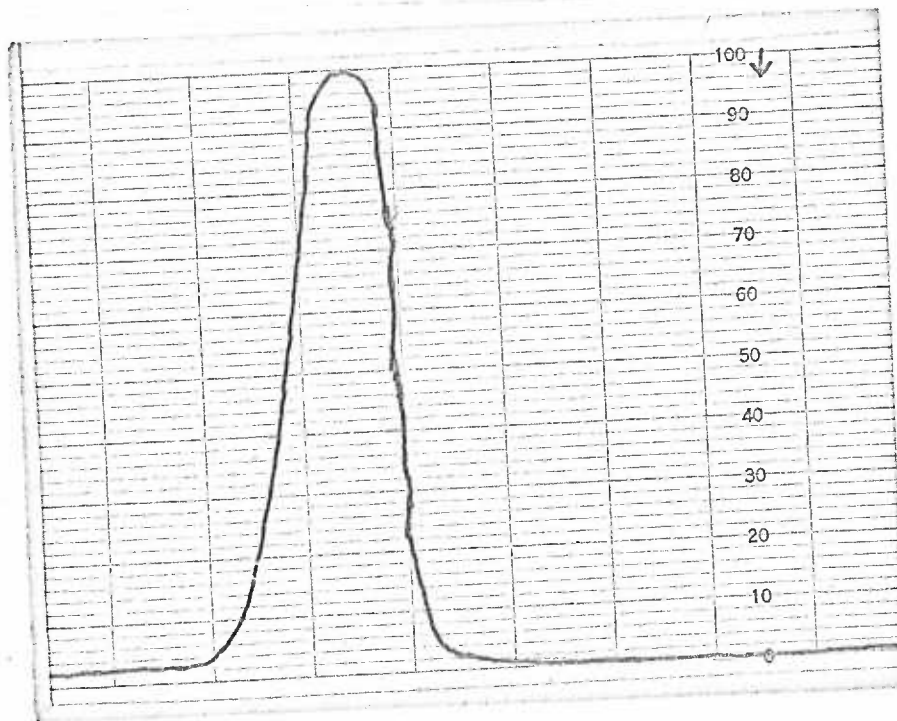Figure 4 (c)

Figure 4 (d)

Figure 4 (e) Mixture 5.

Figure 4 (e)

mixtures 1 and 2 indicates the presence of a small amount of free I
131 in each of these mixtures. The supplier states that approximately
2% of free I 131 will be found in the I 131-labelled human serum albu-
min, and these tracings are not inconsistent with that statement.
The carried free NaI 131 migrated at the same rate as free I 131 in
the mixtures.

The results give no evidence for I 131-human serum albumin de-
iodinization or albumin denaturation by Indocyanine Green.

D. ELECTROPHORETIC, ION EXCHANGE CHROMATOGRAPHY AND TRICHLOROACETIC ACID
ASSAYS FOR FREE I-131 IN INDOCYANINE GREEN AND I-131-LABELLED ALBUMIN
MIXTURES

Two additional methods of checking for free I 131 have been used by
other authors (47, 47).

For comparison, BSP and Evans were also mixed with Radio-Iodin-
ated Human Serum Albumin and studied. Additional, unlabelled human
albumin was added to two of the mixtures to see what effect, if any,
this would have on the binding of I 131. The following six combin-
ations of dyes, isotopes and albumin were included:

Mixture 5: 5 uc of I 131-labelled albumin and 5 mgm Indocyanine
            Green per ml.

Mixture 6: 5 uc I-131-labelled albumin and 5 mgm Evans Blue per ml.

Mixture 7: 5 uc I-131-labelled albumin and 5 mgm BSP per ml.

Mixture 8: 5 uc I-131 labelled albumin and saline.

Mixture 9: 5 uc I-131-labelled albumin, 5 mgms Indocyanine Green,
            and 60 mgms unlabelled human serum albumin per ml.

Mixture 10: 5 uc I-131-labelled albumin, saline, and 60 mgms un-
             labelled human serum albumin per ml.

Glass vials were used in the preparation of all mixtures, with no pre-cautions taken to avoid adsorption onto the glass. Since the mixtures were quite concentrated, all were handled identically, and the amounts of I 131 and diluent were the same in each of the mixtures, adsorption losses were assumed to be minimal, and the same in each of the mixtures.

The six mixtures were assayed by each of the three methods, and were then allowed to stand in the small glass vials at 4° C. for 4 days; aliquots were removed for assaybby trichloroacetic acid method only, and the vials returned to the refrigerator for a total of 14 days before the final assay was performed. In the trichloroacetic acid and ion exchange assays, an aliquot of mixture was counted in the well counter, then treated with trichloroacetic acid, or put through the ion exchange column. The loss in counts, in percent, was then calculated, using the pre-treatment count as 100%. The percent free I 131 was determined on the electrophoresed samples by planimetry measurements on the recorder strips. Results of this study are tab-ulated in Table IV.

TABLE IV

MIXTURE	TCA		ION EXCHANGE	ELECTROPHORESIS
	4 days	14 days	14 days	14 days
5	5.3%	5.2%	9.8%	2.0%
6	4.3%	5.2%	1.0%	5.0%
7	4.0%	4.5%	9.0%	2.0%
8	4.8%	5.4%	1.0%	4.0%
9	5.2%	5.2%	16.0%	.5%
10	4.2%	5.4%		2.0%

These data show no significant increase in deiodination by the trichloroacetic acid method. The only figure showing a substantial increase in free I 131 is the 14 day ion exchange assay of the mixture of Indocyanine Green, Radio-Iodinated albumin and unlabelled albumin. This increase is not substantiated by the other two methods. Mixture 10 was too viscous to go through the ion exchange column after 14 days.

E. DOG INJECTIONS

To study the in vivo behavior of premixed Indocyanine Green and I-131-labelled albumin, a three dog series of experiments were done. Mixture 11 was prepared by diluting a vial of Indocyanine Green containing 25 mgms of dye with 4 ml of water diluent. The I-131-labelled human serum albumin was diluted with saline so that each ml would contain 30 uc of I 131 and 15 milligrams of albumin. 1 ml of the saline-diluted I-131-labelled albumin was added to the vial of Indocyanine Green, so that the resultant mixture contained 5 milligrams of Indocyanine Green, 6 uc of I 131 and 3 milligrams of albumin, per ml.

Enough of mixture 11 was taken from the vial on the first day for the injection of 1 ml into Dog #1, electrophoretic studies, and standard preparation. Remaining Mixture 11 was stored in the original vial at 4° C. until 7 days later, when the procedure was repeated, using Dog #2. One week after the second injection, the process was repeated a third time, using Dog #3. On the same day the injections were done, a small amount of Mixture 11 was subjected to the electrophoretic procedure described to check for free I 131. No increase in free I 131 was found.

Each dog in the series received a constant amount of Indocyanine

Green, 5 mgm, and the amount of isotope would have been reduced by approximately one-half each week. The first dog received about 6 uc, the second, 3 uc, and the third 1½. A counting standard was prepared just prior to animal injections. 6 drops of the mixture to be injected was added to a volumetric flask containing about 90 ml of saline. The amount of dye remaining in the syringe was adjusted so that 1 ml was available for injection through the catheter. The flask with the counting standard was adjusted to 100 ml total volume with saline to be counted at the same time as the serums. A pre-injection sample was drawn to provide serum for blank and standards for the colori- metric study. Post-injection serum specimens were drawn at 1 min, 2 min, 3 min, 4 min, 5 min, 10 min, 20 min, 30 min, and 60 min. Serum was separated and 2 ml placed in counting vials and counted in the well counter. Indocyanine Green concentration was determined spectro- photometrically on each of the post-injection serums.

The calculated plasma volume of Dog #1 was 71 ml/kg, dog #2, 94 ml/kg and dog #3 122 ml/kg.

Percent dye retained was calculated by the reference calculation (Formula #5). The results are given in Table V.

TABLE V

TIME	DOG #1	DOG #2	DOG #3
1 min	255%	262%	297%
2 min	151		234
3 min	142	156	229
4 min	127	145	214
5 min	103	134	202
10 min	51		150
15 min	32	75	
20 min	18		82

30 min			52
60 min	0	14	22

It is noted that the initial percent retention values calculated using the reference equation #5 are high, compared to the percent retention obtained in the Evans Blue-Indocyanine Green studies. The calculated retention of dye increased progressively as the time the dye and the isotope have been mixed and standing at refrigerator temperature increases. The five minute retentions on each dog, it will be seen from Table V, increased from 103% on the first dog to 134% on the second dog a week later, and to 202% on the third dog, after the mixture had been standing for two weeks.

Indocyanine Green in mgms% are shown for each sample in Table VI.

TABLE VI

TIME	DOG #1	DOG #2	DOG #3
1 min	1.4 mgm%	1.1 mgm%	1.5 mgm%
2 min	.65		.9
3 min	.55	.42	.82
4 min	.45	.38	.75
5 min	.38	.34	.70
10 min	.19		.49
15 min	.11	.18	
20 min	.06		.26
30 min			.16
60 min	.0	.03	.07

Figure 5 shows the results of the series of injections in graphic form. It will be noted that there is a rapid initial loss of I 131 counts in the serum of each dog. The loss of counts does not increase significantly with the time Mixture 11 had aged. Total loss of counts in

Figure 5 (a)   Dog # 1.

_____ % dye retained by Formula 5.

___ ___Indocyanine Green in O. D. units at 810 mu

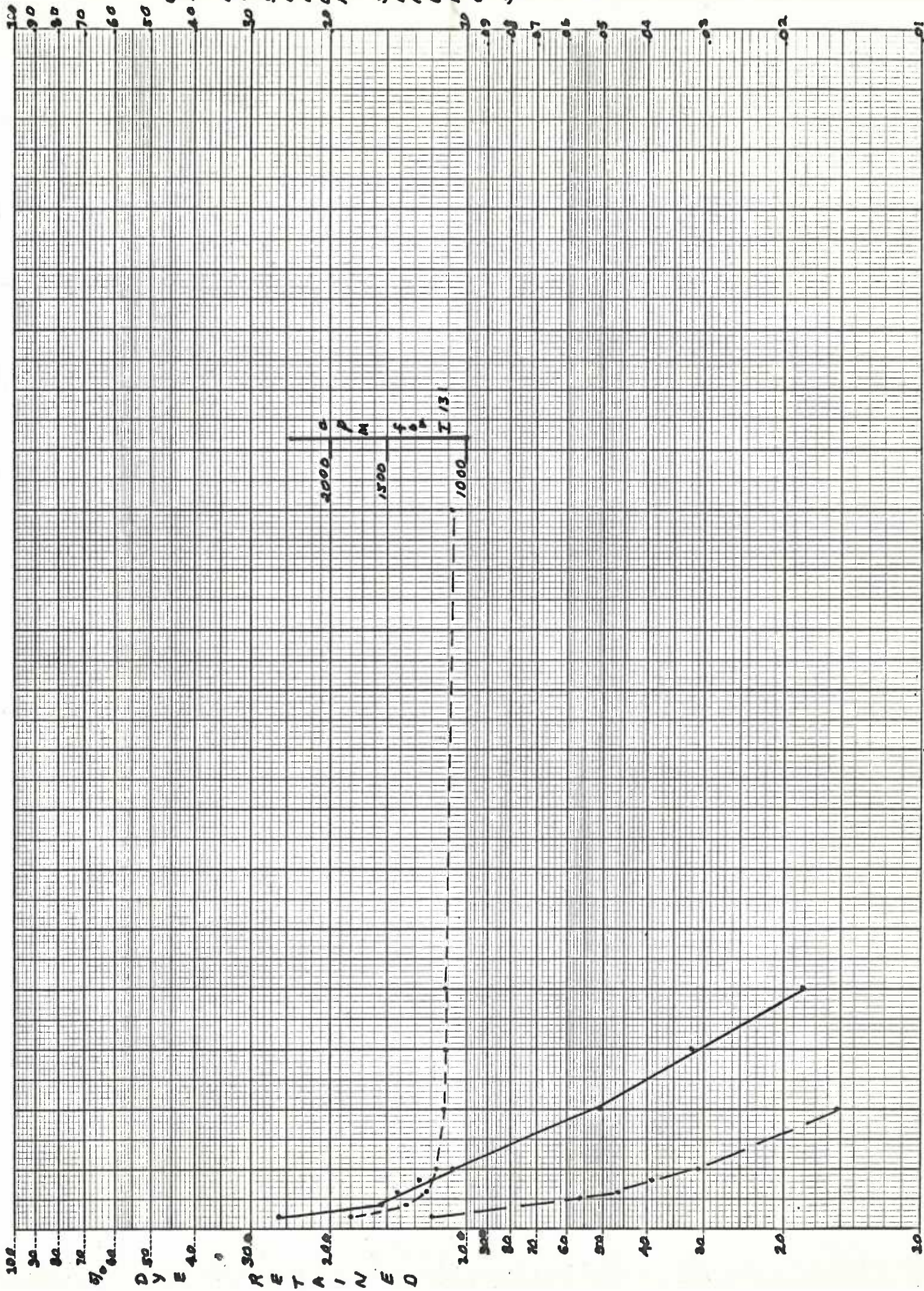_ _ _ _ _ I-131-labelled albumin in counts per minute.
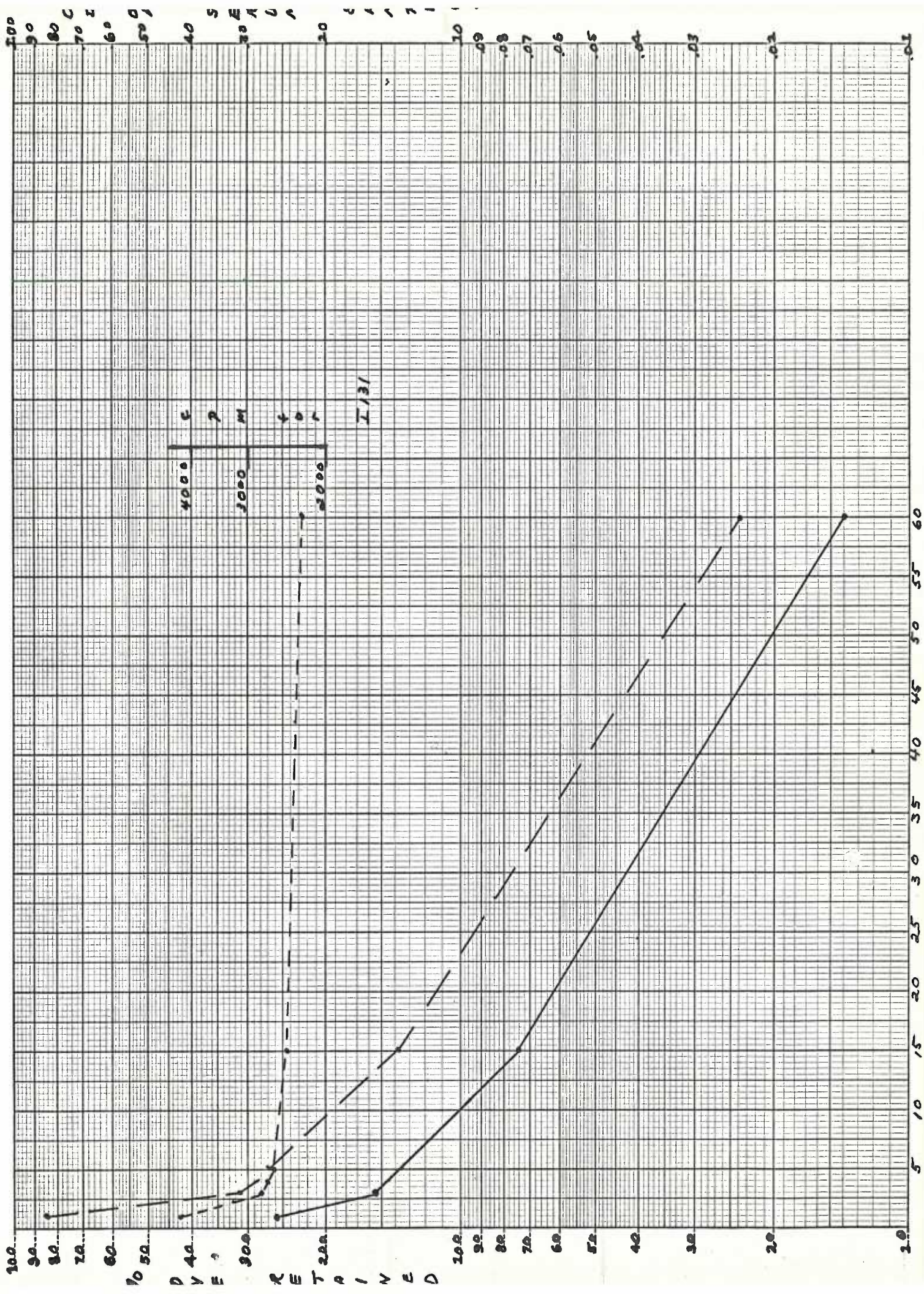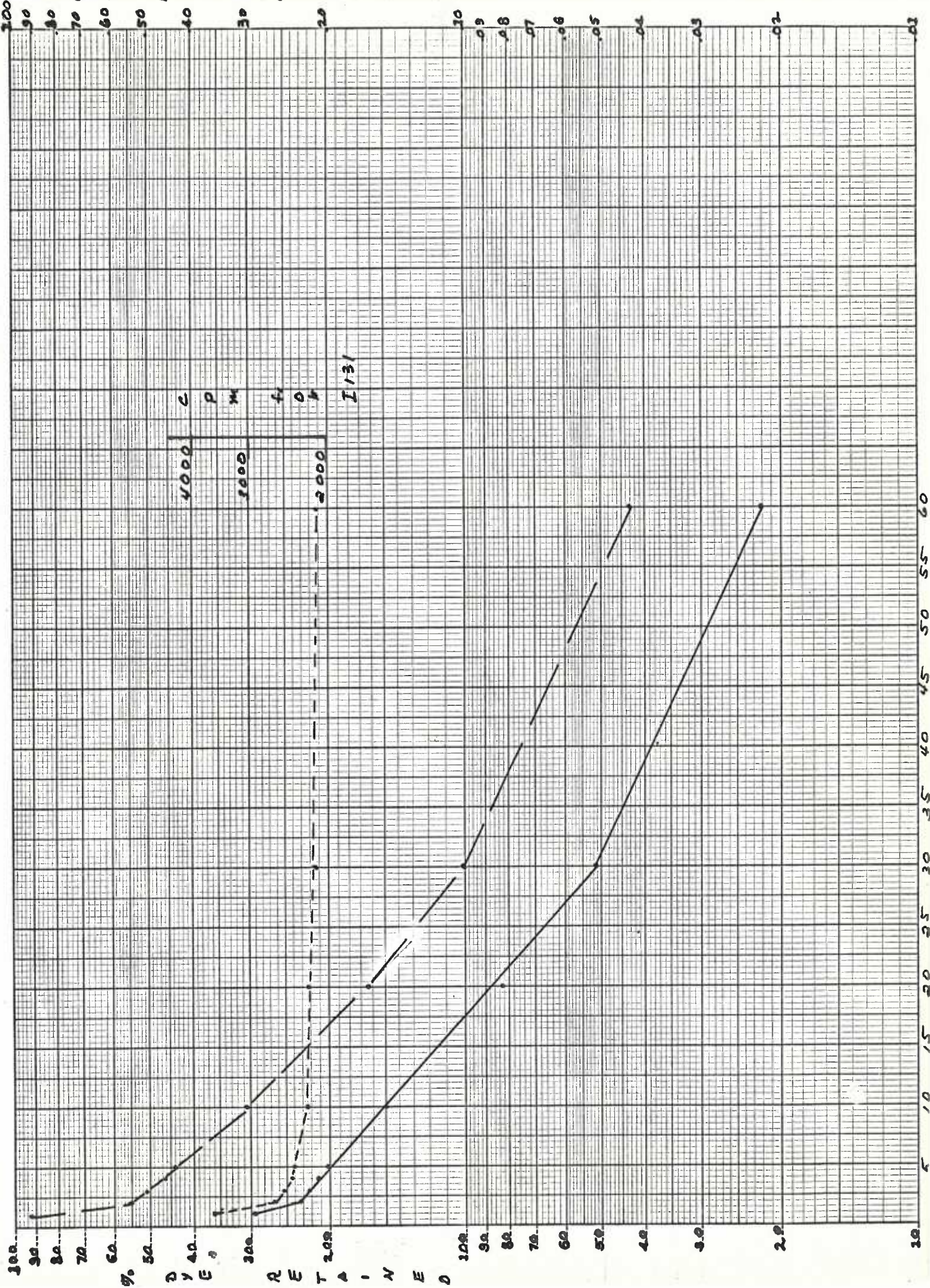
Figure 5 (b)   Dog # 2

I 131

Figure 5 (c)   Dog # 3

the serum of the dogs was 39% for Dog #1, 47% for Dog #2 and 40% for Dog #3. The 1 minute count was taken as 100% in these calculations. The percent retention of Indocyanine Green does increase with time of mixture aging as indicated in Tables V and VI.

These data suggest that pre-mixing of saline-diluted I-131-labelled human serum albumin and Indocyanine Green results in an accelerated disappearance of I 131 from the circulation in the first four minutes post-injection, and that, as the length of time the two substances are mixed together increases, a progressive change in the biological behavior of Indocyanine Green takes place.

F.   APPARENT LOSS OF I 131 FROM CIRCULATION

We considered the possibility that I 131 was being selectively lost on the walls of the indwelling plastic injection catheter, and allowed an aliquot of mixed Indocyanine Green and I-131-labelled albumin to run through a #13 catheter. A 1-250 dilution of material which had not been put through the catheter was compared to a 1-250 dilution of material which had been passed through. A loss of approximately 20% of the counts was observed.

It was also thought that the dilution of the I-131-labelled human serum albumin with saline prior to mixing with the Indocyanine Green might have had an effect on the stability of the labelled albumin. In t he second series of injections, undiluted I-131-labelled human serum albumin was added to the diluted Indocyanine Green.

G.   IN VIVO BEHAVIOR OF IMMEDIATELY PRE-MIXED INDOCYANINE GREEN AND UNDILUTED I-131-LABELLED HUMAN SERUM ALBUMIN

The Indocyanine Green was diluted with the water diluent as before. The amount of undiluted I-131-labelled human serum albumin

necessary to give approximately 5 uc of I-131- was added immediately before the injections. The same shipment of I-131-labelled albumin was used in each of these mixtures, numbers 12, 13 and 14.

Mixture 12 was prepared by diluting 25 mgms of dye with 4.8 ml of water, and adding .2 ml of labelled albumin. Each ml of mixture would contain 5 mgms of dye, 5 uc of I 131, and 2 mgms of albumin.

Mixture 13 also contained 5 mgms of Indocyanine Green, 5 uc of I 131, but 5 mgms of albumin per ml.

Mixture 14 contained 5 mgms of Indocyanine Green, 5 uc of I 131, and 7 mgms of albumin. The discrepancies in the amounts of albumin in the mixtures were not considered to be significant, all of them being in excess of the amount shown to be capable of stabilizing the amount of Indocyanine Green in the vials.

G. INJECTIONS OF MIXTURES 12, 13, AND 14 INTO DOGS #4, 5, AND 6.

The injections of the Indocyanine Green and I 131-labelled albumin were administered through a stainless steel needle into a foreleg vein in these dogs, rather than through the plastic catheter. The mixtures were rinsed in by drawing blood back into the syringe and re-injecting it, as had been done in the earlier series. Drawing of samples and assays of isotope and spectrophotometric determinations were done as previously described. Results were again calculated by Formula #5, and are reported in Table VII.

TABLE VII

TIME	DOG #4	DOG #5	DOG #6	AVERAGE CG RETENTION
1 min	106%	96%	109%	104%
2 min	99	87	103	99
3 min	90	78	100	89

5 min	77		89	78
10 min	53	69	60	53
15 min	35	45	38	34
20 min		30	12	
30 min	13	11.5	10	11.5
60 min	3.7	5.25	5	4.65

Average T½ of the Indocyanine Green disappearance is approximately 8 minutes, in the linear portion of the curve, which extends from 1 minute to 20 minutes.

Figure 6 shows the appearance of the curves on Dog #4. Note the small drop in I 131 counts in the first few minutes post-injection, compared to dogs 1, 2, and 3. It will be seen that the curves for percent dye retained by the reference calculation (Formula 5) and the curves for mgms dye concentration are parallel. This had been true for the later portion of the curves for dogs 1, 2, and 3, but the rapid initial loss of I 131 from the circulation altered the pre-injection ratio of the two substances, thus destroying the validity of the reference calculation.

Figure 7 shows the curves for Dog #5 in the Indocyanine Green-Evans Blue series. The slope of the percent dye retained is very similar to the slope of the percent dye retained with the Indocyanine Green and I-131-labelled albumin in Dog #4 of that series. The loss of the reference materials, I-131 albumin and Evans Blue is also very similar. The curve for percent dye retained is parallel with the Indocyanine Green in O. D. units in both figures.

H. PRELIMINARY ATTEMPT AT IN VIVO LOCALIZATION OF I-131 LOST FROM AGED
   I-131 HUMAN SERUM ALBUMIN-INDOCYANINE GREEN MIXTURES
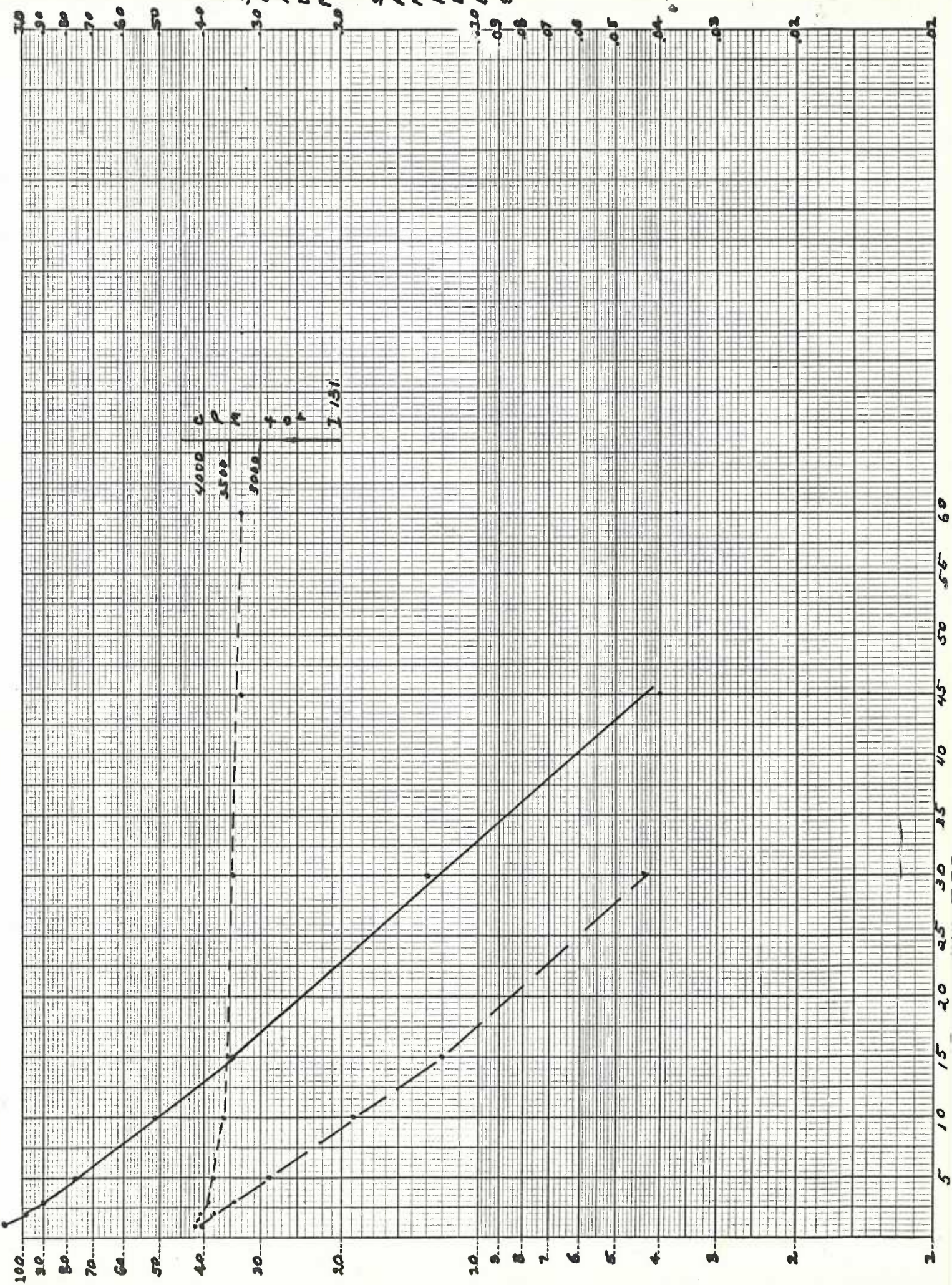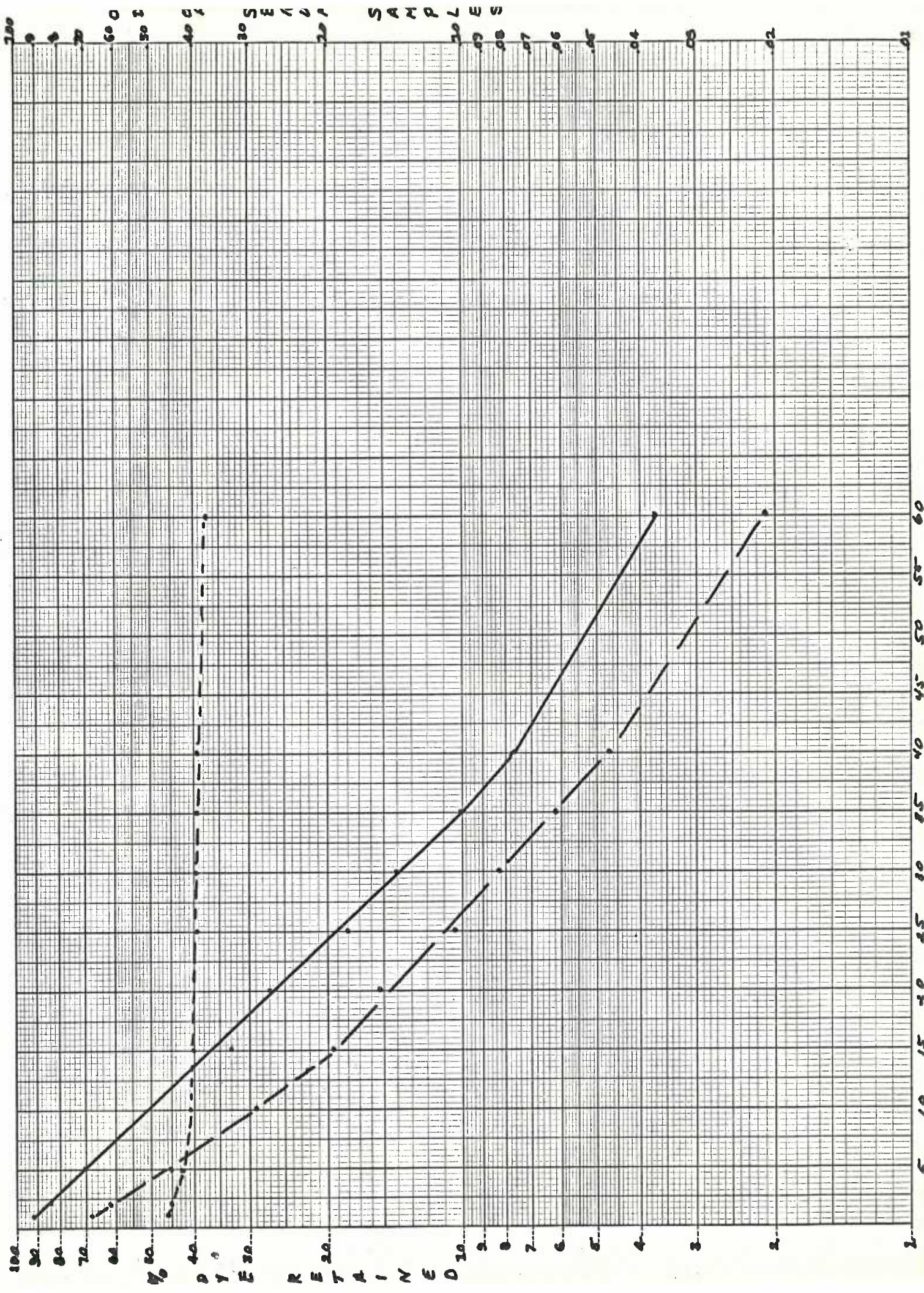
Figure 6. Dog # 4.

Figure 7. Dog # 5. Indocyanine Green-

Evans Blue Series.

Three pairs of dogs were used in a set of three experiments attempting to trace the I-131 that was lost from the circulation of the dogs in the first few minutes after injection of aged Indocyanine Green-I-131 albumin mixture. One dog in each set was injected with a mixture of Indocyanine Green and I-131-labelled albumin, prepared and allowed to stand two weeks as Mixture 11 had done. The other dog was injected with a saline dilution of I-131 of comparable activity.

Because the results of these experiments were considered not to be conclusive or statistically significant, the method and results are not presented in detail. Data from these experiments indicated (1) Increase in I-131 activity excreted in the bile of the dogs injected with the mixture of Indocyanine Green and I-131-labelled human serum albumin as compared with the bile of the dogs injected with saline-diluted I-131 albumin alone. (2) Increase in counts per gram of liver tissue in the dogs injected with the Indocyanine Green-I-131 mixture, as compared with the dog injected with I-131 albumin alone. (3) Using the pyridine-water-butanol (10:6:3) system of Barbier and deWeerdt (48) paper chromatography studies of the bile of the dogs injected with the mixture of Indocyanine Green and I-131-labelled albumin revealed that Indocyanine Green and the source of the I-131 activity migrated together, with no activity left at the point of application. When Indocyanine Green and I-131 were mixed in vitro and planted similarly, some of the activity migrated with the Indocyanine Green, with the major part of the activity still being at the point of application. This finding is compatible with the electrophoretic tracing of Indocyanine Green and NaI 131 which showed a peak (Figure 3c) that appears to be a combination of Indocyanine Green and I-131.

DISCUSSION

The purpose of the reported experimental work was to establish the feasibility of applying the reference approach to Indocyanine Green blood clearance studies using Evans Blue and I-131-labelled human serum albumin as reference substances. Successful application of a reference methodology for Indocyanine Green clearance studies should allow the determination of Indocyanine Green blood disappearance rate by assay of a single blood sample drawn at an appropriate time following injection of the Indocyanine Green-reference material mixture. This would eliminate multiple sampling assays employed by others (40, 42, 43).

The in vitro studies revealed no significant alteration of Indocyanine Green when mixed with Evans Blue or with I-131-labelled human serum albumin for up to three weeks. Neither were there significant alterations observed in Evans Blue or in I-131-albumin in these same mixtures. These results encouraged us to proceed with an investigation of the in vivo behavior of these mixtures.

When Indocyanine Green-Evans Blue mixtures were injected into anesthetized dogs, the individual substances behaved as they would be expected to behave if injected alone. As can be seen from Figure 1, the Evans Blue concentration in the serum of Subject #1 has changed only slightly from the 5 minute value when the 30 minute sample was drawn. The same slow extraction from the serum of dogs is shown in Figure 5. The plasma volumes obtained on the dogs injected with the mixture of Indocyanine Green and Evans Blue compared well with those of Gregerson and Rawson (49), who give average plasma volumes of 52.7 ml per kilogram. Indocyanine Green clearance proceeded as described by Cherrick (38), Hunton (40) and Wheeler, et al (43).

Human injections of Indocyanine Green-Evans Blue mixtures also indicated unaltered behavior of the two substances. Plasma volume on Subject #1 was 39 ml/kg, which is within the normal range as stated by Hutchens (30). Indocyanine Green clearance was similar to that reported by Cherrick (38) and Wiegand (39). Clearance curves plotted from Indocyanine Green mgms% retention, and from values calculated using the reference formula were parallel.

When Indocyanine Green-I-131-albumin mixtures were injected into anesthetized dogs immediately after mixing the individual substances behaved as they would have been expected to if injected individually. The plasma volumes on the dogs injected with this material compared well with those obtained on the dogs injected with the Evans Blue-Indocyanine Green mixture. Indocyanine Green clearance curves were similar to those observed by Wheeler (43). Clearance curves plotted from Indocyanine Green mgms% values and from percent retention values calculated using the reference formula were parallel.

When Indocyanine Green-I-131-labelled albumin mixtures that had been allowed to age from one to 14 days were injected into anesthetized dogs, the substances did not behave the same as would have been expected if they had been injected without premixing. Serum radioactivity showed a marked early drop of approximately 40% in four minutes. Indocyanine Green clearance showed progressive decreased clearance as the injection material aged. Because of the results no human injections were attempted. The altered in vivo behavior of Indocyanine Green and I-131-labelled human albumin after aging as a mixture in the face of no in vitro chemical evidence of alteration is unexplained.

The literature reveals that other investigators have had similar results under closely related conditions. Freinkel, Athens and Breese (50) premixed T-1824 and I-131 IHSA and noted an apparent dilution of radioactivity that exceeded that of the dye and gave a ratio of $\frac{" \text{ T-1824 space }"}{"\text{IHSA space}"}$ that ranged from 0.72 to 0.96. Their experiments were done mostly on humans, although they did a small series of dog experiments to confirm that the same phenomenon occurred in that species as well. Their human subjects were given up to 25 mgms of T-1824 and .4 to 3.0 mgms of albumin labelled with 20 to 60 uc of I-131. Administration of identical amounts of IHSA and T-1824 by separate and simultaneous injection gave dye compartment-isotope compartment ratios that were within a range of 1.108 to .985. These patients had been pre-treated with 20 drops of Lugol's the evening before injection, and it was assumed that urinary excretion of I 131 would more adequately reflect variations in the metabolic breakdown of dyed and undyed IHSA. Urinary excretion, however, was found not to differ significantly among seven subjects given IHSA alone and those given the mixture of dye and isotope. Attempts to demonstrate significant amounts of radioactivity within the red cells or in plasma dialysates during the first 30 minutes were unsuccessful. Ascending chromatograms were prepared in a solvent system of butanol-acetic acid-water (200:30:75), and the radioactivity was found to remain mostly at the origin. Mobile I 131 migrated like an inorganic iodide. These authors inferred that the apparently expanded IHSA space resulted from the early abstraction of an heterogenous fraction of the iodoalbumin. The assumption was substantiated by demonstrating a disparate loss of IHSA from the blood stream within the first ten minutes following injection of IHSA-T-1824 mixtures. They were unable to define the chemical nature of the moiety which is rapidly extracted.

A macromolecular structure is suggested by the negative dialysis and chromatography experiments.

Roberts, Smiley, Sears and Manning (51) measured plasma volumes with mixtures of Evans Blue and I-131-labelled albumin. The mixtures of isotope and dye were given to each of five patients on Monday, Tuesday and Thursday of the same week, at which time the Evans Blue was discontinued, and only I-131-labelled albumin given on Friday and Saturday. In all but one case, the total calculated plasma volume on each of the patients dropped 1000 ml or more on Friday, when the isotope was given alone, as compared to Thursday's value for I-131 determination of plasma volume, when the mixture was given.

Considering our results in the light of the observations of others, a possible explanation of the phenomenon observed presents itself. It may perhaps be initiated by a strong bond formed between Indocyanine Green and albumin when the dye is mixed with I-131-labelled human serum albumin. A complex may be formed, leading to a macromolecular structure which could then be treated as a colloid by the liver, being extracted by the Kupffer cells. Eventual turnover of at least the Indocyanine Green portion of this colloidal particale to the parenchymal cells is indicated by the appearance in the bile of Indocyanine Green. It appears possible from our descending paper chromatography work that some of the I 131 is taken along with the dye. Quantitation of the amount of activity was not attempted. If this is in fact what occurs, the delay in extraction might be due to a blocking action of the reticulo-endothelial system, such as Benacarref alluded to (52).

Barbier and deWeerdt (48) investigated the stability of Indocyanine Green by paper chromatography and infra-red spectroscopy, observing two

fractions of different R_F values by chromatography, and molecular changes in the infra-red spectra. The problem of what alterations may occur in a mixture of Indocyanine Green and I-131-labelled human serum albumin would have required a great deal of additional work.

Our investigations indicate that an evaluation of Indocyanine-Green-Evans Blue mixtures in humans as a test of liver function might be rewarding. Being purely a spectrophotometric procedure, only one instrument is required. The in vitro stability has been established, but pre-mixing of the two dyes and injections after standing have not been investigated.

Indocyanine Green-I-131-labelled human serum albumin mixtures prepared immediately before injection might be useful in assaying human liver function. This method, however, necessitates the use of radio-isotope equipment and would not permit preparation and storage of the diagnostic reagent for future needs.

SUMMARY AND CONCLUSIONS

Basic investigation into the feasibility of use of a mixture of Indocyanine Green and Evans Blue or Indocyanine Green and I-131-labelled human serum albumin as a means of determining liver function has been attempted. Based on spectrophotometry and on dog and human intravenous injections, Indocyanine Green and Evans Blue show unaltered in vitro and in vivo characteristics. Mixed solutions are spectrophotometrically unaltered when stabilizing protein is added for periods up to three weeks.

Following injection of Indocyanine Green and Evans Blue mixture, the disappearance curves plotted from mgm% data and from the percent dye remaining as calculated by the reference formula are parallel, supporting the validity of the spectrophotometric reference methodology.

Based on spectrophotometry, paper electrophoresis and dog injections, Indocyanine Green and I-131-labelled serum albumin show unaltered in vitro and in vivo characteristics when used immediately following mixing, but altered in vivo behavior after the mixture has aged from 1 to 14 days. Approximately 40% of the I-131 activity was lost from the serum of dogs in the first four minutes post-injection, and the retention of Indocyanine Green increased progressively as the mixture aged.

When a freshly mixed solution of Indocyanine Green and undiluted I-131-labelled human serum albumin was used for dog injections, the curves based on mgms% of Indocyanine Green concentration and on the reference calculation of the percent dye remaining are parallel, supporting the validity of the radioisotopic reference methodology.

Because of the exponential clearance of Indocyanine Green from the circulation between 5 and 20 minutes following injection, calculation of

the percent dye remaining at a single time between these post-injection times should adequately define the clearance rate.

These results should serve as an adequate basis for Indocyanine Green spectrophotometric and radioisotopic reference blood clearance studies in normal patients and in those with liver disease to establish their usefulness as a diagnostic procedure. For diagnostic purposes, the spectrophotometric reference procedure may be preferable to the radioisotopic procedure because of the greater stability of the mixtures, and the fact that no isotopic equipment is required.

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