THE NATURE OF INJECTABLE LIVER EXTRACTS

A DISCUSSION ON STANDARDS OF POTENCY AND PURIFICATION

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Since Minot discovered the specific effect of liver in the treatment of pernicious anaemia, and various workers subsequently prepared concentrates suitable for injection, the preparation of such concentrates on a commercial scale has been undertaken in many laboratories. Widely differing methods have been used to make these concentrates or "extracts," with the result that apparently similar products differ from one another to a remarkable degree. The terms used by manufacturers to describe these products also lack uniformity. The fundamental cause of these differences is doubtless the lack of any precise measure of anti-anaemic activity. Although many attempts have been made to devise laboratory tests, these have met with no success, and clinical trials on patients with pernicious anaemia in relapse afford at present the only practicable method of assessing activity.

It is fairly common to refer to a liver extract as being either "crude" or "refined." By the term "crude" we understand an extract that has undergone the smallest amount of processing necessary to render it safe for injection. It will therefore normally contain a comparatively large amount of material other than the active principle, and each millilitre will be derived from a comparatively small weight of original liver (2.5–10 g.).

By the term "refined" we understand an extract that has been derived from a "crude" extract through several additional stages of manufacture, which remove a large proportion of the inert material and very often a part of the active principle also. It will normally contain only a small amount of inert material, and each millilitre will be derived from a much larger weight of original liver (50–200 g.). These descriptions may be useful, but they do not express adequately the differences between extracts, and may even lead to the erroneous belief that all the extracts of any one class are similar to each other.

For several years we have devoted attention to the development of two commercial liver extracts. One would be classed as a "refined" and the other as a "crude" extract. Our investigations have also included laboratory preparation of several highly refined fractions. This work has been carried out in collaboration with a number of pathologists, to whom we are indebted for the necessary clinical tests.

Certain aspects of our work may, we hope, help to clarify some of the obscurity surrounding the nature of liver extracts available to clinicians. To assist the discussion of this work we make use of an arbitrarily defined unit of activity, based on the type of clinical test most frequently used in this country.

Present Methods of Expressing Potencies of Injectable Extracts

There is as yet no standard method of expressing the potency of an extract, and statements made by manufacturers usually fall into one or other of the following three main groups: (a) A stated volume of the extract is derived from a stated weight of liver. (b) A stated volume of the extract given by injection is therapeutically equivalent to a stated weight of liver taken orally. (c) A stated volume of the extract produces adequate haemopoietic response.

Claims for the potencies of liver extracts in terms of the amount of liver used to prepare them (group a) may be very misleading, because varying losses of active principle may occur during the purification process. Thus a highly "refined" extract may contain less activity than a relatively "crude" preparation derived from the same weight of original liver. Our experience of process losses given below illustrates this.

Comparison of extracts by relating their potency to stated weights of liver taken orally (group b) was a useful device when injectable extracts were first coming into use. Then,

most doctors' experience in treating pernicious anaemia was in terms of liver diets, and to express such a relation may have seemed important. Now that injection is the customary form of treatment this type of statement has little value, and is, moreover, misleading—first, because process losses may vary from extract to extract, and, secondly, because the relative efficacies of oral and injectable extracts are not known with any accuracy. Thus, for example, Martindale (1941) quotes a statement that liver given by injection is 20 to 40 times as effective as when given orally, while Minot and Strauss (1943) consider that the probable ratio is nearly 60 to 1.

The statement that a specific volume of an extract is capable of producing an adequate response (group c) clearly provides a more direct basis for the comparison of different extracts. The meaning of a statement of this nature, however, depends on what is meant by an adequate response, and the criteria adopted are not always clearly defined. Even when this has been done, comparison of different extracts may be difficult because the manufacturer may have deliberately understated the potency of his extract in order to make allowance for small batch-to-batch variations in potency.

Criteria of Satisfactory Response

All the clinical tests carried out in conjunction with our investigations have been on cases of classical pernicious anaemia not treated recently with other liver extracts or with hogs'-stomach preparations. These cases had initial red cell counts of not more than $2\frac{1}{2}$ millions per c.mm. As criteria of a satisfactory response it has been required, first, that the red cells should increase over the first two weeks of treatment at least as rapidly as required by the formulae of Isaacs *et al.* (1938), Riddle (1940), and Dyke and Della Vida (1942); and, secondly, that a reticulocyte response satisfying the criteria of Minot *et al.* (1928) should be obtained.

Very occasionally we have encountered cases in which the red cells have risen satisfactorily but the reticulocyte response has not quite satisfied the criteria of Minot *et al.* (1928). In these cases the absence of a second reticulocyte response after a further injection has been taken to indicate a satisfactory initial response. Use of the term "satisfactory response" in this paper means that these conditions have been fulfilled. Thus, the criteria of Dyke and Della Vida (1942) require the following increase in red cells over the first two weeks of treatment:

Initial Red Cell Count	Increase in Red Cells
1.0 mill. per c.mm.	1.45 mill.—i.e., to 2.45 mill. per c.mm.
1.5 " "	1.22 " " 2.72 " "
2.0 " "	1.00 " " 3.00 " "

Preparation and Comparison of "Crude" and "Refined" Extracts

The material used in the preparation of both "crude" and "refined" commercial extracts discussed here is raw finely minced liver which has not been subjected to proteolytic enzyme digestion before extraction. Only the minimum of fractionation procedures necessary to make the product suitable for injection have been employed in the preparation of the "crude" extract, which has consistently given a satisfactory response on a total dose derived from 60 g. of original liver. Responses which just fail to satisfy the defined criteria have been obtained with a dose derived from 40 g. of original liver.

The original "refined" extract was prepared according to the method of Laland and Klem (1936), involving adsorption of the active principle on charcoal and its removal from the adsorbate by means of phenol; it appeared to be a product in which the concentration of solids was reasonably consistent from batch to batch. It was relatively highly purified, the extract from 100 g. of liver containing only 10 to 15 mg. of solids. The extract normally produced a satisfactory response when 4 ml., derived from 200 g. of original liver, was given in a single injection—that is, a satisfactory response was obtained by a solution containing approximately 25 mg. of solids. However, some batches did not give such a good response on this dosage, while others gave a good response with a smaller dose. The reasons for these variations in activity were traced by an extended investigation of the whole process, and they were virtually eliminated by more precise control at all stages of the fractionation. This led to the

production of batches which consistently gave satisfactory responses to a total dose of 4 c.cm., though we also had evidence that a lower dose would suffice in some cases.

The difference between the efficacy of "crude" and "refined" extracts when expressed in terms of original liver will be noted. Whereas "crude" extract derived from 60 g. of liver produced a satisfactory response, "refined" extract from nearly 200 g. of liver was required.

Any or all of three possible causes for the discrepancy might be operative. First, activity might be lost in side-fractions arising during the refining processes; secondly, activity might be destroyed in the later stages of fractionation; thirdly, the haemopoietic activity of liver extracts might be dependent on the simultaneous presence of more than one substance—as postulated, for example, by Jacobson and Subbarow (1941)—and some of these other factors might be removed in the later stages of fractionation. However, investigation showed that appreciable losses of activity were in fact occurring at two stages of the process, and further research indicated a method of avoiding these losses by modifying the conditions of adsorption and elution used in the original Laland and Klem process (1936).

The product of this modified process gave a satisfactory response on a dose derived from 80 g. of liver (all of three cases tested), and the response to lower doses is now being examined. To date, one case has given a satisfactory response on a dose derived from only 60 g. of liver.

It can thus be seen that there is now no discrepancy between the response to "refined" and that to "crude" extract. Moreover, in the modified "refined" extract only 30 to 35 mg. of solids are obtained from each 100 g. of original liver, so that again approximately 25 mg. of solids will give a satisfactory response. Hence the amount of active material extracted from a given weight of liver has been markedly increased without reducing the activity per milligramme of the final extract.

Purification of Liver Extracts

We have recently prepared, by a modification of the Laland and Klem process (1936) involving selective aqueous alcoholic elution of the charcoal adsorbate, a fraction (L.E. 90B) which has produced in two cases of pernicious anaemia satisfactory responses with a total dose of only 5.5 mg. of solids; this dosage was arbitrarily given in 2 ml. of solution. The graphs showing the responses obtained in the two cases are reproduced in Figs. 1 and 2. The tests were carried out by

country recommend, that any liver extract, no matter of what type, should be given in a certain dose volume (e.g., 2 ml. or 4 ml. twice a week) in order to produce a satisfactory remission

FIG. 2

of pernicious anaemia and its allied conditions. Dyke and Della Vida (1942) have adopted it as an arbitrary criterion that any liver extract producing a good response with a total dosage of less than 10 ml. over the first fortnight should be regarded as sufficiently potent for therapeutic use. However, it must be quite clear that with a product like the original "refined" extract described above, for example, the solids content (25 mg.) of 4 ml. of solution could quite easily be concentrated into 1 ml., or even 0.5 ml., or, on the other hand, be diluted to 10 ml. It was decided to make the improved "refined" extract recently developed of such a strength that 1 ml. was derived from 100 g. of liver. At this concentration 0.6 to 0.8 ml. can produce a satisfactory response, as indicated above, but the use of 1 ml. provides a margin of safety that adequately covers any small batch-to-batch variations of potency and any losses on storage. This could obviously have been 0.5 ml., 2 ml., or any other volume convenient for injection.

So far as we have been able to ascertain, no better initial response is likely to be obtained in a normal uncomplicated case of pernicious anaemia by giving larger doses than those necessary to produce a satisfactory response as defined above. In cases complicated by subacute combined degeneration of the spinal cord or nutritional deficiencies larger doses may, however, be required: this can usually be ascertained by careful control of the blood count throughout the period of treatment.

Unit of Activity

We have shown above that different liver extracts should not be compared either in terms of dosage by volume or in terms of the liver from which they are derived, but that the soundest basis of comparison is in terms of their therapeutic activity. Anti-anaemic activity, however, can only be measured by tests on human patients with uncomplicated pernicious anaemia, and the errors involved in such a method of assay are large. Nevertheless, such clinical tests have been adopted as an assay method, albeit a rough one, in America and Canada, and units have been defined in terms of which the activity of all liver extracts must be expressed before they are marketed.

The American (U.S.P.) unit depends on the response given by small daily injections of the extract over a period of 15 days. This response is normally assessed on not fewer than three patients by the U.S.P. Advisory Board, who then assign a unitage to the extract. Thus, if a daily injection of 1/15 ml. produces a response considered satisfactory, the extract is given a rating of 15 units per ml. (*J. Amer. pharm. Ass.*, 1940).

The Canadian unit, only recently adopted, depends on the response produced over the first four weeks of treatment by weekly injections. The unit is defined as one-seventh of the

FIG. 1

Dr. S. C. Dyke, of the Royal Hospital, Wolverhampton, to whom we are indebted for permission to reproduce the charts. The fraction has since been further purified by means of ammonium sulphate precipitation to give material of which 2.8 mg. produced a response just failing to satisfy the criteria.

These fractions compare favourably with the purified fraction described by Karrer (1941), which he reported as possessing "maximal" activity in a single dose containing 15 mg. of solids and "very good" activity in a dose containing 7.5 mg. of solids.

Relation of Volume to Dosage

Some recent medical textbooks (Witts, 1941; Beaumont and Dodds, 1943) indicate, and apparently many clinicians in this

weekly dose of extract required to produce a satisfactory response. Thus, if weekly injections of 0.5 ml. will produce a satisfactory response, the extract is given a rating of 14 units per ml.

For the purpose of expressing relative potencies of extracts in this paper we define an arbitrary unit based on the method of testing more generally used in this country. This unit depends on the production of a satisfactory response over 2 weeks following one or more initial injections given within the first 2 days of treatment. Like the Canadian unit, it is expressed as the number of days of treatment (14) divided by the number of millilitres of extract given. Thus, if a single injection of 1 ml. of an extract produces a satisfactory response over the whole of the first two weeks it is said to contain at least 14 units per ml. If another extract has been shown to produce a satisfactory response on injection of (say) 2 ml. on day 1, followed by a further 2 ml. on day 2, it is said to contain at least 14 divided by 4 = 3½ units per ml. If it can be assumed that a number of small daily injections are utilized as efficiently as the same total dosage given in one or two larger injections at the beginning of treatment, the arbitrary unit defined above is approximately equivalent to the U.S.P. unit.

If it can be found possible to give official recognition to some unit similar to that which we have defined we believe it will be of considerable help both to the clinician and to the manufacturer.

Purification Index

Having expressed the potencies of different extracts in terms of a common unit, the relative degrees of purification can be readily examined by determining their content of solids. A convenient "purification index" (P.I.) is obtained by dividing the number of units of activity in each millilitre of the extract by the solids content, expressed as fractions of 1 g. per ml. Such a method of expressing degrees of purification in terms of arbitrarily defined units per unit weight of solids has long been adopted with other biologically active substances before their isolation in pure form—e.g., several of the vitamins, enzymes, pituitary hormones, antibiotics. In these instances, however, potency could be determined by animal assay or microbiological assay, and by increasing the number of replicates used a more or less accurate estimate of the activity could be obtained. Liver extracts, on the other hand, can be assayed only on human cases, of which but a limited number are available; in consequence, the values so obtained will be subject to considerable experimental error, but they will at least indicate the relative orders of purity of the extracts. A few such figures, calculated from known data, are given below as examples:

Extract	Approx. Minimum Dose giving Optimal Response (ml.)	Units/ml.	Total Solids Content (g./ml.)	Purification Index (units/g.)
Original refined extract ..	2-4	3.5-7	0.0075	500-1,000
Modified refined extract ..	0.6-0.8	17.5-24	0.035	500-700
Crude extract ..	4-6	2.3-3.5	0.25	9-14
American (15 U.S.P.)* ..	1	14.0	0.18	Ca. 80
" ..	1	14.0	0.075	Ca. 200
Experimental fraction ..	2	7.0	0.0027	Ca. 2,600
(L.E. 90B) ..				

* These figures are based on the assumption that the U.S.P. unit is approximately equal to the unit we have defined.

The difference between the "refined" and "crude" extracts is brought out clearly in the above table, "crude" extracts having purification indices of the order of 10, and "refined" extracts (though differing among themselves to a remarkable degree) indices of the order of 100 or more.

The preparation of Dakin and West (1935) has been referred to, though not by the authors themselves, as though it were the actual pure anti-anaemic factor. This, however, gave, at a dose of 80 mg., responses that, judged from the published clinical data, may be regarded as satisfactory in the sense used in this paper. Now, this dose contains approximately 14 units of activity, and is therefore equivalent to a purification index of the order of 200. It seems obvious, from the data obtained with our fraction 90B, that the pure factor must have a purification index of at least 2,500.

General application of this method of expressing the activity of liver fractions would allow papers describing the purification

of the active principle to be more critically compared. This presupposes, however, the existence of a common unit of activity.

Maintenance

It is usually found that the blood of patients who respond satisfactorily to an adequate initial dose is brought rapidly to normal by fortnightly injections equal in amount to the initial injection. Presumably, therefore, the average daily requirement during this phase of treatment is 1 unit. The dosage required for the maintenance of normal red-blood-cell levels is very much more difficult to assess. Before any reliable conclusions can be drawn it is necessary to have observations on a large group of cases carefully controlled over a period of several years.

While it may be argued that the ease and cheapness of treatment with modern liver extracts make the measurement of minimal maintenance requirements a matter of somewhat academic importance, such an investigation might well lead to data of considerable practical value. For example, it is not impossible that the incidence of systemic reactions to the injection of liver extracts may be related to prolonged administration of excessive doses. Further, in this country there is a body of opinion that "crude" extracts have advantages over "refined" extracts for maintenance, though recent American publications have claimed that "refined" extracts are as effective as "crude" extracts both for maintenance and for initial treatment (Evans and Jordan, 1941; Murphy and Howard, 1939).

An investigation of minimal maintenance requirements of the "crude" extract, referred to above, has been reported to us by Dr. J. Mills, of the Royal Berkshire Hospital, Reading, and we gratefully acknowledge his permission to summarize his findings here. In a group of 75 patients with pernicious anaemia, a few of whom showed neurological symptoms, about 90% were maintained at levels of 5 million red cells per c.mm. and 100% haemoglobin for periods ranging from 6 months to more than 2 years on a dose derived from 20 g. of original liver (2 ml.), given at intervals of 4 weeks. Those not maintained by this dosage have, without exception, been satisfactorily maintained by a dose derived from 40 g. of original liver (4 ml.) every four weeks, for periods ranging from 8 to 18 months. Hence a dose of the "crude" extract containing approximately 5 units of activity per month appears to satisfy the need of most patients, while one of 9 units per month satisfies the needs of all.

The dose recommended by American authorities for satisfactory maintenance is usually of the order of one unit of activity per day—i.e., 28 units every 4 weeks. We have some evidence, though as yet inadequate, that a dose containing some 17 units of the newly developed "refined" extract, referred to above, is enough to keep patients' blood normal, but tests with a lower dosage have not as yet been carried out.

It is not impossible that the "crude" extract may contain factors of importance in maintenance which are not measured in tests on patients in relapse. Careful comparison of minimum maintenance requirements of "crude" and "refined" extracts in terms of units as discussed here should provide useful information on this point. Similarly, consideration on a unitage basis of dosage requirements in the treatment of the more complex macrocytic anaemias might lead to a resolution of the divergent views (Wills and Evans, 1938; Foy and Kondi, 1939; Hamilton Fairley, 1940; Trowell, 1941; Sundaram, 1944) which are at present held on the relative efficacy of "crude" and "refined" extracts.

Summary

Statements commonly used in this country to describe injectable liver extracts are criticized.

The chief differences between "crude" and "refined" injectable liver extracts and their uses in the initial treatment and maintenance of cases of pernicious anaemia are discussed.

An arbitrary unit of haemopoietic activity, and a "purification index" based on it, are defined.

Attention is drawn to some advantages which would result from the establishment of an official unit of haemopoietic activity in this country.

The relatively low order of purification of commercial "refined" liver extracts is demonstrated by the description of a laboratory

preparation capable of producing a satisfactory response on a total dose containing only 5.5 mg. of solids.

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THE PROPHYLAXIS OF RICKETS BY SINGLE MASSIVE DOSES OF VITAMIN D

BY

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In an earlier paper (Krestin, 1944) it was shown that rickets still presented a problem in infant entrants to Preston wartime nurseries, for, although severe and advanced degrees of this disease were extremely rare, an early or mild variety was found in 37% of all admissions under 2 years, while in the poorer parts of the town this figure was even higher. Furthermore, it was noted that the free distribution of cod-liver oil for the infants before their admission to the nurseries did not produce any obvious reduction in the incidence of this disorder. Similarly, in a recent report by the Ministry of Health (1944) describing a widespread survey including Great Britain and Ireland, it was found that 73% of the infants with radiologically active rickets had received cod-liver oil or other vitamin D preparation for some period before the investigation, and Corner (1944) found the incidence of the disease in hospital patients actually higher in those who had previously received vitamin D supplements than in the average series. Since the antirachitic value of cod-liver oil is now beyond doubt, and has been shown to be reliable when given regularly and in large enough doses in these nurseries (Krestin, 1945), it is evident that the disappointing results obtained by these mothers in their own homes were due to irregularity of administration and inadequacy of dosage.

For these reasons it seemed desirable to investigate the possibility of a method of preventing the disorder that could be used independently of the mother and yet not require the daily attendance of the child at a clinic or medical centre.

Previous Work

Although Vollmer in 1928 (quoted by Vollmer, 1939) showed that rats which had been fed on a rachitogenic diet could be protected against rickets by a single massive dose of vitamin D, this observation was not applied clinically until 1938, when Harnapp (1938a, 1938b, 1939), in Germany, found that infants under 18 months could be protected during the winter months by single oral doses of 7.5 or 10 mg. of either vitamin D₂ or D₃ dissolved in oil. These results were soon after confirmed in that country by Schwartz (1939), Rietschel *et al.* (1940), Hartenstein (1940), and Heisler (1940), most of whom favoured the view that, whereas 5 mg. was too small, 7.5 to 10 mg. was sufficient for the purpose, and that larger doses were unnecessary. Less constant results were obtained by Windorfer (1938) and Brockmann (1938). More recently Wolf (1943) and Rambar *et al.* (1943), in America, reported the results of using single doses of 15 mg. of "ertron," an electrically activated preparation of ergosterol; although in both cases the numbers of infants followed up for five months or more were small, the results obtained were very satisfactory.

Present Investigation and Results

This investigation concerns full-term infants and children between 2 months and 3 years of age, of average health, and showing no radiological or clinical evidence of rickets on admission to ten day and three residential wartime nurseries in Preston. All lived under similar conditions, received similar diet, and were under constant supervision. The period of observation began during the autumn of 1943, and lasted for 6 months throughout the following winter and early spring. After admission each child was clinically examined and then sent for radiographs of the lower ulnar epiphyses. A single dose of 7.5 mg. of calciferol—i.e., 300,000 i.u. of vitamin D₂ dissolved in 1 c.cm. of arachis oil—was then given by spoon after a milk feed, the amount having been measured in a graduated Record syringe. The oil was readily taken by all ages, and, except for vomiting in one small infant, was not followed by any gastro-intestinal disturbance or other unpleasant effects. Thereafter each child was clinically examined at regular intervals of 4 weeks or less, and at the end of 6 months another radiograph was taken of the wrist. Owing to the distance of the nurseries from the x-ray centre it was not possible, except in a few instances, to have radiographs taken at more frequent intervals. Biochemical examinations of the blood were not practicable. Throughout the period of observation no other vitamin D preparation was given, nor was any child in this series exposed to ultra-violet radiation.

About one-third of the infants had received some cod-liver oil before admission. The doses were small, and often irregularly given. As the subsequent results in these infants did not appear to differ from the results in those who had not previously received any vitamin D preparation, they are not tabulated separately.

At the same time and during a similar period of the previous year two other groups of infants and children living under exactly similar conditions and receiving the same diets were studied. One, a "control" group, received no vitamin D preparation; the other received daily doses of the Ministry of Food cod-liver oil. The results obtained in these two groups have been discussed elsewhere (Krestin, 1945), but the figures are included in the accompanying Table for comparison.

The diagnosis of rickets in these nurseries has been discussed in earlier papers. The radiographic changes at the lower ulnar epiphyses have been clearly described by Wimberger (1923), A. F. Hess (1930), and others, and so require no further discussion here. It should, however, be noted that in mild rickets of short duration obvious alterations in the appearance of the x-ray films may not occur, or may last for so short a time that they may be overlooked unless radiographs are taken at frequent intervals. Craniotabes—i.e., asymmetrical softening of the cranial bones in the region of the lambdoid suture—was found to be a reliable sign in infants older than 3 months, but was rarely found after the sixth month. After the fourth month the most reliable sign, when scurvy could be eliminated, was a definite enlargement of the costo-chondral junctions of the fourth to the tenth ribs. Moreover, this sign was of par-

Table showing Comparison of Results after Antirachitic Prophylaxis with Single Massive Doses of Vitamin D₂ and after Daily Doses of Cod-liver Oil, with a Control Group of Infants

Age Groups:	0 to 6 Months			6 to 12 Months			1 to 2 Years			2 to 3 Years		
	Number Examined	Developed Rickets	Remained Rickets-free	Number Examined	Developed Rickets	Remained Rickets-free	Number Examined	Developed Rickets	Remained Rickets-free	Number Examined	Developed Rickets	Remained Rickets-free
Single oral dose: 7.5 mg. D ₂ = 300,000 i.u. . .	16	2	14	27	1	26	50	0	50	44	0	44
Daily doses of M.O.F. cod-liver oil:												
1 dr. = 700-800 i.u. . .	18	12	6	—	—	—	—	—	—	—	—	—
2 dr. = 1,500 i.u. (approx.) . .	28	3	25	28	2	26	55	6	49	63	0	63
3 dr. = 2,100 i.u. (approx.) . .	—	—	—	15	0	15	11	1	10	—	—	—
4 dr. = 3,000 i.u. (approx.) . .	—	—	—	—	—	—	44	0	44	60	0	60
Controls (no vitamin D) . .	14	12	2	30	18	12	28	3	25	15	0	15