Acta Biotheoretica 25, 2–3: 153–162 (1976)

TOWARDS A CLASSIFICATION OF EVIDENCE IN BIOLOGICAL AND MEDICAL RESEARCH IN RESPECT OF ITS VALIDITY

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(Received 26-VI-1975)

ABSTRACT

An empirical classification of the validity of evidence from experiments in all biological research disciplines is suggested. It could be used both for the practical design of experiments and in the assessment of published papers. It is based on a hierarchy, at the apex of which is the living animal in its normal environment, and at the base is the chemical extract of homogenised tissue.

INTRODUCTION

The assessment of the relative value of evidence is one of the major problems in research in an era in which logic is not generally taught to students of the various biological disciplines. It was considered desirable to attempt classification of experimental findings in a hierarchy. The hierarchy was built in such a way that the most valid evidence is at the apex.

The classification is based on the belief that the biologist aims to understand the structure and behaviour of living organisms, and the biochemical processes occurring within them during life. Therefore, the less they have been manipulated during study, or subjected to agents which would change their microscopic structure or biochemistry, the more likely one is to derive information which reflects the state of the living animal.

The classification is intended to be of practical use in the design of experiments in biology, psychology, medicine, physiology, functional anatomy, cytology, biochemistry and biophysics, and also in the logical analysis of published scientific findings. It is empirically designed, and it is hoped that the desirability of such a classification will be widely accepted, even if the position in the hierarchy of particular studies might not be universally agreed.

(The term 'organism' will be used to cover human beings, animals, plants, bacteria and cells, which can be autonomous, such as egg cells, blood cells and sperms).

THE CLASSIFICATION

Section I
DIRECT OBSERVATIONS OR MEASUREMENTS – in which no assumptions have been interposed between the former and the results of the experiment – in the following systems:

A. *Live organisms*; by definition the most accurate source of information.

(1a), unrestrained organisms in normal environments not statistically significantly affected by being observed or measured;

(2a), unrestrained organisms in normal environments statistically *significantly* affected by being observed or measured;

(3a) and (4a), as (1a) and (2a), but in abnormal environments, the abnormality of which might affect the observations or measurements *significantly*;

(5a) to (8a), as (1a) to (4a), but the organisms are restrained at the time of observation or measurement;

(9a) to (16a) as (1a) to (8a), but the organisms are under stress or pain, which may induce significantly more change in them than restraint alone would do;

B. *Treated organisms*; treatment must be unnatural, by definition.

(17a) to (32a), as (1a) to (16a), but the organisms are anaesthetised or fed with abnormal diets, whose abnormality is not known to alter their appetites in such a way that the observations or measurements might be affected;

(33a) to (64a), as (1a) to (32a), but the anaesthesia, diets, or agents, produce significant effects on the systems being studied;

C. *Isolated limbs or organs*; isolation itself is an unnatural treatment

(65a), in this category, the dying of the organism, or changes post mortem before examination, are known not to affect the observations or measurements significantly;

(66a), limbs or organs separated from dead organisms in which treatment or diets during their life would affect the observations or measurements on the isolated limbs or organs after death more than dying, death or changes post mortem had already affected them;

(67a), isolated limbs or organs deprived in vitro of their normal milieu, regulatory mechanisms or perfusion fluids;

(68a), as (67a), but unphysiological reagents have been added;

D. *Tissues cultures*; tissue has been excised from a young organism, or a primitive one, or a cancerous tissue.
   (69a), tissue cultures which have not been subjected to unphysiological concentrations of natural substances, or to unnatural substances;
   (70a), tissue cultures which have been subjected to unphysiological concentrations of natural substances, or to unnatural substances;

E. *Tissue slices or artificially separated cells*; these tissues usually survive excision for only a few hours.
   (71a), tissue slices or separated cells in physiological media, which have not been subjected to natural unphysiological concentrations, or to unnatural substances;
   (72a), tissue slices or separated cells in media which have been subjected to unphysiological concentrations of natural substances, or to unnatural substances;

F. *Tissue homogenates*: these involve changes of morphology, and re-distribution of subcellular components.
   (73a), crude tissue homogenates in completely physiological media;
   (74a), crude tissue homogenates which have been subjected to unphysiological concentrations of natural substances, or to unnatural substances;
   (75a), tissue homogenates, which have been centrifuged or processed further, which have not been subjected to unphysiological concentrations of natural substances, or to unnatural substances;
   (76a) and (77a), as (74a) and (75a), but which have been subjected to unphysiological concentrations of natural substances, or to unnatural substances;

G. *Dead or fixed tissue*, irrespective of whether the host animal was originally alive or dead.
   (78a), after death or fixation, the tissue has not been subjected to unphysiological concentrations of natural substances, or to unnatural substances;
   (79a), after death or fixation, the tissue has been subjected to unphysiological concentrations of natural substances, or to unnatural substances;

H. *Extracts*; tissue has usually been homogenised; most extractions are done with powerful chemical reagents, which would kill an animal or denature proteins.

(80a), chemical extracts with reagents which are known not to change significantly the system being studied;

(81a), chemical extracts with reagents which probably do not change the tissue significantly.

Section II
OBSERVATIONS OR MEASUREMENTS, in which all major assumptions inherent in the techniques used for the results of the experiments, have been tested:

(1b) to (81b), in the same systems as (1a) to (81a).

Section III
OBSERVATIONS OR MEASUREMENTS, in which all major inherent assumptions are likely to be warrantable, although they have not been tested:

(1c) to (81c), in the same systems as (1a) to (81a).

Section IV
DEDUCTIONS OR EXTRAPOLATIONS, from direct observations or measurements in the systems, as in Sections I–III:

(1d) to (81d)

Section V
DATA DERIVED FROM OTHER STUDIES ON THE SAME SYSTEMS, as in Sections I to IV:

(1e) to (81e).

Section VI
DATA DERIVED FROM BIOLOGICALLY SIMILAR SYSTEMS, as in Sections I to V:

(1f) to (81f).

Section VII
DATA DERIVED FROM ANALOGOUS SYSTEMS, to those in Sections I to VI:

(1g) to (81g).

Section VIII
COMPATIBLE FINDINGS, to those in Sections I to VII, in systems which must be related to them:

(1h) to (81h).

Section IX
SPECULATIONS WHICH ARE TESTABLE, and are related to systems in Sections I to VIII:

(1i) to (81i).

Section X
EXPLANATIONS WHOSE RELATIONSHIP TO SYSTEMS (1a) to (81a) is likely, but has not been demonstrated:

(1j) to (81j).

Section XI
TEACHING MATERIAL, such as diagrams, flow charts, schemata, metabolic maps:

Speculations which are untestable, or explanations which are not incompatible but not necessarily closely related to findings in Sections I to X, cannot be considered as evidence. Furthermore, observations or measurements which have been interpreted with the aid of untestable, grossly uncertain, or unlikely, assumptions have very doubtful value.

DISCUSSION

(The numbers referred to are those in the classification. A simple number without a letter indicates that the comment applies to that particular category in all Sections).

The effects of unnatural environments, restraint, stress, pain and anaesthesia, (1–64), are so well known and amply documented by human biologists, psychologists and biochemists, that it is not necessary to discuss them in detail here. However, they have received insufficient attention in two areas of research. First, in many biochemical experiments, the changes in the biochemistry of the blood and tissues consequent upon these agents could easily affect the results of the experiments. Secondly, research workers involved in experiments concerned with the biochemical effects of learning, memory or conditioning in animals, have rarely succeeded in teaching animals without the use of hunger, stress, or, sometimes, pain; these animals are not normal. Furthermore, one cannot identify separately the effects of learning, for example, from the stress, increased muscular activity, increased sympathetic activity, or hunger, which are usually concommitant with the learning, memory or conditioning.

During the isolation, limbs and organs usually pass through a period during which they are deprived of their blood supplies (65–68). Although an isolated udder can produce milk, an isolated brain can show apparently normal electro-

encephalographic activity, and an isolated limb still contracts reflexly, they usually become oedematous and gradually lose their function. This probably reflects either irreversible change during the isolation, or gradual failure of the perfusion. The latter may arise from biochemical inadequacies of the perfusing fluids, mechanical damage during surgical procedures, or dynamic characteristics of the artificial perfusion systems which do not mimic those of the organ in vivo. Obviously, the shorter the period that elapses between the organ being in its natural environment, and the more similar the artificial perfusion to that in vivo, the more the properties of the isolated organs or limbs will reflect those in the living animal. Furthermore, the addition of any unphysiological concentrations of natural materials, or unphysiological chemicals which are added to the perfusate, will displace the physiological behaviour of the isolated organ.

It is very often assumed that drugs may be given to an animal or added to a tissue perfusate, and have 'specific' effects on only one system within it. During the briefest glance at any metabolic map, it may be observed that every naturally occurring chemical is involved in cycles or pools which are totally related to several others, so that no system can be interfered with in isolation.

If a 'specific' inhibitor is added which affects a particular reaction in a metabolic cycle its initial effect may be to cause accumulation of metabolites 'behind' it in the cycle, but as the system gradually runs down, its ability to produce metabolites before the point of inhibition diminishes. In the opposite sense, an activator may cause 'accumulation' of a metabolite, or 'exhaustion' of the supply of it. The same may be said for the addition of any unnatural product, abnormal concentration of a natural product, or temporary or permanent change of the organ's environment. In a whole, living animal, the effect of all homeostatic mechanisms is to resist any of the latter changes, which produce dire consequences to the organism, as noted by Claude Bernard a century ago.

Tissue cultures have been isolated, but have retained the ability of their cells after incubation in suitably complex media to multiply, and the tissue may grow (69, 70). Until now, with a few rare exceptions – like adult lymphocytes – only tissues from embryos, very young animals, cancerous tissue or undifferentiated cells like fibroblasts, grow in culture. It is generally believed that complex adult healthy tissue will not grow, because of its loss of an unknown mechanism, which may be similar to that seen acting in an uncontrolled manner in cancerous tissue.

Tissue cultures normally pass through a 'depressed' phase after isolation during which they seem to be recovering their ability to grow. This is evidence of some abnormality, albeit reversible. There is also the well-known property of tissue cultures, especially of specialised tissues, to lose the characteristic features of their morphology; this is called 'de-differentiation'.

Tissues slices, (71–72), are often called surviving tissue. After the limbo of incubation lasting 10–30 minutes, they achieve an equilibrium with an artificial incubating medium. During preparation, they have been cut, which involves pressure, exsanguination, shearing and loss of some solids; normal connections are severed. They receive their solutes, oxygen and substrates, by diffusion over a distance of several hundred microns, compared with their state in the living animal, in which these substances come from a distance of only 30 to 50 microns. Nevertheless, there are thousands of whole cells in a single tissue slice.

Homogenisation is normally carried out either with the intention of liberating intracellular materials, like enzymes, or preparatory to centrifuging tissue to separate parts of cells, (73–77). It necessarily involves destruction of the normal microscopic anatomy of cells. Despite much evidence to the contrary, it is generally believed to leave the biochemistry of the cells relatively unchanged, notwithstanding the generation of heat, the mixing of activators and inhibitors, and the change in local environment of every subcellular component.

Furthermore, the general praxis of mixing up parts of cells each containing very diffusible materials to find out where their chemical constituents were before they were mixed is theoretically a very questionable one. Many authorities regard it as the only valid modern way of localising intracellular reactions, but, clearly, it is low down in the hierachy, as the integrity of the cells has been deliberately destroyed. Although the fractions can respire and do biochemical work, they can maintain few transmembrane gradients, or whole animal functions, which a natural historian or zoologist could perceive.

Death of an organism precedes by minutes to days the death of its individual tissues, so that the latter may be excised and studied in equilibrium with a perfusing fluid or incubating medium. However, dead or fixed organs, (78, 79), provide less information about molecular and biochemical processes within living cells. From the moment an animal begins to die, neither it nor its tissues is in equilibrium. The temperature falls, the proteins break down, autolysis occurs, and bacteria and fungi proliferate. Fixation is an attempt to prevent most of these changes.

A dead or fixed tissue does yield information about the anatomy of the tissue, if the effects of dying or fixation have been demonstrated to alter the appearance insignificantly. However, little information can be derived about the chemistry of its living behaviour, as much of the water and fat soluble materials have been extracted during preparation for histology. This is not to gainsay its value in histopathology, in which the tissue from the healthy human being or animal is subjected to the same complex procedures during fixation, embedding, staining, cutting and mounting as that from the ill animal. The conclusions arrived at by histopathologists are usually empirical.

During chemical extraction, (80, 81), different reagents are used to extract

different chemical components. Therefore, each of the latter has a different chemical relationship to the tissue in vivo. One would like to assume that the often powerful extracting agents did not themselves change the components they were extracting, but many of them are strong acids, alkalis, or alcohols, which would damage tissues irreversibly. Before the tissue has been extracted, the animal has been killed; an organ has been exised; it is homogenised; it may be centrifuged. Thus the extract is very far from the living animal in organisation, complexity, time, purity, temperature, pressure, pH, etc. The information derived from it must be correspondingly far away from that sought in observations on the living animal.

Section I deals with direct observations on organisms or their parts. Obviously, in a philosophical sense, when in the calculation of answers one interposes tested assumptions between the direct observations or measurements and the supposed conclusion or result of an experiment, one is moving away slightly from the source of information. However, one is going much farther away when the inherent assumptions of the techniques have not been tested (Section II). This implies the necessity for listing all the assumptions inherent in any technique used, and this must surely apply not only to research in biology, but also in science in general. Biology has a slightly more awkward dimension in that disorganisation or death of tissue is irreversible, whereas in many physical systems, repair or reverse can be engendered. The biggest and most fundamental property of biological tissue is its life, and therefore one should be especially concerned to preserve it in experiments designed to understand living processes.

Observations or measurements in which major inherent assumptions have not been tested represent much poorer quality, however likely the assumptions are to be warranted (Section III). The great danger is the construction of a whole experimental super-structure upon untested foundations.

Deductions and extrapolations both represent guesses based on logic (Section IV). Extrapolations involve projecting expectations beyond findings; they are usually used mainly in linear systems. The farther the extrapolation is from the range of the experimental conditions studied, the less likely it is to be true. It is also a frequent finding in biology – as in physical sciences – that systems often behave non-linearly in extremely high or low concentration ranges. It is also worth noting that many biological responses like growth, excitation, sensation, and homeostasis are non-linearly related to the stimuli which induce them.

Sections V, VI and VII are usually, but not necessarily, lower down the hierarchy. For example, if the data from biologically similar systems are based on assumptions which are more warrantable than that from the initial system one is studying, then they can be more valid. However, in a series of experiments under Sections I–VII which were theoretically of equal validity – if that could be

measured or designed – conclusions from those higher up the hierachy would be more acceptable than those lower down if they were mutually in conflict. The real practical difficulty of making a completely general hierarchy only underlines the necessity for examination of the validity of individual experiments by some such analysis as the present one.

Evidence within Sections VIII, IX and X, depends for its validity upon the closeness of the relationship between the systems whose findings are considered compatible. Untestable hypotheses are of no value to biology or other science, so great efforts must be made to demonstrate the testability, or carry out the test, of any hypothesis.

Teaching material (Section XI) is necessary for the acceptance of findings, which is, willy nilly, part of their 'truth'. However, one can say beyond reasonable fear of contradiction that, when they represent conflicting evidence, the direct observation of an organism is better evidence than a photograph of it; a photograph is better evidence than a diagram; personal examination of each of these lines of evidence is more valid than reading about it.